

## Epidemiology and pathogenesis of influenza

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Influenza A, B and C all have a segmented genome, although only certain influenza A subtypes and influenza B cause severe disease in humans. The two major proteins of influenza are the surface glycoproteins—haemagglutinin (HA) and neuraminidase (NA). HA is the major antigen for neutralizing antibodies and is involved in the binding of virus particles to receptors on host cells. Pandemics are a result of novel virus subtypes of influenza A, created by reassortment of the segmented genome (antigenic shift), whereas annual epidemics are a result of evolution of the surface antigens of influenza A and B virus (antigenic drift). The rapid evolution of influenza viruses highlights the importance of surveillance in identifying novel circulating strains. Infectivity of influenza depends on the cleavage of HA by specific host proteases, whereas NA is involved in the release of progeny virions from the cell surface and prevents clumping of newly formed virus. In birds, the natural hosts of influenza, the virus causes gastrointestinal infection and is transmitted via the faeco-oral route. Virulent avian influenza strains, which cause systemic disease, have an HA that is cleaved by proteases present in all cells of the body, rather than by proteases restricted to the intestinal tract. In mammals, replication of influenza subtypes appears restricted to respiratory epithelial cells. Most symptoms and complications, therefore, involve the respiratory tract. However, systemic complications are sometimes observed and other viral genes besides the HA, including the NA, may be involved in determination of virulence of influenza strains in mammals.

### Introduction

Influenza viruses are segmented, negative-sense, enveloped RNA viruses. There are three types of influenza—A, B and C—which are distinguished by serological responses to their internal proteins. Influenza A viruses can be further subtyped according to the reactivity of their surface antigens, haemagglutinin (HA) and neuraminidase (NA); 15 HA subtypes and nine NA subtypes have been found.

The natural hosts for influenza A viruses are aquatic birds, although various mammals, including humans, horses and pigs, are also hosts. Influenza B is restricted to humans. The natural host for influenza C is also humans, although it has been isolated from pigs.<sup>1</sup> Influenza C causes a less severe illness than either influenza A or B, more akin to the common cold.<sup>2</sup> Influenza A causes the most serious illness in humans, mainly because of its antigenic variability, which allows the virus to escape neutralizing antibodies—the mainstay of human protective immunity against influenza infection. Although antigenic variability

is also seen in influenza B viruses, this is less than is seen with influenza A viruses. Host restriction may play a part in abrogating the pathogenicity of influenza B and C in humans, although the viral genetic factors or host-pathogen interactions contributing to this are undetermined.

Influenza infection occurs in a seasonal pattern and leads to an extensive burden of disease. In healthy young adults, influenza can cause a debilitating febrile illness lasting 1–2 weeks. In patients with pre-existing respiratory or cardiovascular disease, and in the frail elderly, influenza infection can be more serious. One characteristic of seasonal influenza activity is that it leads to excess, or 'hidden', deaths. These are deaths that are not usually attributed to influenza infection, even though many of them are caused by either viral or bacterial pneumonia, secondary to influenza infection. It is estimated that between 5000 and 29,000 excess deaths annually were caused by influenza in the UK between 1975 and 1990—five times the number directly attributed to influenza on death certificates.<sup>3</sup>

## Drift and shift

The antigenic variability of influenza A takes two forms—antigenic drift and antigenic shift. There is a rapid rate of mutation in the influenza genome as a result of the relatively low fidelity of the RNA-dependent RNA polymerase (replicase) of influenza, which also lacks a proofreading capacity (5′–3′ exonuclease activity). Many attenuated and non-viable influenza genotypes are produced by this constant mutation, but selection pressure, such as is exerted by antibody or a new host species, ensures that any new variant that contains some growth advantage over the parent will survive and the population will expand.

### *Drift*

Antigenic drift occurs when the genes encoding the viral surface antigens, the NA and the HA, undergo stepwise mutation, potentially each time the virus replicates. Eventually, these proteins on the virus particle become sufficiently different that host antibodies are unable to neutralize the virus, resulting in a variant capable of causing illness through evasion of the immune response.

### *Shift*

The second, far less frequent, type of antigenic change is called antigenic shift. This occurs when two different viruses, possibly each from a different host species, co-infect a single host, e.g. a pig, which then acts as a ‘mixing vessel’. By reassortment of genome segments, a new virus is created that has elements from both of the original viruses. This process results in unpredictable pathogenicity of the new virus, which may lack the requisite virulence factors (and therefore be attenuated or frankly non-viable) or may possess full virulence for humans, plus a new surface antigen, usually the HA (from an animal host virus). Such a virus has pandemic potential because it may be intrinsically pathogenic in humans and have surface antigens against which the human population lacks any significant immunity. Once a pandemic strain is created, it may change its virulence further as it continues to replicate, adapting to the host as it does so.

As recently as 1997, the pandemic threat was almost realized in Hong Kong, when an avian subtype (H5N1) of influenza A was found to be associated with serious disease in humans.<sup>4</sup> The human H5N1 viruses were found to be closely related to the virulent avian viruses that had been associated with mortality in chickens throughout the New Territories of Hong Kong.<sup>5</sup> Zoonotic transmission of H5N1 from chickens to humans created the conditions for the potential emergence of a reassortant virus in humans, if human influenza had been circulating at the same time.

All of the 15 HA and nine NA subtypes are found in avian species, whereas only three of the HA subtypes (H1, H2 and H3) and two of the NA subtypes (N1 and N2) have

been associated with illness in humans over a long period of time. The Hong Kong H5N1 episode has demonstrated, however, that avian viruses pose a major threat for humans. Birds are a large, mobile, global reservoir of influenza genotypes from which new subtypes of virus can be created that may have pandemic potential; the threat of the next influenza A pandemic remains a question of ‘when’ rather than ‘if’. Influenza B, by contrast, lacks an animal reservoir and therefore does not produce pandemics.

## Human influenza

In 1947, in response to recognition of the antigenic variability of influenza, the World Health Organization (WHO) established an influenza surveillance programme that operates through a network of national and regional centres to monitor global influenza activity. The monitoring ensures that influenza vaccines are updated and contain strains that are closely matched to circulating strains and it serves as an early warning of new pandemic strains. The principal co-ordinating centres for the WHO network are in London, Melbourne, Atlanta and Tokyo, with a further 110 national centres, in 83 countries around the world.

Accumulated surveillance data show that, since 1977 (the last influenza pandemic, caused by an H1N1 subtype), two subtypes of influenza A (H1N1 and H3N2) and influenza B have been co-circulating, with one strain usually predominating. In the 1977 epidemic, the highest attack rates were seen in young people (under 20 years of age). Older people appeared to have a degree of immunity, which was thought to stem from exposure to previously circulating H1 haemagglutinin earlier in the century. This return to circulation of a previously ‘known’ subtype is referred to as antigenic recycling.<sup>6</sup>

### *Interpandemic activity*

In the years between influenza pandemics, the process of antigenic drift continues to produce epidemics of influenza throughout the world. Influenza epidemics nearly always occur during the winter in temperate climates, although the significance of this is not fully understood. The cold, damp conditions in winter may favour virus survival outside the host airway and there may be behavioural influences, such as people spending longer periods together indoors. School children are also thought to play a central role in the spread of influenza, their activities sometimes being described as ‘seeding’ the local community. In equatorial climates, influenza may not have such sharply defined seasonality.<sup>7</sup>

### *Transmission and replication*

Influenza virus is shed into respiratory secretions, which are then coughed or sneezed into the air and transmitted to

the next host. Infection occurs in the cells of the tracheo-bronchial epithelium, where the first cycle of replication takes *c.* 4–6 h. Very high titres of virus are shed during the initial infection period. This, combined with the short incubation period, produces the characteristically explosive nature of influenza outbreaks. Estimates of attack rates in outbreaks vary from 10% to nearly 100%, depending on the type of community, age of individuals, vaccination rates and methods of diagnosis.<sup>7</sup> For example, if seroconversion is used as a method of diagnosis, a significant number of patients are found to be asymptomatic, whereas if clinical illness is used as a method of measuring attack rates, there is the possibility of overestimation of transmission owing to the difficulty of differentiating influenza from other respiratory infections, such as respiratory syncytial virus, that may be circulating at the same time. In addition, diagnosis of influenza using classic culture methods to detect virus may also be difficult to implement. Together, therefore, the difficulties in ascertaining ‘true’ influenza infections adds to the uncertainty of estimating total morbidity associated with influenza. Differences exist between influenza A and B: influenza B has lower attack rates than influenza A in the wider community but occasionally higher attack rates in closed communities.<sup>7</sup>

### Influenza infection

Influenza infection ranges in severity from asymptomatic infection to serious illness with systemic features. The potential sequelae of influenza infection include viral and bacterial pneumonia and contribute to the overall burden of the disease. Acute infection is characterized by abrupt onset of symptoms that include fever (in the region of 38–40°C) or feverishness, chills, cough, headache, myalgia, sore throat, malaise, anorexia and many other non-specific symptoms. None of the acute features are pathognomonic but the constellation of respiratory and systemic symptoms, at a time when influenza activity has already been confirmed in the locality, is likely to lead to accurate diagnosis in 60–70% of medical consultations.<sup>8</sup>

The total extent of sites of influenza replication is still a subject of debate. Although the disease is associated with many systemic symptoms, it appears unlikely that the virus replicates to any great extent outside the respiratory tract in uncomplicated infection. Induction of inflammatory cytokines has been cited as a potential explanation of the systemic features of influenza infection. Infection of peripheral blood leucocytes with a variety of virulent (H3N2) and avirulent (H1N1) influenza viruses resulted in the production of a series of inflammatory cytokines including IL-1 $\beta$ , IL-6, IFN- $\alpha$  and MIP-1 $\alpha$  and indicated that the two strain types induced cytokine release at essentially equivalent rates. Moreover, the serum cytokine levels in infected ferrets did not account for the magnitude of fever recorded during acute infection.<sup>9</sup> However, in a

study of human volunteers experimentally infected with influenza, IL-6 and IFN- $\alpha$  levels in nasal wash correlated with the systemic symptoms.<sup>10</sup> The discrepancy between these data may reflect compartmentalization of cytokines within the body.

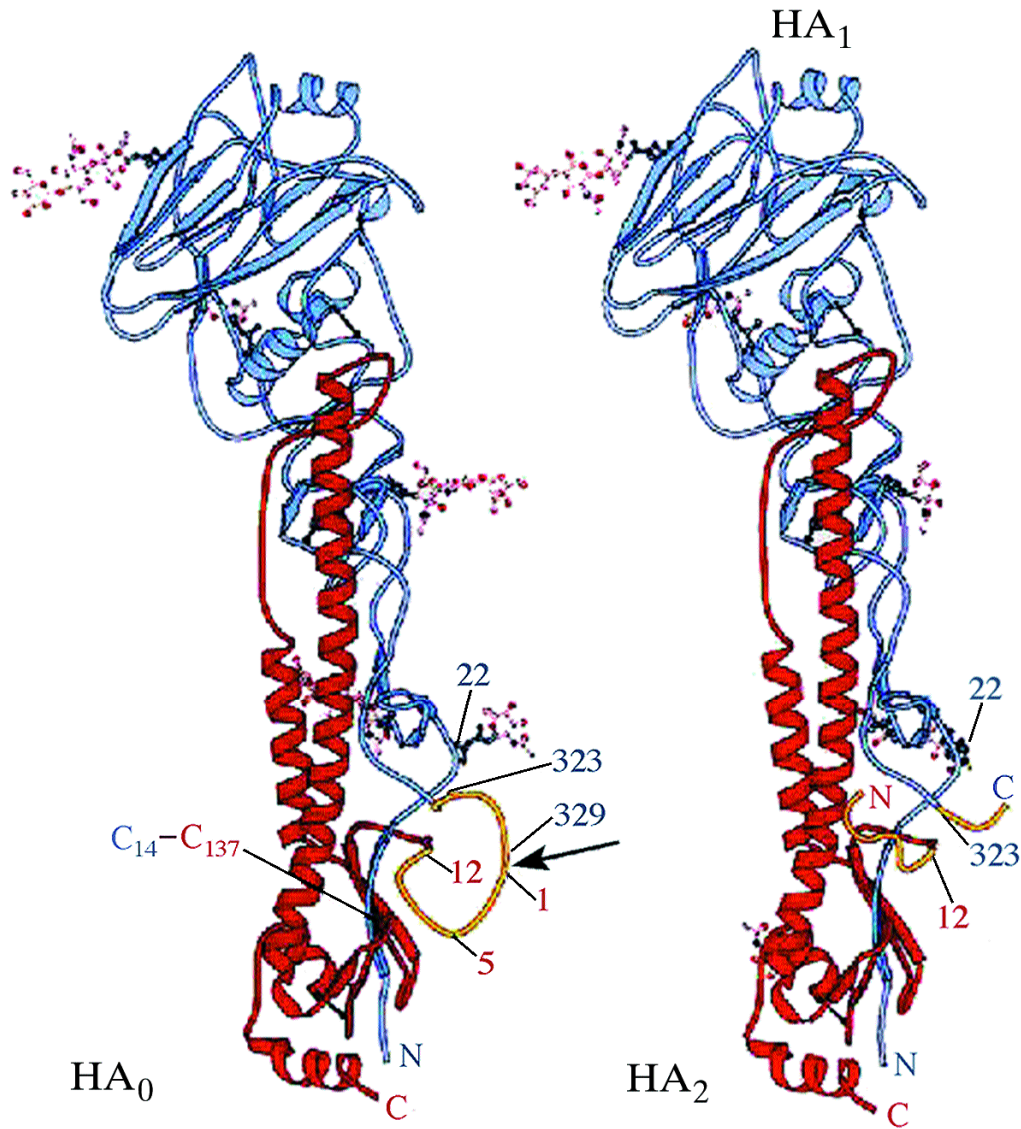
### Pathogenicity of influenza

The precise details of the pathogenicity of influenza virus infection in humans are still incompletely understood. Although many of the molecular determinants of virulence are now recognized, questions remain regarding exactly what makes one virus more virulent than another. The HA is the major determinant of virulence and host range in avians but virus replicative components may also affect species adaptation and pathogenicity in mammals.

### Haemagglutinin

The HA plays a critical role in the pathogenesis of influenza. It is one of the two major antigenic determinants that are recognized by the host’s neutralizing antibodies and its susceptibility to cleavage by host protease determines the range of avian tissues in which the virus can replicate. The HA is a 550 amino acid polypeptide that forms homotrimers (spikes) on the exterior of the influenza virus particle. The nascent HA is directed to the cell membrane in an infected host cell and is anchored to the cell membrane by a short transmembrane region at the C terminus. In this form, the HA is referred to as HA<sub>0</sub> and has not yet undergone the final modification that allows it to acquire full biological activity. This final step involves proteolytic cleavage of a specific region by host enzymes, resulting in two subunits, HA<sub>1</sub> and HA<sub>2</sub>, which are linked by a disulphide bridge. The cleavage of HA<sub>0</sub> occurs close to the point of insertion of HA into the viral membrane (Figure 1).<sup>11</sup> Similar cleavage activation is also seen in other viral polypeptides, such as those of HIV and parainfluenza virus. The nascent HA is also subject to extensive post-translational glycosylation, which may also act as a mechanism for immune evasion.<sup>11</sup>

Once the HA is suitably modified and incorporated into the newly assembled virus particle, its function is to attach the particle to a host cell and to facilitate entry and fusion. Attachment is to terminal sialic acid residues on host cell glycoproteins and this step is followed by cell entry. Once in the host cell, the virus particle enters endosomes that are subject to acidification by cellular proton pumps. At a pH between 5.0 and 6.0, the HA undergoes an irreversible conformational change that exposes the highly conserved N terminus of the HA<sub>2</sub> domain—referred to as the fusion peptide. Exposure of this peptide is critical to allow fusion of viral membrane with cell membranes and activation of the replication complex.



**Figure 1.** Schematic version of the influenza HA monomer before ( $HA_0$ ) and after proteolytic cleavage. The viral membrane is located at the base of the figure and the globular surface of the molecule towards the top. The  $HA_1$  portion of the molecule is shown in blue and the  $HA_2$  portion in red. The proteolytic cleavage site on  $HA_0$  is indicated by an arrow. Figure adapted from ref. 11 with permission. Copyright held by Cell Press.

### *Proteolytic cleavage*

Replication of influenza A in birds usually takes place in the enteric tract; transmission takes place by the faeco-oral route and infection is normally asymptomatic. Differentiation between virulent and avirulent avian influenza A viruses correlates with the sequence of a few amino acids adjacent to the point where the  $HA_0$  is cleaved—the so-called cleavage sequence. These residues form the C terminus of the  $HA_1$  subunit after cleavage. The presence of several basic amino acids at the cleavage sequence of influenza viruses is associated with a high pathogenicity index in birds (Table). Such a sequence allows cleavage of the molecule by cellular proteases widely distributed throughout the body, including the furin and PC6 serine

proteases. The ubiquitous distribution of furin and PC6 in birds allows cleavage activation of the HA in virtually all cell types in the avian host, leading to multiple sites of replication (pantropicity). Viruses lacking a polybasic cleavage site are much more restricted in the range of proteases to which they are susceptible. This in turn restricts their potential replication sites and produces a less serious or asymptomatic infection, confined to the enteric tract.<sup>12</sup>

In humans, influenza A replicates in respiratory epithelial lining cells. Circulating H3N2 strains have a cleavage sequence that lacks the multiple basic amino acids at the C terminus of  $HA_1$ . Theoretically, a sequence of basic residues could eventually be introduced to a human-adapted virus, conferring altered tropism and patho-

**Table.** Protease cleavage site sequence in various influenza strains compiled from references 12 and 13

Virus	Cleavage site sequence	
	HA <sub>1</sub>	HA <sub>2</sub>
Type A		
H1	PSIQYR	GL
H2	PQIESR	GL
H3 <sup>a</sup>	PEKQTR	GL
H4	PEKATR	GL
H5 L <sup>b</sup>	TRRQKR	GL
H5 H <sup>c</sup>	PGAKAKTR	GL
H7 L	ENPKTR	GL
H8 H	KKRKKR	GL
H10	EVVQGR	GL
AH13	PAISNR	GL
H14	PGKQAK	GL
Type B	KLLKER	GL
Type C	TKPKSR	IF

<sup>a</sup> Circulating H3N2 strains have a cleavage site that lacks the multiple basic amino acids.

<sup>b</sup> L, low pathogenicity.

<sup>c</sup> H, high pathogenicity.

genicity, as occurred in H5 viruses in chickens in Mexico in the 1990s. However, this has not been demonstrated yet in human-adapted influenza strains.<sup>13</sup>

Viruses containing polybasic cleavage sites can be highly pathogenic in humans. The H5N1 viruses involved in 18 cases of human illness in Hong Kong in 1997 all contained polybasic amino acids at the cleavage sites.<sup>14</sup> Six out of 18 individuals died, including a child with multisystem failure, although the virus was unable to sustain human to human transmission. The implications of the polybasic HA cleavage site compared with other components of the virus in determining pathogenicity in humans remains to be investigated; there are already some clues in the mouse model that other genes contribute to this.<sup>14</sup>

### *Neuraminidase and plasminogen sequestration*

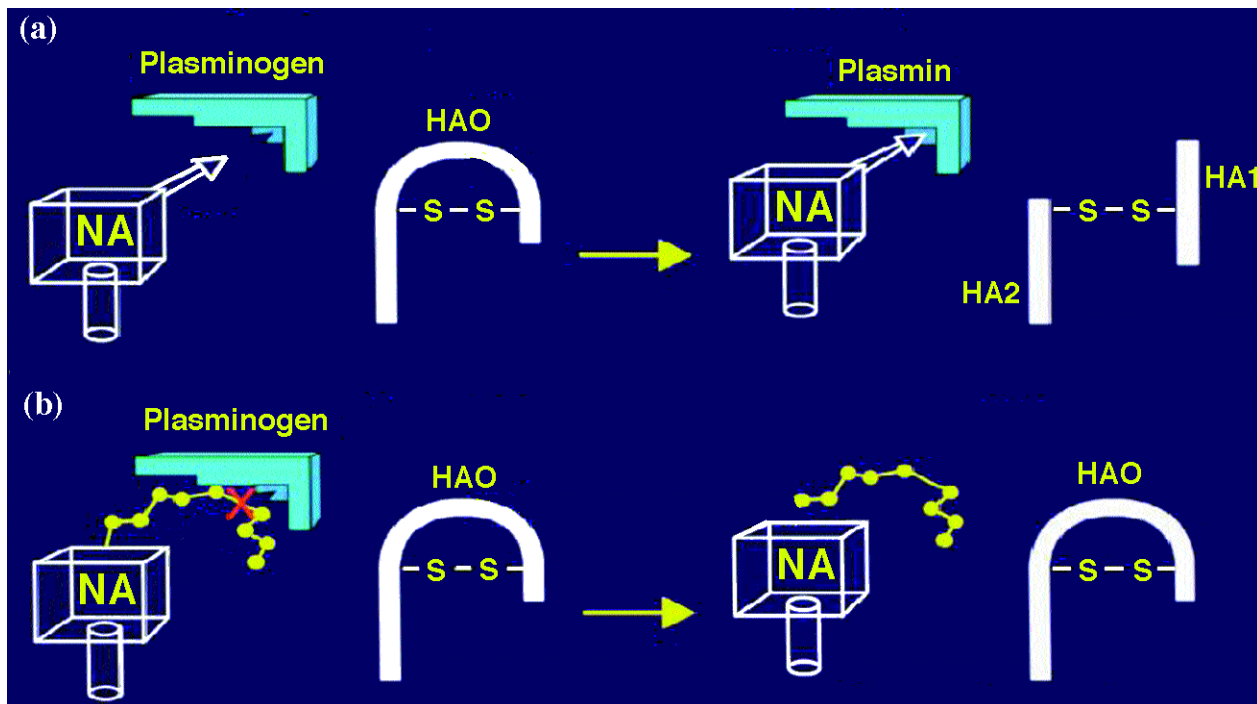
The 1918 'Spanish' influenza pandemic was caused by an extremely pathogenic H1N1 virus that killed at least 20 million people, many of whom were fit young adults. The hypotheses to explain this include the presence of an atypical (for human viruses) cleavage sequence or co-infection or secondary infection with bacteria that provide protease exogenously, thereby increasing the number of sites and extent of influenza replication, as has been suggested by animal models of co-infection. Genetic analysis of archival lung tissue from a young soldier who died of influenza in 1918 indicates that the virus did not possess a

multibasic cleavage site similar to that associated with highly pathogenic avian viruses.<sup>15</sup> This demonstrates that other mechanisms must be sought to explain the observed pathogenicity of this virus. Recently, experimental work implicating NA has provided a framework for explaining these observations. Viral NA plays an essential role in the release of progeny virus particles from the host cell surface, probably by removing the sialic acid residues through which HA binds nascent virions to the cell surface. The NA also prevents viral aggregation and facilitates dispersion of virus through the mucus that coats the respiratory tract epithelium.

The human influenza A virus A/WSN/33 (H1N1) is thought to have descended from the 1918 'Spanish' influenza strain.<sup>16</sup> The virus strain A/WSN/33, derived from the A/WS/33 strain through multiple passage in mouse brain, has lost the requirement for the addition of exogenous serine protease in tissue culture and has acquired neurotropism and high pathogenicity on passage in animals, even though it does not contain a polybasic cleavage site. The novel mechanism put forward to explain the virulence of this virus involves binding of host plasminogen, a serine protease present in serum, by viral NA (Figure 2).<sup>16</sup> The A/WSN/33 strain NA has a C-terminal lysine residue that is capable of binding plasminogen. Mutation of this amino acid abolishes binding of plasminogen and also HA cleavage. It is postulated that elevated levels of the protease bound in proximity to the virus particle allowed cleavage activation of HA directly (Figure 2a). In addition, the loss of a glycosylation moiety at position 146 of NA is also critical to experimental plasminogen activation of HA because it removes steric hindrance to protease binding. Restoration of the glycosylation at position 146 inhibits plasminogen cleavage of HA (Figure 2b). However, the acquisition of virulence by A/WSN/33 on descent from A/WS/33 may be multifactorial because there are at least 18 mutations in the virus compared with its parent strain. Nevertheless, the results suggest that any human N1 virus with a C-terminal lysine without glycosylation at residue 146 of the NA should be regarded as potentially dangerous. Furthermore, this experimental hypothesis offers another possible explanation for the virulence of the 1918 H1N1 virulent virus although it remains to be tested on archival material.

### *Other factors*

Although there are some clear indications that the surface proteins of influenza have pivotal roles in determining pathogenicity, other host and viral factors may well contribute. Studies of influenza in birds indicate that pathogenicity most closely correlates with the HA but the situation in mammals may involve other viral genes that determine host range and tissue tropism. The interactions of influenza A proteins with host cell proteins is far from clear and these may contribute to spread and severity of



**Figure 2.** Schematic representation of plasminogen-activated HA cleavage. (a) Plasminogen binds to a C-terminal lysine (arrow) of NA. Bound plasminogen is activated to plasmin, probably by a cellular enzyme. Plasmin cleaves HA<sub>0</sub> to the HA<sub>1</sub> and HA<sub>2</sub> subunits. (b) If the C-terminal lysine is absent or blocked by a nearby oligosaccharide chain (●—●) plasminogen binding is inhibited and HA<sub>0</sub> remains uncleaved. (adapted from ref 16). Copyright (1999) National Academy of Sciences USA.

illness. Furthermore, influenza A normally replicates in an enteric environment in birds and in a respiratory environment in mammals and the role of accessory and non-structural proteins, such as M2 and NS1, may alter in different cellular environments.

## Conclusions

Influenza is a serious respiratory infection that places a heavy burden of disease on the global population. Many of the effects of influenza are hidden and the lack of effective treatments for the disease has tended to compound this situation. Furthermore, the full pathogenic profile in mammals of this highly labile and species-mobile pathogen remains incompletely understood. Nevertheless, the ultimate arrival of a new pandemic of influenza is a certainty. Experience during the 20th century tells us that we really do not know what to expect from the next influenza pandemic: will the next influenza 'lottery' result in a relatively benign phenotype or can we expect to see a truly virulent pantropic strain with the potential to kill millions?

Fortunately, there have been recent advances in the field of influenza vaccines and antiviral chemotherapy, including a new class of selective anti-influenza agents—the neuraminidase inhibitors. These new agents are highly active against influenza B and all nine influenza A NA subtypes,

which means that they should be effective against any new pandemic strain. In large-scale clinical studies, they have been shown to reduce the severity and duration of symptoms of influenza infection by inhibiting the function of the influenza NA, a critical component of the influenza virus. The first available neuraminidase inhibitor—zanamivir—is delivered by oral inhalation direct to the respiratory tract and offers new opportunities in the management of influenza. It may thus prove useful in the next pandemic.

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