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Molecular Correlates of Influenza A H5N1 Virus Pathogenesis in Mice

JACQUELINE M. KATZ,* XIUHUA LU, TERRENCE M. TUMPEY,† CATHERINE B. SMITH, MICHAEL W. SHAW, AND KANTA SUBBARAO

Influenza Branch, Division of Viral and Rickettsial Diseases, National Center for Infectious Diseases, Centers for Disease Control and Prevention, Atlanta, Georgia 30333

Received 12 June 2000/Accepted 23 August 2000

Highly pathogenic avian influenza A H5N1 viruses caused an outbreak of human respiratory illness in Hong Kong. Of 15 human H5N1 isolates characterized, nine displayed a high-, five a low-, and one an intermediate-pathogenicity phenotype in the BALB/c mouse model. Sequence analysis determined that five specific amino acids in four proteins correlated with pathogenicity in mice. Alone or in combination, these specific residues are the likely determinants of virulence of human H5N1 influenza viruses in this model.

The molecular determinants and related mechanisms that make certain influenza viruses highly pathogenic for mammalian species, including humans, remain poorly understood. Both viral factors and host factors may determine virulence. Numerous studies have shown that influenza virus virulence in mammalian species is a polygenic trait, which may require a critical constellation of genes (4, 21, 32, 33). The phenotypes of virulence and pathogenicity are distinct from that of host range. Influenza viruses that infect one species, such as birds, are often restricted in their ability to replicate in other host species, such as humans, by host range determinants. However, in 1997, highly pathogenic avian H5N1 viruses infected poultry in the live-bird markets of Hong Kong and caused an outbreak of 18 human cases of respiratory illness, including six deaths (6, 8, 30, 34). The majority of individuals who experienced severe illness or died from the H5N1 infection were 13 to 60 years old and had no known risk factors for complications from influenza (5).

The H5N1 viruses are the only highly pathogenic avian viruses that have been documented to cause an outbreak of respiratory disease in humans. An earlier study of humans exposed to chickens infected with an H5N2 virus failed to find any evidence of human infection with this highly pathogenic avian virus (2). The 16 H5N1 viruses isolated from humans during the 1997 outbreak had avian virus genomes; the hemagglutinin (HA) and neuraminidase (NA) genes showed no evidence of adaptive change for humans (3). The outbreak created a new awareness that avian influenza viruses could spread directly from poultry to humans and cause severe respiratory disease in humans, but the molecular basis of the H5N1 virus virulence in humans was not evident.

The BALB/c mouse was previously shown to be a useful mammalian model for the evaluation of human H5N1 virus pathogenesis (11, 13, 17). H5N1 viruses replicate efficiently in the respiratory tract of mice without prior adaptation. Viruses exhibiting high lethality (pathogenicity) replicated in extrapulmonary sites, including the brain, while growth of viruses of low lethality was restricted to the respiratory tract of mice (11, 17). All 16 human H5N1 viruses possessed a multiple basic amino acid motif at the cleavage site between HA1 and HA2 and were lethal for experimentally infected chickens (3, 6, 11, 27, 30). The basic amino acid motif, a key molecular feature of avian viruses of the H5 and H7 subtypes that are highly pathogenic for chickens (23), allows the HA to be cleaved by ubiquitous proteases of the subtilisin family (26) and enables these viruses to replicate systemically in birds. The fact that human H5N1 viruses of low pathogenicity for mice possessed the motif but did not replicate systemically or cause lethal disease in mice (11, 17) suggested that other molecular features are associated with the high pathogenicity of H5N1 viruses in mammalian species. We now investigate the molecular determinants that distinguish 15 H5N1 viruses of high and low pathogenicity in mice.

The origin and outcome of human disease associated with 15 H5N1 viruses isolated from confirmed cases in Hong Kong in 1997 and the antigenic profile and relative pathogenicity of the viruses for mice are shown in Table 1. Viruses were grown in Madin-Darby canine kidney (MDCK) cells and/or the allantoic cavity of 10-day-old embryonated hens' eggs at 37°C for 24 h. Fifty percent egg infectious dose (EID_{50}) titers were determined by serial titration of viruses in eggs and were calculated by the method of Reed and Muench (20). The 50% lethal dose (LD_{50}) of the viruses for 6- to 8-week-old female BALB/c mice (Charles River Laboratories, Wilmington, Mass.) was determined as previously described (17) and used as a marker for pathogenicity. LD_{50} titers were expressed as the EID_{50} value corresponding to 1 LD_{50}. Viruses with an LD_{50} of $>10^{6.5}$ were considered to be of low pathogenicity, while viruses with an LD_{50} of $<10^{3.0}$ were considered to be of high pathogenicity.

Previously, the H5N1 viruses were distinguished into two antigenically and genetically distinct subgroups based on the absence (group A) or presence (group B) of a potential N-linked glycosylation site at residues 154 to 156 (H3 numbering) in the HA1 region of the HA molecule (3, 27). While all five viruses of low pathogenicity for mice belonged to group A, the nine viruses that were highly pathogenic for mice included all seven group B viruses and two group A viruses. A/Hong Kong/156/97 (HK/156), a group A virus, gave an intermediate phenotype of pathogenicity in mice (Table 1). Therefore, the presence or absence of the glycosylation site in HA1 alone could not explain the differences in pathogenicity observed in mice.

The complete nucleotide sequences for all coding regions of all gene segments of nine of the H5N1 viruses were determined by direct cycle sequencing of PCR products generated by reverse transcription-PCR from MDCK cell-passaged (XC1 or XC2) stocks by using gene-specific primer sets as previously described (3; M. Shaw, L. Cooper, X. Xu, et al., submitted for

* Corresponding author. Mailing address: Influenza Branch, Mailstop G-16, Centers for Disease Control and Prevention, 1600 Clifton Road, Atlanta, GA 30333. Phone: (404) 639-3591. Fax: (404) 639-2334. E-mail: JKatz@cdc.gov.

† Present address: USDA/ARS/Southeast Poultry Research Laboratories, Athens, Ga.

TABLE 1. Characteristics and mouse pathogenicity phenotypes of influenza A H5N1 viruses isolated from humans

H5N1 virus	Origin		Case outcome	Passage history[a]	Antigenic group[b]	Amino acids in HA1[c]	Mouse LD_{50}[d]	Mouse pathogenicity phenotype[e]
	Age (yr)	Sex						
HK/481/97	2	Male	Recovered	E2	A	NXA	$10^{1.7}$	High
HK/483/97	13	Female	MV[f]; died	C1E3	B	NXT	$10^{2.4}$	High
HK/485/97	24	Female	MV; recovered	C2E2	B	NXS	$10^{2.9}$	High
HK/491/97	4	Male	Recovered	C1E2	B	NXS	$10^{2.0}$	High
HK/503/97	1	Male	Recovered	C1E2	B	NXS	$10^{2.0}$	High
HK/514/97	25	Female	MV; died	CXE2	B	NXS	$<10^{1.5}$	High
HK/516/97	60	Female	MV; died	C2E2	A	NXA	$<10^{1.5}$	High
HK/532/97	14	Female	Recovered	CXE2	B	NXT	$<10^{1.5}$	High
HK/542/97	19	Female	MV; recovered	CXE3	B	NXS	$<10^{1.5}$	High
HK/156/97	3	Male	MV; died	E3	A	NXA	$10^{5.9}$	Intermediate
HK/486/97	5	Female	Recovered	C2E3	A	NXA	$>10^{6.5}$	Low
HK/488/97	2	Male	Recovered	C1E4	A	NXA	$>10^{6.5}$	Low
HK/507/97	3	Female	Recovered	C1E2	A	NXA	$>10^{6.5}$	Low
HK/538/97	3	Male	Recovered	CXE3	A	NXA	$>10^{6.5}$	Low
HK/97/98	34	Female	MV; died	CXE4	A	NXA	$>10^{6.5}$	Low

[a] Number of passages in a given host cell type: C, MDCK cells; E, embryonated eggs; X, unknown.
[b] As defined in Bender et al. (3).
[c] HA residues 154 to 156 encode a potential glycosylation site defined by NXS/T, where X is not proline. GenBank accession numbers for H5 HA sequences are AF036356 (30), AF046096-97 (27), and AF102671-82 (3).
[d] LD_{50}s were determined by inoculating groups of five lightly anesthetized mice intranasally with $10^{6.5}$ to $10^{1.5}$ EID_{50} of virus in a volume of 50 μl. Mice were checked daily for 14 days, and the LD_{50}s were calculated as previously described (17). LD_{50}s are expressed as the EID_{50} corresponding to 1 LD_{50}.
[e] Viruses with an LD_{50} of $>10^{6.5}$ were considered to be of low pathogenicity, and viruses with an LD_{50} of $<10^{3.0}$ were considered to be of high pathogenicity.
[f] MV, mechanical ventilation required.

publication). This analysis identified five residues that segregated with the mouse pathogenicity phenotype in genes that encoded the NA, matrix (M1) protein, and viral polymerases PB1 and PB2. To confirm this finding, partial sequence analysis was conducted on these four gene segments from the same virus stocks that were used to determine the pathogenicity phenotype in mice (Table 2). Amino acid residues I or T at residue 223 in the NA, K or R at 198 and I or M at 317 in PB1, and K or Q at 355 in PB2 correlated with high and low pathogenicity, respectively, in all 15 viruses analyzed. The amino acid at position 15 (I or V) in the M1 protein correlated with high (I) and low (V) pathogenicity in 14 of the H5N1 viruses. HK/485/97, a virus of high pathogenicity, possessed a unique codon (ACC) relative to all other H5N1 viruses analyzed, encoding a threonine at position 15 in the M1 protein. This result suggests either that threonine at this position is permissive for the high-pathogenicity phenotype or that substitution of this residue alone is insufficient to alter the mouse pathogenicity phenotype of the virus.

Amino acid 223 in the N1 NA corresponds to residue 222 of N2 NA, a conserved framework residue in the enzyme active site in the head of the NA molecule (7). H5N1 viruses of the low-pathogenicity phenotype possess a threonine at this position, creating a potential glycosylation site (N-X-T) in the enzyme active site. Previously, the neurovirulence of mouse-adapted A/WSN/33 virus was correlated with the loss of a

TABLE 2. Nucleotide and deduced amino acid residues in the NA, M1, PB1, and PB2 proteins that correlate with mouse pathogenicity phenotype[a]

H5N1 virus	Mouse pathogenicity phenotype	Nucleotide (nt) or residue (aa) at indicated position									
		NA		M1		PB1				PB2	
		nt 668	aa 223	nt 68	aa 15	nt 617	aa 198	nt 975	aa 317	nt 1090	aa 355
HK/481/97	High	T	I	A	I	A	K	A	I	A	K
HK/483/97	High	T	I	A	I	A	K	A	I	A	K
HK/485/97	High	T	I	ACC	T	A	K	A	I	A	K
HK/491/97	High	T	I	A	I	A	K	A	I	A	K
HK/503/97	High	T	I	A	I	A	K	A	I	A	K
HK/514/97	High	T	I	A	I	A	K	A	I	A	K
HK/516/97	High	T	I	A	I	A	K	A	I	A	K
HK/532/97	High	T	I	A	I	A	K	A	I	A	K
HK/542/97	High	T	I	A	I	A	K	A	I	A	K
HK/156/97	Intermediate	T	I	A	I	A	K	A	I	A	K
HK/486/97	Low	C	T	G	V	G	R	G	M	C	Q
HK/488/97	Low	C	T	G	V	G	R	G	M	C	Q
HK/507/97	Low	C	T	G	V	G	R	G	M	C	Q
HK/538/97	Low	C	T	G	V	G	R	G	M	C	Q
HK/97/98	Low	C	T	G	V	G	R	G	M	C	Q

[a] GenBank accession numbers for nucleotide sequence data are as follows: PB2, AF036363, AF258837, AF258839-40, and AF258843-53; PB1, AF036362, AF258818, AF258820-21, and AF258824-34; PA, AF036361, AF257193, AF257195-96, and AF257199-209; NP, AF036359, AF255744, AF255746-47, and AF255750-67; NA, AF102657-70 and AF296752; M, AF036358, AF255365, AF255367-68, and AF255371-84; and NS, AF036360, AF256178, AF256180-81, and AF256184-94.

glycosylation site at residue 146 in the head of the N1 NA (16). Goto and Kawaoka (12) reported that the absence of this glycosylation site together with the presence of a carboxyl-terminal lysine (residue 453) was associated with the binding of plasminogen by the NA, which facilitated and enhanced HA cleavage and conferred a broader tissue tropism on this virus. However, the molecular features important for this mechanism of virulence have been observed only in the laboratory-derived A/WSN/33 virus and not in any wild-type viruses, including the H5N1 viruses. Other studies have suggested a role for the NA in influenza virus-induced apoptosis, which has been implicated as a mechanism of pathogenicity among influenza viruses (18, 22). Interestingly, the highly pathogenic H5N1 virus HK/483/97 induces peripheral blood lymphocyte depletion and apoptosis in the spleens and lungs of infected mice, whereas HK/486/97, a virus of low pathogenicity, does not (31). Because the specific residues in the PB1, PB2, and M1 proteins that correlated with mouse pathogenicity were not located in any of the defined functional domains of these proteins, their contribution to the mechanism(s) of virulence remains unknown.

Because the human H5N1 viruses are a genetically closely related group of viruses (3, 15), it was possible to associate the five specific molecular markers in the NA, PB1, PB2, and M1 genes with the two distinct phenotypes of pathogenicity observed in mice. To investigate the prevalence of these specific residues in other influenza A viruses, nucleotide sequence alignments for the four gene products were performed using available sequence data (downloaded from the GenBank viral nucleotide database in March and April 2000), including sequences from human, avian, swine, and equine influenza A viruses. No significant distribution of the specific residues associated with high or low pathogenicity for mice in this study was observed, most likely because of the relatively low genetic relatedness of influenza A viruses from different species. Nevertheless, the analysis revealed that residues Thr-223 in the NA and Arg-98 in PB1 were unique to the H5N1 viruses of low pathogenicity and that Glu-355 in PB2 was found only in the human H5N1 viruses of low pathogenicity and in four avian H5N1 viruses isolated from birds in Hong Kong in 1997.

In earlier studies, investigators compared wild-type viruses with either mouse-adapted virus or reassortants derived from parental viruses of distinct pathogenicity phenotypes. In mice, pneumovirulence and neurovirulence were associated with the HA, NA, M, and one or more polymerase genes (4, 21, 32, 33). However, the specific amino acid residues identified in the NA, M1, PB1, and PB2 genes in the present study have not been previously associated with pathogenicity. It is possible that additional molecular markers for pathogenicity were not detected because the findings from complete sequence analysis of nine of the human H5N1 viruses were used to direct partial analysis of the remaining viruses. Mutations in the polymerase genes of influenza viruses have previously been reported to determine host range (1, 29), temperature sensitivity, and, in some instances, attenuation of influenza viruses for mice, ferrets, and humans (25, 28). Mutations in the M protein have been associated with host range, growth, and virulence phenotypes. Growth properties of influenza viruses can be determined by specific mutations in individual gene segments or by the constellation of genes present in the virus.

Although HK/156/97 was shown to be of intermediate pathogenicity in this study, the genotype was that of the high-pathogenicity viruses, a finding consistent with the results of others who have characterized HK/156/97 as being highly pathogenic for BALB/c mice (9, 11, 13, 24). The molecular basis for the apparent attenuation of virulence of the HK/156/97 virus used in this study is unknown, but biological and molecular heterogeneity of this virus isolate has been reported (11, 14). Passage of HK/156/97 in mice was shown to increase the virulence of the virus (13, 14, 24). One other virus, HK/482/97, consistently yielded an indeterminate mouse pathogenicity phenotype and a genotype that consisted of a mixture of the residues associated with high and low pathogenicity in M1, PB1, and PB2. Gao et al. (11) found that HK/482/97 virus passaged exclusively in MDCK cells exhibited low pathogenicity in mice. Therefore, it is possible that the original HK/482/97 isolate, like HK/156/97, was biologically heterogeneous.

While two distinct pathogenicity phenotypes were observed in this inbred mouse model, a broader spectrum of pathogenicity was observed in humans infected with the H5N1 viruses. In particular, the age of an infected individual was an important factor associated with severity of disease (34). Some of the deaths associated with H5N1 infections occurred late in the course of hospitalization, following extended periods of mechanical ventilation and other complications. The mouse pathogenicity phenotype of four viruses failed to correlate with the severity of disease observed in humans. Three of the viruses that were highly pathogenic for mice were isolated from children $\leq$4 years of age who had mild disease. One virus of low pathogenicity for mice was isolated from a 34-year-old woman with systemic lupus erythematosus who succumbed to a lethal H5N1 infection. Therefore, in addition to the general virulence of the H5N1 viruses, age, underlying medical conditions, and other unknown risk factors may have contributed to the severity of disease in humans. Nevertheless, the fact that H5N1 viruses of high pathogenicity induced symptoms of disease similar to those observed in severe and fatal human cases, including viral pneumonia, multiorgan involvement, leukopenia, and death, suggests that the mouse is an appropriate model with which to better understand the molecular basis of influenza virus virulence in mammalian species. However, at present it is not possible to distinguish between molecular determinants responsible for general virulence in mammals and those responsible for specific virulence in mice.

The use of plasmid-based reverse genetics techniques (10, 19) will enable an evaluation of the contribution of each of the specific amino acid residues identified here, either alone or in various combinations, to the pathogenicity phenotype in mice. While it is likely that the polygenic nature of pathogenicity differs among influenza viruses and among host species, the molecular determinants of pathogenicity in this mammalian model may provide a framework for the future identification of influenza A viruses with the potential to cause severe disease.

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