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Review

Progress in identifying virulence determinants of the 1918 H1N1 and the Southeast Asian H5N1 influenza A viruses

Christopher F. Basler*, Patricia V. Aguilar

Department of Microbiology, Box 1124, Mount Sinai School of Medicine, 1 Gustave L. Levy Place, New York, NY 10029, United States

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ABSTRACT

The 1918 pandemic H1N1 influenza virus and the recently emerged Southeast Asian H5N1 avian influenza virus are unique among influenza A virus isolates in their high virulence for humans and their lethality for a variety of animal species without prior adaptation. Reverse genetic studies have implicated several viral genes as virulence determinants. For both the 1918 and H5N1 viruses, the hemagglutinin and the polymerase complex contribute to high virulence. Non-structural proteins NS1 and PB1-F2, which block host antiviral responses, also influence pathogenesis. Additionally, recent studies correlate high levels of viral replication and induction of strong proinflammatory responses with the high virulence of these viruses. Defining how individual viral proteins promote enhanced replication, inflammation and severe disease will provide insight into the pathogenesis of severe influenza virus infections and suggest novel therapeutic approaches.

Contents

* Corresponding author. Tel.: +1 212 241 4847; fax: +1 212 534 1684.
E-mail address: chris.basler@mssm.edu (C.F. Basler).

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1. Introduction: emergence of highly pathogenic influenza A viruses

Three influenza virus pandemics occurred in the 20th century, in 1918, 1957 and 1968, and it is anticipated that additional pandemics will occur. In each of the three 20th century pandemics, avian influenza viruses played a critical role in the emergence of the pandemic strain (reviewed in Lipatov et al., 2004). The contribution of avian viruses reflects the fact that influenza A viruses are maintained in wild birds, through largely asymptomatic infection of the gastrointestinal tract. Because influenza viruses possess segmented genomes, coinfection of the same host by two different viruses can result in the reassortment of viral genes. When reassortment occurs between avian and human influenza viruses, the result can be the formation of antigenically novel viruses that, if efficiently transmitted by the respiratory route from human to human, may become pandemic strains. An alternate route to formation of a pandemic virus, the direct adaptation of an avian influenza virus such that it develops efficient human to human transmission is also possible.

In 1918, the first and most devastating of the 20th century pandemic strains emerged. Whether the virus was the result of the adaption of an avian virus to humans, such that it could efficiently transmit from person to person, or whether it emerged via reassortment, remains uncertain and controversial (Gibbs and Gibbs, 2006; Taubenberger et al., 2006). Regardless, the virus became established in the human population, killing more than 50 million people, and derivatives of this strain continued to circulate in attenuated form, causing outbreaks of seasonal human influenza infection until 1957. In that year, a new virus emerged as a result of a reassortment event such that the resulting virus had acquired new hemagglutinin (HA) and neuraminidase (NA) genes, producing a new H2N2 pandemic strain. An additional reassortment event yielded an H3N2 strain that, in 1968, emerged to cause another pandemic. Although the latter two pandemic viruses caused an increase in influenza morbidity and mortality as they spread around the world, they were much less virulent than the 1918 agent.

No new pandemic influenza A virus has appeared since 1968, although H1N1 viruses reappeared in the human population in 1977 and co-circulate with H3N2 viruses up to the present day. However, in 1997, an H5N1 avian virus emerged in Hong Kong that was lethal for domestic poultry and exhibited the unprecedented ability (for an avian virus) to cause lethal infection in humans, killing 6 of 18 confirmed patients (Centers for Disease Control and Prevention, 1998). (In contrast, an H5N1 avian virus that caused lethal disease in poultry flocks in the United Kingdom in 1991 was not associated with reports of human illness; Wood et al., 1994.) Although culling of poultry ended the Hong Kong outbreak, H5N1 viruses continued to circulate in wild birds and then re-emerged in Southeast Asia in 2003. H5N1 has since been spread through Asia to Africa and Europe by migratory birds and shipments of poultry. To date, more than 300 humans have become infected with the H5N1 virus through exposure to sick domestic ducks and chickens, with a reported case fatality rate exceeding 60% (World Health Organization, 2008). The virus does not spread efficiently from human to human, and only a few instances of direct person-to-person spread have been documented (Yang et al., 2007). However, there is great concern that H5N1 might become highly transmissible among humans, either by adaption of the fully avian virus to mammalian species or through reassortment of the avian virus with circulating human strains, leading to a devastating pandemic.

Both the 1918 virus and the recently emerged H5N1 virus are now the subject of intense laboratory investigation. The reconstructed 1918 agent has proven to be much more virulent than the seasonal H1N1 or H3N2 viruses for a range of animal species, causing lethal illness in mice and ferrets without any prior adaptation (Tumpey et al., 2005a, 2007; Kobasa et al., 2007). The H5N1 avian virus also differs from other avian or human influenza A isolates in being highly pathogenic for a wide range of animal species (e.g. Katz et al., 2000a; Perkins and Swayne, 2002; Keawcharoen et al., 2004; Kuiken et al., 2004; Govorkova et al., 2005; Van Borm et al., 2005). The establishment of standard infection models in mice and ferrets and the development of methods to readily manipulate influenza virus genomes has permitted reverse genetic studies that have evaluated the contribution of individual viral genes to virulence.

In this paper we summarize progress in identifying the contribution of individual viral genes to the pathogenicity of the 1918 H1N1 and the Southeast Asian H5N1 viruses. We first briefly list the functions of individual viral proteins in the replication cycle, then describe laboratory animal models used to assess virulence. We then review the emergence of the 1918 virus and the disease it caused. Finally, we summarize 1918 virus pathogenicity in experimental animals and current knowledge of the contribution of each viral gene to virulence. In the following section, we do the same for the H5N1 virus. We conclude by identifying directions for further research on the pathogenicity of these two agents.

2. Role of individual influenza A viral proteins in virulence

The influenza A virus is an enveloped virus with a genome made up of 8 separate segments of negative-sense single-stranded RNA that encode 11 known proteins. The genomic RNAs are found in ribonucleoprotein complexes (RNPs) in which the viral RNA is coated with the viral nucleoprotein (NP) and is associated with the heterotrimeric polymerase (P) complex. Within the virus particle, the RNPs also associate with the viral matrix protein, M1. Three transmembrane proteins, the hemagglutinin (HA), the neuraminidase (NA) and the ion channel, M2, are embedded in the viral envelope. The proteins and their basic functions are listed in Table 1, and steps in viral replication are shown in Fig. 1.

The virulence of an influenza virus isolate for a given host reflects its ability to enter a host cell, replicate within it, then exit and spread to new cells. Individual viral proteins could contribute to this process in different ways, such as modifying the target specificity of virus binding, facilitating virus entry, enhancing genome transcription and replication, aiding virus assembly and release, or modulating host responses to infection (Table 2).

2.1. Binding specificity of the HA

Influenza A viruses replicate in airway epithelium and are transmitted by the respiratory route (Lowen and Palese, 2007). They bind host cells via their HA, which interacts with sialic acid residues on host cell surface molecules. Human and avian

Table 1
Influenza A virus genes and gene products

General category	Genome segment	Protein(s) encoded	Protein functions
Surface proteins	HA	Hemagglutinin (HA)	Viral attachment and fusion protein
	NA	Neuraminidase (NA)	Virus release
	M2	M2 ion channel	Facilitates viral RNP uncoating
Internal proteins	M1	Matrix (M1) protein	Viral structural protein regulates RNP nuclear import
	NP	Nucleoprotein (NP)	Required for viral RNA synthesis
Polymerase components	PA	PA	Polymerase subunit
	PB1	PB1	Polymerase catalytic subunit endonuclease activity
	PB2	PB2	Polymerase subunit binds caps of host cell mRNAs
Non-structural proteins	NS	NS1	Evasion of interferon responses
		PB1-F2	Proapoptotic factor, possible immune evasion function
		Nuclear export protein (NEP or NS2)	Mediates viral RNA nuclear export

For a complete review, see Shaw and Palese (2007).

influenza virus HAs differ with regard to their specificity for host cell receptors. Human virus HAs preferentially bind receptors with oligosaccharides ending in sialic-acid α-2,6-galactose (Saα-2,6gal) whereas avian viruses preferentially recognize Saα-2,3gal (Rogers and Paulson, 1983; Matrosovich et al., 2000). Recently, evidence has emerged indicating that receptor specificity influences the anatomical site of replication of influenza viruses and could therefore affect the transmissibility of influenza viruses (Shinya et al., 2006). Given the fact that transmissibility is an essential characteristic of a pandemic virus, the acquisition of Saα-2,6gal specificity would appear to be an important event in the generation of an influenza virus with pandemic potential.

Interestingly, Saα-2,6gal predominates on epithelial cells in the human nasal mucosa, paranasal sinuses, the pharynx, trachea and bronchi (Shinya et al., 2006). In contrast, Saα-2,3gal is hardly detected in these cells, but it is found in the non-ciliated cuboidal bronchiolar cells at the junction between the respiratory bronchiole, alveolus and alveolar type II cells. Cumulatively, these studies suggest that receptor specificity contributes to inefficient transmission of H5N1 influenza viruses between humans. In human strains, such as the 1918 virus, receptor specificity has been experimentally demonstrated to influence transmission (Tumpey et al., 2007). However, it must be recognized that other viral factors will also contribute to transmission, although their roles remain to be defined.

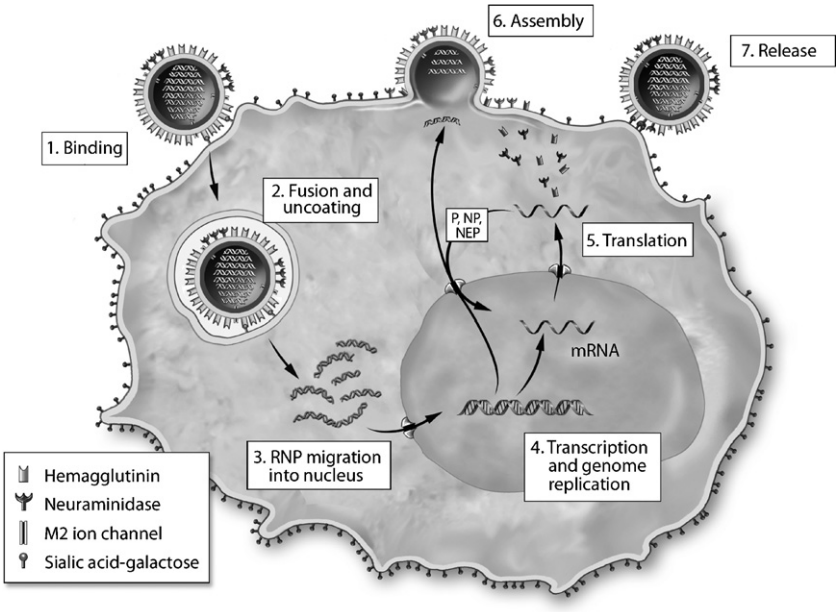

Fig. 1. The replication cycle of influenza A viruses. (1) Binding. Virion HA binds to sialic acid linked to cell-surface glycoproteins or glycolipids. (2) Fusion and uncoating. The virion is endocytosed. Endosome acidification triggers the fusion of the viral and endosomal membranes, releasing viral genetic contents. For HA to act as a fusion protein, it must have been cleaved at a specific site by host proteases. Virus associated M2 ion channel, permits the acidification of the interior of the virus allowing the dissociation of the viral matrix protein, M1, from the viral ribonucleoprotein particles (RNPs) that constitute the viral genome. This uncoating step allows nuclear import of viral RNPs and is inhibited by amantadine and rimantadine, which block the M2 channel. (3) RNA migration into nucleus. Release from M1 allows the RNPs to be released into the cytoplasm and to enter the nucleus where viral RNA synthesis occurs. RNP nuclear import occurs through the interaction of NP with karyopherin alpha proteins. (4) Transcription and genome replication. Within the nucleus, the heterotrimeric viral polymerase complex composed of the PA, PB1 and PB2 proteins and the viral nucleoprotein (NP) are required for viral RNA synthesis. (5) Translation. Viral mRNAs are exported to the cytoplasm and are translated into protein. (6) Assembly and exit. Newly synthesized viral genomes, in the form of RNPs, are exported to the cytoplasm; this requires the viral nuclear export protein (NEP) and the M1 matrix protein. Assembly occurs at the plasma membrane in association with lipid rafts. HA, NA and M2 reach the plasma membrane via the Golgi apparatus. There, the M1 protein and viral RNPs are incorporated into budding particles. HA directs viral budding (B.J. Chen et al., 2007). Incorporation of new viral genomic RNAs with budding particles appears to be mediated by *cis*-acting packaging signals present within each viral RNA segment. (7) Release. Efficient release of new virus particles requires that NA remove sialic acids from glycoproteins and glycolipids on the cell surface or on adjacent virions to prevent aggregation due to HA binding to sialic acids. NA inhibitors, such as the FDA-approved Tamiflu® and Relenza®, inhibit NA activity and impair influenza virus release. Adapted from Beigel and Bray (2008).

Table 2
Changes in function of individual influenza A virus proteins that could affect virulence

Protein	Changes in function that could affect virulence
HA	Altered cleavage site allowing change in tropism; altered receptor specificity allowing increased transmissibility and altered tropism in the airway
NA	Ability to promote HA cleavage
M2	Mutations that confer resistance to amantadine and rimantadine
M1	
NP	
PA	
PB1	Enhanced virus RNA synthesis
PB2	Enhanced virus RNA synthesis in mammalian cells, enhanced polymerase function at high temperature
NS1	Enhanced ability to suppress innate immunity
PB1-F2	Enhanced pro-apoptotic activity
NS2	

2.2. Entry into target cells

As noted above, HA mediates attachment to and entry into host cells, via sialic acid residues present on host cell glycoproteins or glycolipids, and is also the target of neutralizing antibodies (Maines et al., 2005). Following endocytosis of the viral particle, the endosome undergoes acidification. This acidification triggers extensive conformational changes in the HA structure, leading to fusion of the viral and endosomal membranes, thus releasing the viral RNPs into the host cell cytoplasm (Fig. 1). The HA structural rearrangements that mediate membrane fusion at low pH require priming of the HA trimer by cleavage at a specific site (Shaw and Palese, 2007). This cleavage converts the precursor protein HA0 into two disulfide-linked subunits, HA1 and HA2. The cleavage occurs adjacent to the hydrophobic "fusion peptide," which inserts into the endosomal membrane during the fusion process (Shaw and Palese, 2007). Because it determines the type of host proteases capable of priming a given HA, the nature of this cleavage site influences viral tissue tropism and pathogenesis (Steinhauer, 1999). In cell culture, influenza viruses lacking a multibasic cleavage site must be grown in the presence of exogenous protease to facilitate HA cleavage, while viruses with a multibasic cleavage site can grow in the absence of exogenous trypsin. *In vivo*, at least in birds, viruses with a multibasic cleavage site can spread beyond the GI and respiratory tracts. In mouse experiments, the H5N1 multibasic cleavage site was required for virulence and promoted dissemination to the brain, but the contribution of extra-pulmonary virus to disease in mammals is unclear (Hatta and Kawaoka, 2002).

2.3. Modification of genome transcription and replication

As discussed below, high virulence has been associated with increased levels of influenza A virus replication *in vivo*. Viral replication rates could obviously be influenced by the rate at which a virus can replicate its genes and express its mRNAs. Thus, viral polymerase complexes that display increased rates of function could contribute to increased virulence. Influenza virus RNA synthesis is carried out by a heterotrimeric RNA-dependent RNA polymerase, composed of the PA, PB1 and PB2 proteins, and the viral nucleoprotein (NP), which is always found associated with the template RNAs. The negative-sense genomic viral RNAs serve as templates for the synthesis of antigenomic, positive-sense RNAs called cRNA, and act as templates for the synthesis of viral mRNAs. The positive-sense cRNAs serve as template for the production of new vRNAs. On-going cellular mRNA synthesis is required for viral mRNA synthesis, because 5′-capped mRNA primers are cleaved from host cell mRNAs and are used as primers for viral mRNA production.

2.4. Evasion of host antiviral responses

The two viral non-structural proteins, NS1 and PB1-F2, appear to facilitate evasion of host defenses. NS1 antagonizes host interferon (IFN)-α/β responses, allowing the virus to escape from this critical component of innate immunity (Garcia-Sastre and Biron, 2006). The role of NS1 as an antagonist of IFN-responses was demonstrated most clearly when, using a reverse genetics approach, influenza viruses lacking the NS1 gene were generated and shown to be viable but highly attenuated in cells competent to mount an IFN-α/β response (Garcia-Sastre et al., 1998). Consistent with the role of NS1 in suppressing the IFN-α/β response, influenza viruses lacking NS1 induce high levels of IFN and are attenuated in IFN-competent systems, but not in hosts with a deficiency in the IFN response. NS1 may also influence the outcome of influenza virus infection by other mechanisms. For example, NS1 also appears to inhibit the expression of other cytokines, including TNF-α and IL-6 (Newby et al., 2007) and to facilitate evasion of adaptive immune responses (Fernandez-Sesm, 2007). Additionally, some NS1s have also been demonstrated to possess PDZ ligand domains of the type X-S/T-X-V, which can influence pathogenesis (Obenauer et al., 2006; Jackson et al., 2008).

NS1 has been reported to antagonize the host interferon response by mechanisms that prevent IFN-α/β production and that antagonize the function of IFN-induced antiviral proteins (Basler, 2007). NS1 may inhibit activation of innate antiviral responses by sequestering dsRNA, a potential trigger of IFN-α/β responses (Wang et al., 1999). NS1 also inhibits the activation of signaling through RIG-I, an intracellular sensor of virus infection (Hornung et al., 2006; Pichlmair et al., 2006; Basler and Garcia-Sastre, 2007; Mibayashi et al., 2007). Binding to dsRNA alters the structure of RIG-I such that it can transduce a signal leading to the activation of latent transcription factors such as interferon regulatory factor 3 (IRF-3), NF-κB and AP-1 which activate IFNα/β gene expression. NS1 has been shown to interact with RIG-I and to prevent its activation, thus inhibiting IFNα/β gene expression (Pichlmair et al., 2006; Guo et al., 2007; Mibayashi et al., 2007; Opitz et al., 2007). However, NS1 also suppresses innate immunity by targeting host gene expression. NS1 interacts with components of the cellular pre-mRNA processing machinery, including cleavage and polyadenylation specificity factor (CPSF), inhibiting proper 3′-end processing (endonucleolytic cleavage and polyadenylation) of cellular mRNAs (Nemeroff et al., 1998; Chen et al., 1999; Noah et al., 2003; Kochs et al., 2007). The result is an inhibition of the nuclear export of cellular mRNAs, while viral mRNAs formed independently of these cellular functions remain unaffected (Nemeroff et al., 1998; Chen et al., 1999; Noah et al., 2003; Kochs et al., 2007). The ability of NS1 to block host-cell gene expression contributes to the suppression of IFNα/β responses and is important for virulence (Noah et al., 2003; Kochs et al., 2007). Additionally, NS1 has been found to interact with cellular proteins involved in host cell mRNA nuclear export, and this association also appears to contribute to suppression of host cell gene expression (Satterly et al., 2007).

dsRNA also activates cellular enzymes with antiviral activity. Examples include the dsRNA-activated, IFN-induced kinase, PKR. PKR phosphorylates a number of targets including the translation initiation factor eIF-2α, resulting in suppression of translation. A second example is a family of proteins, the 2′,5′-oligoadenylate synthetases (OAS). OAS, once activated, produces 2′,5′-oligoadenylates which activate RNAse L, which in turn cleaves cellular RNAs, also suppressing protein synthesis. NS1 suppresses PKR activation by

sequestering dsRNA and by interacting with PKR (Lu et al., 1995; Tan and Katze, 1998; Zurcher et al., 2000; Li et al., 2006a; Min et al., 2007). Data also suggest that the primary function of the NS1 dsRNA-binding activity is to prevent activation of OAS (Min and Krug, 2006).

The PB1-F2 protein is the most recently discovered influenza A virus protein. It promotes apoptosis and influences pathogenesis. Current models suggest that PB1-F2 promotes the killing of infected macrophages and, in doing so, compromises the ability of the host to mobilize adaptive immune responses (Chen et al., 2001; Conenello and Palese, 2007), although PB1-F2 may also influence viral polymerase function (Mazur et al., 2008). Encoded by the +1 reading frame of the PB1 segment, hence the name PB1-F2 (frame 2), the short (up to 90 amino acids) protein is of variable length and is encoded by many, although not all, influenza A viruses.

PB1-F2 was first identified in a systematic screen for viral proteins produced from alternate open reading frames that could be recognized by CD8+ T cells (Chen et al., 2001). T cells specific for PB1-F2-derived peptides could lyse cells infected with influenza A virus, demonstrating expression of the protein in infected cells (Chen et al., 2001). Indirect immunofluorescence performed on infected cells by using antisera generated against PB1-F2 demonstrated a partial mitochondrial localization, with some of the protein also localizing to the cytoplasm and nucleus (Kobasa et al., 2004). The mitochondrial localization was notable because PB1-F2 was found to promote loss of mitochondrial membrane potential and to induce apoptosis, particularly in human monocytic cells, and to sensitize cells to pro-apoptotic stimuli such as TNFα (Chen et al., 2001; Zamarin et al., 2005). Mitochondrial targeting of PB1-F2 is mediated by largely helical regions in the carboxy-terminus of the protein (Gibbs et al., 2003; Yamada et al., 2004; Bruns et al., 2007), and peptides corresponding to this region of PB1-F2 are able to oligomerize and to permeabilize membranes (Chanturiya et al., 2004; Henklein et al., 2005; Bruns et al., 2007). The ability of PB1-F2 to promote apoptosis appears to be due to the protein's ability to interact with two components of the mitochondrial permeability transition pore complex, adenine nucleotide translocator 3 (ANT3) and the voltage-dependent anion channel 1 (VDAC1). ANT3 localizes to the inner mitochondrial membrane while VDAC1 localizes to the out mitochondrial membrane. PB1-F2 sensitizes cells to apoptosis in an ANT3-dependent manner, and it has been proposed that PB1-F2 promotes permeabilization of the mitochondrial membranes by enhancing formation of the permeability transition pore complex or because interaction with ANT3 and VDAC1 promotes mitochondrial membrane permeabilization by PB1-F2 (Zamarin et al., 2005).

3. Measuring virulence

The 1918 H1N1 virus and the Southeast Asian H5N1 viruses are of great interest because they are highly virulent in humans. For influenza viruses, several experimental systems have been employed as surrogates to gauge potential virulence for humans, including animal and cell culture systems. Most often, parameters of virulence are measured in the context of experimentally infected mammals (e.g. mice, ferrets, non-human primates) and may include various measures of morbidity (weight loss, changes in body temperature, physical appearance, etc.) and mortality. Typically, correlates of virulence such as viral titers in various tissues, histopathology and cytokine levels are also assessed.

In approaching the question of virulence determinants for influenza viruses, it is important to recognize that virulence is a host-dependent phenotype. Mice have most often been used as a model system to characterize and quantify the capacity of influenza viruses to induce morbidity and mortality. However, the mouse model has limitations. Notably, the mouse is not a natural host for influenza viruses; virus is not transmitted from animal to animal, and, more importantly when one is concerned with pathogenesis, most human influenza virus isolates fail to cause disease in mice unless they are first adapted to this species through serial passage. Other animal models that have been employed for pathogenesis studies include ferrets and non-human primates. Additionally, avian species and chicken embryos can serve as systems to measure virulence in birds. Finally, because humans are a host of particular interest to the influenza research community, the behavior of viruses in human cell culture systems, particularly primary human airway cell cultures, has been used as a correlate of virulence.

4. The 1918 H1N1 virus

4.1. Emergence, nature of disease in humans

The 1918 pandemic killed perhaps as many as 50 million individuals (Johnson and Mueller, 2002). The disease associated with the pandemic, particularly after the first relatively mild wave, was notable for several reasons including the number of individuals who died, the age distribution of fatal cases and the pathology of the disease. However, whether the 1918 virus was actually more pathogenic than other influenza viruses could only be known with certainty if the virus was available for study. Because the 1918 pandemic virus was not isolated at the time of the pandemic, direct study of the 1918 virus became possible only when techniques were developed to amplify, by reverse transcription-PCR, viral RNA present in preserved tissue obtained from formalin-fixed pathology specimens or from a cadaver buried in permafrost (Smith et al., 1933; Francis, 1934; Taubenberger et al., 1997; Reid et al., 1999, 2000, 2001; Reid and Taubenberger, 2001). This allowed the entire genome to be sequenced. Once available, the genome sequence allowed the full reconstruction of the 1918 virus (Taubenberger et al., 2005; Tumpey et al., 2005a).

4.2. Host range and infection of laboratory animals

Study of the 1918 virus in several experimental systems has confirmed that it indeed has unique virulence properties that seem to explain the devastating impact of this virus on the human population (Tumpey et al., 2005a; Kash et al., 2006; Kobasa et al., 2007). For example, in the initial study, the reconstructed 1918 virus was able to efficiently kill mice without any prior adaptation to this species while a contemporary human H1N1 virus, influenza A/Texas/36/91 virus (Tx/91) was avirulent (Tumpey et al., 2005a). The 1918 virus also displayed an unusual ability to kill chicken embryos, a characteristic typical of avian influenza viruses not seen in a control human influenza virus (Tumpey et al., 2005a). High virulence in these systems correlated with enhanced replication of the 1918 virus in primary human bronchoepithelial cells, providing evidence that the 1918 virus has unique capabilities in a human system (Tumpey et al., 2005a). In a separate study, the 1918 virus caused severe, sometimes fatal disease characterized by lethargy, anorexia, rhinorrhea, sneezing, severe weight loss and high fever in experimentally inoculated ferrets while the Tx/91 virus did not (Tumpey et al., 2007). The 1918 virus also displays an unusual capacity to cause a lethal disease, characterized by potent proinflammatory responses, in non-human primates (Kobasa et al., 2007). These models, coupled with reverse genetics systems, open the door for the exploration of the molecular determinants of virulence of the 1918 virus.

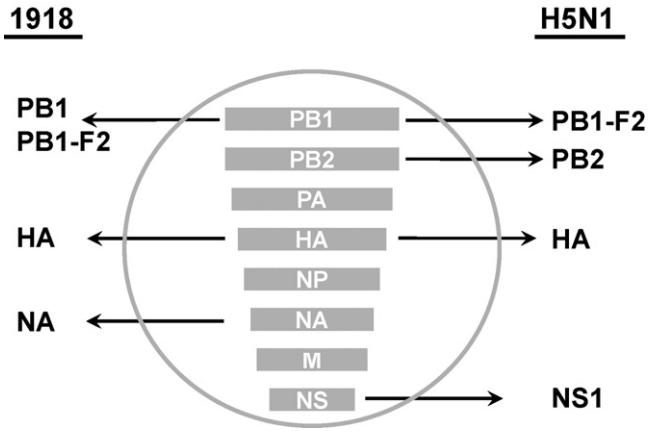

Fig. 2. Virulence determinants in mice of the 1918 and H5N1 avian influenza viruses. The genome of an influenza virus is depicted (center). Gene products implicated as virulence determinants for the 1918 virus (left) and avian H5N1 viruses (right) are indicated. The gene segment encoding each virulence factor is indicated by an arrow. See text for details.

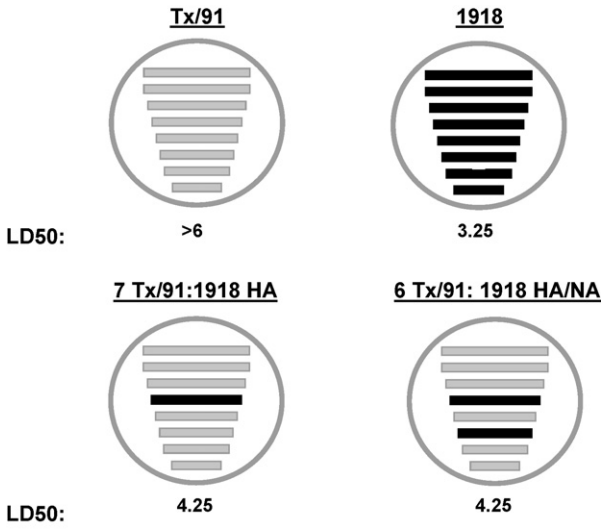

Fig. 4. Lethality in mice of select 1918:Tx/91 reassortant viruses. Figure shows the genetic composition of the parental Tx/91 and 1918 viruses as well as select 1918:Tx/91 reassortant viruses and their $\log_{10}$ LD_{50} by intranasal inoculation in 6–8-week-old BABL/c mice (Pappas et al., 2008). All possible 1:7 viruses were tested; only those that differed significantly from the parental Tx/91 virus are shown.

4.3. Role of individual 1918 genes in virulence

A recent study undertaken to evaluate the contribution of individual 1918 viral genomic segments to virulence identified the HA, NA and PB1 segments as determinants of pathogenicity (Pappas et al., 2008) (Fig. 2). These results build upon previous work that implicated the 1918 HA and NA as virulence factors (Tumpey et al., 2002, 2004, 2005a,b; Kobasa et al., 2004; Conenello et al., 2007). The strategy of this latest study was to generate a series of recombinant influenza viruses in which single 1918 virus segments were replaced with the corresponding segment from Tx/91. These 7 1918:1 Tx/91 "reassortants" were compared to the fully reconstructed 1918 virus for growth in human airway epithelial cells and for their virulence in mice (Fig. 3). The converse experiment was also performed; individual 1918 segments were inserted into a background of 7 Tx/91 segments (Fig. 4). These 1 1918:7 Tx/91 reassortants were then compared to the Tx/91 virus for growth in human airway epithelial cells and for their virulence in mice. Those 7 1918:1 Tx/91 "reassortants" generated by recombinant DNA technology which exhibited decreased virulence would implicate the replaced 1918 gene as a specific determinant of high virulence. Similarly, if a 1 1918:7 Tx/91 virus displayed increased virulence relative to the Tx/91 segment, this would again implicate the 1918 segment as a virulence determinant.

When the 7 1918:1 Tx/91 viruses were compared in mice, virulence differences were readily distinguished based on mortality and weight loss. Mice receiving 10^5 plaque forming units (pfu) of the complete 1918 virus, intranasally, exhibited substantial weight loss and succumbed by day 6 post-infection. Similarly, the 7:1 viruses in which the 1918 PA, PB2, NP, M and NS segments were replaced

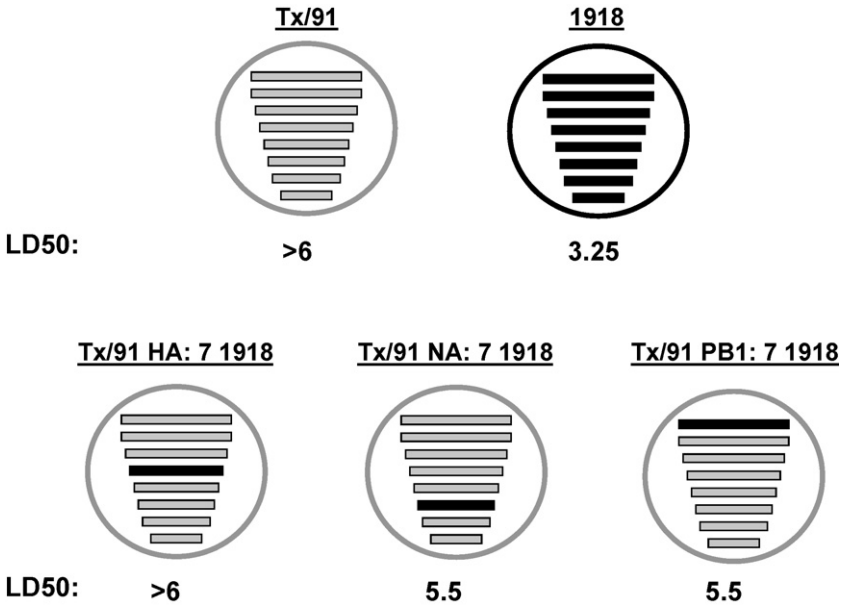

Fig. 3. Lethality in mice of 7:1 1918:Tx/91 reassortant viruses. Figure shows the genetic composition of the parental Tx/91 and 1918 viruses as well as selected 7:1 1918:Tx/91 reassortant viruses and their $\log_{10}$ LD_{50} by intranasal inoculation in 6–8-week-old BABL/c mice (Pappas et al., 2008). All possible 7:1 viruses were tested; only those that differed significantly from the parental 1918 virus are shown.

resulted in 100 percent lethality at the 10^5 dose. Indeed, for each of these viruses mouse LD_{50}s were in the $10^{3.25}$–$10^{3.75}$ range. In contrast, when the PB1, HA or NA segments of the 1918 virus were replaced with their Tx/91 counterparts, the viruses were attenuated such that 10^5 pfu was not lethal and induced less weight loss than was seen with either the 8 1918 gene virus or with the other 7:1 reassortants. For the 7 1918: Tx/91 PB1, Tx/91 HA and Tx/91 NA viruses, mouse LD_{50}'s ranged from $10^{5.5}$ to greater than 10^6. Attenuation of these viruses also correlated with decreased lung titers on both day 3 and day 5 post-infection (Pappas et al., 2008).

For those viruses in which 1918 segments were introduced into the Tx/91 genetic background, addition of the 1918 HA and NA or the 1918 HA alone resulted in viruses which could kill mice and which grew to 100-fold greater titers compared to the parental Tx/91 virus (Pappas et al., 2008). Although the remaining 1:7 viruses did not cause lethal infection in mice, the 1918 PB1:Tx/91 and the 1918 NA:Tx/91 viruses grew to modestly increased lung titers on days 3–5 post-infection relative to the parental Tx/91 virus. Thus, although introduction of the individual 1918 PB1 and NA segments did not fully overcome the attenuated phenotype of the Tx/91 virus in mice, they did confer enhanced replication *in vivo* (Pappas et al., 2008).

As noted above, mice are not natural hosts for influenza viruses. It is therefore informative to obtain correlates of pathogenesis in human systems. The replication of the 1918:Tx/91 reassortant viruses were therefore compared in primary human normal bronchial epithelial (NHBE) cells and in Detroit 562 cells which are derived from a human pharyngeal carcinoma (Temonen et al., 1993). In both culture systems, the 1918 PB1:Tx/91, the 1918 HA:Tx/91 and the 1918 NA:Tx/91 viruses all replicated to lower titers than did the parental 1918 virus or the remaining 7 1918:Tx viruses. For the 1 1918:7 Tx series of viruses, the presence of both the 1918 HA and NA, the 1918 HA alone or the 1918 NA alone conferred enhanced replication in HNBE cells (Pappas et al., 2008). Cumulatively, these data support the view that high virulence correlates with enhanced levels of virus replication in the mouse lung and in human airway epithelial cells, and the view that the 1918 PB1, HA and NA segments each contribute to the high virulence phenotype of the 1918 virus.

4.3.1. HA and NA

It was notable that the HA was the only 1918 segment able to confer a high virulence phenotype upon a virus that otherwise had a Tx/91 background. However, replacement of either 1918 HA or NA with that of Tx/91 was attenuating. These observations are consistent with previous studies implicating the 1918 HA and NA genes as virulence determinants. In an initial study, it was noted that insertion of the 1918 HA and NA segments into the background of influenza A/WSN/33 (H1N1) virus (WSN), a mouse-adapted virus, resulted in a virus which was as lethal to mice as the WSN strain (Tumpey et al., 2002). In contrast, a virus possessing the HA and NA of influenza A/New Caledonia/20/99 (H1N1) virus (New Cal) was non-lethal in mice. Because the New Cal virus was not previously adapted to mice, it was not surprising that the New Cal HA and NA attenuated an otherwise mouse-adapted virus. However, because the 1918 HA and NA were also derived from a fully human virus (i.e. a virus that was never adapted to mice), the absence of attenuation in mice of the 1918 HA/NA virus suggested that these segments encode unique virulence determinants (Tumpey et al., 2002).

These conclusions were further supported by later studies in which viruses possessing two to five 1918 segments were introduced into an otherwise WSN genetic background. In this study, any virus that retained the 1918 HA and NA was highly lethal in mice (Tumpey et al., 2004). However, because these studies again introduced the 1918 HA and NA into the background of a highly mouse-adapted virus, the 1918 HA and NA did not increase virulence above the parental WSN strain. Proof that the 1918 HA or the combination of the 1918 HA and NA could enhance virulence required the introduction of these 1918 genes into the background of a human strain not previously adapted to mice (Kobasa et al., 2004). When this was done, introduction of only the 1918 HA or of both the 1918 HA and NA enhanced virulence as evidenced by lower LD_{50} and increased lung titers relative to the parental, non-adapted virus (Kobasa et al., 2004). Increased virulence due to the presence of the 1918 HA was also associated with increased lung infiltrates and higher levels of chemokines and cytokines (Kobasa et al., 2004). Even in a WSN background, a virus carrying the 1918 HA and NA, which had a LD_{50} equivalent to that of the parental WSN virus and which grew to comparable lung titers as the parental WSN virus, elicited more lung inflammation than the parental strain (Kobasa et al., 2004).

The mechanism by which the 1918 HA acts as a virulence factor in mammals is unclear. In poultry, the presence of a multibasic cleavage site within the HA permits the highly pathogenic avian influenza viruses to disseminate beyond usual sites of replication (Hatta and Kawaoka, 2002). Thus it is notable that the presence of the 1918 NA facilitates plaque formation in the absence of exogenous protease (Tumpey et al., 2005a). This property is reminiscent of the influenza A/WSN/33 (H1N1) virus strain, in which the absence of a specific N-linked glycoslyation site within the WSN NA promotes HA cleavage in the absence of added tryspin (Schulman and Palese, 1977; Li et al., 1993). Evidence suggests that the WSN NA is able to recruit plasminogen to promote HA cleavage (Goto and Kawaoka, 1998; Goto et al., 2001). Although the 1918 virus also plaques on MDCK cells without added protease, there is no conclusive evidence that the 1918 virus replicated systemically in humans during the pandemic, nor do animal experiments support a role for viral dissemination outside the respiratory tract as a contributor to virulence. It is possible that the 1918 HA allows the virus to infect unique cell types or to elicit peculiar host responses, but direct evidence that this is the case is not presently available. The attenuation of viruses in which the Tx/91 NA replaced the 1918 NA may reflect, in part, the need for optimal balance between the neuraminidase activity of the NA and the affinity of the 1918 HA for sialic acid receptors. These two properties must be balanced for optimal virus replication to occur (Wagner et al., 2002).

4.3.2. Receptor usage

In the case of the 1918 pandemic virus, recombinant viruses were generated that differed in the specificity of their HAs for differently linked sialic acids. Viruses possessing SAα-2,6gal preference transmitted in a ferret model efficiently, but those with HAs that displayed either dual SAα-2,3gal/SAα-2,6gal preference or SAα-2,3gal preference were not efficiently transmitted via the respiratory route from ferret to ferret, suggesting that a HA which predominantly binds SAα-2,6-linked sialic acid is needed for optimal transmission, at least for the 1918 virus. Interestingly, however, virulence was not significantly affected by these differences in receptor specificity (Tumpey et al., 2007).

4.3.3. Polymerase complex

The findings that the 1918 PB1 protein makes a major contribution to virulence and enhances virus replication in NHBE cells and in mouse lungs are intriguing, because pandemic influenza viruses of 1957 and 1968 each acquired, by genetic reassortment, both novel viral surface antigens (NA and/or HA) and PB1 segments from an avian source (Scholtissek et al., 1978; Kawaoka et al., 1989). What contribution, if any, these PB1s made to the success of these new viruses as pandemic strains and whether acquisition of the new PB1s influenced virulence of the 1957 and 1968

strains in humans remains unclear. The importance of PB1 in pandemic strains may reflect the role of PB1 as the central component of the trimeric viral RNA-dependent RNA polymerase (Gonzalez et al., 1996; Li et al., 1998, 2001; Ohtsu et al., 2002; Neumann et al., 2004). Given its ability to enhance virus replication, it is notable that the 1918 PB1 protein differs from the conserved avian influenza consensus sequence by only seven amino acid residues (Taubenberger et al., 2005). Previous studies suggest that avian-like PB1 genes might provide increased transcriptional activity of the RNA-dependent RNA polymerase (Naffakh et al., 2000) and thereby enhance virus replication. Alternatively or in addition, the PB1-F2 proteins encoded by PB1 segments may make an important contribution to pandemic virus emergence and to virulence. Perhaps the 1918 PB1 confers high virulence because it encodes a PB1-F2 protein whereas the Tx/91 virus, like other contemporary human H1N1 isolates, does not.

4.3.4. PB1-F2

The influence of PB1-F2 on viral pathogenesis in mouse models of infection was first demonstrated in recombinant viruses that possessed the PB1 segment from influenza A/PR/8/34 (H1N1) virus (PR8) and the 7 remaining genomic segments from influenza A/WSN/33 (H1N1) virus (WSN). In this context, mutations that eliminated expression of PB1-F2 without affecting the PB1 open reading frame were attenuating following intranasal inoculation (Zamarin et al., 2006). When a PB1-F2 knockout was built into a similar recombinant virus that contained a chimeric WSN-PR8 PB1 segment, a virus attenuated relative to either the parental WSN virus or the WSN-PR8 PB1 reassortant virus, loss of PB1-F2 was once again attenuating and resulted in more rapid clearance of the virus from the lungs of infected animals (Zamarin et al., 2006). Similarly, a WSN-background virus carrying the PB1 from a highly pathogenic H5N1 avian influenza virus also displayed attenuation when PB1-F2 expression was absent (Zamarin et al., 2006).

Interestingly, the effect of PB1-F2 upon virulence in the mouse model is context dependent. Knock-out of PB1-F2 in a virus possessing all 8 segments from the WSN background did not reveal any virulence phenotype, (Zamarin et al., 2006). In contrast, mutation of a single amino acid residue (S66N) in the PB1-F2 of the 1918 pandemic influenza virus strain was sufficient to attenuate in mice this otherwise highly lethal virus (Conenello et al., 2007). These differences could be related to the fact that the WSN is a highly mouse-adapted virus whereas the reconstructed 1918 virus was derived from a human infection (Tumpey et al., 2005a), suggesting that PB1-F2s function in a host-specific manner. Additionally, most influenza virus pathogenesis experiments performed in mice measure the impact of what is essentially a primary viral pneumonia, but most human fatalities caused by influenza virus infection are due to secondary bacterial pneumonia. It is thus of great interest that PB1-F2 plays a role in virulence in a mouse model of secondary *Streptococcus pneumoniae* pneumonia following PR8 virus infection. In this system, viruses expressing PB1-F2 caused greater weight loss, more severe pneumonia and a higher fatality rate than a virus that did not express PB1-F2 (McAuley et al., 2007). Strikingly, when a PR8 strain was engineered to express a PB1-F2 identical in sequence to that of the 1918 virus, it replicated to higher titers in cell culture, was more virulent in mice and enhanced secondary bacterial pneumonia relative to the PR8 parent virus (McAuley et al., 2007).

Important questions that remain to be answered include the mechanism by which PB1-F2 influences pathogenesis. Does it act by promoting apoptosis, as has been suggested, and, if so, what are the critical targets of its pro-apoptotic function, and how does this alter the outcome of primary viral or secondary bacterial pneumonia? Additionally, it will be important to demonstrate PB1-F2 function in models other than mice. Also, as described above, the 1957 and 1968 pandemics obtained a PB1 segment from an avian source. Perhaps the PB1-F2 from these avian viruses was somehow critical for the establishment of these viruses in the human population and contributed to virulence in humans. How PB1-F2 might promote the establishment of a pandemic strain remains unclear.

4.3.5. NS1

NS was the first 1918 segment to be built into a recombinant virus (Basler et al., 2001). This was done to test the hypothesis that the 1918 NS1 protein might exhibit an enhanced capacity to suppress host IFN responses and thereby contribute to high virulence. The strategy taken was to insert the entire 1918 NS segment or just the 1918 NS1 coding region into recombinant viruses in which the remainder of the genes were derived from the mouse-adapted WSN strain. When tested by intranasal inoculation into mice, the viruses bearing the 1918 NS1 were actually attenuated relative to the parental WSN virus (Basler et al., 2001). The change in virulence associated with insertion of the 1918 NS1 demonstrated the importance of NS1 in virulence, however, the attenuation may reflect the need for mouse-adaptive changes to enhance the function of a human 1918 NS1 in mice (Basler et al., 2001). Supporting this view, the 1918 NS1 was associated with better suppression of IFN responses in human A549 cells compared with the NS1 of a mouse-adapted influenza A/PR/8/34 (H1N1) virus (Geiss et al., 2002).

As noted above, NS1 proteins often possess potential PDZ ligands, which are short carboxy-terminal peptides that bind to proteins with PDZ domains (Obenauer et al., 2006). PDZ domains mediate protein–protein interactions, and PDZ domain containing proteins often act as scaffolds to regulate large signaling complexes (Sheng and Sala, 2001). Intriguingly, when the NS1 of the WSN strain was altered, such that it had the 4 carboxy-terminal residues of the 1918 virus or those found in H5N1 viruses, the virulence of the virus was enhanced (Jackson et al., 2008). The mechanism by which these apparent PDZ ligand domains influence pathogenesis remains to be determined, but does not appear to be related to altered IFN responses (Jackson et al., 2008).

5. The Southeast Asian H5N1 virus

5.1. Emergence and spread

Until the emergence of H5N1 in Hong Kong in 1997, H5 influenza virus es were only known to circulate in avian species and to cause either mild respiratory illness or lethal disease in poultry (Webster et al., 2006). The recognition of human illness and death arising from H5N1 infection raised alarm and prompted several studies focusing upon the molecular basis for H5N1 virulence in mammals (Claas et al., 1998; Subbarao et al., 1998) (Fig. 2). Limited studies performed on victims of H5N1 infection have suggested that these viruses cause a primary viral pneumonia that can lead to acute respiratory distress syndrome (ARDS) and multi-organ failure (Yuen et al., 1998; de Jong et al., 2006; Taubenberger and Morens, 2006). Fatal H5N1 illness has also been associated with high levels of virus replication and hypercytokinemia (de Jong et al., 2006). Another notable aspect of H5N1 infection is evidence of the spread of virus outside of the respiratory tract, although the role of extrapulmonary virus in pathogenesis is unclear (de Jong et al., 2006).

5.2. Host range and infection of laboratory animals

H5N1 viruses share with the 1918 strain the ability to cause disease in multiple animal species (e.g. Katz et al., 2000a; Perkins

and Swayne, 2002; Keawcharoen et al., 2004; Kuiken et al., 2004; Govorkova et al., 2005; Van Borm et al., 2005). Current highly pathogenic H5N1 viruses display an unusual capacity to cause fatal disease not only in domestic poultry but also in other avian species, including wild waterfowl. They can also kill cats, ferrets and mice (Keawcharoen et al., 2004; Kuiken et al., 2004; Peiris et al., 2004; Sturm-Ramirez et al., 2004; Govorkova et al., 2005). It should be noted, however, that not all highly pathogenic H5N1 avian influenza viruses isolated from poultry exhibited the same virulence in either domestic ducks or in ferrets, but 4 of 4 H5N1 viruses recovered from humans were lethal for ferrets (Govorkova et al., 2005).

5.3. Role of individual H5N1 genes in virulence

Viruses recovered from the 1997 human H5N1 cases were grouped based upon their ability to cause illness and death in mice without prior adaptation (Gao et al., 1999; Lu et al., 1999). Virulence in mice correlated imperfectly with the severity of human infection, suggesting that the mouse model may provide insight into human H5N1 infections (Gao et al., 1999; Lu et al., 1999; Katz et al., 2000b; Hatta et al., 2001). Thus, two isolates from fatal human cases, HK/483 and HK/156, were highly pathogenic for mice, with an LD_{50} less than 1.0 plaque-forming unit (pfu). By contrast, the HK/486 virus, from a non-fatal case, was much less virulent for mice, with an LD_{50} $>6.0 \times 10^4$ pfu (Gao et al., 1999). Comparison of the sequences of the high versus low virulence viruses identified differences that correlated with pathogenicity. The more pathogenic 483 virus caused a systemic infection, whereas the less pathogenic 486 virus was restricted to the lungs (Gao et al., 1999; Katz et al., 2000b). Taking advantage of this difference in virulence, as well as the close genetic relationship between the 483 and 486 isolates, studies were performed implicating the HA and PB2 segments as the main determinants of H5N1 virulence in mice (Hatta et al., 2001; H. Chen et al., 2007).

5.3.1. HA

Studies on single-gene reassortant viruses generated between the 483 and 486 viruses implicated the HA segment as critical for mouse virulence. These observations were further refined, identifying the presence of multiple basic residues at the cleavage site of the HA protein as critical for mouse virulence (Hatta et al., 2001). H5 and H7 avian influenza viruses that are highly pathogenic for poultry possess multiple basic amino acids that are cleaved by ubiquitous furin-like proteases, allowing the virus to replicate beyond its usual sites of infection, the epithelium of the respiratory and gastrointestinal tracts, and to spread systemically (Webster and Rott, 1987; Stieneke-Grober et al., 1992; Horimoto and Kawaoka, 1994; Horimoto et al., 1994). In contrast, viruses whose HA has a single basic residue at the cleavage site are cleaved by proteases with a more restricted tissue distribution, thus limiting viral tropism (Horimoto and Kawaoka, 1994; Hatta and Kawaoka, 2002). Although the multibasic cleavage site is also required for H5N1 viruses to kill mice, how this influences pathogenesis in mammals remains unclear (Hatta and Kawaoka, 2002). As noted, the 1997 human H5N1 isolates were able to disseminate from the airway of experimentally infected mice and reach the central nervous system (CNS). Virus was also detected in the CNS of H5N1-infected ferrets (Maines et al., 2005). However, as in the case of human H5N1 infections, the contribution of extrapulmonary virus spread to disease severity is uncertain, and the mechanism by which the multibasic cleavage site influences mammalian disease remains an unanswered question (Hatta and Kawaoka, 2002).

5.3.2. Receptor usage

Recent studies demonstrated that most H5N1 viruses bind to cells of the lower respiratory tract that are rich in SAα-2,3gal, such as non-ciliated cuboidal bronchiolar cells and type II pneumocytes. This may contribute to the severity of pulmonary lesions observed in human infections and to the restricted replication of the virus impairing human-to-human transmission (Shinya et al., 2006; van Riel et al., 2006). Changes at positions 182 and 192 in the HA have been identified as responsible for the recognition and binding of H5N1 viruses to human-type receptors (Yamada et al., 2006). A few human-derived H5N1 viruses have displayed dual SAα-2,3gal, SAα-2,6gal specificity (Shinya et al., 2006; Yamada et al., 2006); such viruses bind predominantly to epithelial cells in the bronchi (Shinya et al., 2006). Although viruses with these changes have been isolated from infected individuals and replicate in bronchial epithelial cells, they are not known to have been transmitted from human to human, suggesting that the pandemic potential of H5N1 viruses is determined by other viral segments as well.

5.3.3. Polymerase complex proteins

The presence of a lysine at position 627 of the PB2 protein (a characteristic of the 483 H5N1 1997 virus) as opposed to glutamic acid (as in the 486 H5N1 1997 virus) has also been implicated as critical for mouse virulence (Hatta et al., 2001). How this single amino acid substitution confers a host range phenotype is partially understood. In the context of H5N1 viruses, a replication advantage was seen for PB2 627K-containing viruses in mammalian but not avian cells (Hatta et al., 2001; Shinya et al., 2004). In mice, PB2 627K grew to higher titers in the lung, was associated with increased dissemination to different parts of the mouse respiratory system and provoked greater neutrophil infiltration into the lung (Shinya et al., 2004). These phenotypes may reflect an important difference between avian and human influenza virus infections: human influenza viruses generally replicate in the upper airway, which has a temperature of around 33 °C, whereas avian viruses typically replicate in the avian gastrointestinal tract which has a temperature of approximately 41 °C. Avian polymerase complexes were found to exhibit cold-sensitivity in mammalian cells, and this sensitivity is influenced by PB2 and by PB2 residue 627 in particular. Specifically, cold-sensitivity in mammalian cells was reversed by mutation of PB2-627E to K (Massin et al., 2001). At the same time, the PB2-627E to K mutation does not alter viral fitness in avian hosts (Gabriel et al., 2005).

Supporting the importance of PB2 as a determinant of temperature-sensitivity, two H5N1 influenza viruses that differed by only a single amino acid at position 627 of the PB2 (E or K), were isolated from the same patient (Hatta et al., 2007). Hatta et al. found that PB2-627K contributes to efficient replication of H5N1 influenza virus in mammalian cell lines and enables them to grow at the low temperatures of the upper respiratory tract, possibly allowing the virus to spread more efficiently by sneezing and coughing. Thus, it has been suggested that a single lysine mutation in the PB2 protein may also contribute to efficient human-to-human transmission by increasing viral growth in the upper respiratory tract. Other residues within the PB2 have also been proposed to influence virulence in mice, but their exact contribution to pathogenesis has not been clearly defined (Katz et al., 2000b; H. Chen et al., 2007). Additionally, two H5N1 viruses differing by eight amino acids, including an E627K difference, exhibited very similar pathogenicity in either mice or ferrets (Maines et al., 2005). It remains to be defined which host-specific factors, other than temperature, contribute to host-dependent replication and virulence.

5.3.4. PB1-F2

As noted above, the PB1-F2 can influence the virulence of some H5N1 viruses for mice. Thus far, its role has only been studied in the context of a mouse-adapted WSN virus (Conenello et al., 2007). It remains to be demonstrated that PB1-F2 will influence virulence in the context of a complete H5N1 virus.

5.3.5. The NS1 protein

The NS1 gene of the H5N1 1997 isolates appears to have contributed to virulence by helping the virus evade innate immune responses (Seo et al., 2002, 2004). Specifically, 1997 viruses were highly resistant to the antiviral action of IFNs and TNF-α (Seo et al., 2002). Using reverse genetic studies, a reassortant virus that contained the NS1 gene of the A/HK/156/97 virus and other genes of the A/PR/8/34 (H1N1) influenza virus (PR8) was generated and shown to be resistant to the antiviral action of IFN-α, IFN-γ and TNF-α, whereas PR8 was sensitive to these cytokines (Seo et al., 2002). A glutamic acid at position 92 of the NS1 (which is present in the 1997 isolates and more recent H5N1 influenza strains) was required for the inhibitory effect of the H5N1 NS1. These data suggest that NS1 enhances the virulence of these viruses by virtue of its ability to evade the antiviral action of IFNs and TNF-α (Seo et al., 2004). Consistent with these observations, NS1 has also been found to be important for the high virulence of more recent H5N1 viruses for mice (Li et al., 2006b; Jiao et al., 2008). As noted above, H5N1 viruses possess, within their NS1s, PDZ ligand domains that can enhance virulence in mice (Jackson et al., 2008).

6. Immunopathology

A role for HA, the viral polymerase complex and viral immune evasion factors (NS1 and PB1-F2) in virulence are themes common to the 1918 and H5N1 systems, but the question remains how these factors contribute to the disease process. Current data suggest two factors of importance. First, high virulence is associated with substantial levels of virus replication *in vivo*, although it is not yet clear whether this means that replication levels are high in each individual cell or whether the number of infected cells is particularly large. Additionally, how enhanced viral replication contributes to virulence remains to be systematically evaluated. Second, high virulence is associated with potent, perhaps excessive, inflammatory responses, and it is possible that these responses may prove to be the direct cause of the damage seen in severe disease. For example, analysis of specimens from 18 human H5N1 cases revealed high levels of virus replication in the upper airway, with elevated cytokine and chemokine levels and low T cell counts in peripheral blood (de Jong et al., 2006). Similarly, the 1918 virus was lethal for mice and was associated with vigorous proinflammatory responses (Tumpey et al., 2005a). Microarray analysis of lung tissue from 1918 virus-infected mice revealed strong and early activation of immune response genes, a response which was sustained throughout infection and which was accompanied by significant lung pathology (Kash et al., 2006). In a separate study, infection of cynomolgous macaques with the 1918 virus also resulted in severe lung pathology and a dysregulated innate immune response, as deduced from microarray analyses of lung tissue (Kobasa et al., 2007).

While immunopathology correlates with disease severity, it remains unclear whether the poor outcome is a direct result of immune dysregulation and whether dysregulated immune responses are secondary to high levels of virus replication. Studies to define which aspects of the proinflammatory response are beneficial and which are detrimental have only just begun, and are yielding sometimes contradictory results. For example, analysis of 1997 H5N1 virus isolates in knock-out mice suggested that TNF-α promoted influenza virus-induced morbidity, whereas IL-1 contributed to virus clearance (Szretter et al., 2007). However, a study employing a 2004 H5N1 isolate found that inhibition of cytokine responses did not protect mice from lethal infection (Salomon et al., 2007). The contributions of immune responses to 1918 pandemic virus-induced disease have also only just begun (Tumpey et al., 2005b). The fact that the lungs of mice infected with viruses carrying the HA and NA genes of the 1918 pandemic strain showed extensive neutrophil and macrophage infiltration suggested that these infiltrates might contribute to disease. However, when either or both of these cell populations were depleted from mice, an otherwise sublethal dose of virus caused increased disease and death (Tumpey et al., 2005b).

7. Conclusions

The unique properties of the 1918 virus and Southeast Asian H5N1 viruses, particularly their ability to cause severe disease in mice and ferrets without prior adaption, has permitted the studies described above. Coupled with modern molecular techniques, genetic studies have clearly implicated the HA, the viral polymerase and the PB1-F2 proteins as determinants of high virulence for both 1918 and H5N1, and the NS1 protein also influences virulence in both systems. Major gaps in our knowledge remain, however, especially in our understanding of the mechanisms by which these viruses ultimately cause disease. For example, although potent proinflammatory cytokine responses are associated with severe disease, there is no definitive demonstration that they are the cause of death. Further, it is also possible that some components of these responses may prove to be beneficial rather than damaging. Finally, the mechanisms by which specific viral genes or proteins promote virulence have not been clarified. Defining how viral products interact with the host to produce disease will provide insight into influenza pathogenesis and suggest novel antiviral therapies.

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