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Generation of a Highly Pathogenic Avian Influenza A Virus from an Avirulent Field Isolate by Passaging in Chickens

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Highly virulent avian influenza viruses can arise from avirulent strains maintained in poultry, but evidence to support their generation from viruses in wild birds is lacking. The most likely mechanism for the acquisition of virulence by benign avian viruses is the introduction of mutations by error-prone RNA polymerase, followed by the selection of virulent viruses. To investigate whether this mechanism could apply to wild waterfowl, we studied an avirulent wild-swan virus that replicates poorly in chickens. After 24 consecutive passages by air sac inoculation, followed by five passages in chicken brain, the avirulent virus became highly pathogenic in chickens, producing a 100% mortality rate. Sequence analysis at the hemmaglutinin cleavage site of the original isolate revealed a typical avirulence type of sequence, R-E-T-R, which progressed incrementally to a typical virulence type of sequence, R-R-K-K-R, during repeated passages in chickens. These results demonstrate that avirulent viruses maintained in wild waterfowl in nature and bearing the consensus avirulence type sequence R-E-T-R have the potential to become highly pathogenic while circulating in chickens.

The severity of avian influenza virus infections varies considerably with the strain of virus (5). Infections produced by most of these viruses are asymptomatic, although a few highly pathogenic strains (H5 or H7 subtype) can cause systemic "fowl plague" disease, which has been associated with high mortality rates during severe outbreaks in poultry (5). Highly virulent avian influenza viruses have arisen from avirulent viruses in poultry (2, 6, 7). Still lacking, however, is evidence to support the concept that benign viruses carried by wild birds can acquire high virulence after direct transmission to poultry.

Although the pathogenicity of avian influenza viruses is a polygenic trait, the hemagglutinin (HA) surface glycoprotein plays a central role (3, 15). The HA of virulent viruses is cleaved in tissue culture and does not require an exogenous protease for plaque formation. The HAs of virulent viruses differ from those of avirulent influenza A viruses by virtue of possessing multiple basic amino acids at the carboxyl terminus of HA1. This structural feature permits cellular proteases, such as the ubiquitous furin and PC6, which recognize multiple basic amino acids, to cleave the HA and render the virus infectious and able to spread to a variety of organs, leading to systemic infection. By contrast, avirulent-virus HAs do not possess a series of basic amino acids at the cleavage site and are cleaved only by trypsin-like proteases which are secreted from cells in the respiratory or intestinal tract, or both, so that the viruses only produce localized infections, resulting in mild or asymptomatic infections.

The structural requirement for HA cleavage by furin and PC6 has been studied extensively by the selection of variants whose HA cleavabilities were altered during cell culture adaptation without trypsin (13, 16, 19, 21, 25, 27) or by site-directed mutagenesis of the HAs in in vitro expression systems (11, 12, 20, 27). These studies indicated that two structural features, (i) a specific motif consisting of a series of basic amino acid sequence at the cleavage site and (ii) a carbohydrate side chain in the near vicinity, are crucial for determining HA cleavability by the proteases. For the HA to be cleaved completely by endogenous proteases in cell culture, a motif of X-X-R-X-R/K-R (X = a nonbasic residue) must be present at the cleavage site, if a carbohydrate chain is nearby. Otherwise, a motif of R/K-X-R/K-R is adequate.

Among the many outbreaks of disease caused by highly pathogenic avian influenza viruses, one in the United States in 1983 (2) and another in Mexico in 1993 to 1995 (6, 7) were unique in that they were initiated by an avirulent precursor virus that later became highly pathogenic. In the U.S. epizootic, both avirulent and virulent viruses had a series of basic amino acids at the HA cleavage site; however, the latter lost an oligosaccharide side chain in the vicinity of this site due to a single mutation, providing unimpeded access to furin and PC6 and thus a means to acquire high HA cleavability (10). In the Mexican outbreak, the original avirulent virus had a typical avirulence type of sequence at the HA cleavage site, R-E-T-R, which mutated to a virulence type of sequence, R-K-R-K-T-R, during replication in chickens (6, 7). Although these outbreaks demonstrate that virulent viruses can arise from avirulent precursors in poultry, it is still unclear if the latter originated in wild birds, as one might predict from epizootic studies of avian influenza viruses. To address these issues, we passaged an avirulent wild-swan virus in chickens, monitoring the changes in molecular structure and infectivity that accrued during the study.

A/whistling swan/Shimane/499/83 (H5N3) was isolated from wild waterfowl that had migrated to Japan (22). Mardin-Darby

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bovine kidney (MDBK) cells were cultured in Eagle's minimum essential medium (MEM; Gibco) supplemented with 10% newborn calf serum. Chicken embryo fibroblasts (CEFs), prepared from 10-day chicken embryos, were cultured in MEM with 10% calf serum.

The virus was passaged in the brains of chicks five times after 24 serial passages through the air sacs of chicks. The caudal thoracic air sacs of three 1-day-old chicks were inoculated with 0.2 ml of allantoic fluid containing $10^{6.0}$ 50% egg-infectious doses (EID_{50}) of virus. The chicks were sacrificed, and their respiratory organs (lungs and trachea) were collected 3 days postinfection. The serial air sac passages in the group of 1- to 6-day-old chicks (three birds per passage) were done with 0.2 ml of pooled 10% tissue suspensions of infected organs (lung and trachea) every 3 days. Intracerebral serial passages were done with 0.1 ml of brain tissue suspension. Viral isolates were identified by passage number and the organ through which the virus was passaged. For example, the designation 24a5b indicates that the virus was passaged 24 times in air sac and 5 times in brain. Viruses were propagated in the allantoic cavities of 10-day-old embryonated chicken eggs for 48 h at 35°C. The allantoic fluid was harvested and stored at −80°C.

Four- to 6-week-old specific-pathogen-free (SPF) White Leghorn chickens were used to test the infectivity of the passaged viruses. Fifty microliters of each isolate (10^7 to 10^8 EID_{50}/ml) was inoculated intranasally. Signs of clinically significant morbidity, including lethargy, necrosis of the comb, impaired ambulation, and inability to stand, were assessed over 10 days. To study virus replication, we collected organs from three chickens at 3 days postinoculation of virus and determined the virus titers in samples using eggs as the growth medium. All experimental infections were performed in a BL3 containment facility approved for such use by the U.S. Department of Agriculture.

Chicken embryo fibroblast (CEF) cultures were used to assay plaque-forming ability in the presence or absence of trypsin (5 μg/ml), essentially as described by Klenk et al. (14). MDBK cells were also infected with virus, washed to remove unadsorbed virus, and incubated in methionine-free MEM for 30 min at 8 h postinfection, after the addition of radioactive label (Tran[S^{35}] label at 250 μCi/ml; ICN Radiochemicals) to the medium. Cells were lysed with buffer (50 mM Tris-Cl [pH 7.2], 600 mM KCl, 0.5% Triton X-100) and used as antigens in a radioimmunoprecipitation (RIP) assay with anti-H5 monoclonal antibodies.

Partial nucleotide sequences were determined at the HA cleavage (positions 775 to 1021) and glycosylation (position 106 to 178) sites of each virus. Viral RNA was isolated from allantoic fluid containing virus, as in earlier studies (1), and cDNA was synthesized with the use of reverse transcriptase and random hexamers as previously described (9). Direct sequencing by PCR was done with an autosequencer (Applied Biosystems Inc.) in accordance with the manufacturer's protocol. The oligonucleotides used as primers were Uni 12 (AGC AAAAGCAGG), H5H86 (TGCATCGGTTATCATGCAAA), H5H775 (GGAGACTCAGCAATCCCATGAAAAG), and H5HR1021 (CCATACCAACCGTCTACCATTCC).

To determine whether avirulent viruses maintained in wild birds can become highly virulent, we passaged A/whistling swan/Shimane/499/83 (H5N3) (22) in chickens. The intracerebral pathogenicity indexes of this virus ranged from 0.02 to 0.40 (23), indicating low virulence in chickens. Also, the virus failed to kill any 1-day-old chicks or adult chickens after intranasal or intramuscular inoculation. All attempts to propagate this isolate by intranasal, intratracheal, and intracerebral inoculation into 1-day-old chicks were unsuccessful. A more productive strategy was inoculation of the virus into air sacs. During 24 consecutive passages by this route, the virus produced increasingly higher mortality rates in 2-day-old chicks: 0%, original isolate; 10%, isolate 11a (11 passages through air sacs); 50% isolate 18a; 67%, isolate 24a. The virus obtained after 24 passages, isolate 24a was then passaged five times in the brains of chicks, resulting in five passage isolates (24a1b to 24a5b), the last of which produced 100% mortality at 48 h among intracerebrally inoculated 2-day-old chicks. These findings demonstrate that the avirulent avian influenza viruses can become pathogenic during repeated passaging in chickens.

TABLE 1. Acquisition of virulence during serial passages in chickens

Virus[a]	Virulence (no. sick/no. dead/total no.)	Mean time to death (days)	Plaque formation (PFU/ml)[b]		HA cleavability[c]
			With trypsin	Without trypsin	
Parent	0/0/3		3.5×10^4	0	−
11a	0/0/3		8.5×10^2	0	−
18a	0/0/3		6.7×10^2	0	−
24a	0/0/3		2.2×10^3	0	−
24a1b	0/0/3		4.5×10^2	0	−
24a2b	3/3/3	7.0	5.4×10^2	7.5×10^0	−
24a3b	3/3/3	4.4	1.1×10^5	9.1×10^4	+
24a4b	3/3/3	3.8	5.0×10^3	4.3×10^3	+
24a5b	4/4/4	3.7	1.3×10^7	1.0×10^7	+

[a] SPF chickens, 4 to 6 weeks old, were inoculated intranasally with each virus (10^6 PFU) and observed for 10 days.
[b] Plaque-forming ability of the viruses was tested in CEFs.
[c] HA cleavability in the absence of trypsin was tested by RIP assay with anti-H5 monoclonal antibodies.

To determine the virulence of the viral isolates at different passages, we intranasally infected 4-to 6-week-old SPF chickens with the virus and observed them for 10 days. As shown in Table 1, chickens infected with the parental isolate, 11a, 18a, 24a, and 24a1b lacked disease signs throughout the observation period and therefore these isolates were considered avirulent. The 24a2b, 24a3b, 24a4b, and 24a5b isolates, by contrast, produced 100% mortality, indicative of the acquisition of high virulence. In this group, 24a2b was less pathogenic than the other isolates (mean time to death of chickens infected with virus 24a2b, 7.0 days, compared with 3.7 to 4.4 days for chickens infected with other virulent viruses). These data suggest a striking increase in virulence between the first and second passages in brain.

To investigate the correlation of acquired virulence of the viruses correlated with tissue tropism, we determined virus titers in organs from 4- to 6-week-old SPF chickens at 3 days postinfection (Table 2). None of the parental viruses were recovered, and strain 24a was isolated exclusively from the trachea. Isolate 24a2b was recovered from all organs of one of the three chickens tested but only from the lungs and kidneys of the other two. Finally, isolate 24a5b was recovered from virtually all of the organs, including the brain, of all of the test

TABLE 2. Tissue tropism of viruses passaged in chickens

Virus and chicken no.	Virus titer ($\log_{10}$ EID_{50}/g)[a]				
	Brain	Trachea	Lung	Kidney	Pancreas
Parent					
1	—[b]	—	—	—	—
2	—	—	—	—	—
3	—	—	—	—	—
24a					
4	—	2.7	—	—	—
5	—	2.5	—	—	—
6	—	2.5	—	—	—
24a2b					
7	4.3	5.8	3.7	7.2	2.5
8	—	—	1.7	—	—
9	—	—	2.5	1.7	—
24a5b					
10	5.7	5.8	8.7	6.7	—
11	5.7	5.8	7.7	6.5	1.8
12	6.5	5.3	7.0	6.8	2.0

[a] Organs collected at 3 days postinoculation from three chickens intranasally infected with each virus were homogenized for virus titration.
[b] —, no virus was isolated.

chickens. These results indicate a gradual increase in the tissue tropism of viruses during serial passages in chickens, culminating in the acquisition of full pantropicity, a characteristic of highly pathogenic influenza viruses (5). These data also suggest that isolate 24a2b represents a mixture of virulent and avirulent viruses or is intermediate in virulence.

Genetic analyses of the avian influenza virus HAs have demonstrated a relationship between HA cleavability and virulence (3, 15). Thus, we determined the HA cleavage site sequences of the viruses obtained during chicken passages (Table 3). We also investigated the sequence of the potential glycosylation site at position 11, since the presence or absence of a carbohydrate side chain at this site affects HA cleavability (10).

The cleavage site sequence of the original isolate was typical of avirulent H5 strains (R-E-T-R). A point mutation at nucleotide 971 (C to A) was found in the HA gene of isolate 24a, resulting in a T-to-K amino acid substitution at position −2 of the cleavage site. During the first two passages in brain, nucleotide 967 changed from G to A, resulting in an E-to-K alteration at position −3. During the third brain passage, an AGA codon for a basic amino acid residue (R) was inserted at position −4, resulting in a series of basic amino acids, R-R-K-K-R, at the HA cleavage site. These findings clearly show that highly pathogenic influenza viruses can emerge from sequential changes at the HA cleavage site during serial passages in chickens. Analysis of glycosylation site sequences consistently identified the N-S-T potential glycosylation site motif at positions 11 to 13, suggesting that glycosylation is not affected by the same conditions that promote mutations at the HA cleavage site.

Since the plaque-forming ability of avian influenza viruses is correlated with their virulence, we compared this property among isolates obtained during passages in chickens. Neither the original isolate nor 11a, 18a, 24a or 24a1b formed plaques in CEFs lacking trypsin, but they did so in cultures treated with this agent (Table 1). By contrast, isolates 24a3b, 24a4b, and 24a5b were all capable of forming plaques in the absence of trypsin. These findings suggest that the HAs of the original isolate and isolates up to 24a1b possess the nonpathogenic type of cleavability, while isolates 24a3b to 24a5b have the highly pathogenic type. Strain 24a2b formed fewer plaques in cultures without trypsin than in those with trypsin (7.5 versus 540 PFU/ml), which is consistent with its intermediate virulence in SPF chickens.

In MDBK cells, the HAs of the original, 11a, 18a, 24a, 24a1b, and 24a2b isolates were not cleaved in the absence of trypsin whereas those of 24a3b to 24a5b were cleaved into HA1 and HA2 subunits (Table 1 and Fig. 1). These data support the previous conclusions that the degree of HA cleavability of influenza viruses correlates well with their virulence in chickens.

In the present study, we generated a highly pathogenic virus by serially passaging an avirulent waterfowl isolate that replicates poorly in chickens (23). This achievement suggests that any avirulent H5 and possibly H7 viruses circulating in nature could acquire highly pathogenic characteristics, given the proper selective pressure. The original isolate replicated poorly in chicks but demonstrated more efficient growth during subsequent passages in air sacs (Table 2). The mortality rate produced by the 18a virus in 2-day-old chicks was 50% higher than that associated with the original isolate, even though the viruses possessed identical HA cleavage sites. The molecular basis for this observation is unknown, although sequence changes in the HA gene outside the cleavage site may have been responsible. For example, alteration of HA receptor recognition may be needed during the adaptation of waterfowl

TABLE 3. HA cleavage site sequence of viruses passaged in chickens

Virus	Nucleotide sequence	Amino acid sequence[a]	Potential glycosylation site (Asn 11)
Parent	AGA — GAA ACA AGA GGT	R — E T R G	+
11a	AGA — GAA ACA AGA GGT	R — E T R G	+
18a	AGA — GAA ACA AGA GGT	R — E T R G	+
24a	AGA — GAA AAA AGA GGT	R — E K R G	+
24a1b	AGA — GAA AAA AGA GGT	R — E K R G	+
24a2b	AGA — AAA AAA AGA GGT	R — K K R G	+
24a3b	AGA AGA AAA AAA AGA GGT	R R K K R G	+
24a4b	AGA AGA AAA AAA AGA GGT	R R K K R G	+
24a5b	AGA AGA AAA AAA AGA GGT	R R K K R G	+

[a] The dashes are included to adjust the alignment; basic amino acids are underlined. Positions −5 to −1 before each G are shown.

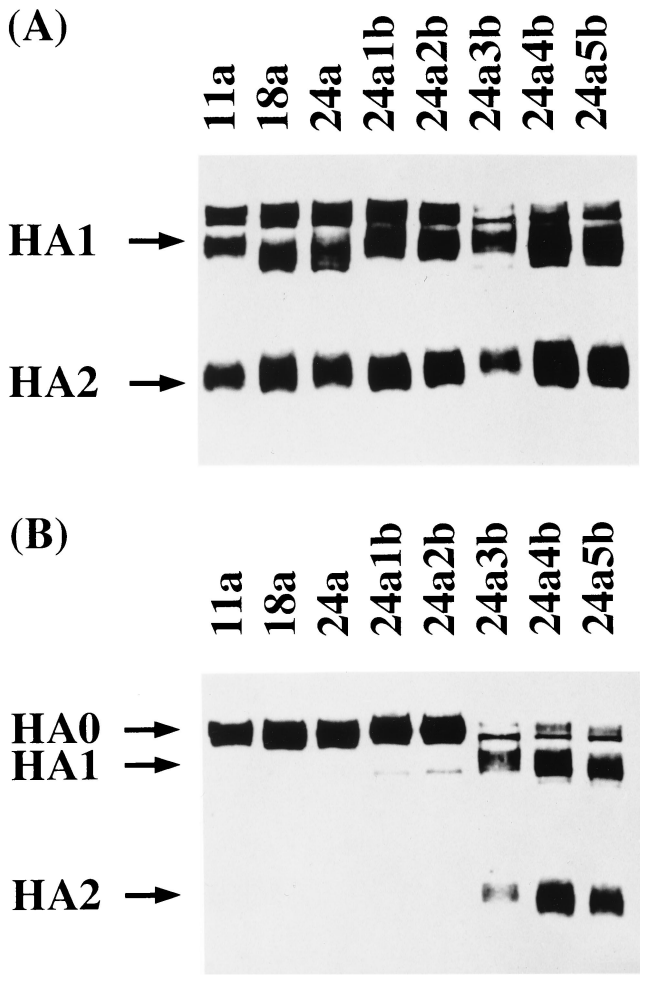

FIG. 1. Comparison of HA cleavability among viruses passaged in chickens. MDBK cells were infected with viruses and incubated in the presence (A) or absence (B) of trypsin (2.5 μg/ml). Cells were then processed for RIP assay as described in Materials and Methods.

virus to chickens, as suggested by Matrosovich et al. (17). Alternatively, mutations in genes other than the HA gene may have contributed to the increased virulence of isolate 18a, but this issue cannot be resolved without comparative genetic analyses of the original isolate and subsequent mutants.

When passaged in chicken brain, isolate 11a failed to replicate (data not shown). Comparison of this virus with isolate 24a, which did grow in brain, revealed the addition of a basic amino acid at position −2 of the HA cleavage site sequence of the latter isolate. This suggests that at least two sequential amino acid changes are needed to convert an avirulent precursor virus to a highly virulent strain. The T-to-K mutation at position −2 may be a minimum requirement for replication of this virus in chick brain. All naturally isolated avirulent H5 viruses have four amino acids in the connecting peptide; most have R-E-T-R (very rarely K-Q-T-R, R-E-T-K, I-G-E-R, or R-E-A-R; 26). Consistent with our finding, no avirulent strains bearing the consensus R-E-T-R sequence became highly pathogenic during either in vitro nor in vivo passages in the laboratory (4, 8, 24).

Is brain passage an absolute requirement for virus conversion to a highly virulent phenotype? Avirulent viruses with uncleavable HA do not replicate in the brain, providing selective pressure for the replication of virulent viruses with cleavable HA. However, we do not know whether continued air sac passage could also result in the generation of virulent viruses. Further studies are needed to clarify this issue.

The conditions under which we generated highly virulent viruses from an avirulent strain are generally not duplicated in nature. However, viruses with low pathogenicity can cause viremia in physically compromised chickens (5). In fact, virus was recovered from the brain of a chick infected with virus 24a by air sac inoculation, although the virus titer was low ($10^{2.75}$ EID_{50}/g). Thus, viruses that grow efficiently in localized sites in poulty, such as the respiratory and intestinal tracts, may occasionally spread to the brain and acquire a virulent phenotype upon subsequent replication, eventually killing millions of susceptible chickens, as occurred in central Mexico in 1994 and 1995 (6, 7).

The decreased mortality rate, mean time to death, and plaque-forming ability of virus 24a2b (Tables 1 and 3) suggest that it represents an intermediate pathogenic stage; however, its HA was uncleaved in the absence of trypsin, possibly reflecting a minor population of mutants with highly cleavable HA. In support of this notion, virus recovered from the brain of a chicken infected with virus 24a2b contained the same highly cleavable HA sequence (R-R-K-K-R) as virus 24a3b.

Although we have focused on the contribution of HA cleavage to virulence acquisition, the data clearly indicate that other factors are involved. For example, the original swan isolate acquired the ability to grow in chickens during air sac passages but lacked mutations in the HA cleavage site, indicating that mutations in other parts of the HA gene or other genes must be responsible. The series of virulence mutants isolated in the present study not only lend insight into mechanisms of virulence acquisition but may also provide a useful tool, in combination with reverse genetics, with which to generate influenza viruses from plasmids (18).

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