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Review

Avian influenza viruses infecting humans

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Abstract. Avian species, particularly waterfowl, are the natural hosts of influenza A viruses. Influenza viruses bearing each of the 15 hemagglutinin and nine neuraminidase subtypes infect birds and serve as a reservoir from which influenza viruses or genes are introduced into the human population. Viruses with novel hemagglutinin genes derived from avian influenza viruses, with or without other accompanying avian influenza virus genes, have the potential for pandemic spread when the human population lacks protective immunity against the new hemagglutinin. Avian influenza viruses were thought to be limited in their ability to directly infect humans until 1997, when 18 human infections with avian influenza H5N1 viruses occurred in Hong Kong. In 1999, two human infections with avian influenza H9N2 viruses were also identified in Hong Kong. These events established that avian viruses could infect humans without acquiring human influenza genes by reassortment in an intermediate host and highlighted challenges associated with the detection of human immune responses to avian influenza viruses and the development of appropriate vaccines.

Key words. Influenza; avian influenza; pandemic; H5N1; genetic features; pathogenesis.

Introduction

The natural hosts of influenza A viruses are avian species, particularly aquatic birds. Influenza A viruses bearing each of the 15 hemagglutinin (HA) and nine neuraminidase (NA) subtypes infect avian species. Most of these infections are not associated with clinical disease in waterfowl, and the viruses are thought to be in evolutionary stasis in these hosts [1]. In contrast, relatively few influenza A subtypes have caused sustained outbreaks of disease in humans; viruses bearing H1, H2 and H3 HA genes and N1 and N2 NA genes have circulated in the human population during the 20th century. H1N1 viruses appeared in 1918 and circulated till 1957; H2N2 viruses replaced them in 1957 and circulated till 1968; and H3N2 viruses appeared in 1968 and continue to circulate till the present time. In 1977, H1N1 viruses reappeared in the human population and have continued to cocirculate with H3N2 viruses. In 1918, 1957 and 1968, influenza A viruses with a novel HA gene (with or without an accompanying novel NA gene) spread around the world through a population that lacked immunity to the novel HA, causing pandemics that were associated with significant morbidity and mortality [2].

The influenza virus genome is composed of 8 segments of negative-sense RNA that encode 10 proteins. The segmented nature of the influenza virus genome confers the ability for genetic reassortment between the genomes of two viruses that coinfect a host cell. Birds that are asymptomatically infected but excrete high titers of influenza virus in their feces serve as a reservoir of influenza A virus genes for the human population [1]. Influenza B and C viruses also circulate in the human population, but are not divided into subtypes and do

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not pose a pandemic threat, because there is little evidence for a nonhuman reservoir for these viruses.
Analysis of the pandemic strains of 1957 and 1968 indicates that they arose due to reassortment between an avian influenza A virus and the circulating human influenza A virus [3]. Experimental evidence also suggested that the infectivity and virulence of wholly avian influenza A viruses were limited in humans [4]. Isolated cases of human infections with avian influenza viruses were reported in the literature [5, 6], but the host range of avian influenza viruses was thought to limit their spread to humans. It was believed that avian influenza viruses per se were not a threat for humans, and that potential pandemic strains would be reassortant viruses that derived one or more gene segments from avian viruses. The recent documentation of human infections with avian influenza A H5N1 [7, 8] and H9N2 [9] viruses in Hong Kong challenged these beliefs. The H5N1 infections were particularly serious [10] and raised concerns about potential pandemic spread. This review deals with the epidemiology, reported clinical illnesses, genetic features, immune responses, pathogenicity and implications of avian influenza A viruses infecting humans, with particular emphasis on the H5N1 viruses isolated in Hong Kong.

Epidemiology and clinical illness

With the exception of the H5N1 outbreak in Hong Kong in 1997 in which 18 cases of clinical illness were documented, information about avian influenza viruses infecting humans is limited to case reports and experimental infections. The available data are summarized below. Seroprevalence studies are discussed in the section dealing with the immune response to avian viruses, since the interpretation of these studies depends on the assays used for the serologic survey.

Experimental infections

Forty healthy human volunteers aged 18–50 years were administered $10^{6.8}$ to $10^{9.2}$ 50% egg infectious doses (EID_{50}) of 10 avian influenza viruses, including viruses of the following subtypes: H1N1, H3N2, H3N8, H4N8, H6N1, H6N2, H9N2 and H10N7. Viruses were recovered from the nasal washings of 11 of the 40 volunteers; these 11 cases were 3 of 14 who had received the H4N8, 2 of 11 who received the H6N1 and 6 of 15 who received the H10N7 viruses. Virus recovery was accompanied by mild local respiratory tract symptoms. Virus recovered from the nasal washings from a volunteer who received the H6N1 virus could not be successfully passed to 5 other volunteers. A serum hemagglutination-inhibition (HI) antibody response to infection was not detected in individuals from whom H4, H6 or H10 viruses were recovered. However, $\geq$4-fold rises in serum HI antibody titers were detected in 1 of 5 avian H1N1, 3 of 6 avian H3N8, 1 of 3 avian H3N2 and 1 of 6 H9N2 virus recipients in whom virus shedding was not observed [4]. Using recovery of virus or detection of an antibody titer rise to define infection, only a proportion of human subjects were infected with these avian viruses, even when they were administered as relatively high-titered inocula. This study, therefore, indicated that avian influenza A viruses were limited in their ability to infect humans.

Clinically significant infections

Infections caused by fowl plague virus (FPV, H7N7). In 1959, a fowl plague-like virus was isolated from blood obtained from a man who was hospitalized with clinically diagnosed infectious hepatitis [11, 12]. The patient was a 46-year-old man who had traveled extensively for 2 months and developed symptoms in the month after his return. Details of the clinical course of the patient's illness were not reported. A virus was isolated from his blood clot on two occasions [11, 12] which, in both instances, was lethal for chicken embryos and for chickens inoculated with the allantoic fluid. The virus isolated from chicken embryos was characterized as a fowl plague virus on the basis of electron microscopy, serologic assays and by tests for pathogenicity in chickens. The virus was antigenically related to FPV and was able to protect chickens from subsequent challenge with FPV. There was some concern about the possibility that FPV was a contaminant in the chicken embryos inoculated with the blood clot; however, the agent was reisolated 6 months later. Campbell et al. indicated that although it was not clear that the agent isolated from this patient's blood was responsible for his clinical syndrome, the case had significant implications for veterinary scientists [12].
Conjunctivitis. Three reports of self-limiting conjunctivitis associated with avian influenza A H7N7 viruses have appeared in the medical literature. The first was a case of keratoconjunctivitis that occurred following an accidental laboratory exposure [13]. The infection occurred in a healthy 24-year-old laboratory technician who was accidentally exposed while she was harvesting allantoic fluids of eggs infected with FPV. About 26 h after exposure, the patient developed conjunctivitis characterized by follicle formation and mucopurulent discharge, and a conjunctival swab yielded FPV in viral culture. The patient's illness was complicated by keratitis, but both the conjunctivitis and keratitis, resolved completely over 2–3 weeks [13].

The second was an H7N7 virus, isolated from a conjunctival swab obtained from a laboratory worker with a case of severe unilateral conjunctivitis, who was sneezed upon by a seal infected with an H7N7 influenza virus, A/Seal/MA/1/80. The patient recovered in about 4 days without further complications [5]. Four other people involved in seal autopsies developed conjunctivitis associated with intense periorbital pain and swelling lasting 4–5 days, but virus cultures were not obtained from these individuals [5].

The third documented incident was an H7N7 virus isolated from a conjunctival swab from a 43-year-old housewife who presented with unilateral conjunctivitis and a swollen eyelid. The patient had recently cleaned out a duck house in which she kept 26 ducks of various species that mingled freely with wild ducks and geese at a nearby lake. There was a history of a possible abrasion caused by a piece of straw in her eye. In this case, too, an uneventful recovery occurred in about 4 days [6]. These reports indicated that avian H7N7 viruses could cause conjunctivitis following direct exposure or inoculation.

H5N1 infections. In May 1997, a previously healthy 3-year-old boy in Hong Kong presented to his physician with a febrile respiratory tract illness. His symptoms worsened on supportive care and aspirin, and he was hospitalized. An influenza A virus was isolated from a throat swab obtained on day 10 of his illness. The patient's condition continued to worsen, and he died on day 16 of complications, including adult respiratory distress syndrome and Reye's syndrome [8].

The virus (fig. 1) could not be typed with reagents designed to identify human H1N1 and H3N2 influenza A viruses but was subsequently identified by reference laboratories as an influenza A H5N1 virus [7, 8]. An epidemiologic investigation of the case was undertaken when the virus was subtyped, but several months had passed since the child's illness and death. The child attended kindergarten, where he was potentially exposed to some chicks and ducklings in a nature court; some of the chicks and ducklings developed yellow diarrhea and died in the weeks preceding the child's illness [8]. Attempts to recover infectious agents from the nature court several months later were unsuccessful.

In November and December 1997, 17 additional cases of laboratory-confirmed H5N1 infections occurred in residents of Hong Kong. Including the first case in May, there were six fatalities. The patients ranged in age from 1 to 60 years, and the fatal cases were in patients 3, 13, 25, 34, 54 and 60 years of age. The clinical features of 12 cases were reported by Yuen et al. [10]. H5N1 virus-infected patients presented with fever and upper or lower respiratory tract syndromes that were clinically indistinguishable from human influenza A H1N1 or H3N2 infections, but the rate of complications was higher. Some of the complications seen were adult respiratory distress syndrome, renal failure, hemophagocytosis, leukopenia and lymphopenia. The risk factors for severe disease included older age, longer duration of symptoms prior to admission, pneumonia, leukopenia and lymphopenia. Although the presence of gastrointestinal (GI) manifestations, liver and renal dysfunction and hematologic disorders suggest wider tissue tropism of the H5N1 virus compared with H1N1 or H3N2 influenza viruses, there was no evidence of viral replication outside the respiratory tract [10].

Several epidemiologic studies were undertaken during the outbreak to identify risk factors for H5N1 disease and to determine whether person-to-person transmission of H5N1 influenza occurred in Hong Kong. A case-control study of 15 patients infected with H5N1 influenza identified exposure to live poultry, by visiting a retail stall or a market selling live poultry in the week prior to onset of illness, as a significant risk factor for the development of H5N1 disease. However, travel, eating or preparing poultry products and recent exposure to persons with respiratory illness were not associated with H5N1 disease [14]. A cohort study of 51 household contacts of 16 H5N1-infected patients

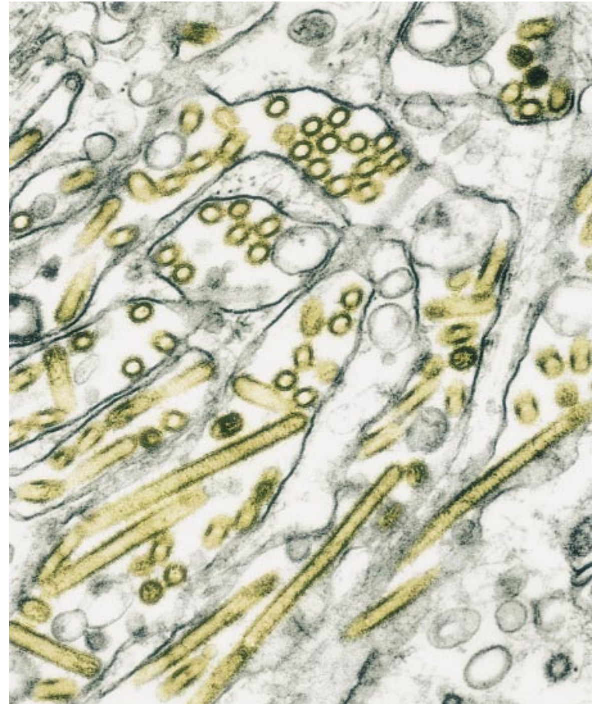

Figure 1. Electron micrograph of human influenza A/Hong Kong/156/97 (H5N1) grown in Madin Darby canine kidney cells. Courtesy of Cynthia Goldsmith and Thomas Rowe, Centers for Disease Control and Prevention (CDC).

identified one individual, with no history of poultry exposure, who likely became infected as a result of person-to-person transmission [15]. Nevertheless, results from cohort studies of co-workers or members of a tour group exposed to case patients provided no evidence for person-to-person transmission of H5N1 virus in these social settings [15]. More convincing evidence for person-to-person transmission came from a retrospective cohort study comparing healthcare workers in Hong Kong who were exposed or not exposed to H5N1-infected patients. Eight of 217 (3.7%) exposed and 2 of 309 (0.7%) nonexposed healthcare workers were seropositive for antibody to H5N1 ($P = 0.01$) [16]. Seroconversion was documented in two healthcare workers, one of whom reported a respiratory illness 2 days after exposure to an H5N1-infected patient. Among the exposed healthcare workers, seropositive individuals were more likely to have bathed a case patient ($P = 0.01$) or changed a case patient's linen ($P = 0.05$) compared with seronegative individuals.

Outbreaks of avian influenza associated with high mortality were reported in three poultry farms in the New Territories of Hong Kong in March 1997, 2 months before the first human case was identified [17]. Chickens on these farms were destroyed. Again, during the outbreak of human H5N1 disease in November and December, there were concomitant outbreaks of severe systemic illness due to H5N1 among chickens in the poultry markets and on farms [18]. The H5N1 viruses isolated from humans and from chickens were highly pathogenic for experimentally infected chickens [8, 17].

In summary, the H5N1 infections in humans were clinically significant and were associated with a high fatality rate. The virus was widespread in poultry and was not efficiently transmitted. The human cases likely resulted from poultry-to-human transmission. The genetic features of the H5N1 viruses are discussed later in this review.

H9N2 infections. In March 1999, H9N2 influenza viruses were isolated from nasopharyngeal aspirates of two children hospitalized in Hong Kong with mild, self-limiting febrile illnesses [9]. The first patient was a 4-year-old girl with a history of eczema and asthma, and the second was a 13-month-old whose past medical history was remarkable for failing to thrive. Both children presented with fever and pharyngitis. The notable laboratory result in the first patient was a mild lymphopenia, while the second patient showed a mild elevation of serum aspartate aminotransferase. In both cases, the illnesses resolved without complications in 5–6 days [9].

The two patients were admitted to different hospitals in different parts of Hong Kong; one patient had a history of possible exposure to chickens in the weeks prior to her illness. H9N2 viruses were known to be circulating among chickens and other avian species in the live bird markets [19]. However, infection of chickens with H9N2 viruses was not associated with the severe morbidity and mortality seen in chickens infected with H5N1 viruses. As many as five additional cases of human H9N2 infections have been reported from China [20]. As in the 1997 outbreak of H5N1 infections in humans, the human H9N2 infections may have been transmitted from birds to humans, but the illnesses associated with the H9N2 infections were mild.

Genetic features of avian influenza viruses infecting humans

The molecular determinants of infectivity, virulence and transmissibility of avian influenza viruses have been the subject of several studies. Determinants of phenotypes, such as virulence and host range, are complex and are likely specified by more than one residue in more than one gene. Several genes have been implicated as determinants of virulence of avian influenza viruses for chickens or mice [21–23]. The polymerase and M genes have been implicated as host range determinants of avian influenza viruses [24–27]. Comments in this review are limited to data regarding these phenotypes in avian influenza viruses infecting primates and humans. Complete genetic analyses of the 10 avian influenza viruses administered to healthy human volunteers or of two of the fowl plague infections are not available [4, 11, 12]. All gene segments of the H7 viruses that caused the second and third cases of conjunctivitis [5, 6] were of avian origin [28], but these cases were localized ocular infections that likely resulted from direct inoculation. The H5N1 viruses isolated in Hong Kong in 1997 have been studied extensively.

Virulence determinants

Two significant molecular motifs have been reported in the HA genes of highly pathogenic avian influenza A viruses (H5 and H7 subtypes): the presence of several basic amino acids in the connecting peptide of the HA [29–32] and the loss of a glycosylation site [33]. The relationship between carbohydrate in the stalk of the HA and the length of the connecting peptide is a critical determinant of cleavability of the HA [34]. The HA molecule must be cleaved into HA1 and HA2 domains for infection to proceed. This cleavage occurs at the arginine residue in the HA1 domain that precedes the first residue (glycine) of the HA2 domain in all HA subtypes [35]. Trypsin-like proteases that cleave the influenza HA and lead to infection of the epithelial cells are present in the respiratory and gastrointestinal tracts of birds [31]. Substitutions to or insertions of basic

amino acids have been observed in the connecting peptide of highly pathogenic avian influenza H5 and H7 HA genes that allow proteases other than trypsin-like proteases to cleave these HA molecules. It is believed that the presence of several basic amino acids in the connecting peptide increases the tissue range of the highly pathogenic avian viruses and results in replication of the virus in multiple organs and severe, usually fatal systemic disease in chickens [31]. As noted below, the HA genes of the H5N1 viruses isolated from chickens and humans in Hong Kong had a multibasic amino acid insertion in the connecting peptide, but the significance of this motif as a virulence determinant for infections in humans is not known.

Determinants of host range

The host range of a virus can be limited due to receptor specificity, which determines attachment to the receptor or release of progeny virions, or at other steps in viral replication. Binding of the HA to its sialic acid receptor is the initial event in influenza infection. There are differences between the receptor specificities of avian and human influenza viruses, which are proposed to determine the host range of avian and human influenza A viruses. Most avian influenza A viruses bind preferentially to the *N*-acetylneuraminic acid-α2,3-galactose linkage, and human influenza A viruses bind the NeuAc-α2,6-galactose linkage on sialyloligosaccharides [36]. The receptor specificity of avian influenza viruses can change during adaptation in pigs [37]; this observation supported the hypothesis that pigs serve as intermediate hosts for adaptation and reassortment of avian and human influenza viruses [38]. The viral NA removes sialic acid, and the specificity of the NA must match that of the HA [39]. The avian N2 NA has acquired the ability to hydrolyze the NeuAc-α2,6-galactose linkage during its evolution in humans [39, 40].

In the 1970s and 1980s, several avian influenza A viruses were evaluated as donors of attenuating genes for potential vaccine candidates [41]. The rationale was as follows: certain avian influenza A viruses, such as A/Mallard/NY/6750/78 and A/Pintail/Alberta/119/78, were restricted in replication in the respiratory tract of squirrel monkeys, and this phenotype was likely determined by the nonglycoprotein genes of these viruses. It was anticipated that reassortant viruses bearing the HA and NA genes of circulating human influenza A viruses and the internal genes from the avian influenza A virus parent would elicit a protective immune response directed against the HA and NA of the human influenza virus and yet would be restricted in replication. In clinical trials, reassortant viruses bearing human H1N1 or H3N2 surface glycoprotein genes and internal genes of A/Mallard/NY/6750/78 were safe and immunogenic in adults and older children [42, 43]. However, when it was observed that the H1N1 reassortant vaccines were associated with significant reactogenicity in young children [44], this approach to the generation of live attenuated influenza vaccines was abandoned [42]. The generation and evaluation of avian-human reassortant influenza A viruses was nevertheless instrumental in identifying some molecular determinants of host range. In order to determine which gene segments were responsible for restriction of replication, a series of reassortant viruses bearing different constellations of avian influenza genes were generated and evaluated in squirrel monkeys. The M and NP genes of the A/Mallard/NY/6750/78 virus, alone or in combination with other avian influenza genes, conferred the phenotype of restricted replication in the respiratory tract of squirrel monkeys [27, 45, 46]. The PB2 gene of the A/Mallard/NY/78 virus conferred the phenotype of restriction of replication in mammalian cells in vitro [26].

Most of the earlier data regarding hostrange and virulence determinants were based on evaluation of reassortant viruses bearing different constellations of influenza virus gene segments. Recent advances in plasmid-based reverse genetics [47, 48] will allow researchers to manipulate the influenza virus genome, leading to more definitive studies on hostrange and virulence determinants.

Source and origin of the H5N1 viruses

Molecular analysis of the H5N1 viruses established that all the genes were derived from avian influenza viruses, and there was no evidence for reassortment with human influenza A viruses. Genetic analysis of cocirculating influenza viruses was undertaken to determine the origin of the genes of the Hong Kong H5N1 viruses.

In 1996, H5N1 viruses were isolated from geese during an outbreak of disease associated with 40% mortality in Guangdong province in China. The H5 HA gene of influenza A/Goose/Guangdong/1/96 was highly related to the HA of the Hong Kong H5N1 viruses (98.8% homology at the nucleotide level and 98.9% homology at the amino acid level, fig. 2). The remaining genes were less closely related (ranging from 90.4% for the NA gene to 97.8% for the NS gene), and the N1 NA lacked the 19-amino-acid deletion seen in the Hong Kong H5N1 viruses [49]. The internal genes of the Hong Kong H5N1 viruses were closely related to those of an H9N2 virus that was isolated from quail in Hong Kong (A/Quail/Hong Kong/G1/97) [19]. Taken together, the molecular data indicate that the Hong Kong H5N1 viruses were reassortant viruses that derived their HA from, or shared an ancestor with, the A/Goose/Guangdong/96 virus and their internal genes from the A/Quail/Hong Kong/97 (H9N2) virus. The origin of the N1 NA gene of the Hong Kong H5N1 viruses remains

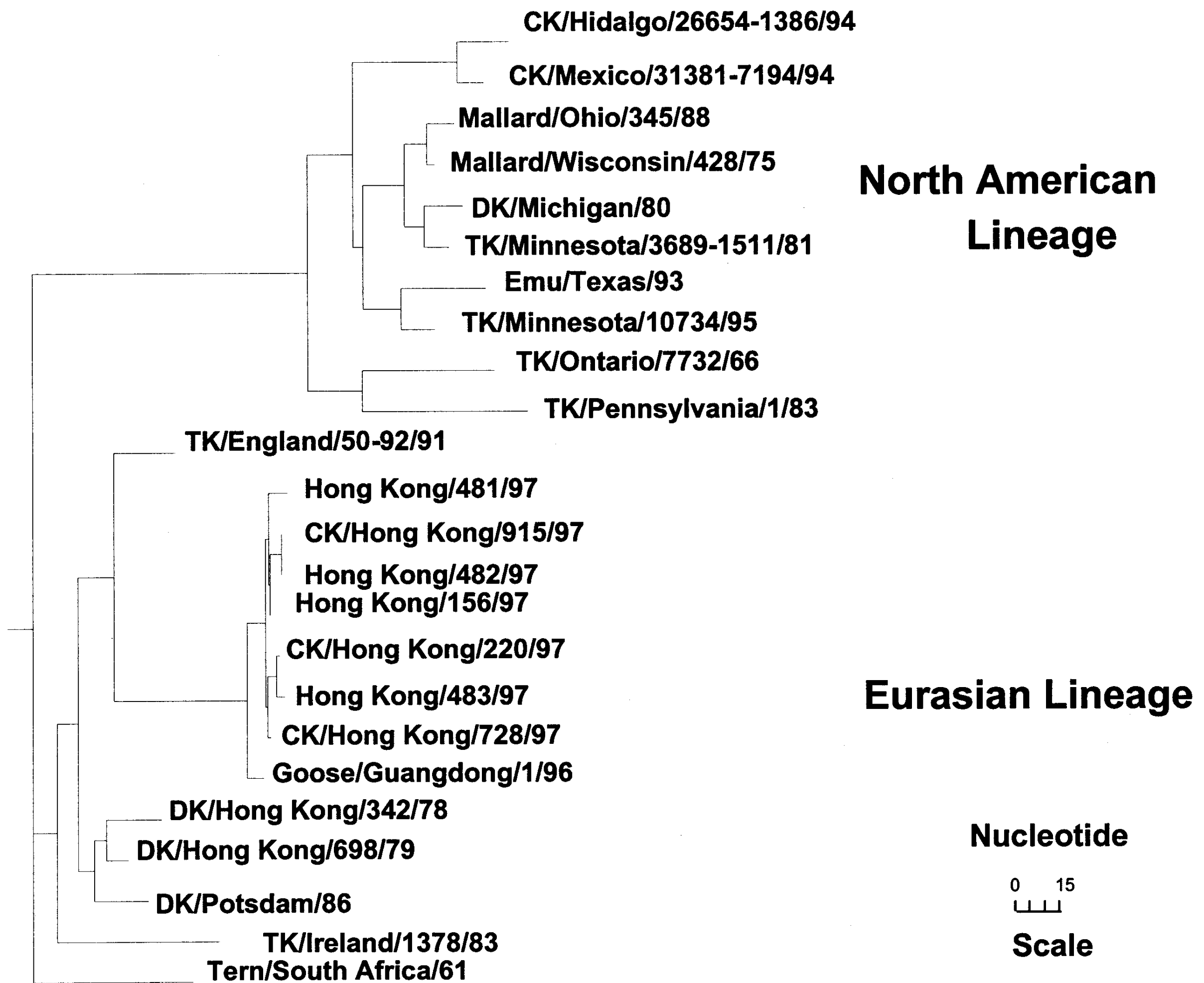

Figure 2. Phylogenetic relationships of the HA1 domain of the hemagglutinin gene of H5 viruses including the Hong Kong H5N1 and A/Goose/Guangdong/1/96 viruses. Version 3.5 of the Phylogeny Inference Package was used to estimate phylogenies from the nucleotide sequences. The tree was generated using neighbor-joining analysis determined by PHYLIP and is rooted to the A/Tern/South Africa/61 virus. Horizontal distances are proportional to the number of nucleotide differences between branch points. Reproduced from Xu et al. [49].

unclear. It is possible that this particular gene constellation determined the ability of avian H5N1 viruses to infect humans.

Genetic features of the Hong Kong H5N1 viruses

All eight of the gene segments of the viruses isolated from humans and chickens in Hong Kong in 1997 were highly related (> 99% sequence identity) at the genetic level [17]. The H5 HA of the H5N1 viruses isolated in Hong Kong had multiple basic amino acids in the connecting peptide (encoding Arg-Glu-Arg-Arg-Arg-Lys-Lys-Arg) [8, 50]. As would be predicted by the presence of the multibasic cleavage site sequence, the viruses isolated from humans were highly pathogenic for experimentally inoculated chickens [8, 17]. The avian and human H5N1 isolates from Hong Kong bound to NeuAc-α2,3-galactose-containing receptors and not to NeuAc-α2,6-galactose containing receptors [39]. Apparently, the receptor specificity of these viruses did not restrict their ability to infect and replicate in humans.

The NA gene of the H5N1 viruses belonged to the avian lineage of N1 NA genes and was notable for a 19-amino-acid deletion in the stalk region [8]. Although transfectant viruses engineered to have shortened NA

stalks displayed altered growth properties [51, 52], and it has been suggested that stalk length could affect the enzymatic efficiency of the NA in different substrates and thereby contribute to altered host range [53], the role of the short NA stalk of the H5N1 viruses in virulence is not yet known. The PB1 gene of the avian and human H5N1 viruses had an additional amino acid at the 3′ end of the protein because the stop codon was one codon further downstream [17] compared with other avian influenza PB1 sequences. The significance of this molecular feature is not known. The PB2, PA, NP and M2 gene products contain amino acid residues previously found only in human influenza viruses [54]. Although these features are notable, and studies to determine the contributions of these residues are ongoing, there are as yet no clear indications of which genes or specific residues in particular gene products of the H5N1 viruses bear the determinants of virulence or host range. The level of genetic identity between the avian and human isolates was consistent with the epidemiologic data [14] indicating that the human infections with H5N1 viruses resulted from avian-to-human transmission, rather than human-to-human transmission. The nature of mutations resulting from adaptation to the human host could not be addressed with the H5N1 viruses, because a single virus isolate was obtained from most of the cases of human H5N1 infection and the human H5N1 viruses, in general, did not transmit from person-to-person.

Genetic features of human H9N2 isolates

The genes of the two human influenza H9N2 isolates from Hong Kong in 1999 were of avian origin [54a]. The nonglycoprotein genes of these viruses were highly related to the genes of the H5N1 viruses isolated from humans and chickens in Hong Kong in 1997 [54a], suggesting that this constellation of genes was also responsible for the ability of these avian influenza viruses to infect humans.

Immune response to avian influenza A viruses in mammalian species

Assay methods

Earlier studies investigating the humoral immune response to avian viruses in mammalian species have relied primarily on HI assays to detect serum antibodies to these viruses. On the whole, these studies have failed to detect serum HI antibody responses to avian viruses of different subtypes in a number of mammalian species, including humans [4, 55, 56].

There are a number of possible reasons for the general failure to detect serum antibody responses in animals or humans infected with avian influenza viruses. First, some avian viruses may be poorly immunogenic and induce low, if any, levels of antibody compared with human influenza A viruses [55, 56]. Second, the traditional HI assay, the gold standard for the detection of antibody to human influenza viruses, may lack sensitivity for the detection of lower-titered or less-avid antibodies induced by avian viruses [57, 58]. Lu et al. [57] demonstrated that subunit HA, but not intact virus, could be used as an antigen in the HI assay to detect antibodies to an avian H2N2 virus. However, neutralizing antibodies were readily detected with intact infectious virus.

The appearance of avian H5N1 viruses in humans in 1997 enabled a direct comparison of the HI assay with the microneutralization assay [58]. The latter was found to be more sensitive in detecting anti-H5 antibodies in infected individuals. The use of subunit HA did not improve the sensitivity of the HI assay. These results suggest that the HI assay may not be suitable for the evaluation of humoral immunity to avian viruses in mammalian species, especially for seroepidemiologic investigations of humans [58]. Seroprevalence studies, which have relied on the HI assay to detect antibodies, must be interpreted with caution.

Seroprevalence studies and humoral immune responses to infection

Profeta and Palladino [59] tested sera from 294 subjects from Milan, who were born between 1900 and 1979, for HI and NI antibodies to five avian influenza viruses. NI antibodies were detected in different birth cohorts against N4, N5, N6, N7 and N9 NAs, but HI antibodies against H4, H7, H8, H11 and H12 HAs were not detected. The significance of the detection of antibody to avian NA subtypes in this population is not clear, but the age dependence suggests that the anti-NA antibody responses may, in part, reflect cross-reactivity with influenza A viruses circulating among humans prior to 1960 [59]. However, other seroprevalence studies have also detected low levels of antibody to avian NA subtypes that cannot be explained by obvious cross-reactivity with N1 or N2 of human viruses [60, 61], raising the possibility of interspecies transmission of influenza viruses.

Shortridge assayed antibodies against avian H1 to H13 HAs by single radial hemolysis, from individuals living in the Pearl River Delta ($n = 400$) and Jiangsu province ($n = 300$) in Southern China, from Taichung, Taiwan ($n = 150$) and from urban Hong Kong ($n = 100$) using the single radial hemolysis (SRH) assay [62]. This assay detects subtype-specific antibody directed against HA and NA, but may also detect antibody to NP which is cross-reactive among influenza A subtypes. Although

this assay lacked specificity for the HA alone, results suggested that the seroprevalence to avian influenza subtypes H4 to H13 ranged from 0 to 38%, depending on the subtype and the population. Antibodies to the H4, H6, H7, H10 and H11 subtypes were most frequently detected. Interestingly, H4, H6 and H10 viruses were also more frequently isolated from domestic ducks in these regions compared with the H5, H8, H9 and H11 subtypes [62].

Earlier studies, which attempted to detect antibody following experimental infection of humans with a number of avian viruses, also used the HI assay [4]. Administration of high doses of avian viruses to human volunteers resulted in limited replication of H4N8, H6N1, and H10N7 viruses, but no detectable serum HI antibody response. The limited replication may have been insufficient to stimulate a detectable primary response in these volunteers, or alternatively, the HI assay may have been unable to detect the low levels of antibody induced by the avian viruses [4]. Similarly, in the case in which fowl plague virus was isolated from the blood of a man with infectious hepatitis, convalescent serum obtained 4 months after illness was negative by HAI tests [11, 12]. More extensive replication of an avian-like H7N7 influenza virus was observed in an individual accidentally infected intraocularly with an H7N7 virus from a seal [5]. The ensuing conjunctivitis lasted for 4–5 days, and substantial titers of virus ($10^{5.0}$/ml) were recovered from the eye on day 2 post-infection. Nevertheless, serum or mucosal (lacrimal) HI antibodies to the H7N7 virus were not detected. Conjunctivitis caused by another avian virus, Newcastle disease virus, also fails to induce a systemic immune response [63].

The emergence of influenza A (H5N1) virus in humans in Hong Kong in 1997 provided a unique opportunity to assess the primary serologic response to the avian virus in infected individuals. The kinetics of the primary serum neutralizing antibody response to avian H5N1 virus (fig. 3) were similar to the previously reported primary response to human influenza A viruses [64]. Neutralizing antibody was generally detected 14 or more days after onset of symptoms. Titers of ≥ 640 were observed in both adults and children, 20 or more days after symptom onset. H5-specific immunoglobulin (Ig)G and IgM responses were detected in a majority of pediatric and adult cases [15]. One case patient with systemic lupus erythematosus failed to generate an H5-specific antibody response to infection, possibly because of her illness or steroid treatment for her underlying illness. With a better understanding of the antibody response to the H5N1 virus in confirmed case patients, it became possible to investigate the seroprevalence of anti-H5 antibody in populations in Hong Kong in 1997. Whereas antibody to H5N1 virus was not detected in the general population in Hong Kong, it was detected in a significant percentage of Hong Kong poultry workers [Bridges C. B., unpublished]. These results were consistent with exposure to live poultry being the main risk factor for infection with H5N1 viruses [14].

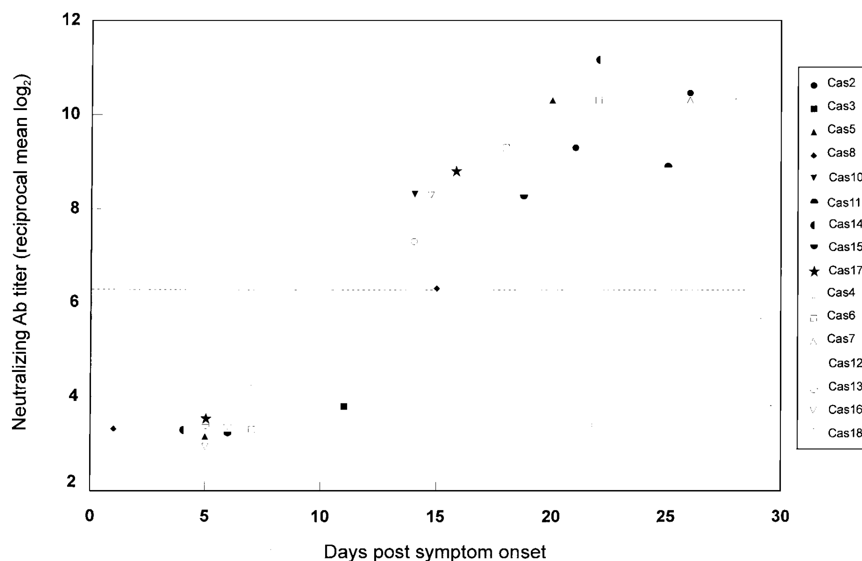

Figure 3. Serum neutralizing antibody response to infection with influenza A (H5N1) virus. Serum samples from 16 H5N1 cases were tested in a microneutralization assay using the HK/156 virus. Values represent the $\log_2$ mean titer of duplicate assays. Closed symbols are case patients aged ≤ 14 years; open symbols are case patients aged > 14 years. The dotted line denotes a titer of $\log_2 6.3 = 80$. Sera with titers of ≥ 80 were considered positive for H5-specific antibody [58]. Serum samples were not collected from case 9, and case 1 was not included in this study.

The microneutralization assay is presently being used to detect antibodies to H9N2 viruses following the recent isolation of avian H9N2 viruses in two children in Hong Kong in March [65]. The H9N2 viruses isolated from the two children were antigenically similar to a virus that had been isolated from poultry in 1997, A/Quail/Hong Kong/G1/97 (G1). However, another antigenically and genetically distinct sublineage of H9N2 viruses, represented by A/Chicken/Hong Kong/G9/97 virus, also circulated in the poultry markets. Preliminary results from studies that used the microneutralization assay to detect antibodies to H9N2 viruses in human populations suggest that the seroprevalence to G1-like viruses in individuals in Hong Kong is low [9]. Peiris et al. [9] detected G1 (H9N2) virus-specific neutralizing antibody titers of 20–80 in 2% of a group of volunteer blood donors in Hong Kong ($n = 150$) and in 0% of 100 control adult sera from the United Kingdom. Seroprevalence to the G9 H9N2 virus was 2 and 3%, respectively, in these same populations. However, in this study, possible cross-reactivity between the G9-like viruses and H2N2 or H3N2 human influenza viruses was not excluded, and confirmatory assays are needed to firmly establish the seroprevalence to G9 virus in human populations.

Cell-mediated immunity (CMI)

There have been relatively few studies on the cellular immune response to avian influenza viruses. Infection of mice with human influenza A viruses generally elicits a dominant class I-restricted cytotoxic T lymphocyte (CTL) response to an epitope on the influenza A nucleoprotein (NP) [66, 67]. NP-specific CTL mediate viral clearance and promote survival in mice given a lethal challenge [68, 69]. Since the NP is largely conserved among influenza A viruses of different subtypes, avian influenza viruses should also possess the NP epitopes recognized by murine class I-restricted CTL. Riberdy et al. [70] investigated the role of antibody and CMI in the protection of mice from lethal challenge with a mouse-adapted avian H3N8 virus. Immunoglobulin expressing ($Ig^{+/+}$) mice, but not congenic $Ig^{-/-}$ mice, previously infected with an H3N2 virus were protected from lethal challenge with the H3N8 virus. Depletion of $CD4^+$ and $CD8^+$ T cells from $Ig^{+/+}$ mice delayed the recovery of the animals but did not affect the survival from lethal challenge. A secondary NP-specific $CD8^+$ T cell response in $Ig^{-/-}$ mice was associated with rapid viral clearance following challenge with H3N2 virus but was only able to control virus infection by a low dose of H3N8 virus. Thus, in this system, cross-reactive antibody provided optimal protection, whereas the efficacy of CMI depended on the virulence of the viral infection. Hioe and Hinshaw detected CTL activity in BALB/c mice immunized with a highly pathogenic H5 virus, A/Turkey/Ontario/7732/66 (Tk/Ont/66; H5N9) [71]. Although class I- and class II-restricted CTL activity was detected in bulk cultures established from the immunized animals, the cell line derived from bulk culture displayed only class II-restricted T cell activity. T cell clones were class II-restricted (I-E^d) $Lyt2^+$ $L3T4^-$ and were specific for the H5 HA. The epitope recognized by the clones was localized to amino acids 158–169 of the H5 HA, which is at the distal tip of the H5 HA and overlapped an antigenic site recognized by H5-specific B cells [72]. These CTL clones failed to reduce mortality when adoptively transferred to mice simultaneously infected with a high dose of the lethal Tk/Ont/66 virus. However, in mice challenged with a lower dose of the H5 virus, transfer of the CTL clones was associated with a modest reduction in lung virus titers and reduced morbidity [72]. The relative contribution of class I- versus class II-restricted CTL in this model system remains to be determined.

In humans, memory CTL responses to influenza A viruses appear to be more broadly directed to epitopes on a number of viral proteins [73]. Recently, Jameson et al. [74] have reported that restimulation of human peripheral blood mononuclear cells with A/Puerto Rico/8/34 (H1N1) virus resulted in CTL activity that was cross-reactive for targets infected with a number of avian viruses of the H1 and H5 subtypes, including two H5N1 (A/HK/156/97 and A/HK/483/97) viruses isolated from humans in Hong Kong in 1997. Using $CD8^+$ T cell lines, it was determined that epitopes on NP, M1, PB1, PB2 or HA proteins were recognized on target cells infected with either H5N1virus. In addition, cytotoxic $CD4^+$ cell lines recognized epitopes on M1 and NS1 of both the H5N1 viruses used in the study [74]. These results indicate that individuals living in an urban US environment possess memory CTL induced by prior infection with human influenza A viruses that recognize epitopes conserved in avian influenza A viruses. A role for memory CTL in the enhanced clearance of virus from infected lungs has been clearly demonstrated in the mouse model [68, 75, 76], and an association between CTL activity and viral clearance in humans has been documented [77]. Nevertheless, five of the six fatal human cases occurred in individuals aged 13 to 60 years, who would likely have had influenza-virus-specific CTL responses induced by prior infection with human influenza A viruses.

Pathogenicity of human H5N1 viruses in mammalian species

Natural infection of mammalian species by avian influenza viruses

It has been proposed that all influenza A viruses that infect mammalian species originate from birds. The

appearance of novel avian viruses in mammals is often associated with outbreaks of severe disease and excess mortality. Such was the case when H7N7 viruses were isolated from dead and dying harbor seals (*Phoca vitulina*) along the Atlantic coast of North America in 1979–80 [78, 79]. An estimated 600 seals died during this outbreak. Additional epizootics of pneumonia in seals have been attributed to avian H4 or H3 viruses [80, 81]. The emergence of an avian H3N8 virus in horses in Northeast China caused up to 20% mortality in some herds [82], but the virus failed to spread and become established in this mammalian host. In contrast, an avian H1N1 virus transmitted to pigs in Europe in 1979 eventually became established as the major influenza virus circulating and causing disease in European pig populations [83]. In fact, viruses of at least 13 avian subtypes have been shown to be capable of infecting pigs following experimental inoculation. Viruses of the H1 to H13 subtype replicated in the upper respiratory tract of pigs for up to 7 days at levels equivalent to those of human and swine viruses yet failed to cause clinical symptoms [55, 56]. Avian viruses clearly exhibit a range of pathogenicity phenotypes when they emerge in mammalian hosts.

Experimental infection of mammalian species with H5N1 viruses

In humans, the H5N1 viruses caused a spectrum of clinical disease from mild respiratory infections to severe and fatal disease. In an effort to better understand the ability of human H5N1 viruses to infect and cause disease in humans, investigators turned to the mouse model that has been widely used in influenza virus research. Unlike the majority of present day human influenza viruses, the Hong Kong H5N1 viruses required no adaptation to the mouse host in order to infect mice by the intranasal route and replicate rapidly and to high titers in the lungs of mice [84–86]. Lung virus titers reached $\geq 10^{6.0}$ 50% infectious doses (ID_{50}) by 24 h post-infection (p.i.), and peak titers ranging between $10^{7.0}$ and $10^{8.2}$ 50% ID_{50} were detected 3–6 days p.i. The human H5N1 viruses could be separated into two distinct phenotypes of pathogenicity. Replication of viruses of low pathogenicity (lethality) was restricted to the respiratory tract, and virus was cleared from the lungs by day 9 p.i. In contrast, the highly pathogenic viruses spread systemically, infected multiple organs including the brain and resulted in the death of mice by 6–8 days p.i. [85, 86]. Immunohistochemical staining of brain tissue detected viral antigen in both glial cells and neurons shortly before the mice succumbed to infection with a highly pathogenic H5N1 strain [85]. Gao et al. reported that all H5N1 viruses tested, regardless of their pathogenicity, caused disease symptoms in mice, including hunched posture, ruffled fur and rapid breathing, as early as 24 h p.i. [86]. However, in studies from this laboratory, these symptoms were only observed in mice infected with the highly pathogenic H5N1 viruses and not in those infected with H5N1 viruses of low pathogenicity. Weight loss, used as a measure of morbidity, also clearly distinguished viruses of high and low pathogenicity. Mice infected with highly pathogenic H5N1 virus A/HK/483/97 began to lose weight 3 days after infection and continued to do so until they died. In contrast, mice infected with an H5N1 virus of low pathogenicity showed only minimal (4%) and transient weight loss on days 5–7 p.i. [87].

In two studies, the ability of human H5N1 viruses to spread systemically to multiple major organs, including the brain, was associated with a lethal outcome to infection [85, 86]. In contrast, Dybing et al. failed to detect virus in extrapulmonary sites in mice lethally infected with A/Hong Kong/156/97 virus and concluded that death was primarily a consequence of the substantial tissue damage that occurred in the respiratory tract, particularly the lungs [88]. Recently, we have shown that infection of mice with highly pathogenic A/Hong Kong/483/97 virus resulted in depletion of lymphocytes in blood, lung and lymphoid tissue together with diminished production of proinflammatory cytokines [87]. These results suggest that destructive effects on the immune system may be an additional and important factor that contributes to the pathogenicity of some H5N1 viruses in this mammalian model system. Dybing et al. [88] demonstrated that other H5 viruses isolated from poultry that were highly pathogenic in chickens, but not BALB/c mice, induced elevated levels of serum transforming growth factor (TGF)-β as early as 8 h p.i. In contrast, the Hong Kong H5N1 viruses that were highly pathogenic in mice failed to induce production of increased levels of TGF-β. This study also highlighted the fact that high pathogenicity for mice was a unique feature of some of the H5N1 viruses isolated in Hong Kong in 1997, and was not a general feature of all H5 viruses that are highly pathogenic for avian species.

H5N1 viruses isolated from chickens and humans in Hong Kong also replicated to modest titers in the upper respiratory tract of weanling pigs infected by the oral and nasal routes [18]. However, viral infection produced no disease, and transmission of H5N1 viruses to contact animals was not detected. Experimentally infected rats also supported modest replication of H5N1 viruses isolated from humans or poultry in Hong Kong. The H5N1 viruses were detected in the lungs of rats on day 3, but not day 5, p.i. and no symptoms of disease were observed.

Ferrets are naturally susceptible to a range of influenza A and B viruses. Hinshaw et al. [55] demonstrated that

ferrets could be infected with avian viruses of the H1, H2, H3, H6, H7 and H10 subtypes. Viruses were detected in nasal wash samples for 3–7 days p.i. High titers of an H1 virus ($10^{6.2}$ EID_{50}/g) were also recovered from the trachea and lungs of ferrets at the peak of viral infection on day 3 p.i. All of the avian viruses tested in this study failed to replicate in the intestinal tract of ferrets and caused no apparent disease in this host. Ferrets were also found to be susceptible to experimental infection with high doses of avian H5 and H6 viruses [89]. Infected animals experienced mild respiratory symptoms but no appreciable rise in temperature. H5 viruses (titer range = $10^{1.8}$–$10^{5.2}$ EID_{50}/ml) and H6 viruses (titer range = $10^{4.0}$–$10^{6.5}$ EID_{50}/ml) were isolated in nasal wash samples collected 2–4 days p.i. There is at present only anecdotal evidence that the Hong Kong H5N1 viruses are more virulent in ferrets. The usual practice of preparing post-infection ferret sera as a serologic reagent was hampered by greater morbidity and mortality in ferrets infected with the H5N1 viruses compared with that observed in ferrets infected with the currently circulating human influenza A and B viruses. A systematic investigation of H5N1 virus pathogenesis in ferrets is currently underway.

Implications for pandemic influenza

Another influenza pandemic will most certainly occur. Before 1997, only three influenza A subtypes, H1, H2 and H3, were known to cause respiratory disease and spread rapidly among humans. The concept of subtype 'recycling' [1, 90] raised the suggestion that the H2 subtype would reemerge in the growing population of individuals with no prior immunity to this subtype. However, the recent events in Hong Kong have created a new awareness that influenza A subtypes that were previously thought to be restricted to avian species have the potential to transmit to humans. Enhanced surveillance for influenza A viruses in avian and mammalian populations, as well as enhanced surveillance for novel influenza subtypes in humans, particularly in southern China, will be key to the early detection and recognition of the next pandemic strain.

The reservoir of influenza A viruses in the aquatic bird populations of the world is the source of influenza A viruses that infect humans, other mammals and domestic poultry. Both the Asian influenza (H2N2) pandemic of 1957 and the Hong Kong (H3N2) pandemic of 1968 were caused by viruses that arose through genetic reassortment between the circulating human virus and an avian virus. The 1957 pandemic strain had derived its HA, NA and PB1 genes from an avian virus, whereas the 1968 H3N2 virus possessed the HA and PB1 genes of avian origin [3, 91]. In both 1957 and 1968 pandemic strains, the HA gene of the avian donor virus belonged to the Eurasian lineage. Until recently, the pig was considered the most likely 'mixing vessel' in which a novel virus would arise as a result of reassortment between avian and human viruses [38]. Pigs express cell surface sialic acid (SA) receptors that recognize the predominant binding specificities found on avian viruses (SA α2,3Gal linkage) as well as those on human viruses (SA α2,6Gal linkage) [37].

Prior to 1997, direct evidence that avian viruses could infect humans was restricted to conjunctival infections of humans by H7 viruses [5, 6] and limited seroepidemiologic investigations [4, 59, 61, 62]. In fact, when an H5N2 virus, A/Chicken/Pennsylvannia/1370/83, caused widespread morbidity and mortality in chickens in the Northeastern United States in the early 1980s, no human infections were identified by attempts to isolate virus or by detection of a serum HI antibody response [92]. In contrast, the 18 documented cases of respiratory disease caused by H5N1 viruses in Hong Kong in 1997 firmly established that direct transmission of highly pathogenic avian H5 virus from poultry to humans could occur. Furthermore, the H9N2 infections identified in two young children in 1999 demonstrated that avian viruses of other subtypes, with low pathogenicity in chickens, could also directly infect and cause respiratory disease in humans [9, 65]. The implications of these findings for pandemic influenza are considerable. We must now recognize that any of the 15 HA subtypes of influenza A viruses found in the aquatic bird reservoirs have the potential to cross the species barrier into humans. It is also possible for humans themselves to serve as a 'mixing vessel' in which an avian virus could reassort with a currently circulating human virus (fig. 4). The result could be a virus with novel surface glycoprotein(s) and a constellation of internal genes that enable the rapid transmission of the virus to a susceptible human population. It is possible that such an event was forestalled in Hong Kong by the destruction of the poultry in December 1997. The fact that H5N1 and H9N2 viruses have emerged in humans in southern China (Hong Kong) is also noteworthy. It has been proposed that this region of the world may be an epicenter for influenza [93]. The large populations of humans, pigs, domestic poultry and waterfowl found in this region may provide optimal opportunity for interspecies transmission of viruses of Eurasian origin.

The development of vaccine candidates and evaluation of vaccine regimens against novel influenza HA subtypes are underway, in preparation for the next pandemic. Candidate vaccines for selected subtypes are under development, and their evaluation in clinical trials should provide important information regarding their optimal use as a key prevention strategy in the event of a pandemic.

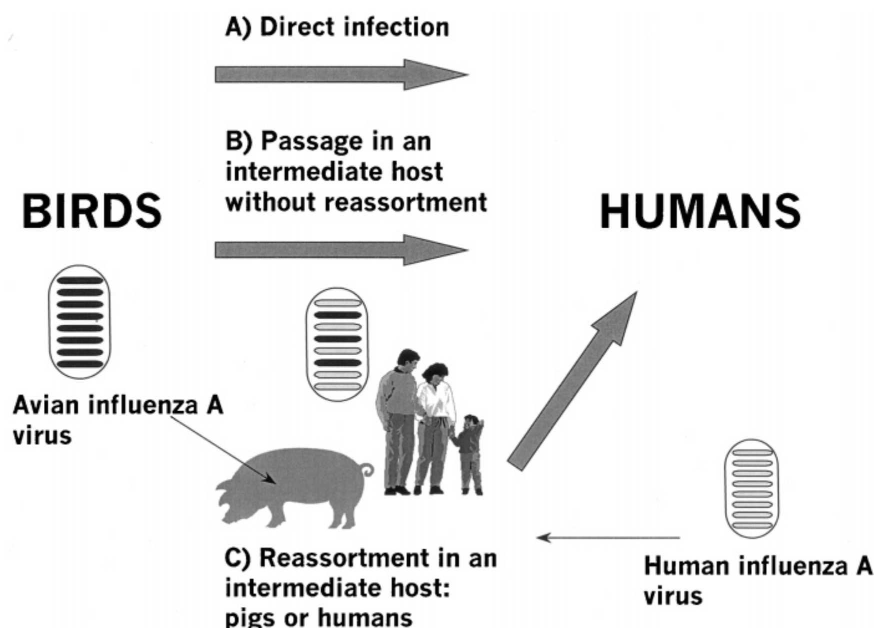

Figure 4. Three proposed routes for the introduction of an influenza A virus bearing an avian hemagglutinin gene, with or without other accompanying avian influenza virus genes, into the human population. (*A*) Direct infection of a human host by an avian influenza A virus, e.g. H5N1 and H9N2 viruses in Hong Kong; (*B*) passage of an avian influenza virus through an intermediate host, without reassortment with a human influenza A virus and (*C*) reassortment of the genomes of an avian and human influenza A virus in an intermediate host such as a pig or possibly a human. Although the intermediate host in which the reassortment event occurred was not identified, the 1957 H2N2 and 1968 H3N2 pandemic strains were reassortant viruses.

Vaccines against avian H5N1 viruses

Current influenza vaccines are prepared from high-growth reassortant viruses that have the desired antigenic characteristics of circulating influenza A viruses. The development of vaccine candidates from the highly pathogenic avian H5N1 viruses have presented special problems due to the higher level of containment, biosafety level (BSL)-3 +, required to work with these strains. Several approaches to the development of a safe and effective vaccine candidate have been pursued. One approach has been the generation of viruses that possess HA genes modified at the cleavage site between HA1 and HA2 to contain a single basic amino acid residue, resembling that of nonpathogenic avian viruses. Using reverse genetics techniques, Li et al. [94] have constructed vaccine candidates that possess the modified 'nonpathogenic' HA and the N1 NA from a human H5N1 strain, together with the internal genes from a 'high-growing' cold-adapted live attenuated master strain, A/Ann Arbor/6/60 (H2N2). It has been proposed that such vaccine candidates could be used either in the preparation of a conventional inactivated vaccine or potentially a live-attenuated vaccine. Two H5N1 recombinant vaccines, prepared in this way, have been shown to be nonpathogenic in chickens and protective against lethal challenge with the homologous wild-type H5N1 virus, although protection against lethal challenge with a heterologous antigenic variant H5N1 virus was reduced [94]. The vaccine candidates were also safe and immunogenic in ferrets.

In a similar approach, Takada et al. generated a vaccine candidate possessing a similarly modified HA with the remaining genes derived from a nonpathogenic H5N1 avian virus [95]. This vaccine candidate was immunogenic in mice following either a single systemic inoculation or multiple intranasal inoculations. The latter route of vaccine delivery was also shown to be protective against lethal challenge with a wild-type H5N1 virus.

A second strategy for H5N1 vaccine design was the use of a 'surrogate' nonpathogenic H5 virus that was antigenically related to the H5N1 viruses isolated from humans in Hong Kong. A conventional formalin-inactivated vaccine prepared with the nonpathogenic A/Duck/Singapore-Q/F119-3/97 (Dk/Sing; H5N3) virus was modestly immunogenic when administered intramuscularly to mice in two doses of 3 μg of HA [85]. Serum HI antibody responses were enhanced by the addition of alum to the vaccine. All mice administered the vaccine, with or without alum, survived a lethal challenge with the highly pathogenic H5N1 virus, whereas only animals that received vaccine with alum were completely protected against infection [85]. In an-

other study, inactivated Dk/Sing vaccine was comparable to an inactivated vaccine prepared with homologous H5N1 virus in its ability to protect mice from lethal challenge, confirming the potential of this approach for the development of a vaccine against a highly pathogenic avian virus [96].

Yet another approach to elicit protection against the highly pathogenic avian viruses is the use of either baculovirus-expressed purified recombinant HA (rHA) protein as a vaccine or expression of the HA in a plasmid vector as a DNA-based vaccine. Both of these strategies obviate the need for biosafety containment, since infectious virus is not used for vaccine production. In animal models, H5 rHA vaccines were immunogenic and protective [97]. However, clinical trials emphasized the dose-dependence of the antibody response to H5 rHA and suggested that relatively high doses were required to achieve a neutralizing antibody response in unprimed healthy adults [Katz J. M., unpublished data]. DNA vaccines have also shown promise in preclinical studies. Gene gun administration of an H5 DNA vaccine completely protected mice from infection and death by homologous Hong Kong H5N1 virus, but did not prevent infection when the vaccine and challenge virus differed in HA1 amino acid homology by 12% [98].

Summary

This review highlights the H5N1 outbreak in Hong Kong, which was the first documented outbreak of human illness caused by avian influenza viruses. The following important points were established as a result of the epidemiologic studies involving the people in Hong Kong who were exposed to and those who were infected by H5N1 viruses and the thorough virologic studies of the viruses isolated from poultry and humans during the outbreak. Avian viruses can directly infect humans, without acquiring human influenza virus genes by reassortment in an intermediate host (or mixing vessel). In fact, a human could serve as a 'mixing vessel' if one were concomitantly infected with a human and avian influenza virus. The H5N1 viruses were poorly transmissible among humans; this property may have been the consequence of the avian influenza virus gene constellation. This outbreak highlighted the need for additional reagents for diagnostic purposes, new assays for the detection of an immune response and the assessment of multiple approaches to vaccine development. The practical difficulties posed by the need to ensure that work with such pathogens could proceed safely, without endangering the health and welfare of humans and animal species, should not be underestimated. The Hong Kong H5N1 outbreak has certainly served to heighten awareness of these issues in surveillance for human infections with avian influenza viruses and in preparing for future influenza pandemics.

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