

Origin of the Pandemic 1957 H2 Influenza A Virus and the Persistence of Its Possible Progenitors in the Avian Reservoir

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Received November 6, 1992; accepted March 9, 1993

H2N2 influenza A viruses caused the Asian pandemic of 1957 and then disappeared from the human population 10 years later. To assess the potential for similar outbreaks in the future, we determined the antigenicity of H2 hemagglutinins (HAs) from representative human and avian H2 viruses and then analyzed the nucleotide and amino acid sequences to determine their evolutionary characteristics in different hosts. The results of longitudinal virus surveillance studies were also examined to estimate the prevalence of avian H2 isolates among samples collected from wild ducks and domestic poultry. Reactivity patterns obtained with a large panel of monoclonal antibodies indicated antigenic drift in the HA of human H2 influenza viruses, beginning in 1962. Amino acid changes were clustered in two regions of HA1 that correspond to antigenic sites A and D of the H3 HA. By contrast, the antigenic profiles of the majority of avian H2 HAs were remarkably conserved through 1991, resembling the prototype Japan 57 (H2N2) strain. Amino acid changes were distributed throughout HA1, indicating that antibodies do not play a major role in the selection of avian H2 viruses. Phylogenetic analysis revealed two geographic site-specific lineages of avian H2 HAs: North American and Eurasian. Evidence is presented to support interregion transmission of gull H2 viruses. The human H2 HAs that circulated in 1957–1968 form a separate phylogenetic lineage, most closely related to the Eurasian avian H2 HAs. There was an increased prevalence of H2 influenza viruses among wild ducks in 1988 in North America, preceding the appearance of H2N2 viruses in domestic fowl. As the prevalence of avian H2N2 influenza viruses increased on turkey farms and in live bird markets in New York City and elsewhere, greater numbers of these viruses have come into direct contact with susceptible humans. We conclude that antigenically conserved counterparts of the human Asian pandemic strain of 1957 continue to circulate in the avian reservoir and are coming into closer proximity to susceptible human populations. © 1993 Academic Press, Inc.

INTRODUCTION

The hemagglutinin (HA) molecule of influenza A viruses is responsible for the binding of virions to host cell receptors and for fusion between the virion envelope and the host cell (Wharton *et al.*, 1989). As such, this glycoprotein represents the dominant surface antigen of the influenza virus virion, subject to intense selective pressure from host immune responses. Although the amino acids making up the receptor-binding site are well conserved, the remainder of the HA molecule is highly mutable, resulting in 14 recognized HA subtypes (termed H1 through H14). The amino acid sequences of these variants differ by at least 30% from each other and are not serologically cross-reactive.

Influenza viruses of the H2 subtype caused a major pandemic in 1957 and then disappeared from humans in 1968. Similar viruses appear responsible for an Asian epidemic in the last quarter of the 19th century, which may have led to the pandemic of 1889–1890 (Mulder *et al.*, 1958). Since influenza A viruses circulat-

ing in aquatic birds are probably the progenitors for most influenza A viruses in other species (Webster *et al.*, 1992), pandemic strains of the H2 serotype would appear to arise from avian prototypes. Yet, there are only isolated instances in which viruses isolated from birds have been genetically related to human H2 viruses. Tumova *et al.* (1975) characterized duck H2 viruses isolated in Germany in 1972–1973 that were closely related to the 1957 pandemic strain; a few years later Shortridge (1979) isolated H2N2 viruses from ducks in southern China. More recently, Schäfer *et al.* (1991) characterized H2N2 viruses similar in antigenic properties to the 1957 pandemic strain.

Although providing suggestive evidence that pandemic H2 influenza A viruses can recycle through avian species, the above observations do not establish which geographical lineage of H2 influenza viruses are the progenitors of those in humans, nor do they establish the absence of antigenic drift in avian H2 influenza viruses. We therefore undertook a detailed study of the

TABLE 1
INFLUENZA A VIRUSES USED IN ANALYSES

Geographic region	Strain	Abbreviation	Source of sequence or virus strain
Human H2N2 viruses			
Asia	A/Singapore/1/57	Singapore 57	This study
	A/Japan/305/57	Japan 57	Gething et al., 1980
	A/Guiyang/1/57	Guiyang 57	Serology only ^a
	A/Korea/426/68	Korea 68	This study
Russia	A/Krasnodar/101/59	Krasnodar 59	This study
Europe	A/England/12/62	England 62	Serology only
	A/Berlin/3/64	Berlin 64	This study
N. America	A/Ann Arbor/6/60	Arbor 60	Herlocher (personal communication)
	A/Berkeley/1/68	Berkeley 68	This study
Avian H2 viruses			
Asia	A/duck/HK/273/78 (H2N2)	Duck HK 78	This study
Russia	A/Pintail/Praimoric/625/76 (H2N2)	Pintail Prai 76	This study
	A/mallard/MT/Y61 (H2N2)	Mall MT 61	This study
Europe	A/duck/GDR/72 (H2N9)	Duck GDR 72	This study
	A/mallard/Potsdam/178-4/83 (H2N2)	Mall Po 83	This study
	A/chicken/Potsdam/4705/84 (H2N2)	Chick Po 84	This study
	A/Peking duck/Potsdam/1689-4/85 (H2N3)	P duck Po 85	This study
	A/Peking duck/Potsdam/648-6/86 (H2N3)	P duck Po 86	Serology only
	A/mallard/ONT/56/76 (H2N3)	Mall Ont 76	This study
N. America	A/gull/MD/19/77 (H2N8)	Gull MD 77	This study
	A/mallard/NY/6750/78 (H2N2)	Mall NY 78	This study
	A/herring gull/DE/677/88 (H2N8)	H Gull DE 88	This study
	A/mallard/ALB/353/88 (H2N3)	Mall Alb 88	This study
	A/chicken/CT/13657/90 (H2N2)	Chick CT 90	Serology only
	A/avian/Mass/25756/90 (H2N2)	Avian Mass 90	Serology only
	A/Guinea Fowl/NJ/26146/90 (H2N2)	G Fowl NJ 90	Serology only
	A/Guinea Fowl/NJ/3070/91 (H2N2)	G Fowl NJ 91	This study
	A/chicken/PA/24826/91 (H2N2)	Chick PA 91	Serology only
	A/chicken/NY/29878/91 (H2N2)	Chick NY 91	Serology only

^a The HA sequence of these viruses was not determined, they were analyzed in serological studies only (Table 2).

evolutionary relationships among H2 influenza viruses to identify the lineage of H2 influenza viruses that infected humans in 1957 and to characterize H2N2 influenza viruses that are becoming more prevalent in wild ducks and domestic poultry.

MATERIALS AND METHODS

Viruses

The influenza A viruses used in this study are listed in Table 1. North American viruses were from the repository at St. Jude Children's Research Hospital; European viruses were from the repository at the Institute of Veterinarian Medicine of the Federal Health Office, Berlin; and RNA from Asian and Russian viruses was kindly provided by Dr. Hiroshi Kida, Sapporo, Japan, and Dr. Svetlana Yamnikova, Moscow, Russia, respectively. The isolates were selected to represent a spectrum of geographic locations, hosts, and dates of isolation. Viruses grown in 11-day-old embryonated chicken eggs were purified, and virion RNA was prepared as previously described (Bean *et al.*, 1980).

Serologic analyses

Monoclonal antibodies to the H2 HA used in this study were previously described by Yamada *et al.* (1984). Hemagglutination inhibition (HI) tests were done as described previously (Webster *et al.*, 1979). Enzyme-linked immunoassays (ELISA) with monoclonal antibodies to hemagglutinin were performed by the method of Voller *et al.* (1977).

Cloning of the HA gene

Full-length cDNA was prepared by reverse transcription of virion RNA as described by Huddleston and Brownlee (1982). A 12-base synthetic primer complementary to the 3' terminus of the virion RNA was phosphorylated with T4 polynucleotide kinase and then used for synthesis of first-strand DNA by reverse transcription of the total virion RNA in the presence of [α -³²P]dATP. Second-strand DNA synthesis was performed with a phosphorylated 13-base synthetic primer complementary to the 3' end of the cDNA and the Klenow fragment of *Escherichia coli* DNA polymerase I. Full-length double-stranded copies of the HA

gene were blunt-end ligated into the *PvuII* site of vector pATX, obtained from C. Naeve (St. Jude Children's Research Hospital, Memphis, TN).

For viruses from Europe, Russia, and Hong Kong (where RNA was available), the first-strand cDNA was prepared by reverse transcription as described below and then amplified by the polymerase chain reaction (PCR) using phosphorylated primers. The PCR product was blunt-end ligated into the *PvuII* site of pATX.

Nucleic acid sequencing

Nucleotides of the cloned HA genes were sequenced by the method of Chen and Seeburg (1985) using alkali-denatured DNA templates. Oligonucleotide primers complementary to the HA gene segment were synthesized on an Applied Biosystems model 280A DNA synthesizer by the solid-phase phosphoramidite method. The reaction products were separated on 6% polyacrylamide-7 M urea 0.4 mm gels.

Sequence analysis

The IntelliGenetics software package (Palo Alto, CA) was used for analysis and transmission of nucleotide sequence data. The A/chicken/Pennsylvania/1/83 (H5N2) nucleotide sequence (Kawaoka *et al.*, 1984; Ohuchi *et al.*, 1989) was aligned with the influenza H2 HAs and used for rooting the nucleotide tree.

Phylogenetic analysis of sequence data was performed with the PAUP software package, version 2.4 (David Swofford, Illinois Natural History Survey, Champaign, IL). This analysis was done by the maximum-parsimony method to generate phylogenetic trees (Fitch, 1971). The shortest (most parsimonious) tree was found by implementing the MULPARS, SWAP = GLOBAL and HOLD = 10 options of PAUP. The total tree length is the sum of all branch lengths.

Nucleotide sequence accession numbers

Sequence data from this article have been deposited with the EMBL/GenBank Data Libraries under Accession Numbers 11125 through 11142.

RESULTS

Antigenic relationships among H2N2 influenza viruses from humans and birds

To assess the antigenic relationships among human and avian H2 viruses, we used a panel of monoclonal antibodies (Mabs) to the HA of Japan 57 and Guiyang 57 in combination with the HI test and ELISA. The majority of Mabs reacted in both the ELISA and HI assays (Table 2), although a substantial number reacted only in the ELISA. This was most evident with the avian viruses and may indicate reduced affinity of binding due to amino acid changes in the epitope.

As shown in Table 2, antigenic differences were evident among human H2 viruses in both the HI and ELISA assays. The results suggested two distinct patterns of reactivity; the human H2N2 influenza viruses that circulated from 1957 to 1960 showed similar reactivity patterns without detectable antigenic drift, whereas extensive drift was detected in England 62 and Korea 68 which reacted with only two or three of the Mabs. There were no counterparts of H2 influenza viruses among avian species during the 1957-1968 interval. Surveillance studies to elucidate the influenza virus gene pool in nature were not begun until the early 1970s; consequently, Duck GDR 72 (H2N2) was the oldest avian H2 influenza virus available for study.

Among avian viruses, Duck HK 78 (H2N2) and the European avian isolate Mall Po 83 (H2N2) showed reactivity profiles similar to those of Singapore 57 (group A), whereas the first avian H2 isolate (Duck GDR 72) and a North American influenza virus isolated 16 years later from a gull (H Gull DE 88) had antigenic properties most akin to those of Korea 68 and England 62 (group B), especially in the HI test (Table 2). The H2 viruses recently isolated from chickens in the United States could also be placed in groups A and B, based on reactivity. Chick NY 91, for example, reacted with the Mab panel much like the human virus strains isolated after 1962, similar to England 62 and Korea 68; whereas Chick PA 91, Chick CT 90, G Fowl NJ 90, and Avian Mass 90 appeared more closely related to Japan 57.

These studies indicate that the majority of H2N2 influenza viruses in feral birds (mallard ducks, gulls) and domestic birds (ducks, chickens, guinea fowl) are antigenically more closely related to the earliest human H2N2 viruses, such as Japan 57, than to the later human viruses, such as Korea 68. This suggests that H2N2 viruses, antigenically similar to the virus that was the progenitor of the Asian/57 pandemic, are still circulating in birds after 35 years. Antigenic analysis cannot resolve whether the second antigenic group (B) of avian H2N2 influenza viruses is of human or avian origin.

Nucleotide and amino acid analyses

Each of the 20 H2 HA genes that was cloned and sequenced was composed of 1773 nucleotides, had a single open reading frame spanning positions 44-1732, and encoded a polypeptide of 562 amino acids (14 in the signal peptides, 326 in the HA1 polypeptide, and 221 in the HA2 polypeptide). Mall Alb 88, Gull MD 77, and Mall Ont 76 had a deletion at position 36 and Pintail Prai 76 and Duck HK 78 had a similar deletion at position 38; both of these positions are in the noncoding region of the HA gene.

All but one of the 20 H2 viruses we investigated had 15 conserved cysteine residues in the HA (Fig. 1). The

TABLE 2
REACTIVITY OF MONOCLONAL ANTIBODIES WITH H2 INFLUENZA VIRUSES IN HI TEST AND ELISA

Virus strain	Mab to Japan 57										Mab to Guiyang 57						Reactivity pattern
	33/1	79/1	61/7	41/4	121/7	67/7	134/4	137/5	112/2	L122/4	18	23	10	4	33	17	
Human viruses																	
Guiyang 57	+	+	+	+	-	+	+	+	+	+	2000 26	128 13	128 26	512 13	1024 6	128 6	A
Japan 57	16 ^a <	256 51	128 3	512 6	128 12	128 3	16 2	128 3	2 <	512 51	+	-	±	±	±	-	A
Singapore 52	+ ^b	+	+	+	- ^c	+	+	+	+	+	+	+	+	+	+	+	A
Krasnodar 59	+	+	+	+	+	+	+	+	+	+	+	+	±	±	±	+	A
England 62	-	-	+	-	-	-	-	-	-	-	-	-	-	+	-	-	B
Korea 68	-	-	+	-	-	-	-	-	+	-	-	-	-	+	-	-	B
Avian viruses																	
Duck GDR 72	± ^d	±	+	±	-	±	-	±	+	+	-	-	-	±	±	±	B
Pint Prai 76	+	+	+	+	+	+	+	+	+	+	+	-	±	±	±	-	A
Mall ONT 76	±	+	+	+	+	+	+	+	-	+	+	-	±	±	±	-	A
Duck HK 78	±	+	+	+	+	+	+	+	±	+	+	+	±	±	±	±	A
Mall Po 83	±	+	+	+	+	+	+	+	±	+	+	-	+	±	±	±	A
P Duck Po 86	±	+	+	+	+	+	+	+	±	+	-	-	±	±	±	±	A
Gull DE 88	±	-	+	±	±	-	-	-	-	+	+	-	-	±	±	-	B
Chick CT 90	+	+	+	+	+	+	+	+	-	+	+	-	+	±	-	-	A
Avian Mass 90	+	+	+	+	+	+	+	+	-	+	+	-	+	±	-	-	A
G Fowl NJ 90	+	+	+	+	+	+	±	+	-	+	±	-	±	±	±	-	A
Chick PA 91	+	±	+	+	+	+	+	+	-	+	+	-	±	±	±	-	A
Chick NY 91	-	-	-	-	-	-	-	-	-	-	-	-	-	±	-	-	B
G Fowl NJ 91	±	+	+	+	+	+	+	+	±	+	+	±	+	±	±	±	A

^a Homologous antibody titers ($\times 10^{-3}$) ELISA titers on top, HI titers below; <, less than 100.

^b (+) ELISA and HI titers less than 10-fold difference from homologous titers.

^c (-) ELISA and HI titers greater than 10-fold below the homologous titers or were undetectable.

^d (±) ELISA titers not different from homologous titers, HI titers greater than 10-fold below the homologous titers or were undetectable.

exception was Arbor 60, with 16 cysteines (Dr. Louise Herlocher, personal communication). The other conserved amino acid residues in the H2 HA were histidine (13 of 14 residues are conserved in all strains analyzed), tryptophan (all 11 residues are conserved), and

proline (17 of the 20 residues are conserved) (Fig. 1). There were seven potential glycosylation sites (Asn-X-Ser/Thr) on H2 HAs, five on the HA1 subunit (positions 25/26, 38, 154, 179, and 300), and two on the HA2 subunit at residues 153 and 217. An exception was found with both of the gull virus strains (gull/MD/77, Gull DE 88), in which a glycosylation site at position 154 on the HA1 subunit was missing, and there was an extra glycosylation site on the HA2 subunit at residue 169 (Fig. 1).

Figure 2 presents results of a phylogenetic analysis of the HA nucleotide sequences in 20 H2 influenza A viruses. The evolutionary tree is rooted to the H5 HA sequence from A/chicken/Pennsylvania/83 because of the high homology (74% overall homology) between H2 and H5 serotypes (Air *et al.*, 1981; Nobusawa *et al.*, 1991). The shortest unrooted tree was 1487 nucleotide changes long. From this analysis, it was possible to divide the H2 HA genes into two host- and geographic site-specific lineages. The major fork in the tree (A) represents a separation of the North American avian lineage from the human and avian lineages of Asia and Europe. The human H2 viruses (F) are clearly separated from the Eurasian avian strains (E and D),

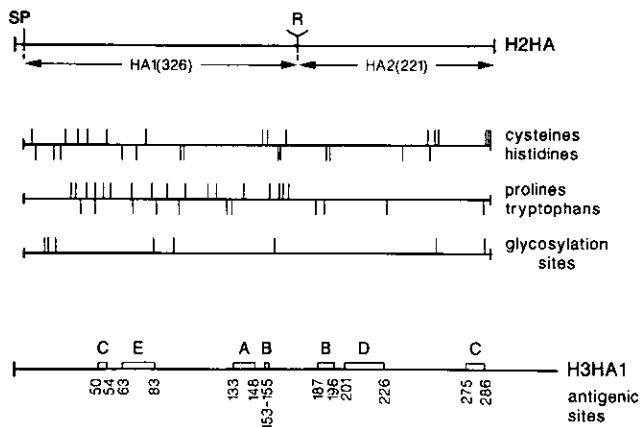


FIG. 1. Linear diagram of the H2 hemagglutinin. SP, signal peptide; R, arginine at the cleavage site. The location of conserved amino acids, glycosylation sites, and antigenic sites (based on the H3 hemagglutinin) are indicated by vertical lines.

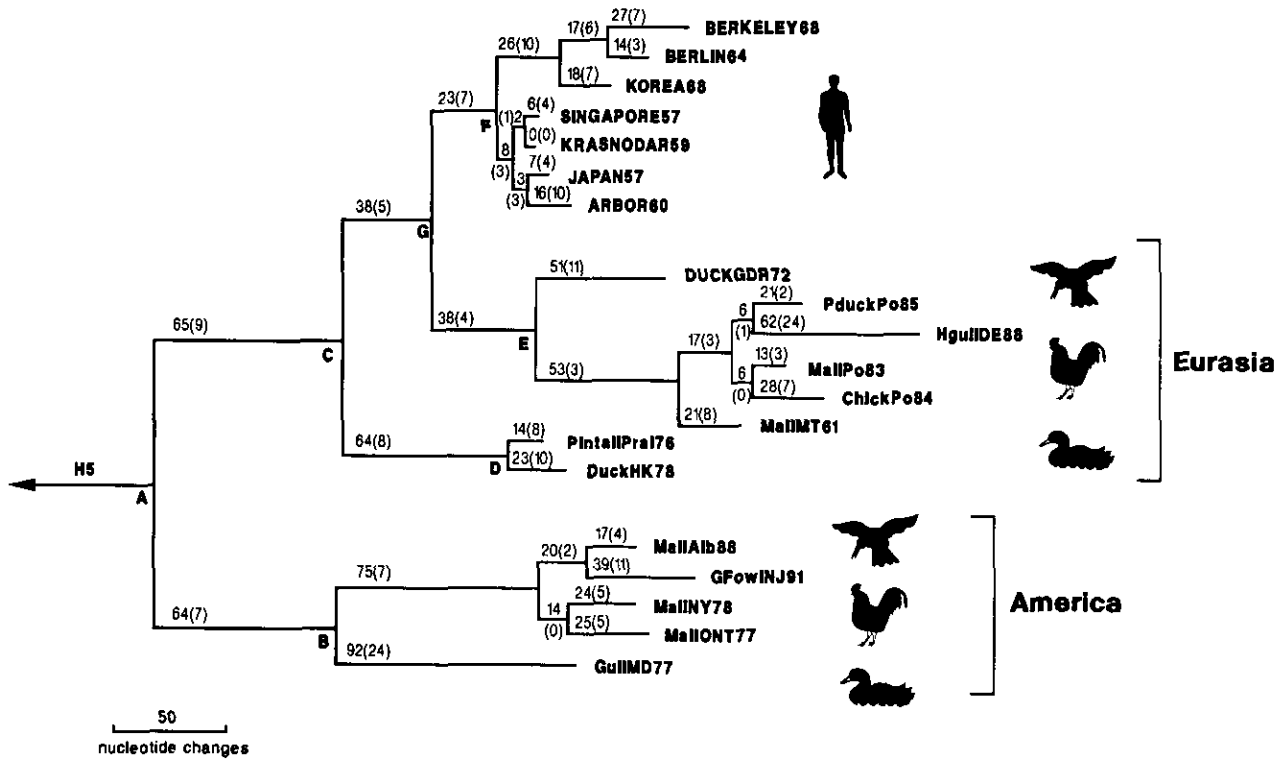


FIG. 2. Phylogenetic tree for H2 influenza A virus HA genes. The tree is rooted to the H5 HA sequence from A/Chicken/Pennsylvania/83. The arrow at the far left indicates the direction of evolution from the root node. Horizontal distances are proportional to the minimum number of nucleotide differences required to join nodes and H2 HA gene sequences; vertical lines are for spacing branches and labels. Numbers on the horizontal lines specify nucleotide branch distances, with amino acid branch lengths given in parentheses; letters after groups of nodes denote discrete separations of lineages. Strain abbreviations are listed in Table 1.

but the European and Asian strains are not clearly separated: Pintail Prai 76 from the former USSR is grouped with DK HK 78 and is separated at C from the European avian strains. This probably reflects the insufficient number of H2 HAs from Asia that were examined in this analysis.

The avian viruses were divided into North American and Eurasian lineages by virtue of their geographic regions of isolation. Nonetheless, Gull DE 88, isolated from a herring gull in the United States (Delaware), belongs to the European H2 lineage, indicating latitudinal transfer of viruses between geographic areas. The human H2 HA sublineage (F) is composed of two groups: human viruses isolated from 1957–1960 and those isolated at later dates.

The amino acid branch lengths are shown in parentheses in Fig. 2, where it can be deduced that the lengths for human strains are relatively longer than those for avian strains. The nucleotide substitutions in the terminal branches that resulted in amino acid substitutions were highest in the early human H2 strains (Singapore 57, Krasnodar 59, Japan 57, Arbor 60), where on the average, 1.6 nucleotide changes resulted in an amino acid change. The later human strains (Berkeley 68, Berlin 64, Korea 68) showed fewer amino acid changes in the terminal branches per nu-

cleotide change (an average of 3.7 nucleotide changes resulted in an amino acid change). For avian influenza viruses in the North American lineage, 4.4 nucleotide changes resulted in an amino acid change.

To determine if the amino acid substitutions in HA1 of the different phylogenetic lineages are clustered or randomly distributed, we calculated the number of changes in the antigenic versus the nonantigenic regions of the molecule using Singapore 57 as the baseline (Table 3). Since the antigenic regions on the H2 influenza virus HA have not been determined, we used the regions identified on the H3 HA (Wiley and Skehel, 1987). The amino acid changes in human H2 influenza viruses were in sites A, B, and D, with no changes in sites C or E (Table 3). There were 19 amino acid changes in the antigenic sites and 18 in the nonantigenic regions of HA1. Since the antigenic sites constitute 92/340 amino acids of HA1, there are 3.9 times as many changes in the antigenic as compared to the nonantigenic portions of HA1. For avian influenza viruses, there were 27 amino acid changes in antigenic areas versus 71 in the nonantigenic regions, yielding similar (1.4 more changes in antigenic areas) numbers of changes in each of these areas of the HA1 molecule. This indicates selection of antigenic variants in the HA molecule of human but not avian H2 influenza viruses.

TABLE 3
IS THE DISTRIBUTION OF AMINO ACID CHANGES CLUSTERED OR RANDOM IN HUMAN AND AVIAN H2 HAs?

Virus in host	Number of nucleotide changes in antigenic sites ^{a,b}				Nonantigenic regions		
	A	B	C	D	HA1	HA2	
Human	6 (2)	5 (4)	0 (2)	8 (2)	0 (1)	18 (34)	14 (20)
Avian	7 (12)	6 (18)	7 (15)	6 (20)	1 (15)	71 (167)	27 (140)

^a As defined by Wiley and Skehel (1987) for H3: A, 133–148; B, 187–196, 153–155; C, 50–54, 275–286; D, 201–226; E, 63–83.

^b Numbers in parentheses are silent changes.

The absence of changes in sites C or E is unexplained and may be due to lack of correlation in structure between H2 and H3.

Evolutionary rates

Influenza viruses of the H2 subtype were first isolated in 1957, circulated in humans until 1968, and then disappeared. The first avian H2 influenza viruses were isolated in 1972 and have continued to circulate in wild and domestic birds. Consequently there is a relatively short history of H2 influenza viruses in humans (12 years), compared with a 20-year history in avian species. The evolutionary rate for human H2 viruses was estimated by plotting the year of isolation for a virus against the branch distance to the ancestral node (F) of the lineage; the slope of the regression line for the plotted points equals the number of nucleotide changes per year. The results were compared with those for the H3 influenza virus over the same time period (Bean *et al.*, 1992).

The estimated evolutionary rate for the human H2 HA was 3.5 nucleotide substitutions per year and approximately 1 amino acid substitution per year (Fig. 3).

By contrast, the evolutionary rate for the human H3 HA was 7.9 nucleotide substitutions per year with 3.4 amino acid substitutions (Bean *et al.*, 1992). We also attempted to determine the evolutionary rate for avian H2 HAs using the same method, but there was so much variation between the branch lengths and the year of isolation that reliable values could not be obtained. Thus, viruses like Duck GDR 72 had more changes than did Mall Alb 88, which was isolated 16 years later.

Surveillance studies of H2N2 influenza viruses

Since the disappearance of H2N2 influenza viruses from humans in 1968, they have been isolated sporadically from domestic and wild ducks (Tumova *et al.*, 1975; Shortridge, 1979; Sinnecker *et al.*, 1983). To determine their prevalence in wild ducks, we examined the results of longitudinal surveillance studies conducted from 1976 to 1991 (Sharp *et al.*, 1992); for domestic poultry, we examined the 1980–1991 records at the National Veterinary Services Laboratories, Ames, IA. In wild ducks, H2 influenza viruses were detected at a low level in 1980 and 1984 (approx. 10 isolates per 1000 samples), accounting for less than 1% of all influenza virus isolates. This prevalence rate increased threefold in 1988. In domestic poultry, serologic and virologic surveillance of influenza viruses also revealed sporadic occurrences of H2 influenza viruses with detection of antibodies in one flock of turkeys in 1984 and one virus isolation in 1985. In 1986, four H2N7 influenza viruses were isolated from turkeys. In 1988, serologic evidence of H2N2 influenza virus infection was found on 22 turkey farms in Minnesota, and on one turkey farm in North Carolina. In 1989, serologic evidence of H2N2 was found on 135 turkey farms in Iowa, Virginia, and Minnesota, and four H2N2 viruses were isolated from two farms in Minnesota. The number of turkey farms where serological evidence of H2N2 infection was found declined sharply in subsequent years (none in 1990 and two in 1991). By contrast, one H2N9 virus was isolated from a duck farm in Delaware in 1990, and two H2N2 viruses were isolated from chicken farms in Pennsylvania in 1991. The dis-

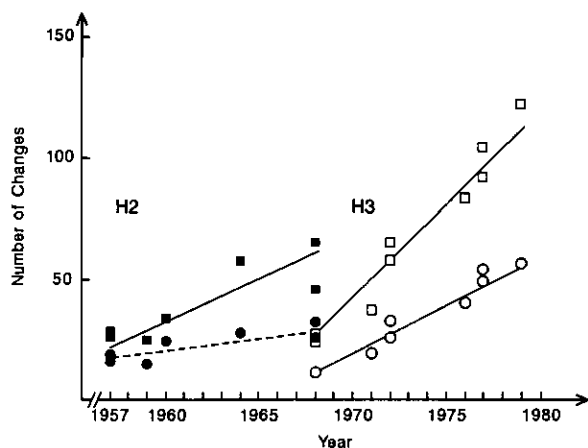


FIG. 3. Evolutionary rates for human H2 and H3 HAs, estimated by regression of year of isolation against branch distance from the common ancestor node of the nucleotide and amino acid phylogenetic trees (Fig. 2). Closed boxes represent H2 nucleotides and open boxes H3 nucleotides and closed circles represent H2 amino acids and open circles H3 amino acids.

ease signs in the H2N2-infected flocks ranged from asymptomatic to mild respiratory disease with a drop in egg production to high mortality in a minority of flocks. Experimental infection of chickens with these H2N2 virus isolates produced no signs of disease.

Additional serologic and virologic evidence of H2 infection of poultry in 1990 and 1991 came from surveillance of live birds in urban markets in Florida, New York, and New Jersey, where 19 H2N2 influenza viruses were isolated in 1990 and 9 in 1991. These viruses came from apparently healthy chickens, ducks, guinea fowl, and from the water and feces in markets that provide live birds directly to the public. Thus, although H2 influenza viruses have not been isolated from wild ducks since 1988, their prevalence in domestic poultry has increased and they have continued to circulate in these hosts for the past 4 years.

DISCUSSION

A central aim of this study was to find the origin of the H2 influenza viruses that infected humans in 1957 and determine whether similar viruses continue to circulate in avian reservoirs. Antigenically closely related H2 viruses circulated in the human population from 1957 through 1961, but by 1962, antigenic variants had begun to emerge that reacted with only a few of the Mabs to the HAs of two 1957 strains. This divergence did not correlate with findings in avian H2 strains, where the majority of viruses continued to show cross reactivity with the human strains from 1957. This supports the hypothesis that the human Asian pandemic strain of 1957 originated from avian strains and that counterparts of these antigenically conserved viruses continued to circulate in birds through 1992. A minority of avian influenza viruses show reactivity patterns similar to those of human strains isolated after 1962; Duck GDR 72, for example, reacted with only a few of the Mabs in our panel by ELISA. From antigenic analysis we could not determine whether this virus was derived from humans or is an antigenically distinct avian strain. However, phylogenetic analysis indicates that Duck GDR 72 represents a lineage distinct from the human isolates. The absence of antigenic drift in avian H2 genes is consistent with findings for avian H3 HA genes (Bean *et al.*, 1992). Avian species do produce humoral and cell-mediated responses to influenza virus infection, but antibody responses are not long lasting (Kida *et al.*, 1980). Thus, antibody titers may be too low to permit selection of variants, or antibodies may not play a dominant role in selection of antigenic variants of avian H2 strains, as has been found with influenza B viruses (Air *et al.*, 1990).

The results of our phylogenetic analysis identify three distinct lineages of H2 HAs, one in humans and

two in avian species. The avian lineages are separated on the basis of geographic regions of isolation, the primary separation involving North American and Eurasian birds. The only exception is the H2 of HGull DE 88, which belongs to the European lineage yet was isolated in the United States (Delaware). The avian H2 influenza viruses that have recently become prevalent in domestic poultry (G Fowl NJ 91) are derived from the H2 influenza viruses in wild ducks in North America.

Human H2 influenza viruses form a separate lineage in the phylogenetic tree, indicating that there was probably a single introduction of an H2 gene into humans before 1957. The human H2 viruses are most closely related to Eurasian avian viruses and are most distant from the North American avian H2 viruses. This suggests that the human pandemic strain of 1957 was derived from a Eurasian avian virus. The Asian/57 pandemic virus reportedly derived three genes from an avian precursor (Scholtissek *et al.*, 1978; Kawaoka *et al.*, 1989), including the HA, NA, and PB1 and other genes from a late human H1N1 strain.

The avian H2 hemagglutinin most closely related to the human Japan 57 strain is Duck GDR 72; the nucleotide homology is 93% and amino acid homology is 95.2%. This is lower than was found between avian and human H3 hemagglutinins (Bean *et al.*, 1992) where the nearest avian strain is 98.8% and 99.2% homologous in nucleotides and amino acids with the human strains. The close relationship of avian and human H3 hemagglutinins was based on analysis of many H3 avian strains and further analysis of H2 strains may show closer relationships.

The earliest stages in the evolution of the human lineage appear to have been under greater selective pressures than the later branches as judged by their ratios of coding to noncoding changes (Fig. 2). Initially 1.6 nucleotide changes resulted in amino acid changes. Later 3.7 nucleotide changes were required per amino acid change. This may reflect early stages of adaptation of the virus to humans or perhaps the response of the virus to the first appearance of antibody in the human population after the initial pandemic. The overall lower rate of mutation of the H2 when compared with the H3 may reflect a greater ability of the H3 HA to tolerate mutations and still maintain viability, or it could reflect differences in the immune response of the human population to the different viruses. If the H2 HA is less tolerant of mutation, this could explain short survival time of the H2 virus in humans.

Attempts were made to correlate the significant antigenic changes found in human H2 HAs after 1962 with changes in the amino acid sequence. Mabs 79/1, 67/7, and 137/5 did not combine with human viruses isolated after 1962 (England 62; Korea 68) or with some avian strains (H Gull DE 88, Chick NY 91); this failure

correlates with an amino acid change at residue 188 of threonine → alanine in site B (H3 antigenic site). The failure of Mab 33/1, 41/4, and L22/4 to react with the late-emerging human viruses correlates with sequence changes of glycine → alanine at residue 200 and threonine → alanine at residue 214. This in turn correlates with site D on the H3 hemagglutinin molecule.

The appearance of significant numbers of H2 influenza viruses in domestic avian species after an increase in their prevalence in wild ducks in 1988 raises the possibility of their reintroduction into humans. We do not know the molecular changes in H1, H2, or H3 that permit transmission of avian influenza viruses to humans, but the appearance of H2 virus in humans at the end of the 19th century (Muider *et al.*, 1958) and again in 1957 indicates that these subtypes have the capability to be transmitted to humans and to spread in these hosts. Since H2 viruses have not been detected in humans for 24 years, the population of susceptible potential hosts is large. The presence of H2N2 influenza viruses in live bird markets in New York and Florida and on turkey farms in Minnesota brings these viruses into close contact with humans. Currently, we are not aware of evidence either for or against transmission of these H2N2 viruses to humans, nor have they been isolated from pigs, unlike the H1N1 and H3N2 viruses. These negative findings may reflect the lower prevalence of H2N2 viruses in wild and domestic avian species before 1988. In view of the increasing prevalence of avian H2N2 viruses and their proximity to susceptible human populations, we recommend regular surveillance of pigs and humans for interspecies transmission of these viruses.

ACKNOWLEDGMENTS

We thank Scott Krauss for excellent technical assistance, John Gilbert for assistance with scientific writing, and Dayna Anderson for manuscript preparation. We acknowledge Dr. Clayton Naeve, Molecular Resource Center, for preparation of oligonucleotides and Pat Eddy for computer support. This work was supported by U.S. Public Health Research Grant AI-29680 and AI-08831, Cancer Center Support (CORE) Grant CA-21765, and American Syrian Associated Charities (ALSAC).

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