Avian Influenza

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The current epidemic of H5N1 highly pathogenic avian influenza in Southeast Asia raises serious concerns that genetic reassortment will result in the next influenza pandemic. There have been 164 confirmed cases of human infection with avian influenza since 1996. In 2004 alone, there were 45 cases of human H5N1 in Vietnam and Thailand, with a mortality rate over 70%. In addition to the potential public health hazard, the current zoonotic epidemic has caused severe economic losses. Efforts must be concentrated on early detection of bird outbreaks with aggressive culling, quarantines, and disinfection. To prepare for and prevent increased human cases, it is essential to improve detection methods and stockpile effective antivirals. Novel therapeutic modalities, including short, interfering RNAs and new vaccine strategies that use plasmid-based genetic systems offer promise, should a pandemic occur.

Introduction

Avian influenza is an infectious disease of birds caused by specific strains of influenza A. Avian influenza strains were not recognized to infect species other than birds and swine until 1996, when a single case of conjunctivitis was reported, and 1997, when the first significant human outbreak occurred in Hong Kong. Since 1996, there have been three significant human outbreaks and 164 confirmed human infections with avian influenza. Highly pathogenic avian influenza (HPAI) in domestic poultry has reached epidemic proportions in eight Southeast Asian countries, resulting in the loss of hundreds of millions of chickens, through infection or culling, and huge economic losses. Mutation of the genome and/or genetic reassortment resulting in antigenic shift in avian viruses sets the stage for the emergence of a highly pathogenic strain with pandemic potential. It has been estimated that the next influenza pandemic will result in 207,000 deaths and 734,000 hospitalizations in the United States alone [1]. Avian influenza, especially H5N1, is the infectious agent that is most likely to cause a pandemic, leading to massive fatalities worldwide.

Virology

The influenza viruses, types A, B, and C, belong to the family Orthomyxoviridae. Influenza A is the only type with veterinary significance. The virus is enveloped and contains a genome of eight RNA segments. The segmented genome allows viruses from different species to recombine RNA segments, leading to reassorted virus with surface proteins to which humans are nonimmune. These major changes in glycoproteins, called antigenic shifts, are associated with epidemics and pandemics. Minor changes, or antigenic drifts, are usually associated with annual outbreaks. Antigenic drift results from high mutation rates in hemagglutinin and neuraminidase genes, caused by the poor proofreading ability of RNA polymerase, resulting in amino acid substitutions and quasi-species with optimal chances for environmental adaptation.

Influenza A has two major antigenic surface proteins embedded in the membrane, hemagglutinin (HA) and neuraminidase (NA), that are essential for viral attachment and cellular release. HA enables attachment to new cells via binding to sialic acid residues on respiratory epithelial cells. HA monomers are cleaved by trypsin-like enzymes, which results in a conformational change that activates membrane fusion and entry into acidified endosomes. Cleavability and invasion are impaired by having a carbohydrate side chain at this cleavage site and enhanced by having multiple basic residues directly upstream from this site. HA variability, including extensive post-translational glycosylation, allows evasion of host immune responses. After viral replication, progeny virions are bound to the host cell and cleaved by neuraminidase to liberate new virions.

There are three subtypes of hemagglutinin (H1–H3) and two subtypes of neuraminidase (N1 and N2) that circulate in humans. Avian viruses have 15 HA and nine NA antigenic variants. Other influenza viral proteins are nucleoprotein, the nucleocapsid structural protein, membrane proteins (M1 and M2), polymerases (PA, PB1, and PB2), and nonstructural proteins (NS1 and NS2). NS1 is believed to have anti-interferon properties (Fig. 1).

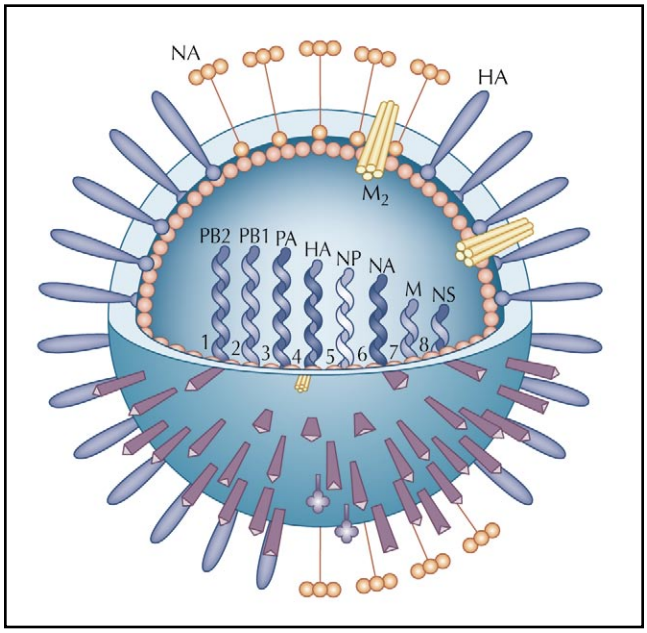

Figure 1. Diagram of influenza A demonstrating hemagglutinin (HA) and neuraminidase (NA) surface proteins and eight genes. M—membrane proteins; M2—matrix protein; NP—nucleoprotein; NS—nonstructural proteins; PA, PB1, PB2—polymerases.

Epidemiology

Avian influenza was first recognized as "fowl plague" in 1878. The first avian virus, subtype H5N3, was isolated in 1961 from a South African tern. Avian influenza virus is found in waterfowl, shorebirds, and gulls. Swine, horses, humans, and sea mammals are aberrant hosts in which increased virulence occurs. Although chicken and turkey isolates were grouped with avian isolates, it may be more appropriate to consider these species as aberrant hosts [2]. Cats have also been found to acquire infection through direct inoculation and/or consumption of infected poultry [3].

Avian influenza viruses have coevolved with their waterfowl hosts, mostly wild ducks, and are relatively avirulent in this bird population. Virus is shed in large quantities by infected waterfowl in saliva, nasal secretions, and feces into bodies of water, providing an ideal mode of perpetuation in the environment. Fecal-to-oral transmission is the most common form of spread between birds. Wild birds are not usually sick, but transmit influenza to other birds. Domesticated birds become infected through direct contact with infected birds, water, or feed, or through people, vehicles, or cages used to move birds from one farm to another. International spread occurs through the live poultry trade and by migratory wild birds that carry infection over long distances. Two additional important reservoirs for spread of avian influenza are live bird markets and commercial swine facilities. The continuing supply of poultry in markets enhances the opportunity for viral mutation and spread to commercial flocks. The close human-to-bird contact facilitates transmission and the opportunity for genetic reassortment.

Poultry have high rates of infection with low virulence virus (up to15% in ducks and geese) that results in mild-or-no illness. Domestic poultry, such as chickens and turkeys, are especially susceptible to epidemics of HPAI, which have a mortality rate of 90% to 100%. Avian H2, H5, H6, H7, H8, H9, and H10 subtypes are most likely to be transmitted to humans. H1N1 and H3N2 subtypes have caused outbreaks in pigs and H7N7 and H3N8 in horses.

The subtypes that routinely cause human influenza are H3N2, H2N2, H1N1, and H1N2. H1N1 caused the Spanish influenza pandemic of 1918 that killed 20 million people and the less lethal 1977 Russian pandemic. Antigenic shift without gene reassortment caused the latter pandemic, which represented reemergence of a strain to which the population was not immune. This isolate was almost identical to H1N1 strains from the 1950s. The H2N2 pandemic in 1957 and the H3N2 pandemic in 1968 had hemagglutinin and PB1 changes similar to those found in avian strains and are thought to have arisen from human–avian reassortant virus.

Human Cases

Human cases of avian influenza have been increasing in frequency. It is unclear whether this represents heightened awareness and surveillance or an actual increase in transmission. Most human cases resulted from contact with infected poultry or contaminated surfaces; rare person-to-person spread has been documented.

The first reported case of human infection with avian influenza occurred in 1996 in a woman who developed H7N7 conjunctivitis [4]. The source was her collection of ducks that mingled with wild waterfowl. In 1997 in Hong Kong, H5N1 infected a 3-year-old boy who died of pneumonia and Reye's syndrome [5]. This virus killed six of 18 infected humans, marking the first confirmed human outbreak with avian influenza. Person-to-person transmission was serologically documented in health care workers.

Prior to 1996, infection was not thought to be transmissible directly from birds to humans, owing to the hypothesized requirement for infection of an intermediate host, such as swine, that has both the human respiratory epithelium receptor (α2-6 sialic acid) and the avian (α2-3 sialic acid) receptor [6]. Swine are thought to be an ideal vehicle for reassortment of viruses with avian and human components, with the potential to generate pandemic strains.

In 1999, an active surveillance system among the live bird markets of Hong Kong revealed H9N2 viruses in the poultry population with hemagglutinin cleavage genes identical to those from the 1997 H5N1 outbreak, indicating a persistent heterogeneous pool for reassortment of pathogenic genes [7]. In 2001, the World Health Organization adopted the Global Agenda for Influenza Surveillance and Control to increase surveillance, knowledge of disease burden, vaccine use, and pandemic preparedness.

Table 1. Human cases of avian influenza*

Year	Place	Subtype	Source	Human Cases	Mortality, %
1996	United Kingdom	H7N7	Domestic ducks	One case of conjunctivitis	0
1997	Hong Kong	H5N1	Poultry	18 infected, six deaths; rare PTP transmission	33
1999	China and Hong Kong	H9N2	Poultry	Two children, no deaths	0
2002	Virginia	H7N2	Poultry	One person with documented seroconversion	0
2003	Hong Kong	H5N1	Travel to China	Two family members, one death; both contacts of a third case who died of an undiagnosed respiratory illness	50
2003	Netherlands	H7N7	Poultry	89 cases: 78 conjunctivitis, five influenza with conjunctivitis, two influenza, four "others"; one death in a veterinarian; possible PTP transmission	0.01
2003	Hong Kong	H9N2	Unknown	One child, survived (CDC); three children, survived (WHO)	0
2003	New York, U.S.	H7N2	Unknown	One adult, survived	0
2004	Thailand	H5N1	Poultry	17 cases, 12 deaths; one case of PTP transmission	70.5
2004	Vietnam	H5N1	Poultry	28 cases, 20 deaths	75
2004	British Columbia, Canada	H7N3	Waterfowl	Two influenza, three conjunctivitis; 10 unconfirmed cases	0
2004	Egypt	H10N7	Poultry	Two cases in infants, no deaths; father of one is a poultry merchant	0

*Data reported by the US Centers for Disease Control and Prevention (CDC) and World Health Organization (WHO).
PTP—person-to-person.

Since 1996, 164 documented infections with avian subtypes have occurred in humans (Table 1). Person-to-person transmission was documented serologically in the 1997 Hong Kong outbreak and the Netherlands outbreak. Serologic data from the Netherlands outbreak showed that at least 50% of persons exposed to infected poultry had antibody to H7. The seroprevalence of persons who were close contacts of infected poultry workers was 59% [8].

The last confirmed avian influenza human case in 2004 was a 16-year-old Vietnamese girl, and was associated with an active poultry outbreak. During 2004, there were 45 cases of avian influenza in Asia and 32 fatalities. Of the 15 avian influenza virus subtypes, H5N1 is of particular concern because the virus mutates quickly, has a documented propensity to acquire genes from viruses infecting other animal species, and causes severe disease in humans. It has been demonstrated that a series of genetic reassortments caused the outbreak of H5N1 in 1997, avian outbreaks in 2001 and 2002, and the 2004 H5N1 outbreak in Southeast Asia. Domestic ducks in China are thought to play a role in generation and maintenance of this subtype.

Swine influenza (H3N2) has been recognized for decades, and spread to turkeys has been documented. Since 1979, human H3N2 has circulated along with avian H1N1 in swine, and reassortant virus was detected. The human H3N2 subtype is now circulating in swine in southern China, raising the risk of reassortment with endemic avian virus [9•]. Although such reassortant virus in swine has not been documented as the cause of any epidemic, it was detected in a small number of children in the Netherlands [10]. Figure 2 shows the species specificity and possibilities for genetic reassortment within and between species.

Pathogenesis

In humans, clearance of influenza A is linked to the appearance of circulating interferons (IFN) and tumor necrosis factor-α (TNF-α). Patients experimentally infected with H1N1 had elevated levels of interleukin–6 (IL-6) and IFN-α in nasal lavage fluid that peaked at day two and correlated with viral titers, temperature, and symptoms. TNF-α and IL-8 peaked later, after symptoms and viral shedding had decreased [11]. Patients with H5N1 disease studied in 2003 had very high levels of inflammatory cytokines including interferon-induced protein-10 and monokine-induced by IFN-γ. Monocyte chemotactic protein-1, RANTES, and interleukin 8 were not higher than in uncomplicated, non-H5N1 influenza. Two autopsied cases of fatal H5N1 infection from 1997 showed a reactive hematophagocytic syndrome with elevated levels of inflammatory cytokines (IL-6, TNF-α,

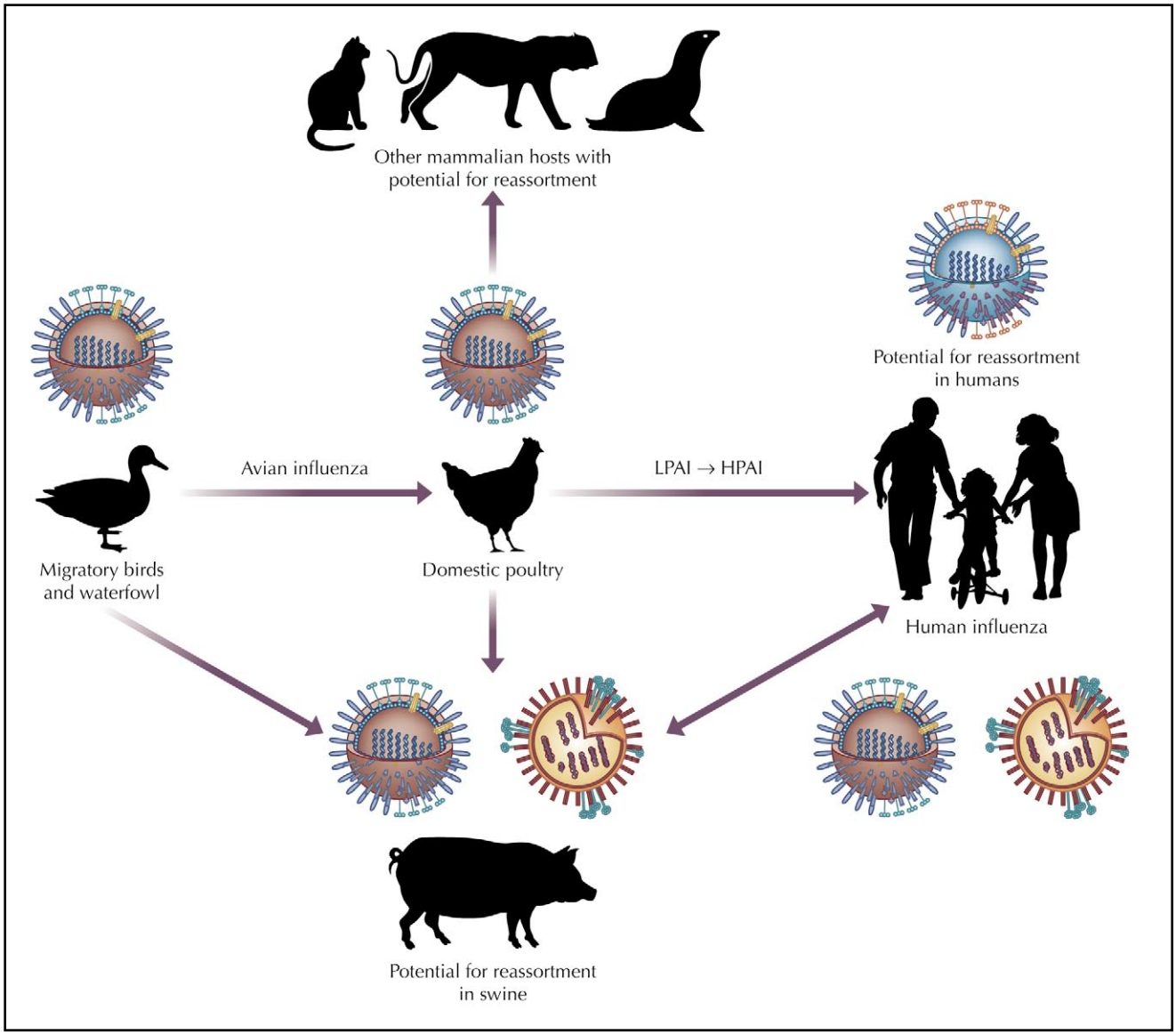

Figure 2. Potential mechanisms of H5N1 influenza A reassortment resulting in a virus that is highly pathogenic to humans. Reassortment could take place in swine, humans, and, possibly, other mammals, such as wild and domestic cats. HPAI—highly pathogenic avian influenza; LPAI—low-pathogenic avian influenza.

and IFN-γ). H5N1 was isolated only from the respiratory tract of patients, suggesting that high levels of cytokines generated in response to contain the virus caused the widespread disease with limited viral detection [12]. H5N1 also induced a higher transcription of proinflammatory cytokines, especially TNF-α, compared with H3N2 and H1N1 in human primary monocyte-derived macrophages. The levels of TNF-α were comparable to levels in macrophages exposed to lipopolysaccharide [13•]. Pigs infected with H1N1 carrying the *NS* gene from H5N1 had more severe disease and a longer course, suggesting that the *NS* gene confers resistance to IFNs and TNF-α, resulting in viral escape from their natural antiviral effects [14]. In human macrophages, the human H5N1 isolates from 1997 were more potent inducers of proinflammatory cytokines, especially TNF-α, than human H1N1 or H3N2 subtypes [15••]. These studies all suggest that increased levels of proinflammatory cytokines play a major role in the pathogenicity of H5N1 in humans.

The HA of HPAI possesses multibasic amino acid sequences that can be cleaved throughout the body by tissue proteases as opposed to human strains in which cleavage by trypsin-like proteases occurs in the respiratory and enteric tracts. The former leads to an infectious virus that can spread to multiple organs and lead to systemic infection. By contrast, avirulent virus produces limited infection in the respiratory or intestinal tracts. In a mouse model, Hatta et al. [16] demonstrated that high cleavability of the 1997 isolate caused by a mutation in the PB2 protein was essential for lethal infection.

Human infection with avian subtypes depends upon the ability of virus to bind cellular receptors. Avian viruses are tropic for ciliated and human viruses for nonciliated respiratory epithelium. Infection of nonciliated human epithelial cells is essential for replication of avian virus and may decrease the fitness of these subtypes, explaining the low rates of human infection and person-to-person transmission. Alteration of receptor specificity with ciliated human respiratory epithelium permissive to both avian and human viruses is needed to generate a pandemic virus [17].

There is also evidence that low pathogenic avian viruses can mutate within the avian host into a highly pathogenic strain. Ito [18] demonstrated that serial passage of an avirulent swan influenza virus through air sacs and chick brain inoculation led to development of a highly pathogenic virus. The original virus grew poorly in chicks, but the new isolate had a mortality rate 50% higher, suggesting that with the proper selection pressure, virulent virus could arise naturally in wild birds.

Clinical Features

Avian

Most birds are asymptomatic. In some flocks, the only evidence of infection is seroconversion. Infection may cause a respiratory, enteric, central nervous system, or reproductive illness. Decreased egg production and decreased oral intake are the first signs of infection. Other symptoms include coughing, sneezing, ruffled feathers, swollen heads, and diarrhea. With HPAI, birds may die quickly without manifesting any clinical illness. HPAI with subtypes H5 and H7 is responsible for the current zoonotic epidemic in Southeast Asia.

Human

The symptoms of avian influenza in humans are variable. In the current experience from Thailand, the median time from exposure to onset of illness was three days. The mildest syndrome is a self-limited upper respiratory tract infection. The typical human influenza syndrome of fever, cough, sore throat, and myalgia may also occur. Conjunctivitis, with or without respiratory tract symptoms, is associated with the H7 subtype.

In the 1997 Hong Kong H5N1 influenza outbreak involving 18 patients, exposure to live poultry was the most significant risk factor for infection. Reported findings included conjunctivitis, diarrhea, sore throat, pneumonia, acute respiratory distress syndrome, multisystem organ failure, and death. Rapid deterioration was common, and patients with lower respiratory involvement required intubation within several days. The case fatality rate was 33% in 1997 and over 70% in the current 2004 Asian H5N1 outbreak. Reported laboratory abnormalities include lymphopenia, transaminase elevation, and abnormal coagulation assays [5,19•]. Chest radiograph findings were variable, including diffuse infiltrates and lobar consolidation. Severe disease was associated with elevated age, delayed hospitalization, lower respiratory tract involvement, and leukopenia or lymphopenia. As opposed to other forms of influenza, superimposed bacterial pneumonia has not been reported in avian influenza in humans [20••]. A recent case of avian influenza with fever and diarrhea in the absence of respiratory symptoms has been reported from Thailand [21], which broadens the symptom complex in human infection.

Other mammalian species

Fatal avian influenza has recently been confirmed in two tigers and two leopards in a Thailand zoo that were fed fresh chicken carcasses from a local slaughterhouse. The animals developed fever and respiratory distress. Reverse transcriptase polymerase chain reaction (RT-PCR) for H5N1 was positive in lung samples. These findings extend the host range of avian H5N1 and raise the question as to whether wild cats can serve as hosts for adaptation of the virus to mammals [22]. Domestic cats, known to be infected through horizontal transmission or by feeding on wild or domestic birds, are another possible host for viral reassortment [3].

Diagnosis

The diagnosis of avian influenza should be considered in persons with atypical pneumonia, upper respiratory diseases with and without conjunctivitis, diarrhea, and fever in association with a travel and epidemiologic history of avian exposure.

Diagnostic tests for influenza include viral culture, serology, rapid antigen testing, PCR, and immunofluorescence. Viral culture in embryonated chicken eggs with HA and NA subtyping is the gold standard for diagnosis. Culture is labor-intensive and time consuming, and requires biosafety laboratories. Rapid diagnostic tests are available that detect avian influenza, but tests differ in the types of influenza viruses they can detect. The specificity and sensitivity of rapid tests that detect H5N1 are lower than viral culture. Molecular techniques such as RT-PCR and real-time reverse transcriptase-PCR (RRT-PCR) are highly sensitive. The RRT-PCR is a rapid, one-step assay that detects H5 and H7 subtypes and has been used in commercial flocks [23]. The nucleic acid sequence-based amplification with electrochemiluminescent detection uses H5N1 virus isolated from blood or anal swabs of chickens and has a sensitivity equal to viral culture [24].

Serologic testing, including virus neutralization, ELISA and Western blotting, is useful for epidemiologic surveillance studies and retrospective diagnosis. Hemagglutination inhibition (HI) tests are insensitive in detecting H5 and H7 after natural infection or vaccination due to the different sialic acid receptors. The use of horse erythrocytes increased the detection of HI subtypes in one study [25].

Prevention and Vaccines

The availability of vaccine and anti-influenza drugs is limited by insufficient production capacity. Vaccine production takes advantage of the virus' ability to reassort, using six specific gene segments derived from the H1N1 A/Puerto Rico/8/34 strain that enable growth in chicken eggs and gene segments containing the necessary antigenic determinants. Natural reassortment takes place within embryonated chicken eggs, and strains with the required HA and NA genes are selected. This process takes approximately 6 months and requires a steady supply of eggs. Unfortunately, H5 and H7 cannot be grown in chicken eggs due to their ability to accumulate basic residues at the site of hemagglutinin cleavage, which is lethal to chicken embryos [26,27••].

Plasmid-based reverse genetic systems could expedite production of H5 and H7 vaccine seed strains. Virus with copies of each of the eight RNA segments could be generated from individually cloned cDNA to viral RNA. Removal of additional HA site amino acids facilitates generation of vaccine in eggs. These segments are added to the plasmid encoding the genes of the base vaccine and added to cell lines [28]. This process produces reassortants carrying several different HA and NA glycoproteins. The African green monkey kidney cell line, Vero, is one of the few cell lines that meets all criteria for international use and is WHO-approved. Vaccine manufacture has been hampered by safety issues and fear of litigation over adverse events and disease in laboratory personnel exposed to strains to which they have no immunity.

Low-pathogenic avian influenza veterinary vaccine decreases morbidity, mortality, and viral shedding in domestic poultry but does not eradicate virus entirely. It is important to differentiate vaccinated-infected from vaccinated-uninfected livestock, which is known as the DIVA strategy [29]. This strategy was used successfully during the Italian H7N1/ H7N3 epidemic in 2000 to 2003. An inactivated oil emulsion heterologous vaccine was used with field type HA and a different NA; antibody to field type NA was used as a marker of disease exposure. As of October 2004, Hong Kong required vaccination of all poultry, and was free of H5N1 during the Chinese poultry outbreak in early 2004. China and Indonesia use selective vaccination in endemic areas, but Thailand declined [30].

The key to containing a bird outbreak is culling, or rapid destruction of infected birds with proper disposal of carcasses, followed by quarantining and disinfection. Use of personal protective equipment is mandatory by all persons involved in culling activities. Avian influenza is killed by heat and common disinfectants, but can survive in manure for at least 3 months in cool temperatures. All organic material must be completely removed and disinfected. Preventing direct contact between free flying birds and domestic poultry is a harder, but crucial, step in containing infection.

The US Centers for Disease Control and Prevention advises travelers to countries in Asia with documented H5N1 outbreaks to avoid poultry farms and contact with animals in live markets. Humans presenting with an unexplained or severe respiratory illness within 10 days of travel to an endemic area should be considered for respiratory isolation. Avian influenza viruses are heat-inactivated (70°C), and there is no evidence that transmission occurs secondary to consumption of cooked poultry.

Treatment

There is limited experience regarding treatment of avian influenza in humans [12,19•,31•,32]. Of 12 reported cases, two received no specific therapy (one died), seven received antiviral alone (4 amantadine, 3 oseltamavir, with five deaths), and three received antivirals with steroids (2 oseltamavir and steroid, 1 amantadine and steroid, with two deaths).

Oseltamavir, a neuraminidase inhibitor (NI), is efficacious against H5N1 and H9N2 viruses in mice at doses of 1 mg/kg/day (even when administered up to 36 hours after exposure), and in canine kidney cells [33]. Zanamavir, another NI, was effective in preventing H9N2 infection in doses of 50 mg/kg. Zanamavir decreased the morbidity from H6N1 and H5N1 infection, decreased viral titers in lung, and prevented viral spread to brain in the mouse model [34]. Oseltamavir should be effective in prophylaxis, but there are no available data. Amantadine and rimantidine resistance is widespread among H5 subtypes and has been reported in birds that received prophylaxis with these drugs. In the event of a pandemic, the current supply of effective anti-influenza drugs would not be sufficient for containment.

Short interfering RNAs (siRNAs) are a novel investigational class of molecules. SiRNAs are synthetic RNA duplexes that cause transient interference of gene expression in a sequence-specific manner. SiRNAs have been shown to inhibit influenza A production in cell cultures and embryonated chicken eggs. Intravenous or intranasal administration of siRNAs in a polycation carrier decreased virus production in mice before and after infection. SiRNAs specific for genes encoding nucleocapsid protein and components of PA and PB1 block accumulation of all viral RNAs, which makes them as potent as current antivirals for treatment of influenza [35•].

Conclusions

The large reservoir of epidemic H5N1 in Asia represents an ongoing danger to human health worldwide. Although aggressive attempts at controlling outbreaks among poultry, more sensitive influenza detection methods, increasing effective antiviral supplies, increasing efficient modes of vaccine development, and new drugs, such as siRNAs, are promising, many believe that an influenza pandemic, likely from H5N1, is inevitable. Cohabitation of birds, swine, and humans in parts of Asia provide

the potential for gene reassortment and development of pandemic virus with efficient dissemination. The low transmission rate could quickly change if a shift in receptor specificity of the avian virus to nonciliated cells occurs. Avian influenza viruses must also be considered to be potential weapons for bioterrorists. Although a pandemic may be inevitable, vigorous efforts in vaccine development, detection, and treatment must be pursued to decrease the chances of its occurrence and decrease morbidity and mortality if an epidemic occurs.

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This is an interesting paper in which the novel technology of short interfering RNAs as potential new anti-influenza agents is discussed.