

H5N1 influenza: A protean pandemic threat

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Infection with avian influenza A virus of the H5N1 subtype (isolates A/HK/212/03 and A/HK/213/03) was fatal to one of two members of a family in southern China in 2003. This incident was preceded by lethal outbreaks of H5N1 influenza in waterfowl, which are the natural hosts of these viruses and, therefore, normally have asymptomatic infection. The hemagglutinin genes of the A/HK/212/03-like viruses isolated from humans and waterfowl share the lineage of the H5N1 viruses that caused the first known cases of human disease in Hong Kong in 1997, but their internal protein genes originated elsewhere. The hemagglutinin of the recent human isolates has undergone significant antigenic drift. Like the 1997 human H5N1 isolates, the 2003 human H5N1 isolates induced the overproduction of proinflammatory cytokines by primary human macrophages *in vitro*, whereas the precursor H5N1 viruses and other H5N1 reassortants isolated in 2001 did not. The acquisition by the viruses of characteristics that enhance virulence in humans and waterfowl and their potential for wider distribution by infected migrating birds are causes for renewed pandemic concern.

influenza A | human | avian | virus evolution | cytokine

Avian influenza viruses pose significant threats to animal and human health. They are a source of genetic diversity that permits the emergence of pandemic influenza by means of genetic reassortment with prevailing human influenza viruses (1). In recent years, purely avian influenza viruses of subtypes H5N1 and H7N7 have crossed the species barrier to directly cause fatal disease in humans in Hong Kong and Holland (2, 3). Viruses with the gene constellation of the human A/Hong Kong/156/97 (H5N1/97) isolate have not been seen since the 1997 outbreak ceased concurrent with the slaughter of 1.5 million poultry in Hong Kong. However, its precursors, the A/Goose/Guangdong/1/96 (Gs/Gd/96)-like (H5N1) viruses, have continued to circulate in southern China (4, 5) and, more recently, have reassorted with other influenza viruses of aquatic avian origin to generate a series of reassortants that have caused disease outbreaks in terrestrial poultry (6). Some of the reassortant viruses found to have acquired a deletion in the stalk of the neuraminidase (NA) molecule that is thought to be associated with adaptation to chickens (6, 7).

Human disease associated with the H5N1 "bird flu" outbreak in 1997 was unusually severe (2, 8), for reasons that have remained unclear. H5N1/97 viruses were shown to induce the overproduction of proinflammatory cytokines (9) and to evade their antiviral effects (10). We report the reemergence of H5N1 viruses that are pathogenic for both humans and waterfowl and show that their genotypes are related to their proinflammatory cytokine induction phenotypes.

Methods

Virus Surveillance, Isolation, and Characterization. Prospective surveillance of influenza viruses in humans, live poultry retail markets, and farms in Hong Kong is described in ref. 6. As of December 2002, wild birds that were found dead in Hong Kong nature reserves and parks were also investigated. Specimens obtained from humans were inoculated into MDCK cells, and avian specimens were cultured in embryonated chicken eggs. Virus isolates were identified and subtyped by using reference antisera. Two 2003 human

H5N1 isolates and representative 2002 viral isolates from poultry and wild birds (Table 1) were characterized antigenically and genetically. Antigenic characterization was done by hemagglutination inhibition tests with polyclonal antisera and mAbs. The mAbs were to the hemagglutinin (HA) of A/Chicken/Pennsylvania/1370/83 (H5N2) (6).

The virus isolates were characterized genetically by sequencing RT-PCR-amplified DNA fragments of each gene segment and by performing genetic and phylogenetic analysis, as described in ref. 6. We analyzed the following nucleotide sequences: HA 64–1,589; NA 23–1,426; nucleoprotein (NP) 44–1,545; nonstructural (NS) 41–858; matrix (M) 25–1,005; PB1 21–1,449; PB2 1,003–2,247; and polymerase (PA) 34–2,210. A total of 120 avian H5N1 virus isolates were genotyped in 2002. We selected 10 isolates representing the most frequent genotypes for detailed analysis (Table 1). Other sequences that were used for comparison were obtained from GenBank.

Pathogenicity in Mice. We inoculated groups of 10–14 6-week-old BALB/c mice intranasally with 50 μ l of PBS-diluted allantoic fluid containing $\approx 0.5 \times 10^{6.5}$ egg 50% infective dose (eID₅₀) of virus, and we recorded body weight and mortality daily, as described (11). Three mice from each group were killed on day 5 after inoculation. Virus was titrated in lung, brain, kidney, liver, and spleen homogenates, and titers were expressed as log₁₀ eID₅₀ in 0.1 ml of tissue homogenate. Virus was titrated similarly in mice that died during the experiment.

Tumor Necrosis Factor α (TNF- α) Induction in Primary Human Macrophage Cultures. Differentiated human monocyte-derived macrophages were separated from buffy coats of blood obtained from healthy donors, placed in 24-well tissue culture plates, and inoculated with influenza viruses at a multiplicity of infection of 2, as described in ref. 9. The research protocol to obtain human blood was approved by the ethics committee of the University of Hong Kong. RNA was extracted from the cells 3 h after inoculation, and mRNA encoding TNF- α and IFN- γ -inducible protein 10 (IP-10) was assayed by real time quantitative RT-PCR with a LightCycler (Roche, Mannheim, Germany), as described in refs. 9 and 12. Primers used for detecting IP-10 mRNA were as follows: forward, 5'-CTGACTCTAAGTGGCATT T-3'; reverse, 5'-TGATGGCCTTCGATTCTG-3'. Secreted TNF- α and IP-10 were assayed in culture supernatants that were harvested 6 h after inoculation by specific ELISA (R & D Systems), according to the manufacturer's instructions.

Results

Epidemiology. In early December 2002, dead ducks, geese, and swans were observed in Penfold Park, a public park in Sha Tin (New

Abbreviations: eID₅₀, egg 50% infective dose; HA, hemagglutinin; M, matrix; NA, neuraminidase; NP, nucleoprotein; NS, nonstructural; TNF- α , tumor necrosis factor α ; IP-10, IFN- γ -inducible protein 10.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. AY575869–AY575916 and AY576368–AY576415).

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Table 1. Influenza A H5N1 viruses characterized in this study

| Virus | Date isolated | Source | Genotype |
|---------------------|---------------|---|----------------|
| A/HK/212/03 | Feb. 14, 2003 | Human, Nasopharyngeal aspirate, 33-year-old male | Z ⁺ |
| A/HK/213/03 | Feb. 14, 2003 | Human, Nasopharyngeal aspirate, 9-year-old boy | Z ⁺ |
| A/Gs/HK/739.2/02 | Dec. 4, 2002 | Canada goose (<i>Branta canadensis</i>), Penfold Park | Z ⁺ |
| A/Eg/HK/757.3/02 | Dec. 11, 2002 | Dead egret (<i>Egretta garzetta</i>), Penfold Park | Z ⁺ |
| A/Gh/HK/793.1/02 | Dec. 17, 2002 | Dead gray heron (<i>Ardea cinerea</i>), Lok Ma Chau | Z |
| A/Dk/HK/821/02 | Dec. 18, 2002 | Dead Rosy-billed Pochard duck, Kowloon Park | Z |
| A/Ck/HK/31.4/02 | Jan. 10, 2002 | Dead chicken, retail poultry market | B |
| A/Ck/HK/61.9/02 | Jan. 16, 2002 | Chicken feces, retail poultry market | Z |
| A/Ck/HK/YU777/02 | Apr. 18, 2002 | Chicken feces, retail poultry market | Z |
| A/Ck/HK/96.1/02 | Feb. 3, 2002 | Dead chicken, farm | Y |
| A/Ck/HK/sv409.1/02 | Feb. 9, 2002 | Dead chicken, farm | Y |
| A/Ph/HK/sv674.15/02 | Feb. 6, 2002 | Pheasant feces, retail poultry market | X |

Abbreviations used in virus names are as follows: Ck, chicken; Dk, duck; Eg, egret; Gh, gray heron; Gs, goose; HK, Hong Kong; Ph, pheasant.

Territories, Hong Kong). Postmortem examination revealed disseminated disease in ducks and geese, with pathologic changes in the lungs and brain. This incident had been preceded by deaths of little egrets (*Egretta garzetta*) that roosted in Penfold Park. These birds are largely a resident population, which are augmented by migratory birds in November and December. H5N1 influenza virus was isolated from the dead waterfowl that were found in the park and from a dead little egret that was found near the park.

In mid-December 2002, an unusual number of dead ducks were observed in Kowloon Park in the densely populated Tsim Tsa Tsui area of the Kowloon Peninsula in Hong Kong, and H5N1 viruses were isolated from the dead birds. The outbreak spread to ducks, geese, swans, and flamingos. H5N1 viruses were also isolated from two wild gray herons (*Ardea cinerea*, migrant birds that winter in Hong Kong) that were found dead in the Lok Ma Chau nature reserve (New Territories, Hong Kong). The H5N1 outbreak in the Hong Kong nature parks and the pathogenicity in ducks of the isolated viruses are described in greater detail in ref. 13.

In February 2003, influenza A virus of the H5N1 subtype was isolated from a 33-year-old man (A/HK/212/03) and his 9-year-old son (A/HK/213/03), on their return to Hong Kong after travel to Fujian Province in mainland China. The man died of “viral” pneumonia, whereas the boy recovered uneventfully. Another member of this family, an 8-year-old girl, had died of a pneumonic illness while she was in Fujian. The two human H5N1 virus isolates and representative recent H5N1 virus isolates from the retail markets, farms, parks, and dead wild birds were selected for antigenic, genetic, and immunological characterization.

Phylogenetic Analysis. To establish the genetic relationship between the recent human H5N1 isolates and recent isolates from poultry in the Hong Kong retail markets, farms, and parks, we fully sequenced and analyzed the representative viruses (Table 1).

In 2001, H5N1 viruses from five distinct genotypes (see genotypes A–E in Fig. 3, which is published as supporting information on the PNAS web site) were identified in Hong Kong retail markets and farms (6). Of viruses isolated from 2001 (i.e., genotypes A–E), only the genotype B viruses were isolated in 2002. In addition, this genotype was not detected after February 2002. Three genotypes, designated X, Y, and Z, emerged in retail markets and farms after January 2002 (Table 1 and Fig. 3). These three genotypes obtained novel gene constellations by acquiring novel internal protein genes from avian influenza viruses of unknown origin (Fig. 1 and data not shown). All genotype X, Y, and Z viruses had the 20-aa deletion (positions 49–68) in the stalk of the NA that was reported (6) in genotype A viruses of 2001, but the genotype B viruses did not. Genotypes B, Y, and Z viruses, whose NS gene is derived from a common donor, shared a 5-aa deletion in the NS1 protein (positions

80–84) that was reported previously in H5N1 viruses isolated in 2001 (6). However, the PB2, PB1, PA, M, and NS genes of genotype X viruses differed from those of the H5N1 viruses of 2001, and no deletion was observed in the NS gene.

Four viruses isolated from wild birds and birds from two different parks were fully sequenced in this study (Table 1). The virus isolated from a dead wild egret (A/Eg/HK/757.3/02) in Penfold Park in December 2002 was genetically indistinguishable from viruses that killed resident ducks and geese (A/Gs/HK/739.2/02) in the park. The six internal protein gene segments of this virus were consistent with genotype Z (Figs. 1 and 3), but the NA gene did not encode a 20-aa deletion in the NA stalk region. This genotype was similar to genotype Z and was, therefore, designated genotype Z⁺. Thus, genotypes Z⁺ and Z differed primarily on the basis of the 20-aa deletion in the NA stalk. Viruses isolated from a dead wild gray heron (A/Gh/HK/793.1/02) and from a dead Rosy-billed Pochard duck (A/Dk/HK/821/02) had the characteristic deletion in the NA stalk region and belonged to genotype Z, like other contemporary viruses that were isolated from poultry in the retail markets.

The H5N1 viruses isolated from the 33-year-old man (A/HK/212/03) and his 8-year-old son (A/HK/213/03) in 2003 were almost identical in all gene segments (data not shown). All A/HK/212/03 gene segments were of avian origin and appeared to be related most closely to the genotype Z⁺ and Z viruses first detected in 2002. Like A/Gs/HK/739.2/02 (genotype Z⁺), A/HK/212/03 had no deletion in the NA stalk. Therefore, overall, the genotypes of the human isolates A/HK/212/03 and A/HK/213/03 were most similar to A/Eg/HK/757.3/02 and A/Gs/HK/739.2/02 that were isolated during the Penfold Park outbreak.

Antigenic Analysis. The antigenic relationships of the virus isolates were analyzed by hemagglutination inhibition tests with polyclonal and monoclonal antisera. The results showed that recent H5 isolates had undergone antigenic drift compared with H5N1 viruses of 1997–2001. This effect was greatest in the genotype Z and Z⁺ viruses that were isolated in 2002 and in the human A/HK/212/03 and A/HK/213/03 viruses that were isolated in 2003 (Table 2). Most H5N1 viruses isolated from 2001 onward (genotypes A, B, Y, Z, and Z⁺) showed little or no reactivity to mAbs 406/7, whereas CP58 reacted poorly or not at all with virus genotypes Z, Y, and Z⁺ that were isolated in 2002. Interestingly, CP24 discriminated between genotype Y (reactive) and genotypes Z and Z⁺ (poorly reactive). The human A/HK/212/03 and A/HK/213/03 viruses reacted weakly with all mAbs except CP24.

Genetic Analysis. The HA gene of A/HK/212/03 encoded multiple basic amino acids at the HA1–HA2 connecting peptide (RERRRKKR↓G), like highly pathogenic avian influenza viruses

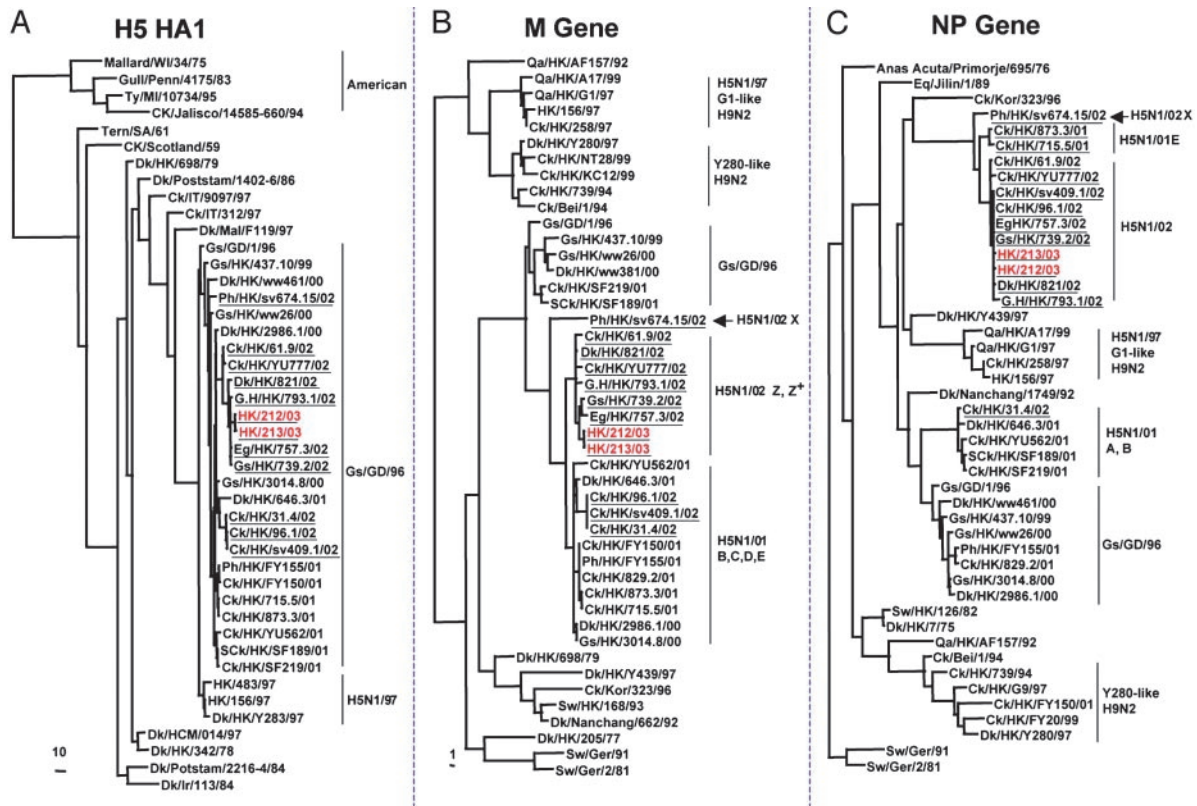


Fig. 1. Phylogenetic trees for the H5 HA1 (a), M (b), and NP (c) genes of influenza A viruses. The nucleotide sequences were analyzed with PAUP by using a maximum-parsimony algorithm. Nucleotides 70–1,033 of the HA1, 46–982 of the M, and 46–905 of the NP genes were analyzed. The HA1 tree is rooted to A/Japan/305+/97 (H2N2). The M and NP trees are rooted to A/Equine/Prague/1/56 (H7N7). The lengths of the horizontal lines are proportional to the minimum number of nucleotide differences required to join nodes. Names and abbreviations of viruses are as listed in Table 1 and ref. 6. Other sequences are available in GenBank. G1-like H9N2 and Y280-like H9N2 refer to the A/Quail/Hong Kong/G1/97-like and A/Duck/Hong Kong/Y280/97-like lineages of H9N2 viruses, respectively (27).

and contemporary H5N1 viruses (5). The HA glycosylation sites were similar to those of human H5N1/97 viruses as well as contemporary avian H5N1 viruses. The HA gene of the genotype Z⁺ viruses that we analyzed (including the human isolates) had

amino acid substitutions at the residue before 134 (Ser-Leu) and at residue 193 (Lys-Arg) (H3 numbering is used throughout), which are at the receptor binding site and adjacent to the receptor binding site, respectively (14, 15). The amino acid residues Gln-226 and

Table 2. Hemagglutinin inhibition titers showing the antigenic interrelation of H5N1 viruses isolated in Hong Kong from 1997 to 2003

| Virus antigen | Genotype | Sheep | Chicken | Duck | mAb | mAb | mAb | mAb |
|--------------------|----------------|------------------|-----------------------|-----------------------|---------|---------|---------|----------|
| | | anti-A/HK/156/97 | anti-A/Gs/HK/437.4/99 | anti-A/Dk/HK/739.2/02 | CP24 | CP46 | CP58 | CP406/07 |
| A/Ck/PA/1370/83 | — | 2560 | 640 | <40 | ≥12,800 | ≥12,800 | ≥12,800 | ≥12,800 |
| A/HK/156/97 | H5N1/97 | 5120 | 5120 | 40 | 3,200 | ≥12,800 | ≥12,800 | ≥12,800 |
| A/Gs/HK/437.4/99 | Gs/Gd | 5120 | 5120 | 80 | 1,600 | ≥12,800 | ≥12,800 | ≥12,800 |
| A/Gs/HK/ww28/00 | Gs/Gd | 5120 | 2560 | 80 | 3,200 | ≥12,800 | ≥12,800 | 800 |
| A/Ck/HK/822.2/01 | A | 2560 | 640 | <40 | 1,600 | <100 | ≥12,800 | 100 |
| A/Ck/HK/YU562/01 | B | 1280 | 640 | <40 | 3,200 | 6,400 | ≥12,800 | 200 |
| A/Ph/HK/FY155/01 | C | 1280 | 640 | <40 | 400 | 3,200 | ≥12,800 | 1,600 |
| A/Ck/HK/31.4/02 | B | 1280 | 320 | <40 | 200 | <100 | 6,400 | 100 |
| A/Ck/HK/sv409.1/02 | Y | 1280 | 320 | <40 | ≥12,800 | 200 | 200 | <100 |
| A/Ck/HK/96.1/02 | Y | 320 | 160 | <40 | ≥12,800 | <100 | 400 | <100 |
| A/Dk/HK/821/02 | Z | <40 | 320 | 40 | <100 | <100 | <100 | <100 |
| A/Ck/HK/61.9/02 | Z | 40 | 640 | 40 | <100 | 1,600 | <100 | <100 |
| A/Ck/HK/YU777/02 | Z | 40 | 640 | 40 | <100 | 3200 | <100 | <100 |
| A/Gh/HK/793.1/02 | Z | 40 | 640 | 40 | 100 | <100 | <100 | <100 |
| A/Gs/HK/739.2/02 | Z ⁺ | 80 | 1280 | 80 | 800 | <100 | <100 | <100 |
| A/Eg/HK/757.3/02 | Z ⁺ | 80 | 640 | 160 | 400 | <100 | <100 | <100 |
| A/HK/212/03 | Z ⁺ | 160 | 2560 | 320 | 1,600 | 100 | 400 | 200 |
| A/HK/213/03 | Z ⁺ | 160 | 2560 | 640 | 1,600 | 100 | 400 | 200 |

Underlined numbers indicate titers to prototype viruses.

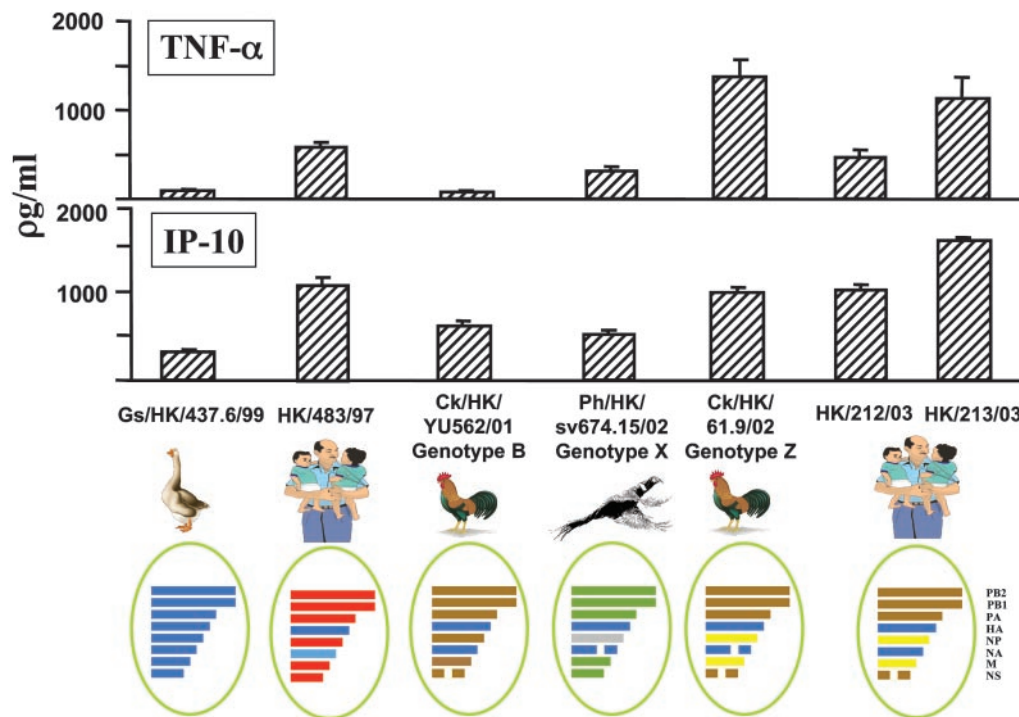


Fig. 2. Expression of TNF- α and IP-10 protein in primary human macrophages infected *in vitro* with H5N1 viruses of the indicated genotypes. TNF- α and IP-10 were assayed in culture supernatants 6 h after inoculation with virus at a multiplicity of infection of 2.0.

Gly-228, which are crucial for binding to receptors that have the avian-specific α -2,3-NeuAcGal sialic acid linkage, were unchanged in these viruses (14). However, there was a unique Ser227Asn substitution within the receptor-binding site of the human A/HK/212/03 isolate that was not found in A/Gs/HK/739.2/02 (Z⁺ genotype) or any of the other avian viruses that were analyzed. A Ser227Ile substitution at this position has been shown (16) to alter the virulence of human H5N1/97 viruses in mice. When compared with H5N1 genotypes A–E that were isolated in 2001 (6), the genotype Z⁺ viruses (including the human isolates) had 10 aa changes in the HA. Five of these mutations were located at the globular head of HA1 and might be relevant to antibody binding. By using the H3 numbering system (14, 15), three of these mutations were at positions 129 (Asn-Ser), 142 (Leu-Gln), and 193 (Lys-Arg). The two other mutations were adjacent to amino acids 126 and 134, respectively (Ser-Asn, –1 of 126; and Ser-Leu, –1 of 134).

We identified a unique amino acid substitution (Ser31Asn) in M2 in both of the human H5N1 viruses but not in the viruses obtained from Penfold Park (genotype Z⁺). This mutation is associated with amantadine resistance (17). The infected boy had received two doses of amantadine before specimens were collected for virus isolation; the father had not received amantadine. Some genotype B and Y viruses also carry Asn at this position.

Lys-627 in PB2 and Glu-92 in NS1 have been proposed to be important determinants of the virulence of H5N1/97 viruses in mammals (16, 10). The A/HK/212/03 and A/HK/213/03 viruses had neither mutation.

Induction of Proinflammatory Cytokines in Primary Human Macrophages. The H5N1/97 viruses associated with human disease induced a massive proinflammatory cytokine response in primary human macrophages *in vitro* (9). To investigate whether the recent human H5N1 isolates have this phenotype, we compared viruses isolated from humans in 1997 (A/HK/483/97) and 2003 (A/HK/212/03 and A/HK/213/03) with a virus representing the parental

A/Gs/Gd/96-like (H5N1) lineage (A/Gs/HK/437.6/99) and with other contemporary H5N1 reassortants isolated from poultry. The human influenza virus A/HK/54/97 (H1N1) and lipopolysaccharide were used as low and high cytokine inducers, respectively. As previously reported, the human H5N1/97 virus induced high TNF- α expression, whereas the human H1N1 virus (data not shown) and A/Gs/HK/437.6/99 induced low TNF- α expression (Fig. 2). Induction of IP-10 followed a similar pattern. Inactivation of the virus by UV irradiation reduced cytokine induction markedly (data not shown).

Among the recent avian H5N1 reassortants, A/Ck/HK/YU562/01 (genotype B) induced a low TNF- α response, A/Ph/HK/sv674.15/02 (genotype X) induced an intermediate response, and A/Ck/HK/61.9/02 (genotype Z) induced a high response. Genotypes X and B induced intermediate-level expression of IP-10, whereas genotype Z induced high-level expression of IP-10. A/HK/212/03 and A/HK/213/03 were high inducers of both TNF- α and IP-10 expression. The results of the mRNA assays were consistent with the results of the protein assays (data not shown). These findings suggest that the human H5N1 viruses of 2003 have a high cytokine-inducer phenotype like that of the 1997 human H5N1 isolates. The genotype Z virus isolated from poultry also shared this phenotype, and thus, the TNF- α -inducing phenotype appears to be related to pathogenicity in humans.

Viral Pathogenicity and Organ Tropism in Mice. In a mammalian model, we compared the pathogenicity of two representative viruses that were isolated early in 2002 from chickens, virus isolated from a dead Canadian goose (A/Gs/HK/739.2/02), and virus isolated from a human in 2003 (A/HK/213/03) (Table 3). All of the viruses replicated to high titers in the lungs of mice without adaptation; three of the four viruses that were tested were also isolated from the brain. Neurological signs (hind-leg paralysis, paresis, and disorientation) were observed in mice inoculated with two of the viruses but not with A/HK/213/03. All of the viruses were lethal to one or more mice; however, a high inoculating dose of virus was used.

Table 3. Pathogenicity of avian and human H5N1 viruses in mice

| Viruses* | Deaths | Days to death | Lung titer | Brain titer | Kidney titer | Paralysis |
|------------------|--------|---------------|------------|------------------|--------------|----------------------|
| A/Ck/HK/61.9/02 | 7/7 | 8 | 4.8 ± 0.29 | No | No | No |
| A/Ck/HK/31.4/02 | 2/7 | 9 | 3.6 ± 0.38 | 1.8 [†] | No | Yes (1/9) |
| A/Gs/HK/739.2/02 | 3/11 | 10 | 2.8 ± 0.5 | 3.25 ± 0.25 | No | Yes (3/8, 3/9, 3/10) |
| A/HK/213/03 | 10/11 | 11 | 4.5 ± 0.5 | 4.0 [†] | No | No |

Groups of mice were inoculated intranasally with 50 μ l of allantoic fluid containing $\approx 0.5 \times 10^{6.5}$ eID₅₀ of virus. Titers are expressed as the mean \pm SD log₁₀ eID₅₀ per 0.1 ml of tissue homogenate. Deaths are expressed as the number of deaths per number inoculated, and paralysis is expressed as the number of mice with paralysis per number of days after inoculation.

*Infectivity in embryonated chicken eggs for all viruses was 10^{7.5} eID₅₀ per 0.1 ml.

[†]Virus was detected and titered in the brain tissues of one paralyzed or dead mouse.

There were interesting differences in pathogenicity between the human A/HK/213/03 (H5N1) isolate and its genotypically closest precursor, the avian virus A/Gs/HK/739.2/02 (H5N1). The mortality rate was 90% in mice inoculated with the human virus but only 30% in mice inoculated with the same dose of the avian virus (see Fig. 4, which is published as supporting information on the PNAS web site). Morbidity differed as well: mice infected with the human isolate lost as much as 40% of their body weight before death, whereas mice infected with A/Gs/HK/739.2/02 lost only \approx 10% of their body weight and recovered rapidly (Fig. 4). Both viruses replicated in the lungs, but the titer of the human isolate was 62% higher than that of the goose isolate. These findings show that the human isolate is more pathogenic in mice than the goose isolate.

Discussion

It remains unknown what viral and human genetic characteristics allowed the transmission of avian influenza viruses to humans. The human H5N1 isolates have not acquired gene segments from influenza viruses that are established in humans. Amino acid residues at positions 226 and 228 of the receptor binding pocket of HA1 appear to determine binding affinity to cell surface receptors and to influence the selective binding of the virus to avian (sialic acid α -2,3-NeuAcGal) or human (sialic acid α -2,6-NeuAcGal) cell surface receptors. The human A/HK/212/03 and A/HK/213/03 isolates retain the signature associated with avian receptor binding, but they have a unique amino acid substitution (Ser227Ile) within the receptor binding pocket that was not present even in the closely related A/Gs/HK/739.2/02 (genotype Z⁺) virus. Although the biological significance of this change is unclear, a Ser-to-Ile substitution at this position has been shown (16) to alter the virulence of human H5N1/97 viruses in mice. Interestingly, the A/HK/213/03 and A/Gs/HK/739.2/02 showed markedly different pathogenicity in mice.

The HA1 region of the 2001 and 2002 H5N1 isolates differed at 10 aa. Five of the amino acids are located in the globular head of the HA1 molecule, and three of the amino acids (positions 129, -1 of 134, and 142) are within the known antigenic sites A and B. The mAbs CP46 and CP58, which reacted with H5N1/97 viruses, with the precursor A/Gs/HK/437.4/99 virus, and with many of the reassortants that emerged in 2001, were not reactive with the 2002 isolates of genotypes Y, Z, and Z⁺. These mAbs bind epitopes defined by HA1 antigenic site B, and their binding is affected by mutations at positions 129 and 131 (124 and 126 in H5 numbering) (18). The observed antigenic drift may be the result of selection pressure exerted by vaccination or by naturally acquired immunity in the avian host. Alternatively, the HA of these viruses may be derived from variants within the naturally diverse ecosystem of H5N1 viruses.

Both the A/HK/212/03 and A/HK/213/03 isolates had a Ser31Asn substitution in M2, which is associated with amantadine resistance. Some genotype B and Y viruses also carried this mutation. Similar mutations associated with amantadine resistance have been reported in porcine influenza viruses (19), and all of the

early human H1N1 isolates before 1940 with a similar substitution are amantadine resistant (R.G.W., unpublished data). It remains unknown why such resistance has emerged and persisted in the influenza viruses of these populations in the absence of known amantadine exposure. The possibility that animal feed contains amantadine-like molecules should be investigated. These findings suggest that amantadine is not a therapeutic option for human H5N1 disease. One of the patients who experienced H5N1 disease recently recovered completely after treatment with amantadine, although the role of amantadine in his recovery is not clear (20).

The clinical findings in the 2003 fatal case of H5N1 disease in the 33-year-old man are reminiscent of clinical findings observed in the 1997 cases (8, 20). Progressive primary viral pneumonia, lymphopenia, a raised liver transaminase level, and reactive hemophagocytosis were observed in these patients. The host factors and viral factors that contributed to interspecies transmission and pathogenicity in these cases remain to be defined.

The pathogenicity in mice of the recently emergent H5N1 genotypes, including the human virus isolates, is relevant. All genotypes isolated in 2001 (6) and 2002, as well as the human isolates from 2003, replicated efficiently in mouse lungs without prior adaptation but caused mortality only at a high virus dose (6). Highly pathogenic neurotropic variants of most studied genotypes can be selected and isolated from mouse brain after a single passage in the lungs (11). However, genotype B viruses (e.g., A/Ck/HK/YU562/01) and Gs/Gd/1/96-like viruses (e.g., A/Gs/HK/437.6/99) were not detected in mouse brain (6, 11). Interestingly, the avian Gs/HK/739.2/02 and human A/HK/213/03 isolates had markedly different phenotypes despite their genetic similarity. A/Gs/HK/739.2/02, the presumed precursor of A/HK/213/03, caused a lower rate of mortality, and the mice that died showed neurological signs, such as paralysis. In contrast, the higher rate of mortality caused by human A/HK/213/03 virus was associated with bronchopneumonia rather than paralysis (Table 3). Clearly, not only can the H5N1 viruses be highly pathogenic in mammals, but a small number of genetic differences can influence the character of the disease markedly.

In primary human macrophages, the human H5N1 virus isolates of 1997 were more potent inducers of proinflammatory cytokines, including TNF- α , than the established human H1N1 or H3N2 influenza virus subtypes (9); this difference may be related to the pathogenic potential of the viruses. Whereas the precursor Gs/Gd/1/96-like H5N1 viruses (e.g., A/Gs/HK/437.6/99) were low inducers of TNF- α and IP-10 in this model, the A/HK/213/03 and a Z genotype virus (A/Ck/HK/61.9/02) were high inducers of both cytokines. The two 2003 patients with H5N1 disease had unusually high serum levels of IP-10 (20). The parallel between the cytokine induction phenotype *in vitro* and the pathogenicity of an H5N1 virus in humans is striking and may be pathogenetically relevant. The current dominance in chickens of the Z genotype, a high cytokine inducer, suggests that human infection with these H5N1 viruses may lead to clinically severe disease.

Studies of the H5N1/97 viruses (A/HK/483/97 and A/HK/486/97) indicated that the internal protein gene constellation, rather than the genes encoding surface proteins, was the essential determinant of the high TNF- α -inducing phenotype (9). Because none of the internal protein genes of the A/HK/212/03 or A/Ck/HK/61.9/02 (genotype Z) viruses are phylogenetically related to those of the 1997 H5N1 isolates, the genetic basis of their high TNF- α induction remains unclear. Cheung *et al.* proposed that the NS gene contributes to the high TNF- α -inducing phenotype of the H5N1/97 viruses (9). The A/HK/212/03 virus and genotypes Z⁺, Z, Y, A, B, and C viruses have a 5-aa deletion (residues 80–84) in the NS1 protein that is not seen in H5N1/97 or A/Gs/HK/437.6/99 viruses, but this deletion does not appear to be related to the high TNF- α -inducing phenotype. Mutations at position 627 of PB2 and at position 92 of NS1 have been reported to be important determinants of the virulence of H5N1/97 viruses in mammals (10, 16). However, neither the recent human H5N1 isolates nor the genotype Z⁺ viruses contain these mutations. Therefore, the high cytokine- and chemokine-inducing phenotype appears to be associated with overall gene constellation rather than an individual gene segment or mutation.

Although influenza viruses have been isolated from a broad range of wild aquatic birds (21), highly pathogenic avian influenza viruses have been reported in wild birds on only three occasions (22–24). In aquatic birds, the natural hosts of influenza viruses, infection is usually asymptomatic and localized to the intestinal tract. Even the H5N1/97 viruses caused no disease in experimentally infected ducks (25). Although wild birds were thought to introduce avian influenza viruses into poultry flocks, the viruses were presumed to become highly pathogenic only after transmission to poultry (26). The isolation of highly pathogenic H5N1 viruses in wild birds is, therefore, an important finding, particularly in view of the concurrent H5N1 outbreaks in Hong Kong farms (2002 and 2003) and parks (2002) and the peak migratory season of wild birds. The ability of recent H5N1 isolates (i.e., those from Kowloon and Penfold Parks) to cause disseminated disease in ducks is unprecedented and reflects their unusual virulence (13). Recently, we reported the two-way transmission of influenza viruses

between terrestrial and aquatic poultry (27). The unusual virulence of recent H5N1 isolates in waterfowl could, therefore, be explained by the prolonged adaptation of these viruses, especially the genotype Z viruses from Kowloon Park, in another avian reservoir, such as land-based birds.

Our findings suggest that H5N1 viruses are actively reassorting and crossing interspecies-host barriers, moving from aquatic poultry to land-based poultry and, more recently, to wild terrestrial birds and man. Isolation of H5N1 viruses from wild terrestrial birds implies that these viruses are already widespread in the region and that surveillance and control of H5N1 infection in humans and poultry are needed in the wider region. The rapid global spread of severe acute respiratory syndrome (SARS) illustrated the severe impact of a new viral respiratory illness that can be transmitted from human to human. A pandemic influenza strain is likely to be far more transmissible than the SARS agent and is unlikely to be controllable by isolation, quarantine, or travel advisories. Thus, the reemergence of H5N1 disease in humans is a cause for renewed pandemic concern and a call for preemptive action, especially in view of the recent reports of human H5N1 disease in Thailand and Vietnam by the World Health Organization (available at www.who.int/csr/don/2004_01_22/en). There is an urgent need for intensified global influenza surveillance, for assessment of factors that allow the transmission of avian viruses to humans, and for development of effective vaccines against H5-subtype influenza virus for both humans and animals.

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