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Cooperation between the Hemagglutinin of Avian Viruses and the Matrix Protein of Human Influenza A Viruses

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To analyze the compatibility of avian influenza A virus hemagglutinins (HAs) and human influenza A virus matrix (M) proteins M1 and M2, we doubly infected Madin-Darby canine kidney cells with amantadine (1-aminoadamantane hydrochloride)-resistant human viruses and amantadine-sensitive avian strains. By using antisera against the human virus HAs and amantadine, we selected reassortants containing the human virus M gene and the avian virus HA gene. In our system, high virus yields and large, well-defined plaques indicated that the avian HAs and the human M gene products could cooperate effectively; low virus yields and small, turbid plaques indicated that cooperation was poor. The M gene products are among the primary components that determine the species specificities of influenza A viruses. Therefore, our system also indicated whether the avian HA genes effectively reassorted into the genome and replaced the HA gene of the prevailing human influenza A viruses. Most of the avian HAs that we tested efficiently cooperated with the M gene products of the early human A/PR/8/34 (H1N1) virus; however, the avian HAs did not effectively cooperate with the most recently isolated human virus that we tested, A/Nanchang/933/95 (H3N2). Cooperation between the avian HAs and the M proteins of the human A/Singapore/57 (H2N2) virus was moderate. These results suggest that the currently prevailing human influenza A viruses might have lost their ability to undergo antigenic shift and therefore are unable to form new pandemic viruses that contain an avian HA, a finding that is of great interest for pandemic planning.

New pandemic human influenza A viruses can be created when reassortment causes the hemagglutinin (HA) gene of the prevailing human strain to be replaced by the allelic gene of an avian influenza A virus; such reassortment occurred in the 1957 and 1968 influenza pandemics (6, 11). An important question needs to be answered: are all 15 subtypes of avian HA compatible with human strains, i.e., could reassortment involving any of the avian HAs result in the formation of new pandemic human influenza A viruses? In past pandemics, only HA subtypes 1, 2, and 3 were found in human influenza A viruses (8). Our concern is whether other HA subtypes should be expected in the next pandemic virus and whether preparation against such future, novel strains should now be made.

In our previous studies of the rescue of temperature-sensitive mutants, the HA gene of the fowl plague virus (FPV; H7N1) did not segregate with the human virus matrix (M) gene (10). Reassortants carrying the human HA and FPV M genes were obtained only under strong selection pressure with specific antisera. However, these reassortants replicated to only very low virus titers and they formed turbid and fuzzy plaques. Such inefficient reassortants are expected to be neutralized by antibodies before they cause any signs of disease; therefore, it is unlikely that they would become dominant pandemic human influenza A virus strains. For example, live vaccine strains are obtained with defects in that they replicate efficiently only at low temperatures and/or are temperature sensitive and therefore grow only to low titers at normal temperatures. In contrast, reassortant viruses containing the HA genes of avian influenza virus and the M gene of FPV, and vice versa, were easily obtained and replicated efficiently (9). Thus, the products of the human HA and FPV M genes do not appear to be compatible, although avian HAs cooperate efficiently with FPV M gene products. A similar observation was made by Tian et al. (14). Those scientists studied influenza viruses created by the reassortment of an avian virus and a human virus in squirrel monkeys, and they found that the M gene and the nucleoprotein (NP) gene are the main determinants of host restriction.

With these observations in mind, we developed an experimental system with which to investigate both the compatibility between human virus M gene products and various avian HA subtypes and their abilities to create efficient reassortants.

MATERIALS AND METHODS

Viruses and cells. The egg-grown influenza A viruses (see Tables 1 to 3) were from the repository at St. Jude Children's Research Hospital, Memphis, Tenn. The viruses were plaque purified in Madin-Darby canine kidney (MDCK) cells, and stocks were produced in 10-day-old embryonated chicken eggs. The number of PFU (plaques per milliliter of allantoic fluid) was determined for each stock. MDCK cells were either singly or doubly infected at a multiplicity of infection (MOI) of about 10 for each virus by a corresponding dilution of the stock. The virus yields after the selection procedure (see below) were somewhat dependent on the relative MOIs of the human and avian viruses. Therefore, to obtain comparable and reproducible results from double-infection experiments, we used the same virus dilutions from the same stocks of allantoic fluids. If the ratio of the MOIs of the human and avian viruses was 1:10, then the plaque yield after selection was nearly five times higher than it was when equal MOIs were used. If the ratio of the MOIs of the human and avian viruses was 10:1, the plaque yield decreased by a factor of about 5.

The MDCK cells were routinely subjected to passage in Eagle's minimal

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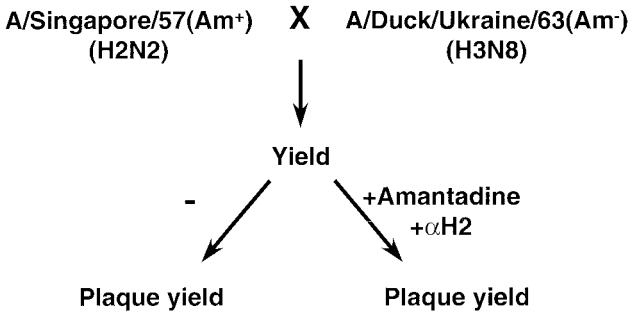

FIG. 1. Experimental design of double infection of MDCK cells and selection of influenza virus reassortants that carry the HA gene of the avian virus and the M gene of the amantadine-resistant variant of the human Singapore influenza virus. The hyperimmune antiserum (αH2) was directed against the HA of the human Singapore virus.

essential medium (MEM; Sigma, St. Louis, Mo.) containing 5% fetal bovine serum. Amantadine (1-aminoadamantane hydrochloride)-resistant variants were obtained by three passages of the virus in MDCK cells cultured in the presence of 2 μg of amantadine (Sigma)/ml. Plaques were chosen from a plaque test, and the virus particles underwent further plaque purification in the presence of amantadine (13). Finally, purified isolates were injected into 10-day-old embryonated chicken eggs to obtain a stock of infectious allantoic fluid. The amantadine-resistant variants used in these studies had the same growth characteristics concerning virus yield and plaque size in MDCK cells as the wild-type viruses.

Virus growth, plaque tests, and selection procedure. MDCK cells were singly or doubly infected (see Fig. 1). The cells were overlaid with MEM containing 1 μg of tosylsulfonyl phenylalanyl chloromethyl ketone-treated trypsin (Worthington Biochemical Corporation, Lakewood, N.J.)/ml and 4% bovine serum albumin (2 ml per well). After incubation for 20 h, supernatants were collected and used without further treatment (no selection) or they were serially diluted into a solution containing antiserum against the HAs of the human strains (selection). We used hyperimmune antisera raised in goats to the isolated HA. They were directed against the HAs of the human viruses as listed in Tables 1 to 3. The hyperimmune antisera were diluted in phosphate-buffered saline (PBS; dilution ratio, 1:100 or 1:200). These serum concentrations neutralized the human viruses completely. After 1 h on ice, MDCK cells were infected for use in the plaque test by using 0.9% agar, 0.5 μg of tosylsulfonyl phenylalanyl chloromethyl ketone-treated trypsin/ml, and 4% bovine serum albumin in MEM, as described by Appleyard and Marber (1). For amantadine selection, the overlay medium contained 4 μg of amantadine/ml. After 3 days at 37°C, plaques intended for further purification were chosen and dissolved in 1 ml of PBS; those not intended for further purification were visualized and counted by discarding the agar overlay and staining the cells with 0.1% crystal violet containing 10% formaldehyde. All experiments were replicated at least twice.

Sequencing. This procedure has been described by Guan et al. (4). In brief, viral RNA was extracted from infective allantoic fluid by using a RNeasy Mini Kit (Qiagen, Valencia, Calif.). We used specific primers to perform reverse transcription and to amplify by PCR a region of approximately 500 nucleotides that encode the region surrounding the ion channel of the M2 protein (primer sequences are available upon request). PCR products were purified by using a QiaQuick PCR purification kit (Qiagen) and sequenced by using synthetic oligonucleotides produced by the Hartwell Center for Bioinformatics and Biotechnology at St. Jude Children's Research Hospital. Reactions were performed with Rhodamine Dye-Terminator Cycle Sequencing Ready Reaction kits and AmpliTaq DNA polymerase FS (Perkin-Elmer Applied Biosystems, Foster City, Calif.). Samples were subjected to electrophoresis and analyzed on a Perkin-Elmer model 377 DNA sequencer. The sequences obtained were compared with known sequences of human and avian M genes.

RESULTS

Experimental system for testing cooperation between M gene products of human viruses and HAs of avian viruses. In doubly infected MDCK cells, the appearances and sizes of plaques obtained without selection were very heterogeneous. Many large, clear plaques resembling the parent viruses were mixed with smaller and very tiny plaques, some of which were turbid and fuzzy. This spectrum of appearance of the plaques represents the large number of possible reassortants that resulted from the infection of a host cell with two viruses that had segmented genomes. After the selection procedure, the parent viruses were removed and only reassortants that contained the M gene of the human virus and the HA gene of the avian virus remained (Fig. 1). If the virus yield is high and large plaques prevail after selection in repeated experiments, cooperation between the human M gene product(s) and avian HAs in reassortants in the large plaques is regarded as being optimal. In contrast, if the virus yield is low and only small, turbid, and fuzzy plaques are formed, cooperation is regarded as being bad.

Reassortment between the amantadine-resistant human PR8 strain and amantadine-sensitive avian influenza A viruses. We have used two different A/PR/8/34 (H1N1) (PR8) isolates from our collection, an early isolate from the late 1960s that forms relatively small plaques (1 mm in diameter in MDCK cells) and a very recent isolate that forms plaques of 3 mm in diameter. There was no significant difference in the outcomes of the results. MDCK cells were doubly infected with the recent human PR8 strain and various avian influenza A viruses with different HA subtypes (Table 1). After selection with amantadine and hyperimmune antiserum against the HA of PR8, only reassortants that carried the M gene of the human virus and the HA gene of the avian virus remained (Fig. 1). In most cases, selection pressure caused a slight (no more than 10-fold) reduction in the virus titers, and many of the remaining plaques were the same size as those formed by the parent viruses. For example, after double infection with the human PR8 and the A/Duck/Ukraine/63 (H3N8) viruses, the plaque

TABLE 1. Plaque yields (PFU) and maximum plaque diameters after a 20-h single or double infection with the human PR8 and avian influenza A viruses

Virus strain(s)	PFU (maximum plaque diam [mm])	
	No selection	Selection[a]
A/PR/8/34 (H1N1)[b]	2×10^8 (3)	$<10^3$
A/Mallard/Potsdam/178-4/83 (H2N2)	2×10^8 (5)	$<10^4$
A/Mallard/Potsdam/178-4/83, PR8	5×10^7 (5)	6×10^5 (5)
A/Duck/Ukraine/63 (H3N8)	2×10^8 (6)	$<10^4$
A/Duck/Ukraine/63, PR8	8×10^7 (6)	4×10^7 (6)
A/Duck/Hong Kong/Y264/97 (H4N8)	1×10^7 (4)	$<10^3$
A/Duck/Hong Kong/Y264/97, PR8	2×10^7 (3)	2×10^5 (3)
A/Duck/Singapore/3/97 (H5N3)	1×10^8 (2)	$<10^3$
A/Duck/Singapore/3/97, PR8	1×10^8 (3)	6×10^6 (2)
A/Gray Teal/Australia/1/79 (H7N8)	2×10^7 (3)	$<10^3$
A/Gray Teal/Australia/1/79, PR8	2×10^7 (5)	1×10^6 (5)
A/Chick/Germany N/49 (H10N7)	3×10^7 (3)	$<10^3$
A/Chick/Germany N/49, PR8	2×10^7 (4)	3×10^6 (4)
A/Duck/Hong Kong/P50/97 (H11N9)	5×10^7 (2)	$<10^3$
A/Duck/Hong Kong/P50/97, PR8	3×10^7 (2)	4×10^6 (3)
A/Mallard/Astrachan/263/82 (H14N5)	4×10^7 (4)	$<10^3$
A/Mallard/Astrachan/263/82, PR8	4×10^7 (4)	4×10^6 (4)
A/Wedge-tailed Shearwater/Australia/79 (H15/N9)	2×10^8 (4)	$<10^4$
A/Wedge-tailed Shearwater/Australia/79, PR8	5×10^7 (3)	8×10^6 (3)

[a] Anti-H1 antiserum (1:100 dilution in PBS) was used to select against human HA, and amantadine (4 μg/ml in the agar overlay) was used to select against avian M genes.

[b] The PR8 virus is naturally amantadine resistant (5).

TABLE 2. Plaque yields (PFU) and maximum plaque sizes after single or double infection of MDCK cells with the human Singapore and avian or swine influenza A viruses

Virus strain(s)	PFU (maximum plaque diam [mm])	
	No selection	Selection[a]
A/Singapore/57 (H2N2)[b]	2×10^8 (2)	$<10^2$
A/Oystercatcher/Germany/87 (H1N1)	4×10^6 (1.5)	$<10^3$
A/Oystercatcher/Germany/87, Singapore	1×10^6 (2)	2×10^2 (0.1)
A/Duck/Alberta/35/76 (H1N1)	4×10^7 (2)	$<10^3$
A/Duck/Alberta/35/76, Singapore	1×10^8 (2)	2×10^4 (0.2)
A/Duck/Ukraine/63 (H3N8)	2×10^8 (6)	$<10^4$
A/Duck/Ukraine/63, Singapore	2×10^8 (6)	1.3×10^8 (6)
A/Duck/Hong Kong/Y264/97 (H4N8)	3×10^7 (4)	$<10^3$
A/Duck/Hong Kong/Y264/97, Singapore	1×10^7 (4)	1×10^6 (5)
A/Duck/Singapore/3/97 (H5N3)	2×10^8 (3)	$<10^3$
A/Duck/Singapore/3/97, Singapore	2×10^8 (3)	2×10^5 (0.5)
A/Chick/Germany N/49 (H10N7)	5×10^7 (2)	$<10^5$
A/Chick/Germany N/49, Singapore	8×10^7 (2)	1.5×10^5 (0.2)
A/Duck/Hong Kong/P50/97 (H11N9)	3×10^8 (3)	$<10^3$
A/Duck/Hong Kong/P50/97, Singapore	2×10^8 (4)	2×10^7 (4)
A/Mallard/Astrachan/263/82 (H14N5)	2×10^7 (4)	$<10^4$
A/Mallard/Astrachan/263/82, Singapore	3×10^7 (4)	5×10^4 (0.4)
A/Wedge-tailed Shearwater/Australia/79 (H15/N9)	8×10^8 (6)	$<10^4$
A/Wedge-tailed Shearwater/Australia/79, Singapore	8×10^7 (4)	5×10^5 (0.4)
A/Swine/Germany/81 (H1N1)	8×10^7 (3)	$<10^4$
A/Swine/Germany/81, Singapore	2×10^7 (3)	2×10^6 (3)

[a] Anti-H2 antiserum (1:200 dilution in PBS) was used to select against human HA, and amantadine (4 μg/ml in the agar overlay) was used to select against avian and swine M genes.
[b] An amantadine-resistant Singapore variant was used.

yields obtained under selection pressure differed only slightly (approximately twofold) from those obtained in the absence of such pressure and most of the remaining plaques were the same diameter as those formed by the A/Duck/Ukraine/63 virus. These findings indicated that the replacement of the human virus H1 HA with the avian H3 HA leads to the development of reassortants that replicate well. Only after double infection with A/Mallard/Potsdam/178-4/83 (H2N2) or A/Duck/Hong Kong/Y264/97 (H4N8) and selection was the virus yield relatively low (about 2%); however, large plaques remained. After selection, at least one large plaque was isolated from most of the doubly infected cells, including those infected with A/Mallard/Potsdam/178-4/83 or A/Duck/Hong Kong/Y264/97 (Table 1). Each of these plaques was purified at least four times before it was injected into an embryonated egg. Viral RNAs isolated from the infectious allantoic fluids were used to sequence the M2 gene that was present in the plaque-forming virus; in all cases, the M2 gene was derived from the human PR8 virus. These findings indicated that the HAs of most of the avian viruses tested were able to efficiently reassort with the M gene product(s) of the human PR8 virus.

Reassortment between an amantadine-resistant variant of the human Singapore strain and amantadine-sensitive avian influenza A viruses. In a second set of experiments, MDCK cells were doubly infected with an amantadine-resistant variant of the human A/Singapore/57 (H2N2) (Singapore) strain and amantadine-sensitive avian influenza A viruses. After double infection and selection, examination of the supernatants of the MDCK cells revealed that only a few avian viruses formed reassortants with the human Singapore strain effectively (Table 2). For example, the A/Duck/Ukraine/63 (H3N8) strain produced high yields of large plaques seen in doubly infected MDCK cells after selection; however, most of the other avian strains tested, whose HAs cooperated efficiently with the M gene products of the human PR8 strain (Table 1), produced low titers (the titer of the selected virus was less than 1% of the titer of the unselected virus) and small, turbid, fuzzy plaques. In addition to the A/Duck/Ukraine/63 (H3N8) virus, two HAs of recent isolates from Hong Kong, A/Duck/Hong Kong/Y264/97 (H4N8) and A/Duck/Hong Kong/P50/97 (H11N9), efficiently complemented the human Singapore strain.

Interestingly, although the HAs of the two avian H1N1 viruses (Table 2) did not cooperate well with the M gene product(s) of the human H2N2 virus, the HA of the avian-like swine virus (H1N1) did. This finding suggests that an avian virus has to cross the species barrier and infect a lower mammal before its H1 HA can efficiently replace the H2 HA of the human Singapore virus. Thus, only after an avian H1N1 virus forms a stable lineage in a mammalian host is the avian HA able to efficiently cooperate with the M gene product(s) of the human Singapore virus.

MDCK cells were doubly infected with the human Singapore virus and with one of the three efficiently complementing avian viruses (A/Duck/Ukraine/63, A/Duck/Hong Kong/Y264/97 [H4], or A/Duck/Hong Kong/P50/97 [H11]) or the H1N1 swine virus. After selection, large plaques were purified and injected into embryonated eggs. Sequencing revealed that these reassortants contained the amantadine-resistant M gene of the human Singapore virus.

Reassortment between an amantadine-resistant variant of the human Nanchang strain and amantadine-sensitive avian influenza A viruses. Because the two early human viruses, PR8 and Singapore, showed such differences in complementation by avian HAs, we tested a more recently isolated human virus, A/Nanchang/933/95 (H3N2) (Nanchang). Of the avian HAs that we tested, none efficiently cooperated with the M gene product(s) of the recent human isolate (Table 3). In seven of the eight avian influenza viruses tested, the difference between titers of viruses that were under selection pressure and those that were not was at least 100-fold and the remaining plaques were small. After infection with the A/Oystercatcher/Germany/87 virus and selection, no plaques were visible at a virus dilution of 1:100. The avian-virus-like A/Swine/Germany/81 virus was also unable to efficiently form reassortants with the human Nanchang virus. Even after double infection with the A/Duck/Ukraine/63 virus and the Nanchang isolate and selection, the virus titer decreased by 20-fold, and the maximum diameter of the remaining plaques was only half of that of the plaques that were not subjected to selection pressure. Thus, the cooperation between the HAs of the tested avian viruses and the M gene product(s) of the most recent human influenza virus isolate was worse than that between the HAs of avian viruses and the M gene product(s) of the human strain isolated in 1957.

Possible pitfalls of using this system of MDCK cells doubly infected with human and avian influenza A viruses. In some cases, especially with double infection of MDCK cells with the human Singapore strain and avian influenza A viruses, we obtained large plaques where we did not expect them. We found two different explanations for the appearance of these large-plaque formers. First, partial M gene heterozygotes formed and persisted through subsequent passages in the pres-

TABLE 3. Plaque yields (PFU) and maximum plaque sizes after single or double infection of MDCK cells with the human Nanchang and avian or swine influenza A viruses

Virus strain(s)	PFU (maximum plaque diam [mm])	
	No selection	Selection[a]
A/Nanchang/933/95 (H3N2)[b]	6×10^7 (4)	$<10^2$
A/Oystercatcher/Germany/87 (H1N1)	6×10^5 (2)	$<10^3$
A/Oystercatcher/Germany/87, Nanchang	2×10^6 (3)	$<10^3$
A/Swine/Germany/81 (H1N1)	1×10^8 (4)	$<10^3$
A/Swine/Germany/81, Nanchang	4×10^7 (4)	1×10^5 (0.3)
A/Mallard/Potsdam/178-4/83 (H2N2)	3×10^8 (6)	$<10^4$
A/Mallard/Potsdam/178-4/83, Nanchang	2×10^8 (6)	1×10^6 (2.5)
A/Duck/Ukraine/63 (H3N8)[c]	2×10^8 (6)	$<10^4$
A/Duck/Ukraine/63, Nanchang	8×10^7 (6)	4×10^6 (3)
A/Duck/Hong Kong/Y264/97 (H4N8)	2×10^7 (4)	$<10^4$
A/Duck/Hong Kong/Y264/97, Nanchang	1×10^7 (4)	1×10^5 (0.6)
A/Duck/Singapore/3/97 (H5N3)	6×10^7 (3)	$<10^3$
A/Duck/Singapore/3/97, Nanchang	6×10^7 (3)	4×10^4 (0.4)
A/Gray Teal/Australia/1/79 (H7N8)	8×10^6 (3)	$<10^3$
A/Gray Teal/Australia/1/79, Nanchang	8×10^6 (3)	1×10^4 (0.4)
A/Chick/Germany N/49 (H10N7)	6×10^7 (4)	$<10^4$
A/Chick/Germany N/49, Nanchang	4×10^7 (4)	1×10^5 (0.2)
A/Duck/Hong Kong/P50/97 (H11N9)	2×10^7 (3)	$<10^3$
A/Duck/Hong Kong/P50/97, Nanchang	1×10^7 (3)	3×10^4 (0.3)

[a] Anti-H3 antiserum (1:100 dilution in PBS) was used to select against human HA, and amantadine (4 μg/ml in the agar overlay) was used to select against the avian and swine M genes.
[b] An amantadine-resistant Nanchang variant was used.
[c] The anti-H3 antiserum used did not neutralize the A/Duck/Ukraine/63 (H3N8) virus.

ence of amantadine. However, if amantadine was not present in the agar overlay medium of only one passage, most of the large plaques in the subsequent passage became amantadine sensitive. We observed this phenomenon several times. It happened, for example, when MDCK cells were doubly infected with either A/Chick/Germany N/49 and Singapore or A/Duck/Hong Kong/Y264/97 and Nanchang, as shown in Table 4. After three passages, with the last passage occurring without amantadine, we sequenced the M genes from a large plaque and found that they were of avian virus origin and that they did not encode a corresponding mutation leading to amantadine resistance in the ion channel region of the M2 protein (5).

The second explanation for the unexpected appearance of large plaques is that a rare amantadine-resistant variant of the avian influenza viruses formed during the experiment by spontaneous mutation and overcame the selection pressure. The variant then grew to relatively high titers. This type of variant remained amantadine resistant, even after the release from selection pressure. This happened only twice during all of our experiments. Sequencing of the M gene revealed that the M2 protein of these amantadine-resistant isolates was that of the avian virus and that it contained a corresponding amino acid replacement at position 30 or 31, a known cause of amantadine resistance (5). Where such inconsistencies were present, the experiments were repeated several times. In these repeated experiments, large plaques were not seen again. Therefore, in questionable cases, it is essential to thoroughly analyze large plaques and sequence their M genes to ensure that the plaques were formed by true reassortment.

DISCUSSION

In the process of pandemic planning, antigenic shifts caused by reassortment must be anticipated. Therefore, it is essential to determine whether all of the avian HAs can cooperate efficiently with the gene products of the prevailing human influenza A virus to form a reassortant that contains an avian HA; such reassortment events occurred in 1957 and 1968 (6, 11). Here we report the development of a system to test the cooperation between avian HAs and the M gene products of the human virus (Fig. 1). After double infection of MDCK cells with an amantadine-resistant human influenza A virus and an amantadine-sensitive avian influenza A virus, we used specific antisera to select against the human HA and amantadine to obtain reassortants that carry the avian HA gene and the human M gene. Because the M gene encodes a viral component that is one of the determinants of species specificity (10, 14), this experimental system also examines whether the avian HA gene is compatible with the genome of the human influenza A virus. After the application of selection pressure, a high virus yield and large plaques indicate that the avian HA cooperates efficiently with the gene products of the human virus; a low virus yield with small, turbid plaques that are fuzzy indicates that the avian HA does not cooperate effectively with the gene products of the human virus. Although growth in MDCK cells might not in all cases reflect a corresponding growth in vivo, most of such reassortants probably grow to low titers in humans and are eliminated by neutralizing antibodies before

TABLE 4. Results of plaque-to-plaque passages after double infection of MDCK cells with a human and an avian influenza A virus and selection[a]

Virus strains[b]	Passage no.	Selection procedure	PFU (maximum plaque diam [mm])
A/Chick/Germany N/49, Singapore[c]	1	Anti-H2, amantadine	1×10^3 (2)
	2	None	7×10^3 (2) plus 5×10^3 (0.2)
	3	None	1.5×10^3 (2.5)
	3	Amantadine	<10
A/Duck/Hong Kong/Y264/97, Nanchang[d]	1	Anti-H3, amantadine	3×10^3 (3)
	2	None	2×10^4 (3.5) plus 2×10^4 (0.6)
	3	None	1×10^4 (3.5)
	3	Amantadine	6×10^1 (3) plus 4×10^3 (<0.1)

[a] One large plaque from each infection was further analyzed in subsequent passages. For the plaque-to-plaque passages, the plaque fluids (1 ml) were serially diluted from 10^{-1} to 10^{-4}.
[b] Dilution, 1:1,000.
[c] Three 2-mm-diameter plaques were seen among 250 small plaques.
[d] Three 3-mm-diameter plaques were seen among 15 small plaques.

they can induce any symptoms. These reassortants are not expected to constitute a human pandemic influenza A virus.

The number of avian influenza A viruses investigated here is too small to assess whether all viruses of the same HA subtype can donate their HA genes to create novel (efficient or weak) reassortants with the M gene of the human virus. At this time, we can make such a statement only for the individual viruses tested, which include avian viruses that are currently prevalent in Hong Kong and China.

To cooperate with the human M gene products, the avian HA needs physical contact with them. The M2 protein and HA are both embedded in the viral envelope, and therefore they must be in physical contact with one another; however, we do not know whether these two proteins interact, let alone how such an interaction might influence their functions. Enami (2) has suggested that the carboxy terminus of the HA might be in contact with the M1 protein during virus assembly. Whatever the interactions are, substantial differences exist in the levels of cooperation between the avian HAs and at least one of the M gene products of human viruses isolated during different decades.

Phylogenetic analyses have revealed that the avian influenza A viruses are in phylogenetic stasis as long as they remain in an avian host (12, 15), a finding that indicates that these viruses have been perfectly adapted to the bird population for a long time. However, human influenza viruses have been under strong selection pressure since an avian influenza A virus was introduced into the human population around 1900 and subsequently formed a stable lineage (12, 15). At the branch of the human viruses, the PR8 virus is still relatively close to its avian root; this close relationship explains why the avian HAs can cooperate with the M gene products of the human PR8 virus quite well. The PR8 strain has been passaged many times in different hosts, and this may affect the outcomes of such reassortment experiments. However, we have compared the earliest PR8 isolate available in our collection with our most recent PR8 isolate and we did not obtain significant differences in our results. In contrast, the human Nanchang virus is far removed from its avian root; thus, the HAs of the avian viruses did not efficiently cooperate with the M gene products of the Nanchang virus to create a highly virulent reassortant. The human Singapore virus, which has an intermediate phylogenetic position, formed moderately efficient reassortants with the avian viruses tested. This observation must be considered with respect to the human influenza B and C viruses. Human influenza B and C viruses are also rooted to avian influenza A viruses (3); however, they are now regarded as leftovers of former branches of human influenza viruses and they no longer form reassortants with any influenza A viruses.

Our findings may be relevant for pandemic planning. Although most of the avian influenza A viruses that we tested can donate their HA genes in forming reassortants with the most recently isolated (1995) human virus that we evaluated, none of these reassortants grew to high titers. As outlined above, such poorly replicating viruses are not expected to be pandemic in the human population, in spite of the fact that neutralizing antibodies against such viruses with novel surfaces are not present in the human population. However, one must consider to what extent partial heterozygotes, which can survive for at least several passages under a specific selection pressure (Table 4), might help to create a better-growing reassortant by introducing corresponding mutations. The viruses tested were isolated from different species of birds, and one was an avian-virus-like swine virus. These viruses were isolated during different years and in different parts of the world. Therefore, it is not very likely that the next human pandemic virus will be created by an antigenic shift (reassortment). Rather, the next pandemic may be caused by an avian virus or an avian virus-like swine virus that enters the human population in toto, without reassortment.

The species barrier that prevents avian influenza A viruses from infecting humans is quite high. Although avian influenza viruses from bird markets in southern China are able to infect humans, it is rather unlikely that these viruses form a stable lineage in humans. On the other hand, the species barrier preventing swine viruses from infecting humans or birds is relatively low (12, 15). Therefore, the European swine virus is a more likely candidate to cause the next human influenza pandemic (7) that would mimic the pandemic of 1918 and 1919. Another possible scenario for a pandemic involves a human virus that has remained in a hidden reservoir for many years. Its reappearance could cause a pandemic resembling that of 1977, when an H1N1 virus of 1950 infected mainly people born after 1957 worldwide. A human H2N2 virus is a likely candidate for this type of reappearing virus, because these viruses could infect humans born after 1968, who do not have antibodies against an H2 virus. Although the creation of the next pandemic influenza virus by reassortment cannot be excluded, these considerations should be used to set preferences for pandemic preparedness.

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REFERENCES

1. **Appleyard, G., and H. B. Marber.** 1974. Plaque formation by influenza viruses in the presence of trypsin. J. Gen. Virol. **25:**351–357.
2. **Enami, M.** 1996. The role of the hemagglutinin and neuraminidase glycoproteins in influenza A virus assembly, p. 328–332. *In* L. E. Brown, A. W. Hampson, and R. G. Webster (ed.), Options for the control of influenza III. Elsevier, Amsterdam, The Netherlands.
3. **Gammelin, M., A. Altmüller, U. Reinhardt, J. Mandler, V. R. Harley, P. J. Hudson, W. M. Fitch, and C. Scholtissek.** 1990. Phylogenetic analysis of nucleoproteins suggests that human influenza A viruses emerged from a 19th century avian ancestor. Mol. Biol. Evol. **7:**194–200.
4. **Guan, Y., K. F. Shortridge, S. Krauss, and R. G. Webster.** 1999. Molecular characterization of H9N2 influenza viruses; were they the donors of the "internal" genes of H5N1 viruses in Hong Kong? Proc. Natl. Acad. Sci. USA **96:**9363–9367.
5. **Hay, A. J., A. J. Wolstenholm, J. J. Skehel, and M. H. Smith.** 1985. The molecular basis of the specific anti-influenza action of amantadine. EMBO J. **4:**3021–3024.
6. **Kawaoka, Y., S. Krauss, and R. G. Webster.** 1989. Avian-to-human transmission of the PB1 gene of influenza A viruses in the 1957 and 1968 pandemics. J. Virol. **63:**4603–4608.
7. **Ludwig, S., L. Stitz, O. Planz, H. Van, W. M. Fitch, and C. Scholtissek.** 1995. European swine virus as a possible source for the next influenza pandemic? Virology **212:**555–561.
8. **Potter, C. W.** 1998. Chronicle of influenza pandemics, p. 3–18. *In* K. G.

Nicholson, R. G. Webster, and A. J. Hay (ed.), Textbook of influenza. Blackwell Science Ltd., Oxford, England.
9. **Rott, R., M. Orlich, and C. Scholtissek.** 1979. Correlation of pathogenicity and gene constellation of influenza A viruses. III. Non-pathogenic recombinants derived from highly pathogenic parent strains. J. Gen. Virol. **44:**471–477.
10. **Scholtissek, C., R. Rott, M. Orlich, E. Harms, and W. Rohde.** 1977. Correlation of pathogenicity and gene constellation of an influenza A virus (fowl plague). I. Exchange of a single gene. Virology **81:**74–80.
11. **Scholtissek, C., W. Rohde, V. von Hoyningen, and R. Rott.** 1978. On the origin of the human influenza subtypes H2N2 and H3N2. Virology **87:** 13–20.
12. **Scholtissek, C., S. Ludwig, and W. M. Fitch.** 1993. Analysis of influenza A virus nucleoproteins for the assessment of molecular genetic mechanisms leading to new phylogenetic virus lineages. Arch. Virol. **131:**237–250.
13. **Scholtissek, C., G. Quack, H. D. Klenk, and R. G. Webster.** 1998. How to overcome resistance of influenza A viruses against adamantane derivatives. Antivir. Res. **37:**83–93.
14. **Tian, S.-F., A. J. Buckler-White, W. T. London, L. J. Reck, R. M. Chanock, and B. R. Murphy.** 1985. Nucleoprotein and membrane protein genes are associated with restriction of replication of influenza A/Mallard/NY/78 virus and its reassortants in squirrel monkey respiratory tract. J. Virol. **53:**771–775.
15. **Webster, R. G., W. J. Bean, O. T. Gorman, T. M. Chambers, and Y. Kawaoka.** 1992. Evolution and ecology of influenza A viruses. Microbiol. Rev. **56:**152–179.