THE FACTORS OF VIRULENCE OF INFLUENZA A VIRUS

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Summary. – This review deals with factors that influence the virulence of influenza A virus. A high genetic and antigenic variability is demonstrated in a broad spectrum of influenza A viruses ranging from avirulent to lethal ones. Influenza A viruses have caused several epidemics and pandemics in the past and present that have cost millions of lives worldwide. Therefore these viruses still belong to the most important ones with strong impact on whole human population. Here, we discuss the latest findings concerning the role of individual viral proteins, interaction of the virus with the host immune system, and interspecies transmission and evolution of the virus. It is important to elucidate the genetic background of virulence of influenza A viruses, mechanisms involved in crossing the interspecies barrier, and mechanisms of destruction of the host cell by these viruses, and to identify the factors influencing the interaction between these viruses and the host immune defense system. This knowledge should help to estimate the threat of influenza A viruses with human pandemic potential to human population.

Key words: influenza; influenza A virus; interspecies transmission; pandemic potential; pathogenesis; virulence

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Abbreviations: CRM = cellular export factor; Gal = galactose; HA(s) = hemagglutinin(s); HAEC = primary human adenoid epithelial cells; HIV = Human immunodeficiency virus 1 and/or 2; IFN = interferon; IL = interleukin; LAP = latency-associated peptide; M1 = matrix protein; MHC = major histocompatibility complex; NA = neuraminidase; NeuAc = N-acetylneuramic acid; NeuGc = N-glycolylneuramic acid; NS = nonstructural; PKR = protein kinase R; RBS = receptor-binding site; SA = siallic acid; SARS = severe acute respiratory syndrome; TGF = tumor growth factor; vRNP = viral ribonucleoprotein

1. Introduction

The virulence is a quantitative expression of pathogenicity of a given organism or virus for certain animal species (Ferenčík *et al*., 2002). Virulence can be described as "the capacity to produce lesions or symptoms in a given tissue or host". It depends upon the interaction between a virus or its components and an individual cell type, specific tissue or whole organism; this combination of factors confers upon a virus the ability to replicate and spread in a host population (Baigent and McCauley, 2003). Virulence of influenza viruses is multigenically determined (Brown, 2000; Reina, 2002).

2. Role of virus surface glycoproteins in virus virulence

2.1 Specificity of binding of viral hemaglutinins (HAs) to cell receptors and its modulation by glycosylation and sialylation

HA is required for binding of the virus to the cell receptor and for fusion of host endosomal membrane with the virus. Role of HA and neuraminidase (NA) in determining influenza virus virulence is summarized in Fig. 1. The first step in influenza virus life cycle is the binding of HA to the receptor of the host cell. The receptor-binding site (RBS) of HA binds to its receptor, sialic acid (SA) attached to galactose on the surface of the host cell. The RBS is a pocket located at the distal end of each subunit of HA. The residues forming' the pocket (Tyr 98, Trp 153, His 183, Glu 190, and Leu 194) are largely conserved among subtypes (Wilson *et al*., 1981), while residues surrounding the conserved pocket vary during the antigenic drift of viruses prevalent from year to year (Steinhauer and Wharton, 1998).

SA is 5-amino-3,5-dideoxy-D-glycero-D-galacto-nonulosonic acid. The amino group is always substituted with either an N-acetyl or N-glycolyl group, yielding N-acetylneuraminic (NeuAc) or N-glycolylneuraminic (NeuGc) acid, respectively, while the hydroxyl groups can be substituted by acetyl, lactoyl, methyl, sulfate or phosphate residues (Suzuki *et al*., 2000). Influenza viruses recognize both species of SA, which are attached to galactose in SAα-2,3-Gal or SAα-2,6-Gal linkages (Steinhauer and Wharton, 1998; Skehel and Wiley, 2000; Baigent and McCauley, 2003). Ability of the viruses to replicate in different host species is influenced by SA species, linkage type in the host and amino acids at positions 226 and 228 in the RBS. Gln 226 correlates with the SAα-2,3-Gal receptor specificity in avian viruses, while Leu 226 with the SAα-2,6-Gal specificity in human viruses of H2 and H3 but not H1 subtype (Steinhauer and Wharton, 1998; Vines *et al*., 1998; Ito and Kawaoka, 2000; Skehel and Wiley, 2000). The comparison of amino acid sequences of influenza A viruses from different hosts revealed six amino acids around the RBS (Ala 138, Glu 190, Leu 194, Gly 225, Gln 226, and Gly 228); these amino acids are highly conserved in avian viruses but bear substitutions in human viruses. This finding suggests that mutations at these positions are required for adaptation of the avian virus HA to human hosts (Matrosovich *et al*., 2000).

The binding specificity of SA, receptor and RBS of HA determine the host range of given virus and its replication site. Human tracheal epithelium contains predominantly NeuAcα-2,6-Gal, duck intestine NeuAcα-2,3-Gal and swine tissues both (Ito and Kawaoka, 2000; Skehel and Wiley, 2000; Baigent and McCauley, 2003; Landolt *et al*., 2003). The fact that swine tissues contain both SA species and both species of linkage of SA to galactose means that pigs can be infected by both avian and human viruses. This makes pigs a mixing vessel for influenza viruses, provides conditions for gene segment reassortment and wipes off the interspecies barrier (Ito *et al*., 1998). Moreover, Matrosovich *et al*. (2004) have found that whereas human viruses infect preferentially nonciliated cells, avian viruses as well as an egg-adapted human virus variant with avian virus-like receptor specificity infect mainly ciliated cells. This pattern correlated with the predominant localization of receptors for human viruses (2-6-linked SA) on nonciliated cells and for avian viruses (2-3-linked SA) on ciliated cells. These findings suggest that avian influenza viruses can infect the human airway epithelium and explain how human population could be infected with an avian influenza virus without its prior adaptation, as it has repeatedly happened since 1997 in South-Eastern Asia.

2.2 Role of viral NA in enzymatic cleavage of SA residues and its contribution to virus virulence

Another glycoprotein that has the ability to modulate the virulence of influenza A viruses is NA. The basic function of NA is to cleave the linkage of SA to HA and enable the release of infectious progeny viruses so they can infect other cells. For a virulent virus, receptor-binding properties of HA should be functionally compatible with the cleavage specificity of NA (Hulse *et al*., 2004) and the stalk length of NA since the release of virus from the cell surface requires cleavage of the receptor by NA. Incompatibility between HA and NA can restrict the virulence of reassortant viruses, while some combinations of HA and NA are associated with infection of certain host species. NA can cleave SA in α-2, 3- and α-2, 6-linkages. Although the mechanism of substrate specificity is not well understood, it is known that the specificity of N8 NA is associated with the amino acid at position 275, close to the active site and with the glycosylation at Asn 144 (Saito and Kawano, 1997; Baigent and

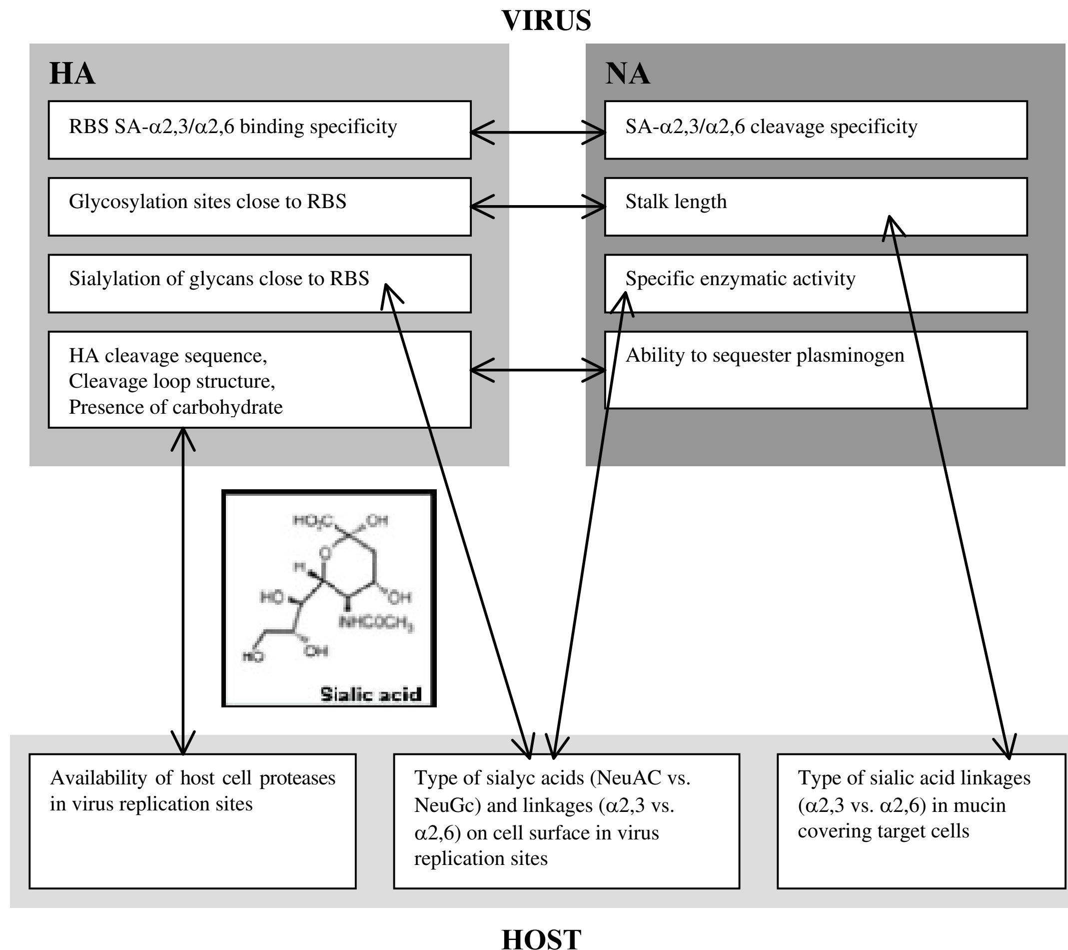

Fig. 1

Role of HA and NA in determining influenza virus virulence

According to Baigent and McCauley (2003), modified.

McCauley, 2003). Kobasa *et al*. (1999) have identified two amino acid residues at positions 275 and 431, close to the active site of N2 NA, which are involved in the ability of NA to recognize NeuAcα-2,6-Gal and NeuGc, respectively. The cleavage specificity of NA should be compatible with major linkage type in the host species.

The stalk that holds the active site above the virion envelope varies in sequence and length. Deletions of up to 28 amino acids and insertion of up to 41 amino acid in the stalk region did not abolish formation of infectious progeny viruses (Luo *et al*., 1993). Campitelli *et al*. (2004) have shown that a NA stalk deletion occurs in N1, N2 and N3 NAs in avian viruses isolated from terrestrial or raised aquatic birds (including chickens, turkeys, quail, pheasants, teal and chukar), but this deletion does not occur in wild avian strains. A similar study done by Banks *et al*. (2001) with a much broader spectrum of viruses including highly pathogenic avian influenza virus strains circulating in Hong Kong poultry in 1997 gave the same results. The mechanism of deletion of NA stalk region during transmission from wild to domestic birds can present an important step towards the comprehension of further interspecies transmission, especially from birds to humans, and emergence of potentially pandemic influenza viruses.

On the other hand, in studies carried out by Castrucci and Kawaoka (1993), a stalkless mutant of A/WSN/33 (H1N1) was highly attenuated in mice and, moreover, it replicated only in respiratory organs, while the parent virus caused a systemic infection in mice. Therefore it seems that the deletion of stalk region of NA leads to the attenuation of the virus for mammals but not birds. The attenuation of the virus through the deletion of the entire NA stalk raises the possibility of use of such a virus as live human vaccine.

NA participates in the acquisition of virulence through its capacity to bind to plasminogen (a serine protease present in the serum) and increase the concentration of activating proteases (Zambon, 1999; Reina, 2002). Goto and Kawaoka (1998) have demonstrated that human influenza viruses can become pantropic by utilizing a ubiquitous protease, plasmin, for HA cleavage. Plasmin is associated with an inhibitor, α2-antiplasmin. Plasminogen is converted to an active protease, plasmin, by cellular plasminogen activators. Plasminogen binds to NA by its C-terminal Lys and then is converted to plasmin, which cleaves HA into HA1 and HA2 subunits that become incorporated into progeny virions.

To summarize, the virulence requires both effective release of the virus from infected cells and efficient binding of the virus to new target cells. This is achieved by compensating changes in the receptor affinity of HA (regulated by amino acid changes in the RBS and/or glycosylation/sialylation near the RBS) and effective enzyme activity of NA (regulated by amino acid changes in the active site and NA stalk length) to ensure an optimal balance in the activities of these two molecules (Baigent and McCauley, 2003; Hulse *et al*., 2004).

2.3 Fusion activation and stability of HA

The next step in the life cycle of influenza virus is its entrance into the host cell and following fusion of the host endosomal membrane with the virus envelope. For this purpose, the precursor of each HA monomer has to be cleaved into two chains, HA1 and HA2, to expose the fusion peptide. Cleavage of HA is an important determinant of influenza virus pathogenicity, because it is essential for infectivity of the virus particle and spread of infection in the host organism (Ward, 1997). Mammalian and non-pathogenic avian influenza virus strains possess HAs that are usually cleaved in a few cell types only. As a consequence, these viruses cause local infection. Pathogenic avian strains have HAs that are cleaved in a broad range of host cells and consequently cause systemic infection. For HAs, in which cleavage is restricted, the linker usually consists of a single Arg (Lys, a monobasic cleavage site), while highly cleavable HAs generally have multiple basic residues (a multibasic cleavage site) in this position, forming the consensus sequence R-X-K/R-R (R is arginine, K is lysine, and X is a nonbasic amino acid) (Wood *et al*., 1993; Garten and Klenk, 1999; Zhirnov *et al*., 2002a). Comparison of complete HA1 coding sequences of 21 isolates demonstrated that an increased pathogenicity was directly associated with acquisition of an Arg-Lys insertion or nucleotide substitution at the HA cleavage site. The insertion of polybasic amino acids resulted in an altered 3-D structure around the cleavage site that possibly made it more accessible to proteases with different structural requirements at their active site (Zambon, 2001). A multibasic cleavage site exists in HA of influenza A virus subtypes H5 and H7 (Zhirnov *et al*., 2002a). Senne *et al.* (1996) have studied sequences of 76 influenza A virus isolates from 1993–1994. All highly pathogenic H5 and H7 isolates had multiple basic amino acids adjacent to the HA cleavage site and most had basic amino acids in excess of the proposed minimum motif B-X-B-R (B is a basic amino acid, Arg or Lys, X is a nonbasic amino acid, and R is Arg) associated with high pathogenicity. Of the nonhighly pathogenic viruses studied, 35 of 38 for H5 and 30 of 31 for H7 conformed to the motif B-X-X-R and B-X-R, respectively. Two nonhighly pathogenic H5 viruses had the motif X-X-X-R at the cleavage site and one had the motif B-X-X-K (K is Lys). Also Horimoto and Kawaoka (1995) have shown that naturally occurring virulent viruses have multiple basic amino acid motifs at the HA cleavage site, while most avirulent viruses do not.

Controversial with the facts mentioned above are results of genetic analysis of archival lung tissue from a young soldier who had died of influenza in 1918. They indicate that the highly pathogenic 1918 H1N1 virus did not possess a multibasic cleavage site in HA0. This finding has been confirmed with other archival materials analyzed from the same period (Zambon, 2001). Similar results were obtained by Suarez *et al*. (1998).

It has already been noted above that the HA0 precursor of the main functional HA glycoprotein requires cleavage by host proteases before infectious virus particles are formed. HA0 proteins of avian influenza viruses of low virulence for poultry are limited to cleavage by host proteases such as trypsin and trypsin-like enzymes and thus restricted to replication at sites in the host where such enzymes occur, i.e. the respiratory and intestinal tracts. Such enzymes include plasmin, kallikrein, urokinase, thrombin, blood clotting factor Xa, acrosin, tryptase Clara, mini-plasmin, proteases from human respiratory lavage, and bacterial proteases from *Staphylococcus aureus* and *Pseudomonas aeruginosa* (Garten and Klenk, 1999; Zhirnov *et al*., 2002a). Tryptase Clara shows recognition specificity for the sequence Q/F-X-R found at the HA cleavage sites of H1, H2 and H3 subtypes (Skehel and Wiley, 2000). Zhirnov *et al*. (2002) have shown that primary human adenoid epithelial cells (HAEC) infected with an influenza A virus containing a single arginine in the HA proteolytic site are capable of cleaving viral HA without adition of exogenous trypsin. It appears that such cleavage may occur in HAEC both for newly synthesized HA molecules (cleavage from within) and for the HA of incoming (input) virions (cleavage from without). A persistent and important question in influenza virus pathogenesis has been the origin of influenza virus activating protease in respiratory epithelium; whether it is

synthesized directly in virus-infected ciliated epithelial cells or produced by specialized cells, such as Clara cells. Zhirnov *et al.* (2002) have speculated that such a hypothetical enzyme may be similar in terms of intracellular transport and turnover to those in the subtilisin-like family that perform the intracellular cleavage of viral glycoproteins containing multibasic proteolytic sites in the *trans*-Golgi area. This influenza virus protease-activating enzyme would have to possess specificity for single-arginine proteolytic sites of HA.

In contrast, virulent viruses appear to be cleaved by a ubiquitous protease(s) which remains to be fully identified; it seems to be one or more protein-processing subtilisin-related endoproteases, of which furin is the leading candidate. This enables these viruses to replicate throughout the animal, damaging vital organs and tissues (Capua and Alexander, 2002; Hilleman, 2002). Subtilizin-like proteases generally recognize multibasic cleavage sites; they are unable to cleave monobasic cleavage sites (non-pathogenic influenza viruses) (Zambon, 2001). Subtilisin-like endoproteases cleave influenza virus HAs intracellularly in the constitutive exocytic pathway (Garten and Klenk, 1999; Zhirnov *et al*., 2002).

Another feature crucial for determining HA cleavability by proteases is the presence or absence of a carbohydrate side chain in near vicinity of the cleavage site. Non-pathogenic viruses have a carbohydrate side chain in the stalk region of the HA. The presence of the carbohydrate chain in the stalk region of the HA close to the protease cleavage site appears to affect the accessibility of the cleavage site by proteases. This steric hindrance seems to modulate the cleavability of the HA, so that only a restricted range of proteases can act on the sequence (Zambon, 2001; Zhirnov *et al*., 2002). HAs are glycosylated at 4–11 sites in the head and stem. Glycosylation and sialylation close to RBS of HA regulate release of avian viruses from cells, and thereby contribute to the virulence and tissue tropism (Baigent and McCauley, 2003). The loss of a glycosylation site on the HA represents natural mechanism of acquiring virulence. Also a study with the virus A/Phillippines/1/82 showed that a successive loss of potential glycosylation sites at residues 165 and 246 was associated with a significant stepwise increase in the pathogenicity of this virus for mice (Ward, 1997).

3. Ability of the virus to replicate in the host cell

Only reassortants with a set of genes that function efficiently together in a particular host species emerge as viable viruses. The ability of the virus to replicate in the host cell can be influenced by the following viral proteins: polymerase proteins, M proteins and nonstructural (NS) proteins.

3.1 Polypeptides of the polymerase complex

The polymerase complex of influenza A virus consists of the subunits PB1, PB2 and PA. In both animal model and tissue culture, the PB2 polypeptide, encoded by the RNA segment 1, confers a strong influence on host range. A reverse genetics approach identified a single amino acid substitution, Glu 627→Lys, in PB2 as responsible for difference in virulence (Hatta *et al*., 2001; Govorkova *et al*., 2005). Subbarao *et al.* (1993) have identified the residue 627 of PB2 as an important determinant of virulence of influenza A viruses for primates, but it appeared not to correlate directly with the virulence for mice (Gao, 1999). The replication of influenza A viruses in avian cells was the same, no matter the residue 627 was Glu or Lys. This substitution in the avian virus PB2 did not affect virus tropism for different mouse organs, but it enhanced its ability to support efficient virus replication in mouse model cells in general (Shinya *et al*., 2004).

The region between the residues 362 and 581 contained key amino acid substitutions to promote the replication in Vero cells and single amino acid changes at the positions 647 and 701 also contributed to the phenotype (Yao *et al*., 2001). The same working group has found out that the PB2 genes from the HK/97 H5 viruses which were able to replicate in humans and mice contained changes in PB2 at the residues 199, 627, 661, and 667, and that a substitution of lysine by glutamine at the residue 355 also correlated with high mouse pathogenicity. Also Katz *et al*. (2000) have shown that Lys or Arg 198, Ile or Met 317 in PB1, and Lys or Gln 355 in PB2 correlated with high and low pathogenicity, respectively. However, other polypeptides than PB2 in the viral replication complex also influence the host range and their compatibility is important (Subbarao, 1993; Zambon, 2001).

The analysis of reassortants allowed to suggest that changes in the HA and polymerase genes (most likely PB1) are necessary for the acquisition of virulence by the A/USSR/90/77 virus in the course of adaptation to mice, while the changes in the genes NA and NS are irrelevant (Kaverin *et al*., 1989).

3.2 M proteins

The RNA segment 7 encodes two polypeptides, M1 matrix protein that lines the viral lipid membrane and associates with vRNP, and M2, a proton channel. The sequence analysis revealed 10 sites with amino acids specific for either avian or human influenza strains; the amino acid sequence differences were greater in M2 (7 sites) than in M1 (3 sites). However, it remains unknown which of them contributes to host range restriction (Baigent and McCauley, 2003).

The amino acid changes resulting in increase of virulence are distributed over the M1 gene at the amino acid positions 15 to 227 and often fall in areas of unknown function. The studies of the M1 gene of the virus that caused the 'Spanish flu' epidemic suggest that variation of its sequence plays an important role in virulence, but that the virulence cannot be predicted directly from the M1 sequence (Reid *et al*., 2002).

Mutations in the M1 protein control the virulence possibly via its role in virus growth rate (Brown and Bailly, 1999). The M1 gene of mouse-adapted virus A/FM/1/47 has the ability to increase growth and virulence as a consequence of the mutation from ACC to GCC and consequently the substitution of threonine at position 139 by alanine. There was also a silent mutation in the RNA segment 7 from TTT to TTC, both encoding phenylalanine at the position 32 (Smeenk and Brown, 1994). An increased virulence for mice was determined by two specific amino acid substitutions in the M1 protein, Ala 41→Val and Thr 139→Ala. Such changes are likely to affect the pH-dependent association/dissociation of M1 with viral ribonucleoprotein, as well as with growth and virulence (Ward, 1996, Gao *et al*., 1999).

The possible use of mutation of M1 in development of a live influenza virus vaccine was suggested by Liu and Ye (2005). They have demonstrated that a double mutation (Arg 101→Ser, Arg 105→Ser) in M1 gene of A/WSN/33 resulted in temperature sensitivity and attenuation in mice. In their challenge studies, the mice immunized by infection with the double mutants mentioned above were fully protected from lethal challenge with A/WSN/33.

3.3 NS proteins

The RNA segment 8 of influenza A viruses encodes two proteins, NS1 and NS2. Whereas NS1 is encoded by a collinear mRNA transcript, NS2 is encoded by a spliced mRNA (Lamb and Krug, 1996). The changes in the NS gene in neurovirulent strains cause alterations in the mRNA secondary structure that mask the 3'-splice site, and correlate with reduced splicing of the NS gene in these strains (Ward, 1996). The NS1 gene of human influenza viruses functions as an antagonist of IFN-α/β (Seo *et al*., 2004). Moreover, Stasakova *et al*. (2005) have demonstrated that NS1 protein is involved in the inhibition of pro-inflammatory cytokines, such as TNF-α, IL-6, CCL3 (MIP-1α), IL-1β and IL-18, and is essential for the virulence of influenza A virus (Garcia-Sastre *et al*., 1998). The NS1 protein of influenza A virus down-regulates the IFN response in the human lung epithelial cell system and supports the hypothesis that this viral protein has an inhibitory effect on IFN and NF-κB pathways (Geiss *et al*., 2002; Donelan *et al*., 2003) and counteracts the PKR-mediated antiviral response (Bergmann *et al*., 2000). Donelan *et al*. (2003) have demonstrated a crucial role for amino acid residues located within the second α-helix of the NS1 protein of influenza A virus in preventing IFN-α/β secretion in virus-infected cells and in viral replication and pathogenicity for the host.

In the study of the NS gene segment of pandemic influenza virus, transfectant viruses were constructed containing either the 1918 NS1 alone or the entire 1918 NS segment. The resulting viruses, although growing to comparable titers in MDCK cells, paradoxically displayed a reduced virulence for mice as compared with control H1 viruses (WSN isolated in 1933 and PR8 isolated in 1934). The attenuation in mice of the 1918 NS segment demonstrates that NS1 influences the virulence for mice. The 1918 NS1 differed from that of WSN in 11 amino acids. Seven of them (amino acids 3, 22, 81, 114, 124, 224, and 227) were shared by PR8 and WSN. The amino acid differences between the 1918, WSN, and PR8 NS segments may be important for the adaptation of these strains to mice and likely account for the observed differences in virulence in the experiment where only NS1 was changed (Basler *et al*., 2001).

The NS2 (NEP) protein is a phosphoprotein that binds to the M1 protein which associates with viral ribonucleoproteins (vRNPs) and takes part in the nuclear export of the vRNP (Enami, 1997).This protein contains a highly conserved nuclear export signal motif in its N-terminal region (amino acids at the positions 12–21) which is thought to be required for nuclear export mediated by a cellular export factor (CRM). In the study with mutant viruses of A/WSN/3 it was demonstrated that the M14Y mutant, whose *in vitro* replication was appreciably slower than that of wild-type virus, showed highly attenuated growth properties in mice. These results suggest that this site would be a lucrative target for virus attenuation in the development of a live attenuated influenza vaccine (Iwatsuki-Horimoto *et al*., 2004).

4. Ability of the virus to destroy the host cell

Influenza viruses induce apoptosis in the cells that are permissive for viral replication and in the cells that do not support viral replication (Schultz-Cherry *et al*., 2003).

Apoptosis is a physiologic process that helps to preserve cellular homeostasis during normal tissue turnover. When cells die from apoptosis, the chromatin first aggregates next to the nuclear membrane. Then, apoptotic bodies of nuclear material are formed and often absorbed by intact adjacent cells. Another feature of apoptosis is the fragmentation of DNA into oligonucleosomes (Ito *et al*., 2002). Influenza virus induces apoptosis in a number of cell types including macrophages, lymphocytes, MDCK, Mv1L and HeLa cells (Morris *et al*., 1999; Schultz-Cherry *et al*., 2003; Stasakova *et al*., 2005), monocytes (Fesq *et al*., 1994), and bronchial and bronchiolar epithelial cells (Mori *et al*., 1995). Human

influenza A virus can also induce apoptosis in a mouse model (Ito *et al*., 2002).

Influenza virus infection induces apoptosis in cultured cells with an augmented expression of Fas (APO-1/CD95) (Takizawa *et al*., 1999; Ito *et al*., 2002; Schultz-Cherry *et al*., 2003). To induce apoptosis, some steps of virus replication are needed, since β-propiolactone- or UV-inactivated viruses do not induce apoptosis (Morris *et al*., 1999). Schultz-Cherry *et al*. (2001) have shown that apoptosis occurs in MDCK cells early in the course of virus replication. The proteins NA, NS1, PB1-F2 (Lowy, 2003; Takizawa, 2003) and M1 (Zhirnov *et al*., 2002) play a major role in influenza virus-induced apoptosis.

Viral NA is a candidate for the inducer of apoptosis in cells because anti-NA but not anti-HA antibodies inhibited activation of TGF-β (a known inducer of apoptosis). NA induces apoptosis by direct and indirect mechanisms. Indirectly, NA activates TGF-β *in vivo* and *in vitro*. TGF-β is a multifunctional growth factor that induces apoptosis in various cell types. Except this function, it influences various cellular, physiological and immunological processes. In the immune response, TGF-β acts as both an immunosuppressive agent and a potent proinflammatory molecule through its ability to attract and regulate inflammatory molecules, induce cytokine secretion and stimulate the T-helper-cell type I phenotype. TGF-β is secreted by virtually all cells as a biologically inactive protein, a latent TGF-β. The latter consists of N-terminal latency-associated peptide (LAP) that remains noncovalently associated with the C-terminal mature TGF-β molecule. The release of mature TGF-β from LAP is thought to be necessary for binding to cellular receptors and induction of biological response. The *in vivo* studies have shown that viral NA directly activates latent TGF-β, probably via the enzymatic cleavage of the sialic acid residues located on the LAP. This effect is not restricted to influenza viruses, as paramyxoviruses also contain NA and activate latent TFG-β. Increased TGF-β levels due to virus infection induce apoptosis in cells, suggesting a potential role of TGF-β in pathogenesis (Schultz-cherry and Hinshaw, 1996). Morris *et al*. (1999) have shown that some apoptosis still occurred despite neutralization of NA activity. The neuraminidase activation of TGF-β cannot be the sole mechanism for apoptosis induced by influenza virus.

Zhirnov *et al*. (2002) have observed that NS1 has the ability to bind double-stranded RNA and thus to prevent the intracellular dsRNA-activated PKR. PKR is a common link in the signaling pathways leading to IFN induction and apoptosis. NS1 interferes with the IFN-PKR system and directly inhibits PKR. The above observations indicate that NS1 has an IFN-dependent antiapoptotic potential and down-regulates the apoptotic response in influenza virus-infected cells. NS1 inhibits the activation of PKR by up-regulation of a cellular PKR inhibitor, binding directly to PKR, and inhibiting IFN regulatory factor 3 (Schultz-Cherry *et al*., 2001; Stasakova *et al*., 2005).

Another mechanism of induction of apoptosis is caspase activation. Caspases are activated by various stimuli including viral infection, calcium release and alteration of mitochondrial potential (Lin *et al*., 2002). Multiple apoptotic signals may first activate initiator caspases (caspase-8 and -9), followed by activation of downstream caspases (caspase-1, -3 and -6) (Julkunen *et al*., 2001).

The caspases are a family of cysteine-dependent aspartate-directed proteases; they are prominent among death proteases. Caspases are synthesized as inactive zymogens that become activated by scaffold-mediated transactivation or cleavage via upstream proteases in an intracellular cascade. Regulation of caspase activation and activity occurs at several levels: (i) zymogen gene transcription is regulated, (ii) antiapoptotic members of the Bcl-2 family and other cellular polypeptides block a proximity-induced activation of certain procaspases, and (iii) certain cellular inhibitor of apoptotic proteins can bind to and inhibit active caspases.

Once activated, caspases cleave a variety of intracellular polypeptides including major structural elements of the cytoplasm and nucleus, components of the DNA repair machinery, and a number of protein kinases (Earnshaw *et al*., 1999; Lin *et al*., 2002).

A characteristic feature of M1 protein, binding to caspase-8 is probably controlled by a N-terminal domain, which shows similarity with a baculovirus p35 protein displaying caspase-inhibitory properties. It should be noted that during the apoptosis initiation process caspase-8 undergoes an auto-aggregation and activation via its interaction with an effector DED-domain of cellular adaptor FADD protein molecule, which is associated with apoptosis Fas-receptor. Comparison of primary structures of FADD DED-domain and influenza A virus M1 protein revealed a 35% homology between the and the FADD DED-domain (aa 44–67) and the M1 N-terminal region (aa 25–40). This FADD region plays an important role in proapoptotic aggregation and activation (signal transduction) of caspase-8. It can be assumed that M1 can interfere with the interaction between caspase-8 and FADD as a result of direct interaction with caspase-8 and owing to its similarity with the FADD DED-domain, followed by FADD-dependent auto-aggregation and activation of caspase-8. It is still unclear whether the supposed interaction and M1 interference with caspase-8 would have pro- or anti-caspase nature (Zhirnov *et al*., 2002).

PB1-F2 induces apoptosis in influenza virus-infected cells (Chen *et al*., 2004). PB1-F2 is proposed to kill host immune cells responding to influenza virus infection, either internally via virus infection or externally in certain situations when the protein is released from infected cells under certain circumstances. Addition of synthetic PB1-F2 to planar

phospholipid bilayer membranes causes an increase in their conductance and decreases their lifetime. This novel influenza virus proapoptotic protein forms potential-dependent lipidic pores in planar phospholipid bilayers that initiate the breakdown of bilayer structure (Chanturiya *et al*., 2004).

The ability of the virus to either promote or inhibit apoptosis contributes to its virulence and the severity of apoptosis is likely to reflect a balance between the activities of viral and cellular factors.

5. Interaction of the virus with the host immune defense system

Virus virulence depends partly upon the ability of the virus to stimulate an immune response in the host, since the latter contributes to the severity of disease symptoms and to the ability of the virus to evade or suppress the immune response of the host. Both humoral and cell-mediated immunity play a role in control of influenza A virus infection (Baigent and McCauley, 2003).

Major protection against influenza virus infection is mediated by neutralization antibodies directed against viral envelope glycoprotein, HA (Thomas *et al*., 1998; Knossow *et al*., 2002). HA antibodies restrict the virus by neutralization of infectivity and the NA antibodies restrict the virus spread by interfering with release of progeny virus from the host cell (Hilleman, 2002).

In general, an antibody can control an infection *in vivo* in two ways: (i) by impairing the ability of infectious virus to spread the infection to new host cells (virus neutralization), and (ii) by impairing maturation and release of progeny virus from infected host cells (yield reduction).

In vitro, the virus neutralization activity is mediated by HA-specific antibodies that prevent attachment of the virus to the host cell and, according to some authors, also inhibition of intraendosomal fusion (Varečková *et al*., 2003). The yield reduction activity can be assessed by using antibodies that are capable of reacting with live infected cells but lack virus neutralization activity *in vivo* at physiological conditions. Examples are M2- and NA-specific antibodies (Gerhard, 2001).

If an individual in last few years underwent an infection with a related influenza virus strain the produced HA antibodies can protect him from an infection or at least moderate its course. Secretoric IgA antibodies neutralize virus in upper respiratory tract, while serum IgG antibodies protect from pneumonia. Together with the production of antibodies the cell-mediated response (T-lymphocytes, CD 4^+ and CD 8^+) is activated (Čiampor *et al*., 1998).

The cell-mediated immunity is based on CD 8^+ T cell responses, which usually appear within 3–4 days after infection. CD 8^+ T cells detect and lyse virus-infected host cells and their specificity may be directed against peptides derived from any viral protein, but NP as the most important. CD 4^+ T cells are of significant importance in facilitating both humoral and cellular immune responses and exerting cytolytic effects, though to a lesser extent than CD 8^+ T cells (Hilleman, 2002).

The MHC class-I-restricted CD 8^+ T cell response is still considered of paramount importance for clearing an established influenza virus infection in an immunologically intact host. Support for this has come from studies showing that (i) a vigorous virus-specific cytotoxic T cell response can be detected in infected lungs, (ii) the rise of this response coincides with virus clearance, and (iii) adoptive transfer of activated CD 8^+ T cells reduces virus titers in an infected recipient (Gerhard, 2001). The cell-mediated immunity is often subtype- or even intersubtype-cross-reactive.

6. Host-adaptation, evolution and interspecies transmission of the virus

Experimentally, most influenza viruses isolated from one species do not replicate in another, but some can cross the species barrier (Baigent and McCauley, 2003). Efficient interspecies transmission of influenza viruses is a result of combination of viral, cellular and environmental factors and may represent an important factor of virulence.

Perez *et al*. (2003) have identified 7 amino acids at certain positions on H9 HA corresponding to the adaptation of H9 viruses to land-based birds. Quail, chicken and duck viruses had these 7 amino acids at the same positions on HA. One of these amino acids (at the position 146) near RBS is likely to influence the binding of HA to the SA receptor. Two basic amino acids were found at the positions-4 (Arg or Lys) and -2 (Ser) of the HA1-HA2 cleavage site, a situation characteristic of chicken and quail viruses. These results suggest that H9 viruses that contain Asp-2 at the HA1-HA2 cleavage site replicate poorly in chickens and quail. The remaining 4 amino acids apparently occupy positions that have not been recognized as important host range markers, although three of them (109 in HA1 and 135 and 160 in HA2) could influence the pH of fusion of HA with the host endosomal membrane.

The H5N1 viruses isolated from humans in Hong Kong represent the first known direct transmission of avian influenza virus that has caused severe respiratory disease and death in humans (Zhou *et al*., 1999; Perez *et al*., 2003; Ungchusak *et al*., 2005). Shinya *et al*. (2004) have detected a Glu 627→Lys mutation in PB2 in the same group of viruses that supported viral replication in mouse cells. It needs further examination, but there is the possibility that Lys 627 in PB2 of H5N1 influenza viruses is among other factors a prerequisite for direct transmission of influenza viruses from birds to humans.

Avian influenza viruses have been directly transmitted, without recombination and apparent mutation, to other mammals including horses (H3N8), seals (H7N7), and pigs (H1N1), causing epidemics in these species (Baigent and McCauley, 2003).

Since 1997, direct transmissions of avian influenza viruses to humans without previous adaptation have been recorded. These infections often caused diseases with severe course and high lethality. Transmission of avian influenza viruses to humans could cause a pandemic spread of a totally novel influenza virus subtype in human population in a near future. We observe that a crossing of interspecies barrier has, in general, severe medical-epidemiological consequences: crossing of interspecies barrier was identified in coronaviruses (severe acute respiratory syndrome, SARS), hantaviruses (hemorrhagic fever), retroviruses (HIV-1, HIV-2) and filoviruses (Ebola). This and other data (not shown) make crossing of interspecies barrier one of the most important conditions of severity of various virus diseases.

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