Chapter 13

Influenza

Influenza is a highly contagious, acute respiratory illness afflicting humans. Although influenza epidemics occur frequently, their severity varies *(1)*. Not until 1933, when the first human influenza virus was isolated, was it possible to define with certainty which pandemics were caused by influenza viruses. In general, influenza A viruses are more pathogenic than are influenza B viruses. Influenza A virus is a zoonotic infection, and more than 100 types of influenza A viruses infect most species of birds, pigs, horses, dogs, and seals. It is believed that the 1918–1919 pandemic originated from a virulent strain of H1N1 from pigs and birds.

The natural reservoir of influenza viruses was identified as wild aquatic birds, from whose populations viruses with new surface proteins could emerge through reassortment. However, it is still not possible to predict how and when new influenza strains will emerge or how virulent a new strain will prove *(2)*.

Currently, influenza A viruses of subtypes H1N1 and H3N2 and influenza B viruses of two antigenically distinct hemagglutinin lineages are present in human populations. The occurrence of sporadic avian influenza subtypes (e.g., H9, H7, and particularly H5) in human populations have led to widespread concerns about the possibility of an influenza pandemic.

Since 1889, at least five major pandemics have been recorded, when new hemagglutinin and/or neuraminidase subtypes have been introduced into human populations. The pandemic of 1918–1919 was by far the worst of its kind. It was followed by pandemics of decreasing severity in 1957 (Asian flu; subtype H2N2), 1968 (Hong Kong flu; subtype H3N2), and 1977 (Russian flu; subtype H1N1).

13.1 Pathophysiology

Based on the antigenic differences between their nucleoprotein (NP) and matrix (M) protein antigens, the influenza viruses are divided into types A, B, and C *(1)*. The influenza A viruses are further divided into subtypes.

The influenza viruses are single-stranded RNA viruses and share structural and biological similarities. The viral RNA core consists of 8 gene segments surrounded by a coat of 10 (influenza A) or 11 (influenza B) proteins. Immunologically, the most important surface proteins are hemagglutinin and neuraminidase, as the influenza viruses are typed on the basis of these proteins. For example, influenza A subtype H3N2 expresses hemagglutinin 3 and neuraminidase 2. The most prevalent human influenza A strains are H1N1 and H3N2.

Although the morphologic characteristics of the influenza viruses are a genetic trait, the spherical morphology appears to be dominant on passage in chicken embryos or tissue culture systems. The most distinct feature of the influenza virions is the presence of a layer of spikes projecting radially outward over the surface. The surface spikes are of two distinct types, corresponding with the hemagglutinin and neuraminidase components of the virus.

13.1.1 Hemagglutinin

The hemagglutinin (HA) is the surface glycoprotein, which accounts for approximately 25% of viral protein and is distributed evenly on the virion surface. It is responsible for the virus's binding to the host receptor, internalization of the virus, and subsequent membrane-fusion events within the endosomal pathway in the infected cell. Furthermore, HA is also the most abundant antigen on the viral surface and harbors the primary neutralizing epitopes for antibodies *(1)*.

Structurally, HA is a glycoprotein consisting of two polypeptide chains, HA1 and HA2. HA1 and HA2 are linked by a single disulfide bond, and each HA "spike" contains three of these HA1 and HA2 chains. Furthermore, HA contains up to seven oligosaccharide chains (six in HA1 and one in HA2) linked to asparagine. The majority of these carbohydrate chains are on the lateral surface of the trimer. No obligatory function has been assigned to these side chains *(1)*.

13.1.2 Neuraminidase

The neuraminidase (NA) exists as a mushroom-shaped spike containing a hydrophobic region by which it is embedded in the viral membrane in the opposite way to the HA. NA is the second subtype-specific glycoprotein on the influenza virion and is composed of a single polypeptide chain *(1)*. It is not evenly distributed on the virion surface but rather found in patches. The principal biologic role of NA protein of the influenza A virus is the cleavage of the terminal sialic acid residues that are receptors of the virus's HA protein. Removal of these residues from the surface of infected cells and from newly formed viruses will prevent the budding viruses from clumping to each other or to the cell surfaces. In addition, the ability to cleave sialic acid is also thought to help the virus to penetrate mucus *(3)*.

Because NA functions largely in the release of newly formed viral particles, antibodies against it do not prevent initial infection. However, they sharply limit its spread and therefore, in humans, selection favors NA variants with mutations that hinder antibody recognition (*antigenic shift*) *(3)*.

13.1.3 Nucleoprotein

The nucleoprotein (NP) is one of the type-specific antigens of influenza viruses that distinguish among types A, B, and C viruses *(1)*. The nucleoproteins are basic proteins that constitute the backbone of the helical internal complex and have a putative role in transcription and replication. During infection, the NP accumulates in the nucleus, and karyophilic sequences have been identified that are partially conserved among the influenza A, B, and C viruses.

13.1.4 Matrix Proteins (M)

The RNA segment 7 of influenza A viruses codes for two proteins M1 and M2 *(1)*. The basic organization of RNA segment 7 is present in all influenza A and B viruses sequenced.

The M1 protein is a virion structural protein that is intimately associated with the lipid bilayer. It is believed to be a multifunction protein having a role in the downregulation of the activity of the virion-associated transcriptase. It is located in the nucleus, cytoplasm, and plasma membrane of infected cells. Passively transferred monoclonal antibodies to the M1 protein did not confer resistance to infection.

The M2 protein of influenza A virus is an integral membrane protein that is expressed on the surface of infected cells with an extracellular domain *(1)*. The M2 protein is present in high copy number in infected cells and thus appears to be actively excluded from the virions.

13.2 Influenza A (H1N1) Pandemic of 1918–1919

In just 8 months, the 1918–1919 pandemic known as the "Spanish influenza" killed between 20 million and 40 million people worldwide *(2)*. The virus causing this influenza pandemic was not isolated at the time. However, its enhanced severity, multiple waves in just 1 year, and its predilection for the young and healthy all suggested that this influenza pandemic was unique. As the most deadly influenza virus ever experienced, the 1918 strain offers a unique opportunity to understand the connection between genotype and virulence *(2)*.

From preserved autopsy samples of two U.S. soldiers and from the frozen lungs of an Inuit woman, fragments of the deadly virus have been isolated, copied, and analyzed. The strains from these three cases have been named: (i) A/South Carolina/1/18; (ii) A/New York/1/18; and (iii) A/Brevig Mission/1/18. For the first time, it has become possible to test hypotheses about where the 1918 influenza virus came from and what made it so deadly *(2,3)*.

Advances in molecular biology techniques have allowed scientists to get a closer look at the virus that caused the 1918 pandemic *(3–7)*. *It was defined as influenza virus A H1N1 strain.*

13.2.1 Structural Elucidation of the 1918 Influenza A Hemagglutinin

The crystal structure of the 1918 influenza HA has been elucidated from a second human subtype (H1) derived from reassembling the extinct 1918 influenza virus *(8, 9)*. In addition, two closely related HAs in complex with receptor analogues have also been determined *(9)*. These two related HAs may explain how the 1918 HA, while retaining receptor binding site amino acids characteristic of an avian precursor HA, was able to bind human receptors and how, as a consequence, the virus was able to spread in human populations *(9)*.

The primary event in influenza infection is the binding of the virus to the host receptor. The crystal structure of HA has shown that its receptor-binding site is situated in a shallow pocket in the membrane-distal HA1 domain in each subunit of the HA trimer *(8, 9)*.

The nature of the receptor's sialic acid linkage to the vicinal galactose is the primary determinant in lung epithelial cells that differentiates avian viruses from mammalian. Sialic

acids are usually found in either α2,3- or α2,6-linkages to galactose, which is the predominant penultimate sugar of the *N*-linked carbohydrate side chains. The binding preference of a given HA for one or other of these linkage types correlates with the species specificity for infection (*species barrier*). Thus, avian viruses preferentially bind to receptors with an α2,3-linkage to galactose, whereas human-adapted viruses are specific for the α2,6-linkage *(9)*. For example, the HAs of all 15 antigenic subtypes found in avian influenza viruses bind preferentially to sialic acid in α2,3-linkage, and it is this form of the sialosaccharide that predominates in the avian enteric tract where these viruses replicate. Human viruses of the H1, H2, and H3 subtypes that are known to have caused pandemics in 1918, 1957, and 1968, respectively, recognize α2,6-linked sialic acid, the major form found on cells of the human respiratory tract *(9)*.

Because an avian origin has been proposed for the HAs of swine and human viruses, changes in the binding specificity of HAs will be required for cross-species transfer.

13.2.2 Characterization of the 1918 Influenza Neuraminidase Gene

The complete coding sequence of the 1918 influenza virus A neuraminidase gene has been determined and compared with other N1 subtype NA genes, including nine N1 newly sequenced strains *(3)*. In general, the 1918 NA shares many sequences and structural characteristics with avian strains, including the conserved active site, wild-type stalk length, glycosylation sites, and antigenic sites. Phylogenetically, the 1918 NA gene appears to be intermediate between mammals and birds, suggesting that it was introduced into mammals just before the 1918 pandemic *(3)*.

The active catalytic site of the NA protein consists of a pocket in the top surface of each subunit of the tetrameric protein. The pocket contains 15 charged amino acids that are conserved in all influenza A viruses *(3)*.

13.2.3 Characterization of the 1918 Influenza Virus Polymerase Genes

The influenza A viral heterotrimeric polymerase complex (PA, PB1, PB2) is known to be involved in many aspects of viral replication and to interact with host factors, thereby having a role in host specificity. Recently, an additional small open reading frame has been identified that codes for a peptide (PB1-F2) that is thought to play a role in the virus-induced cell death. It is not yet clear how the polymerase complex must change to adapt to a new host. However, a single amino acid change in PB2, E627K, was shown to be important for adaptation in mammals.

The polymerase protein sequences from the 1918 human influenza virus differ from avian consensus sequences at only a small number of amino acids; that is consistent with the hypothesis that they derived from an avian source shortly before the 1918 pandemic. However, when compared with avian sequences, the nucleotide sequences of the 1918 polymerase genes have more synonymous differences than expected, suggesting evolutionary distance from known avian strains *(10)*.

In 2005, the sequence and phylogenetic analyses of the complete genome of the 1918 influenza virus were determined *(10)*. The data suggested that the 1918 virus was not a reassortant virus like those of the 1957 and 1968 pandemics. More likely, the 1918 virus was an entirely avian-like virus that had adapted to humans.

One interesting feature found in the polymerase complex of the 1918 virus and subsequent human isolates as well was the presence of a Lys residue at position 627. This residue has been implicated in host adaptation and has been previously shown to be critical for high pathogenicity in mice infected with the 1997 H5N1 virus *(11)*.

13.2.4 Virulence of the 1918 Influenza A Virus

One of the characteristics of the 1918 influenza pandemic was its unusual virulence, reflected in the dramatic increase in the severity of the illness and the prevalence of pneumonic complications *(2,3)*. The virulence of the influenza viruses is a complicated function of the genetic characteristics of the virus itself, the immune status of the infected person, and the dose and route of transmission. The severity of the 1918 pandemic suggested that both the HA and NA were *antigenically novel* as supported by sequence and phylogenetic analyses of both 1918 HA and NA proteins *(5)*, as well as that the virus had not circulated widely in the human population before spring 1918 *(3)*.

The relationship between virulence and the genetic structure of the influenza virus is complex. There have been several examples where simple changes in a single gene resulted in dramatic changes in virulence. Thus, one of these changes is the insertion of basic amino acids in the HA cleavage site, which will allow the virus to grow in many tissues outside its normal host cells. Although this change has been found in the H5 and H7 subtypes in birds, it *was not found* in the 1918 HA *(5)*. In another change observed in the NA gene, the loss of a glycosylation site at amino acid 146 in WSN/33 contributed to making the virus exceptionally virulent as well as neurotropic in mice. This change was also *not observed* in the 1918 virus strain *(3)*.

13.3 Asian Influenza A Pandemics of 1957 and 1968

Although milder than the 1918 Spanish H1N1 influenza pandemic, both the 1957 Asian influenza H2N2 pandemic and the 1968 Hong Kong influenza H3N2 pandemic caused significant morbidity and mortality worldwide (1,2).

The 1957 influenza pandemic was caused by a reassortant virus that was derived from the HA (H2), NA (N2), and PB1 (polymerase basic protein 1) genes from an avian influenza virus infecting ducks and the remaining gene segment from the previously circulating human H1N1 virus (12–14).

The H3N2 virus that caused the 1968 pandemic consisted of avian HA (H3) and PB1 genes in a background of other internal protein genes of the human H2N2 virus that was circulating at the time (12–14). The presence of an avian HA H3 glycoprotein made the reassortant virus antigenically novel to humans, and it spread in the susceptible human population causing a pandemic (12, 13).

Studies on the origin and evolutionary pathways of the PB1 genes of influenza A viruses responsible for the 1957 and 1968 human pandemics and the variable or conserved region of the PB1 protein have shown that the evolutionary tree constructed from nucleotide sequences suggested that (i) the PB1 gene of the 1957 human pandemic strain, A/Singapore/1/57 (H2N2), was probably introduced from avian species and was maintained in humans until 1968; (ii) in the 1968 pandemic strain, A/NT/60/68 (H3N2), the PB1 gene was not derived from the previously circulating virus in humans but probably from another avian virus; and (iii) a current human H3N2 virus inherited the PB1 gene from an A/NT/60/68-like virus (12). Nucleotide sequence analysis also showed that the avian PB1 gene was introduced into pigs. Hence, transmission of the PB1 gene from avian to mammalian species is a relatively frequent event. Comparative analysis of deduced amino acid sequences disclosed highly conserved regions in PB1 proteins, which may be key structures required for PB1 activities (12).

The RNA of the human influenza virus Singapore (H2N2) strain has been labeled *in vivo* by phosphorus-32 and separated by polyacrylamide gel electrophoresis into eight segments, which were correlated to the corresponding gene functions and/or proteins (2). The base sequence homology between the individual genes (segments) of the H2N2 virus and those of different influenza A strains has been determined by molecular hybridization. Segments 1, 5, 7, and 8 of the Singapore strain exhibit a base sequence homology of almost 100% compared with those of the FM1 strain (HlNl), whereas the homology between the other segments was significantly lower (24% to 76%). For the Singapore and Hong Kong (H3N2) strains, all segments except that coding for the HA (24%) exhibit a homology close to 100%. The ^{32}P-labeled segment 4 (HA gene) of the avian influenza A strain duck Ukraine (Hav7Neg2) showed a homology of 92% to Hong Kong, whereas the homology of at least two other segments was significantly lower. These results were interpreted as an indication that the H2N2 subtype is derived from the HlN1 subtype by a recombination event retaining four H1N1 segments, whereas the other four segments were gained from another yet unknown strain. The H3N2 subtype is presumably derived from a H2N2 subtype, retaining seven segments of the H2N2 subtype, whereas the gene coding for the HA is obtained from the duck Ukraine or another highly related strain (13).

13.4 Avian Influenza A (H5N1)

An unprecedented epizootic avian influenza A (H5N1) virus that is highly pathogenic has crossed the species barrier in Asia to cause human fatalities and thus poses an increased threat of pandemic (15). In 1997, an avian subtype, H5N1, was first described in Hong Kong. The infection was confirmed in only 18 people, but 6 of them died. Subsequent, although sporadic, cases of avian (bird flu) influenza continued to be recorded, mainly in southern China, but also in other regions of Southeast Asia. The H5N1 influenza, in nearly all cases, has been transmitted to humans from birds. Other routes of transmission include possibly environment-to-humans, and limited, nonsustained, human-to-human transmission. Transmission to felids has been observed by feeding raw infected chicken to tigers and leopards in zoos in Thailand and to domestic cats under experimental conditions. Transmission between felids has been found under such conditions.

Because of the poultry outbreaks and bird-to-human transmission, hundreds of new cases of avian influenza have been reported, stretching from Southeast Asia (mainly China, Vietnam, Thailand, and Indonesia) through Mongolia, Kazakhstan, and Russia to Turkey, raising the concern that a slight mutation may convert subtype H5N1 into a strain that would be easily transmitted from human to human. To date, human-to-human transmission of influenza A virus (H5N1) has been suggested in several household clusters (11) and in one case of apparent child-to-mother transmission (16). There has been a WHO report suggesting that local virus H5N1 strains (northern Vietnam) *may be adapting to humans (17)* (http://www.who.int/csr/resources/publications/influenza/WHO_CDS_CSR_GIP_2005_7/en/). However, epidemiologic and virologic studies will be needed to confirm these findings.

13.4.1 Pathogenesis

Studies of isolates of avian influenza A (H5N1) from patients in 1997 have shown that the virulence factors included (i) the highly cleavable hemagglutinin that can be activated by multiple cellular proteases; (ii) a specific substitution in the polymerase basic protein 2 (Glu627Lys) that enhances replication; and (iii) a substitution in nonstructural protein 1 (Asp92Glu) that confers increased resistance to inhibition by interferons and tumor necrosis factor α (TNF-α) *in vitro* and prolonged replication in swine, as well as greater elaboration of cytokines, particularly TNF-α, in human macrophages exposed to the virus *(15)*.

Since 1997, studies of influenza A (H5N1) have indicated that these viruses continue to evolve, including (i) changes in antigenicity and internal gene constellations; (ii) an expended host range of avian species and the ability to infect felids; (iii) enhanced pathogenicity in experimentally infected mice and felids, in which they caused systemic infections; and (iv) increased environmental stability *(15)*.

Phylogenetic analyses have demonstrated that the Z genotype has become dominant *(18)* and that the virus has evolved into two distinct clades, one encompassing isolates from Cambodia, Laos, Malaysia, and Vietnam, and the other isolates from China, Indonesia, Japan, and South Korea *(17)*. Recently, a separate cluster of isolates has appeared in northern Vietnam and in Thailand, which included variable changes near the receptor-binding site and one fewer arginine residue in the polybasic cleavage site of the hemagglutinin *(12)*. However, the importance of these genetic and biologic changes with respect to human epidemiology or virulence is uncertain.

13.4.2 Host Immune Responses to Avian Virus A (H5N1)

The relatively low frequency of influenza A (H5N1) illness in humans despite widespread exposure to infected poultry has suggested that the *species barrier* to acquisition of this avian virus is substantial. Clusters of cases in family members may be caused by common exposures, although the genetic factors that may affect a host's susceptibility to disease will require more studies *(15)*.

The innate immune responses to influenza A (H5N1) may contribute to disease pathogenesis. In the 1997 outbreaks, elevated blood levels of interleukin-6, TNF-α, interferon-γ, and soluble interleukin-2 receptor were observed in individual patients *(19)*, and in patients in 2003, elevated levels of the chemokines interferon-inducible protein 10, mono-cyte chemoattractant protein 1, and monokine induced by interferon-γ were found 3 to 8 days after the onset of illness *(20)*. Recently, plasma levels of inflammatory mediators (interleukin-6, interleukin-8, interleukin-1β, and monocyte chemoattractant protein 1) were found to be higher among patients who died than among those who survived, and the average levels of plasma interferon-α were about three times as high among patients with avian influenza A who died as among healthy controls *(15)*. Among survivors, specific humoral immune responses to influenza A (H5N1) are detectable by microneutralization assay 10 to14 days after the onset of illness.

13.4.3 Clinical Features

The clinical spectrum of influenza A (H5N1) in humans has been based on descriptions of hospitalized patients *(15)*. The frequencies of milder illness, subclinical infections, and atypical presentations (e.g., encephalopathy, gastroenteritis) have not been determined, but case reports indicated that each have occurred. Most of the hospitalized patients were previously healthy young children or adults.

Incubation. The incubation period of the avian influenza A (H5N1) may be longer compared with those of other known human influenzas—in most cases it is within 2 to 4 days after exposure but occasionally up to 8 days *(15)*. The case-to-case intervals in household clusters have generally been 2 to 5 days with a upper limit between 8 and 17 days (possibly resulting from unrecognized exposure to infected animals or environmental sources).

Initial Symptoms. Most patients have initial symptoms of high fever (38°C or higher) and an influenza-like illness with lower respiratory tract symptoms *(21)* (http://www.who.int/csr/disease/avian_influenza/guidelines/Guidelines_Clinical%20Management_H5N1_rev.pdf). Upper respiratory tract symptoms are manifested only occasionally. Also, unlike patients with infections caused by avian influenza A (H7N7) viruses, patients with avian influenza A (H5N1) rarely have developed conjunctivitis *(22)*. Diarrhea, vomiting, abdominal pain, pleuritic pain, and bleeding from the nose and gums have also been reported early in the course of illness. Watery diarrhea may precede the respiratory manifestations by up to 1 week *(15)*.

Clinical Course and Management. Lower respiratory tract manifestations develop early in the course of the disease—respiratory distress, tachypnea, and inspiratory crackles are common. Sputum production is variable and occasionally bloody. Nearly all patients have clinically apparent pneumonia. Progression to respiratory failure has been associated with diffuse, bilateral, ground-glass infiltrates and manifestations of acute respiratory distress syndrome (ARDS).

Multiorgan failure with signs of renal dysfunction, and sometimes cardiac compromise including cardiac dilatation and supraventricular tachyarrhythmias, have been common *(15)*.

Most hospitalized patients with avian influenza A (H5N1) have required ventilatory support within 48 hours after admission, as well as intensive care for multiorgan failure and sometimes hypotension. Empirical treatment with broad-spectrum antibiotics, antiviral agents—alone or with corticosteroids—has been tried in most patients, although their effects have not been rigorously assessed *(15)*. Early initiation of antiviral drugs appears to be beneficial *(11)*.

Mortality. Death has occurred an average of 9 or 10 days after the onset of illness (range, 6 to 30 days), and most patients have died of progressive respiratory failure *(15)*. The mortality rate among hospitalized patients has been high although the overall rate has probably been much lower *(17)*. In contrast with 1997, when most deaths occurred among patients older than 13 years of age, recent infections have caused high rates of death among infants and young children (89% among children younger than 15 years of age in Thailand).

13.5 Avian Influenza A (H9N2)

Since the late 1990s, several cases of human infections with the avian influenza A (H9N2) virus have been reported *(23)*. However, despite concerns after the initial cases of human infections with the H9N2 strain occurred, no virulent outbreak of human H9N2 infection did occur. As with influenza A (H5N1) virus outbreaks, there has been considerable apprehension that a virulent H9N2 strain might still mutate to allow human-to-human infection and that such a strain might also possess the triad of infectivity, lethality, and transmissibility.

13.5.1 *Molecular Characterization of Avian Influenza A (H9N2) Virus*

The avian H5N1 influenza virus that was transmitted from poultry to humans in 1997 and caused high mortality in both species is unusual in having a large proportion of amino acid substitutions in all gene products except in the surface antigen, thus suggesting that the H5N1 virus may be a *reassortant (24)*. Phylogenetic and antigenic analyses of the H9N2 and H5N1 viruses and a quail H9N2 virus, all isolated from Hong Kong, provided evidence that the H5N1 and H9N2 influenza viruses were indeed reassortants, and that the quail H9N2 virus may have been the internal gene donor *(25,26)*. The reassortment between N5N1 and N9N2 had occurred

prior to the human infection by H5N1 in 1997. Further results have indicated the presence of multiple lineages of H9N2 viruses in Asia and at least three distinguishable subgroups in Hong Kong poultry *(25)*, as well as that H9N2 influenza viruses possessing H5N1-like internal genomes continue to circulate in poultry in southeastern China *(27)*.

In another study *(28)*, the H9N2 influenza viruses were found to have receptor specificity similar to that of human H3N2 viruses. In addition, the neuraminidase of poultry H9N2 viruses has mutations in its hemadsorbing site, a characteristic resembling that of human H2N2 and N3N2 viruses, but differing from that of other avian viruses. These *peculiar features* of the surface glycoproteins of H9N2 viruses from Hong Kong suggest an *enhanced propensity for introduction into humans* and emphasize the importance of poultry in the zoonotic transmission of influenza viruses.

13.6 NIAID and Influenza Research

The influenza infection (flu) is a contagious respiratory illness that can cause mild to severe illness, which at times can lead to death. The best way to prevent influenza is by getting a flu vaccination each year. According to statistics by the CDC, every year in the United States, on average: (i) 5% to 20% of the population will contract flu; (ii) more than 200,000 people are hospitalized from flu complications; and (iii) about 36,000 people die from flu *(29)* (http://www. cdc.gov/flu/keyfacts.htm). Recent studies have revealed that children 6 to 23 months of age have a substantially higher risk for influenza-associated morbidity *(30,31)*. Despite the high annual rates of influenza in children, preventive vaccines are given infrequently *(27)*. For the influenza season 2003–2004, 92 pediatric deaths were reported to the CDC (http://www.cdc.gov/mmwr/preview/mmwrhtml/mm5253a4.htm).

Upon recommendations of the Blue Ribbon Panel on Influenza Research *(32),* major goals and objectives of the NIAID's Influenza Program involve research in the following areas (http://www3.niaid.nih.gov/about/directors/congress/2004/02122004.htm):

- *Basic Biology.* NIAID supports many basic research projects aimed at understanding how the influenza virus replicates, interacts with the host, stimulates an immune response, and evolves into new strains. Results from these studies lay the foundation for the design of new antiviral drugs, diagnostics, and vaccines.
- *Antiviral Drugs.* NIAID currently supports the identification, development, and evaluation of new antiviral drugs against influenza, including the screening of new drug candidates to see if they have activity against the virus

both in laboratory cells and in animals. NIAID is also focused on developing novel broad-spectrum therapeutics intended to work against many influenza virus strains; some of these target viral entry into human cells, whereas others specifically attack and degrade the viral genome. Development and evaluation of a combination antiviral regimen against potential pandemic influenza strains is also now under way.

- *Diagnostics*. NIAID supports the development of rapid, ultrasensitive devices to detect influenza virus infection. Although early in development, these devices will allow detection of newly emerging viral mutants and discrimination between different antigenic subtypes.

- *Surveillance and Epidemiology*. The threat from influenza, like virtually all emerging and re-emerging infectious disease threats, is global in scope. For this reason, in recent years NIAID has expanded its activities in other countries. Through a contract for pandemic influenza preparedness, NIAID supports a long-standing program in Hong Kong to detect the emergence of influenza viruses with pandemic potential in animals. Under this program, scientists had detected the re-emergence of highly pathogenic H5N1 avian strains in this area in 2002 and 2003 and were instrumental in the early detection and characterization of the SARS coronavirus in 2003. This approach has underscored the concept that research on one type of infectious disease often supports or can be applied to research on other types of infectious diseases, whether newly emerging, re-emerging, or deliberately introduced.

- *Vaccine Development and Evaluation*. Because influenza is so easily transmitted, effective vaccines are essential to controlling annual influenza epidemics. The current egg-based system used to produce licensed influenza vaccines—despite being reliable for more than 40 years—can still be improved. Limitations of the current system include (i) a lengthy manufacturing process; (ii) the need to select which virus strains will be in the vaccine at least 6 months in advance of the influenza season; (iii) the need to produce nearly 90 million doses of a new influenza vaccine each year; and (iv) the need for hundreds of millions of fertilized chicken eggs to manufacture the vaccine. The early decision about which strains to include in the influenza vaccine will not always be correct, and the long lead time required to produce the vaccine makes midstream corrective action impossible. Additional limitations may also include allergenicity of eggs in some individuals, and the inability to use eggs for propagating viruses lethal to chickens. NIAID is currently supporting several research projects aimed at developing vaccines that can be manufactured more rapidly, are more broadly cross-protective, and are more effective. The use of reverse genetics—a genetic tool developed by

NIAID-supported scientists—holds the promise of more rapid generation of high-yielding vaccine candidates that match the anticipated epidemic strain. Reverse genetics can also be used to turn highly pathogenic influenza viruses into vaccine candidates more suitable for manufacturing of vaccine by removing or modifying certain virulence genes; laboratories around the world are using the technique to prepare vaccine candidates against the H5N1 viruses emerging in Asia. NIAID also is funding the development of new influenza vaccine technologies. Recently, NIAID supported a Phase II clinical trial of a new influenza vaccine produced in a cell culture system as an alternative to manufacturing the vaccine in eggs. Another approach has focused on improving the effectiveness of current inactivated virus vaccines by giving increasing doses of influenza vaccine to elderly individuals, the population that frequently accounts for up to 90% of influenza deaths each year in the United States. NIAID is also funding the development of new technologies for the production of influenza vaccines; these involve DNA-based approaches and broadly protective vaccines based on influenza virus proteins that are shared by multiple strains of the influenza virus.

13.6.1 Research Programmatic Developments

- *The Broad Agency Announcement (BAA)*. BAA was issued in 2006 to continue and to expand influenza surveillance activities, such as the *Pandemic Preparedness in Asia* contract held by the St. Jude Children's Research Hospital in Memphis (N01-AI-95357), to carry out surveillance and characterization of avian influenza viruses with pandemic potential in Hong Kong, Thailand, Vietnam, and Indonesia.

- *Vaccine Development and Optimization*. Major accomplishments of the program include:

  (i) NIAID's continuing support for the development of novel vaccination strategies for the elderly. Thus, a study conducted to assess the immunogenicity and reactogenicity of a current U.S. vaccine formulation at increased doses in the elderly population (65 years and older; n=202) has shown that at the increased dose (60 μg), higher levels were observed for the mean serum hemagglutination inhibition and neutralizing antibody levels (44% to 71% and 54% to 79%, respectively) compared with the standard dose (15 μg) of the vaccine *(33)*. Increasing the antigen content of inactivated vaccines may provide a straightforward approach to improving protection in the elderly.

(ii) In 2006, a multicenter clinical trial to evaluate the safety and immunogenicity of trivalent inactivated influenza vaccine (produced by CSL, Ltd.) was completed (DMID-06-0016) (http://vaccines.stanford.edu/completed_studies.html).

(iii) In 2006, NIAID initiated or completed a number of multicenter Phase I/II clinical trials to study the safety and immunogenicity of H5N1 A/Vietnam/1203/2004 inactivated influenza vaccines from pharmaceutical companies (Sanofi-Pasteur, Novartis/Chiron, and Baxter). The studies, carried out in children, adults, and the elderly, were aimed at investigating dose ranges, routes of administration, and the use of adjuvants (e.g., aluminum hydroxide, MF59) (http://www.clinicaltrials.gov). Results from the Sanofi-Pasteur vaccine trial showed that the vaccine was well tolerated, and 54% of the subjects (n = 99) receiving the higher dose (90 µg) achieved a neutralizing antibody response to the vaccine at serum dilutions of 1:40 or greater, whereas only 22% of the subjects (n = 100) who received the 15-µg dose developed a similar response to the vaccine *(34)*.

(iv) Results from a Phase I randomized, double-blind clinical trial to assess the safety and immunogenicity of a 2-dose schedule (administered on days 0 and 28) of 4 dose levels (3.73, 7.5, 15, and 30 µg hemagglutinin) of inactivated influenza A/chicken/Hong Kong/G9/97 (H9N2) vaccine with and without adjuvant have shown that the combination of MF59 adjuvant with a subunit vaccine was associated with improved immune responses to the H9N2 virus *(35)*. The adjuvanted vaccine was immunogenic even after a single dose, raising the possibility that 1-dose vaccination strategy may be attainable with the use of adjuvanted vaccine.

(v) NIAID's Vaccine Research Center (VRC) has undertaken an initiative to develop a protective vaccine that is effective against multiple influenza strains (see Section 13.8). The proposed approach is to incorporate both conserved and variable genes into DNA and adenoviral (AdV) vectors that can be readily produced by existing methodologies. In particular, the VRC has been involved in developing three new vaccines, each comprising a single plasmid DNA encoding hemagglutinin protein from H1N1, H3N2, and H5N1 subtypes isolated from recent human outbreaks of influenza. Adenoviral construct expressing the same inserts are also being constructed. In addition, protein subunit vaccines based on production from insect and mammalian cells are being developed and tested.

- *Surveillance and Influenza Genetics.* In 2004, NIAID initiated the *Genome Sequencing Project*, which is providing influenza sequence data, thereby enabling scientists to further study how influenza viruses evolve, spread, and cause disease. To this end, in 2006, the *Global Initiative on Sharing Avian Influenza Data (GISAID)*, an international consortium to promote data sharing, was created. By March 2006, 1,553 human and avian isolates were completely sequenced and released to the public (GenBank).

- *Immunity to Influenza.* NIAID is currently involved in supporting a robust program that will further broaden the knowledge regarding immunity to influenza, as follows:

  (i) *NIH Tetramer Facility at Emory University*. Since 1999, the tetramer facility has prepared nearly 2,400 unique tetramers, of which 459 have been directly related to monitoring T-cell functions against infectious diseases; of those, 45 tetramers were produced specifically for influenza-related studies.

  (ii) *Immune Epitope Database and Analysis Resource (IEDB) at La Jolla Institute of Allergy and Immunology*. In 2005, IEDB became publicly available (http://www.immuneepitope.org). The IEDB contains extensively curated information from the published literature on antibody and T-cell epitopes, as well as tools to predict antibody and T-cell epitopes or visualization/mapping of epitopes onto known protein structures. There are currently 17,868 unique epitopes within the database, including all published influenza antibody and T-cell epitopes.

  (iii) *Modeling Pulmonary Immunity*. This program, conducted at the University of Pittsburgh, involves analysis of innate and adaptive immunity in the lungs and draining lymph nodes of mice (various ages) either infected with or vaccinated against influenza viruses. The computational models to be developed may be used to simulate human innate responses to adjuvants or immune modulators, as a method for screening novel compounds against influenza.

  (iv) *Biodefense Immune Modeling.* Scientists from the University of Rochester will conduct a comprehensive examination of B- and T-cell–mediated immunity to influenza vaccination in healthy adults and influenza A infection or vaccination in mouse model systems. The major goal of these studies is to produce computational models capable of predicting human immune responses to natural variants or genetically engineered influenza viruses and can be used to test novel vaccine strategies or immune modulators *in silico*, prior to further testing in animal models and humans.

(v) *Pathways in the Interferon Signaling Cascade.* This NIAID-supported program is aimed at creating computational models to decipher the type 1 interferon signaling networks in primary human dendritic cells that are modulated by viral proteins.

13.6.2 Centers of Excellence for Influenza Research and Surveillance

NIAID has had a long history of supporting research activities to provide more effective approaches to controlling influenza virus infections. These activities include both basic and applied research on the influenza virus basic biology and replication, pathogenesis, immunology, epidemiology, and clinical research to develop new and improved diagnostics, antiviral drugs, and vaccines. Because of the ever-present threat of an influenza pandemic, NIAID has initiated a program to establish NIAID *Centers of Excellence for Influenza Research and Surveillance (CEIRS)* to support the research agenda of the *HHS Pandemic Influenza Plan.* The overall goal of this program is to provide the government with the information and public health tools and strategies needed to control and lessen the impact of epidemic influenza and the increasing threat of pandemic influenza.

The activities undertaken by the NIAID Centers of Excellence for Influenza Research and Surveillance will lay the groundwork for developing new and improved control measures for emerging and re-emerging influenza viruses, including determining the prevalence of avian influenza viruses in animals in close contact with humans, understanding how influenza viruses evolve, adapt, and transmit, and identifying immunologic factors that determine disease outcome. In the event of an urgent public health emergency involving the emergence and rapid spread of an influenza pandemic in humans, the network of centers will also develop and implement a *NIAID Pandemic Public Health Research Response Plan.*

Each of the NIAID Centers of Excellence for Influenza Research and Surveillance has a focus on one or both of the following research areas:

- *Research Area 1: Animal Influenza Surveillance.* This is designed to conduct prospective international and/or domestic surveillance of animal influenza for the rapid detection and characterization of influenza viruses with pandemic potential.
- *Research Area 2*: *Pathogenesis and Host Response Research.* This is designed to enhance understanding of the molecular, ecologic, and environmental factors that influence pathogenesis, transmission, and evolution of influenza viruses, as well as to characterize the protective immune response.

On March 30, 2007, NIAID awarded six Centers of Excellence for Influenza Research and Surveillance to the following institutions:

- Emory University: Research Area 2: Pathogenesis and Host Response Research
- Mount Sinai School of Medicine: Research Area 2: Pathogenesis and Host Response Research
- St. Jude Children's Research Hospital: Research Areas 1 and 2: Animal Influenza Surveillance; and Pathogenesis and Host Response Research
- University of California, Los Angeles: Research Area 1: Animal Influenza Surveillance
- University of Minnesota: Research Area 1: Animal Influenza Surveillance
- University of Rochester: Research Area 2: Pathogenesis and Host Response Research

13.6.3 NIAID Involvement in International Influenza Research

(i) NIAID has awarded two contracts for the production of inactivated H5N1 vaccine to Aventis Pasteur (Swiftwater, Pennsylvania) and Chiron (Liverpool, United Kingdom).
(ii) NIAID is also supporting the production of inactivated H9N2 vaccine manufactured with and without adjuvant by Chiron (Sienna, Italy).
(iii) *Clinical Trials of Pandemic Influenza Vaccine.* In 2003, NIAID conducted a Phase I/II clinical trial to evaluate increasing doses of inactivated influenza vaccine made by using the H9N2 virus isolated in 1999 from two infected children in Hong Kong. Immunogenicity assays have been generated and data analysis is ongoing. The preliminary data showed that the vaccine was well tolerated.

In 2004, NIAID expanded its "Pandemic Preparedness in Asia" contract to St. Jude Children's Research Hospital (N01-AI-95357). Activities conducted under this expansion include (i) establishing animal influenza surveillance sites in Asia; (ii) generating high-yielding vaccine candidates against influenza strains with pandemic potential accompanying reagents; (iii) supporting an international animal surveillance training course in Hong Kong; and (iv) studying newly emerging influenza strains infecting swine in the United States. In 2004, NIAID launched the *Influenza Genome Sequencing Project,* which will rapidly provide influenza sequence data to the scientific community to enable further studies of how the influenza viruses evolve, spread, and cause disease, and which may

ultimately lead to improved methods of treatment and prevention.

13.7 Influenza Vaccine Research

During the past several years, there have been increasing reports of direct transmission of avian influenza viruses to humans (36–38). Furthermore, the continuing outbreaks of H5N1 influenza virus infections in avian species and humans in several countries (36,39,40) has emphasized the considerable threat posed by highly pathogenic avian influenza (HPAI) and low pathogenic avian influenza LPAI viruses to human health (14). This, coupled with the difficulty to predict which subtype of avian influenza virus will cause the next human pandemic means that an ideal vaccine would elicit an immune response that protects the host from infection with a broad range of influenza viruses from the same or different subtypes (14).

The HA and NA glycoproteins of influenza viruses undergo genetic and antigenic variation to escape the immune response (14,41,42). Whereas the presence of neutralizing antibodies specific for the HA glycoprotein at systemic or mucosal sites of infection would provide immediate protection against infection with influenza viruses, the clearance of human influenza viruses depends mainly on cell-mediated immunity (43). Although antibodies specific for the NA glycoprotein do not neutralize infectivity, they restrict virus replication by preventing the release of new virus particles, a process that requires viral NA proteins. Therefore, antibodies specific for NA can decrease the severity of the disease (44, 45). Epitopes recognized by cytotoxic T lymphocytes (CTLs) are present on NP, PB2, and PA proteins of human influenza viruses. Therefore, if a virus with a new HA and/or NA glycoprotein emerges in the human population, cell-mediated immunity directed against the highly conserved internal proteins could have a role in protection at the time of a pandemic (14). The principle underlying the currently licensed vaccines against human influenza viruses is the induction of protective antibodies specific for the HA glycoprotein of the predicted epidemic strain. The concentration of HA glycoprotein in licensed, inactivated virus vaccines for seasonal influenza is standardized, but the concentration of NA glycoprotein is not standardized (14).

Although most influenza vaccines are designed to induce HA-specific antibody responses to protect the host from infection, the biology of avian influenza viruses presents several unique challenges compared with human influenza viruses. These challenges include the presence of different subtypes of HA and NA glycoproteins and the genetic and antigenic diversity within each subtype (14).

Whereas the antigenic diversity has consequences for pandemic vaccines that must be considered in the design of a protective vaccine, not all of the 16 HA and 9 NA subtypes of avian influenza viruses have similar pandemic potential. Although HPAI H5N1 viruses are the main focus of global attention, LPAI H9N2 viruses are also widespread in poultry in Asia (46) and HPAI H7 viruses have caused large outbreaks in poultry in Europe (47), North America (48), and South America (49). Although HPAI viruses cause morbidity and mortality in poultry, HPAI viruses might not be intrinsically more likely to cause a human pandemic than would LPAI viruses. To this end, there are no known examples of a pandemic caused by an H5 or H7 HPAI virus, although virologic data are limited to those from the three influenza pandemics that occurred in the past century (38).

Because of this uncertainty, it would be highly desirable to develop vaccines against each of the subtypes of avian influenza virus, although the order of development can be prioritized on the basis of epidemiologic data (14).

A comparison of the predicted protein structures of HA glycoprotein subtypes 1 to 15 has led to the classification of these subtypes into four different clades: clade 1 (H1, H2, H5, H6, H11 and H13), clade 2 (H8, H9 and H12), clade 3 (H3, H4 and H14), and clade 4 (H7, H10 and H15) (51).

Phylogenetic analysis of the genes encoding certain subtypes of HA glycoprotein reveals a separation into lineages that correspond with the geographic separation of the birds that they infect. These genetic lineages are referred to as the Eurasian and North American lineages, and they generally correspond with the flight paths of migratory birds (51–53). Viruses from these two lineages might also be antigenically distinguishable, but the consequences of these genetic and antigenic differences for vaccine development are not known (14).

Circulating human influenza viruses undergo rapid mutation owing to the low fidelity of the viral RNA-dependent RNA polymerase (54). Antigenic drift occurs when the genes encoding the HA and/or NA glycoproteins undergo stepwise mutations, resulting in variant viruses with amino acid changes at one or more antibody-binding sites of HA and/or NA (55) that allow the viruses to evade neutralization by antibodies generated as a result of previous natural infection or vaccination. The internal protein genes of avian influenza viruses are not under positive immune selection in waterfowl and shorebirds. However, the use of veterinary vaccines to protect poultry from infection with avian influenza viruses might drive evolution of the HA glycoprotein if such vaccines do not induce sterilizing immunity (14).

The viral determinants of pathogenicity of avian influenza viruses in humans are multigenic. Further studies are required to understand how the pathogenicity of avian influenza viruses affects the infectivity and transmissibility of these viruses in humans and to establish whether these factors have implications for vaccine design (14).

13.7.1 Types of Influenza Vaccines

Inactivated virus vaccines and live attenuated virus vaccines that are being developed for pandemic influenza are based on technologies that are licensed for the existing seasonal human influenza vaccines (14). Vaccines based on various other platforms, such as live virus vectors expressing influenza virus proteins and DNA vaccines, are also being developed and have shown promise in preclinical studies (see Table 2 in Ref. 14).

The currently licensed vaccines against human influenza viruses are produced in embryonated chicken eggs, and the manufacturing process can take 6 to 9 months (14). Consequently, for vaccines that are based on the currently licensed technologies, the availability of embryonated eggs is a crucial factor, and if the pandemic virus causes widespread morbidity and mortality in poultry, the supply of embryonated eggs might be compromised. Therefore, alternative substrates, including mammalian cell lines, such as Madin-Darby canine kidney (MDCK) cells and Vero cells, have been developed for the production of influenza viruses for use in vaccines. To this end, considerable progress was made in the development of vaccines based on inactivated influenza viruses and live cold-adapted influenza viruses grown in these cell lines in microcarrier fermentors (56–58).

Influenza A viruses replicate in several experimental animals, including chickens, mice, cotton rats, ferrets, hamsters, guinea pigs, and non-human primates. The use of mouse models for the study of influenza is limited because intranasally administered influenza A viruses do not cause symptoms of respiratory tract disease in mice, although some influenza A viruses are lethal in some other animal models (14). Ferrets are generally thought to be the best available model for influenza research. Unlike mice, ferrets develop fever, rhinorrhea, and sneezing after infection with intranasally administered human influenza viruses and the virus replicates in the respiratory tract of these animals. Seronegative ferrets develop a strain-specific immune response to human influenza viruses. Currently, preclinical studies of pandemic influenza vaccines are carried out in mice and ferrets (14).

13.7.1.1 Inactivated Virus Vaccines

In preclinical studies, parenterally administered, inactivated whole-virus H9 and H5 subtype vaccines were shown to be effective in mice against challenge with homologous and heterologous viruses (14,59–63). Recombinant H5 influenza viruses—which contain a modified HA glycoprotein, a wild-type NA glycoprotein from the 1997 or 2003 H5N1 viruses or from an LPAI H5N3 virus, and internal protein genes from

the PR8 H1N1 influenza virus (A/Puerto Rico/8/34) that confer high yield in eggs—have been generated by reverse genetics (64–68). The removal of the multibasic amino acid motif in HA that makes the HA0 precursor of HPAI viruses highly cleavable attenuated the virus for infection of chickens, mice, and ferrets without altering the antigenicity of the HA glycoprotein (38). Two doses of these inactivated virus vaccines provided complete protection from lethal challenge with homologous and heterologous H5N1 viruses in mice and ferrets (64–68).

Data from Phase I clinical trials of inactivated virus vaccines against H9N2, H5N3, H5N1, and H2N2 viruses have been reported and other vaccines are still under evaluation (see Table 2 in Ref. 14). Studies that were carried out to date indicated that inactivated split-virion vaccines against avian influenza viruses—in which the virions were disrupted or split by detergent treatment and the surface glycoproteins were then partially purified—were not optimally immunogenic (69) and required multiple doses (70) or the inclusion of an adjuvant (71–74) to induce a protective immune response (14).

Whole-virus vaccines are more immunogenic than are split-virion vaccines, but they are likely to be more reactogenic (75). Adjuvants are required to increase the immunogenicity of inactivated virus vaccines and to decrease the concentration of viral proteins that is required to induce protective immunity, and several adjuvants for this purpose are under investigation, including aluminum salts, the squalene-oil-water emulsion (MF59), and other proprietary compounds (14).

An inactivated whole-virus H9N2 vaccine was found to be immunogenic in individuals who had circulating antibodies induced by prior exposure to H2N2 viruses that cross-reacted with H9N2 viruses, but the vaccine was not immunogenic in individuals who were born after 1968, when H2N2 viruses stopped circulating in humans (76). This observation is consistent with findings from studies of an H1N1 vaccine in 1976–1977, when prior exposure to H1N1 viruses that had circulated in the population earlier ("priming") was found to be a determinant of the response to vaccination (75, 77). These studies also emphasized the need for two doses of vaccine in "unprimed" individuals. In other studies of vaccines against H9N2 viruses, aluminum hydroxide and MF59 adjuvants improved immunogenicity (71, 73).

Inactivated virus vaccines prepared from recombinant PR8 viruses that consisted of a modified HA glycoprotein and wild-type NA glycoproteins from H5N1 viruses isolated in 2004 were evaluated as *subvirion vaccines* or whole-virus vaccines, with or without adjuvants (68,72,78,79). The subvirion vaccines were safe and well-tolerated in healthy adults, and the antibody response that was induced could be enhanced by increasing the dose of antigen used or by the addition of an adjuvant (72, 79). A whole-virus vaccine was

also well-tolerated by humans, and when administered with an adjuvant, this vaccine was immunogenic at a lower dose than that of the subvirion vaccines *(74)*. However, the available data indicate that inactivated H5 influenza virus vaccines are poorly immunogenic and require a large concentration of HA glycoprotein or co-administration with an adjuvant to achieve the desired antibody response *(14)*.

13.7.1.2 Live Attenuated Virus Vaccines

Live attenuated, cold-adapted influenza virus vaccines against human influenza viruses elicit both systemic immunity and mucosal immunity at the primary portal of infection *(14)*.

These vaccine strains are generated by the reassortment of a wild-type influenza virus carrying the HA and NA genes of interest with a cold-adapted donor AA (H2N2) influenza virus (A/Ann Arbor/6/60), which was generated by serial passage of the wild-type AA virus at successively lower temperatures *(80)*. The temperature-sensitive, attenuated, cold-adapted donor AA virus has five mutations in three gene segments that contribute to the temperature-sensitive or attenuation phenotype *(81)*, and the virus has a high degree of phenotypic and genotypic stability *(82)*. Candidate live attenuated virus vaccines against H9N2 and H5N1 avian influenza viruses generated on this cold-adapted donor backbone using reassortment and plasmid-based reverse genetics, respectively (see Fig. 3 in Ref. *14*), were safe and effective in mice and ferrets *(83–85)*. Phase I clinical evaluation of these vaccines is currently in progress *(14)*.

Generally, live attenuated virus vaccines must retain some infectivity to be immunogenic. Hence, virus shedding during clinical testing of these vaccines must be closely monitored *(14)*. There are potential challenges in the development of live attenuated virus vaccines for pandemic influenza, namely, (i) to generate reassortant viruses that are sufficiently infectious when the HA glycoprotein is derived from an avian influenza virus, in particular if the HA used has a preference for α2,3-linked oligosaccharides; (ii) to reproducibly achieve the desired level of viral attenuation with different combinations of HA and NA genes; and (iii) to minimize the risk of reassortment with circulating human influenza viruses. The evaluation of live attenuated virus vaccines of different subtypes in preclinical studies in appropriate animal models and in clinical studies will address the first two challenges. The standard approach of preclinical evaluation that is applied to vaccines against human influenza viruses might not be uniformly applicable to avian influenza viruses, because the infectivity, immunogenicity, and protective efficacy of avian influenza viruses of different subtypes have not been studied extensively *(86)*. The risk of reassortment of the live attenuated vaccine virus with human influenza viruses

during clinical trials can be minimized by conducting vaccine studies in isolation units when human influenza viruses are not circulating in the community. In the event of an influenza pandemic, the potential benefits of a live attenuated virus vaccine will have to be balanced against the risks associated with it, and this type of vaccine will only be introduced judiciously when a pandemic is imminent *(14)*.

13.7.1.3 Recombinant Subunit, DNA, and Vectored Vaccines

The use of recombinant or expressed proteins of the influenza virus in a vaccine is an attractive option for vaccine development because these approaches do not require handling of HPAI or infectious viruses for vaccine production *(6)*.

Preclinical studies of recombinant HA, NA, and M2 proteins as vaccine antigens (see Table 2 in Ref. *14*) showed that the proteins were poorly immunogenic and required multiple doses *(87)* or the inclusion of adjuvants *(88, 89)* for improved immunogenicity and efficacy. DNA vaccines encoding the HA and NA glycoproteins of avian influenza viruses or conserved internal virus proteins, such as matrix proteins and nucleoproteins, induced protective immunity in mice and chickens *(90–93)*. The protective efficacy of a nucleoprotein-encoding DNA vaccine was increased by a booster vaccination in the form of a recombinant replication-defective adenovirus (rAdV) expressing the nucleoprotein *(94)*. In two recent studies, intramuscular or intranasal immunization of mice with a human rAdV vaccine expressing the influenza virus HA glycoprotein induced both humoral and cell-mediated immune responses and conferred protection against challenge with the wild-type virus in mice and chickens *(95, 96)*. A recombinant baculovirus-expressed H5 glycoprotein subunit vaccine was well tolerated but was poorly immunogenic in humans, indicating the need for an adjuvant *(97)*. The production of recombinant proteins and DNA vaccines is safe and economical, but clinical studies of their safety and immunogenicity in humans are awaited *(14)*.

13.7.1.4 Universal Influenza Virus Vaccines

An ideal influenza vaccine would be effective against a range of virus subtypes and could be useful during pandemic and interpandemic periods. One approach to creating a universal vaccine would be to target an antigenically stable protein or an antigenically stable part of a variable protein that is essential for virus replication *(14)*. The high degree of conservation of the M2 protein makes it a prime candidate for a universal influenza vaccine. The M2 protein induced cross-reactive immunity that decreased the severity of disease in animal models after challenge with wild-type virus *(98, 99)*.

However, the emergence of immune-escape mutants of the M2 protein in mice in the presence of specific antibodies raised concerns regarding the usefulness of the M2 protein as a target for a universal vaccine *(100)*. Clinical studies would be required to evaluate the immunogenicity of the M2 protein in humans *(14)*.

It has been suggested that the use of the NA glycoprotein, which is less variable than the HA glycoprotein, to induce cross-protective immunity should be explored *(101)*. NA-specific immunity in mice provides significant cross-protection against antigenically distinct viruses of the same subtype *(102, 103)*. Although NA-specific antibodies do not prevent infection with influenza viruses, they decrease the severity and duration of illness in humans by limiting the release and spread of the virus *(44, 45)*.

Furthermore, if common immunogenic epitopes are identified within the four clades of HA glycoprotein subtypes, HA-based immunogens could induce widely cross-reactive immunity *(104)*. Alternatively, genetically engineered viruses that have several conserved immunogenic epitopes on the viral envelope could be developed and evaluated for use as a universal influenza vaccine *(101)*. Recombinant viruses expressing chimeric HA glycoproteins have also been described recently *(105)*. Although universal influenza vaccines are still in preclinical development, the potential benefits of such vaccines are so great that strategies to develop them must be encouraged *(14)*.

13.7.2 Immunogenicity of Pandemic Influenza Vaccines

The evaluation of vaccines against potential pandemic strains of avian influenza viruses presents a unique challenge because vaccines developed against these viruses can only be evaluated for safety and immunogenicity, and not for protection, in clinical trials because challenge studies to assess the efficacy of the vaccines cannot be undertaken in humans *(14)*. In addition, when the immunogenicity of candidate pandemic vaccines is assessed, the data can be difficult to interpret because specific information on the nature and magnitude of the antibody response that correlates with protection is lacking. If a vaccine is immunogenic, it might be possible to assess its efficacy by testing the vaccine in a large group of people who are at high risk from infection with avian influenza virus, such as poultry farmers in areas with severe epizootics *(14)*.

Serum and mucosal antibodies can independently mediate immunity to influenza viruses. Live viruses and inactivated virus vaccines differ in the induction of protective antibodies, but there are no standardized methods for evaluating the mucosal antibody response. The conventional assay for assessing the immunogenicity of a human influenza vaccine is the *hemagglutination-inhibition assay*. Although the standard hemagglutination-inhibition assay, which uses chicken or turkey erythrocytes, is relatively insensitive for the detection of antibodies specific for H5N1 viruses, there is a modified assay using horse erythrocytes that was found to be more sensitive because horse erythrocytes exclusively express the α2,3-linked oligosaccharide side chains that are preferred for binding by avian influenza viruses. However, the horse erythrocyte hemagglutination-inhibition assay has not been well standardized, and the antibody titers determined by this assay that correlate with protection are not known. Therefore, the choice of assays by which the immune response is assessed poses a practical challenge for the evaluation of pandemic influenza vaccines *(14)*.

An alternative to the hemagglutination-inhibition assay that might be more biologically relevant is a neutralization assay, in which the ability of antibodies to neutralize the infectivity of the avian influenza virus is assessed *(14)*. Using paired sera from individuals infected with H5N1 influenza virus in 1997 in Hong Kong collected at the acute and convalescent stages of infection, a neutralizing antibody titer of 1:80 was shown to be indicative of infection with an H5N1 virus *(106)*. However, it is not known whether this antibody titer correlates with protection from re-infection. Other barriers to the use of the neutralization assay are the requirement for appropriate biosafety containment measures, as the assay requires handling of the infectious virus, and the fact that the test has not yet been standardized *(107)*.

13.8 Recent Scientific Advances

- *Sialidase Fusion Protein as a Novel Inhibitor of Influenza Virus Infection.* A recombinant fusion protein (DAS181) composed of a sialidase catalytic domain derived from *Actinomyces viscosus* was fused with a cell surface–anchoring sequence. When applied topically via inhalation, DAS181 effectively removed the influenza viral receptors, sialic acids, from the airway epithelium *(108)*. By effectively cleaving the sialic acid receptors used by both the human and avian influenza viruses, DAS181 prevented the virus from binding to and entering the host cells. This is an innovative antiviral strategy because DA181 acts on the receptors used by the influenza virus rather than targeting the virus itself.

- *Large-Scale Sequence Analysis of Avian Influenza Isolates.* Avian influenza is a significant human health threat globally because of its potential to infect humans and result in a global influenza pandemic; however, very little sequence information for avian influenza virus (AIV) has been publicly available. A more comprehensive collection of publicly available sequence data for AIV is

necessary for research in influenza to understand how flu evolves, spreads, and causes disease to shed light on the emergence of influenza epidemics and pandemics and to uncover new targets for drugs, vaccines, and diagnostics. A team of NIAID-supported scientists has released genomic data from the first large-scale sequencing of AIV isolates, doubling the amount of AIV sequence data in the public domain *(109)*. These sequence data include 2,196 AIV genes and 169 complete genomes from a diverse sample of birds. The preliminary analysis of these sequences, along with other AIV data from the public domain, revealed new information about AIV, including the identification of a genome sequence that may be a determinant of virulence. This study provides valuable sequencing data to the scientific community and demonstrates how informative large-scale sequence analysis can be in identifying potential markers of disease.

- *Architecture of Ribonucleoprotein Complexes in Influenza A Virus Particles.* Data from transmission electron microscopy of serially sectioned virions of influenza A viruses have shown that the ribonucleoprotein complexes (RNPs) of the virus are organized in a distinct pattern: seven segments of different lengths surrounding a central segment *(110)*. Furthermore, the individual RNPs are suspended from the interior of the viral envelope at the distal end of the budding virion and are oriented perpendicular to the budding tip. These findings have argued against a random incorporation of RNPs into virions, supporting instead a model in which each segment contains specific incorporation signals that would enable the RNPs to be recruited and packaged as a complete set. The selective mechanism of RNP incorporation into virions and the unique organization of the eight RNP segments may be crucial to maintaining the integrity of the viral genome during repeated cycles of replication *(110)*.

- *Structure and Receptor Specificity of the Hemagglutinin from an H5N1 Influenza Virus.* The hemagglutinin structure at 2.9 Å resolution from a highly pathogenic Vietnamese H5N1 influenza virus (Viet04) has been elucidated *(111)*. Its structure was found to be more related to that of the 1918 *(8)* and other human H1 hemagglutinins than to a 1997 duck H5 hemagglutinin. Glycan microarray analysis of the Viet04 virus revealed an avian $\alpha 2,3$ sialic acid receptor binding preference. Introduction of mutations that can convert H1 serotype hemagglutinins to human $\alpha 2,6$ receptor specificity only enhanced or reduced affinity for the avian-type receptors. However, mutations that can convert avian H2 and H3 hemagglutinins to human receptor specificity, when inserted onto the Viet04 hemagglutinin framework, permitted binding to a natural $\alpha 2,6$ glycan, thereby suggesting a path for this H5N1 virus to *gain a foothold in the human population (111)*.

- *NS1-Truncated Modified Live-Virus Vaccine.* Swine influenza viruses (SIVs) naturally infect pigs and can be transmitted to humans. Furthermore, in the pig, genetic reassortment to create novel influenza subtypes by mixing avian and human influenza viruses *is possible*. Therefore, a vaccine against SIV and inducing cross-protective immunity between different subtypes and strains circulating in pigs will be highly advantageous. To this end, an H3N2 SIV (A/swine/Texas/4199-2/98) (labeled TX98) containing a deleted NS1 gene expressing a truncated NS1 protein of 126 amino acids (NS1/126) was attenuated in swine. Subsequently, 4-week-old pigs were vaccinated with the TX98 NS1/126 modified live-virus (MLV) vaccine. The highly attenuated MVL completely protected against challenge with the homologous SIV *(112)*. Vaccinated pigs challenged with the heterosubtypic N1N1 virus demonstrated macroscopic lung lesions similar to those of the unvaccinated H1N1 control pigs. Remarkably, vaccinated pigs challenged with the H1N1 SIV had significantly less microscopic lung lesions and less virus shedding from the respiratory tract than did unvaccinated, H1N1-challenged pigs. Furthermore, all vaccinated pigs developed significant levels of hemagglutination inhibition and enzyme-linked immunosorbent assay titers in serum and mucosal immunoglobulin A antibodies against H3N2 SIV antigens *(112)*.

- *Immunization by H5 Avian Influenza Hemagglutinin Mutants with Altered Receptor Binding Specificity.* Scientists from NIAID's VRC have developed a strategy to generate vaccines and therapeutic antibodies that could target predicted H5N1 mutants before these viruses evolve naturally. This advance was made possible by creating mutations in the region of the H5N1 hemagglutinin protein that directs the virus to bird or human cells and eliciting antibodies to it *(113)*.

- *Protective Immunity to Lethal Challenge of the 1918 Pandemic Influenza Virus by Vaccination.* Using the genetic sequence information for the 1918 flu virus, VRC scientists have created plasmids—small strands of DNA designed to express specific characteristics—carrying genes for the virus's hemagglutinin protein, the surface protein found in all flu viruses that allows the virus to stick to a host cell and cause infection *(114)*. The researchers created two types of plasmids: one to reflect the HA found in the original 1918 flu virus; the other an altered HA protein designed to attenuate the virus. Mice were then injected with a DNA vaccine containing both types of plasmids to determine whether they would generate immune responses to the 1918 virus. The researchers found significant responses both in terms of the production of T cells and the production of neutralizing antibodies.

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