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Avian-virus-like receptor specificity of the hemagglutinin impedes influenza virus replication in cultures of human airway epithelium

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Abstract

A non-optimal receptor-binding specificity of avian influenza viruses is believed to hamper their replication in humans; however, the magnitude of this restriction remains undefined. Here we generated recombinant viruses, R1 and R2, that differed solely by two amino acids in the receptor-binding site of their hemagglutinin (HA). R1 harbored the original HA of the pandemic human virus A/Hong Kong/1/68 (H3N2), whereas R2 was the L226Q/S228G HA mutant with avian-virus-like receptor specificity. In differentiated cultures of human tracheo-bronchial epithelial cells, R1 preferentially infected non-ciliated cells, whereas R2 predominantly infected ciliated cells indicating that cell tropism was determined by the viral receptor specificity. In the course of multi-cycle replication in these cultures, R2 spread less efficiently and grew to 2–10-fold lower titers than did R1. These results for the first time estimate the level of receptor-dependent restriction of avian influenza viruses in human airway epithelium. They support a theory that alteration of the receptor specificity of an avian virus could facilitate its human-to-human transmission.

Keywords: Influenza; Pandemics; Host range; Reverse genetics; Sialic acid; Receptor specificity, Cell tropism; Replication efficiency, Human airway epithelial cultures

Introduction

Influenza pandemics originate from birds which perpetuate influenza A viruses with all known hemagglutinin (HA) and neuraminidase (NA) subtypes (reviewed by Webster et al., 1992; Cox and Subbarao, 2000). Fortunately, the avian viruses cross the species barrier to humans only rarely. Although this host-range restriction depends on many viral genes (reviewed by Klenk and Rott, 1988; Baigent and McCauley, 2003; Webby et al., 2004; Horimoto and Kawaoka, 2005), it can be partially overcome by gene reassortment between avian and human viruses. However, as any pandemic virus must carry the HA from a non-human virus to escape the herd immunity in the population, it is particularly important to understand what minimal changes in the avian HA are needed for the emergence of a new pandemic strain.

A poor fit of avian viruses to cellular receptors in humans is one potential HA-mediated restriction mechanism (reviewed by Skehel and Wiley, 2000; Matrosovich et al., 2006). Avian influenza viruses bind to cell-surface receptors containing terminal sialyl–galactosyl residues linked by 2–3-linkage [Neu5Ac(α2–3)Gal], whereas human viruses bind to receptors which contain terminal α2–6–linked sialyl–galactosyl moieties (Paulson, 1985; Nobusawa et al., 1991; Connor et al., 1994; Matrosovich et al., 1997). The earliest studied virus strains from the 1918, 1957, and 1968 pandemics displayed a human-virus-like preference for 6-linked sialic acid receptors and decreased binding to 3-linked receptors (Paulson, 1985; Connor et al., 1994; Matrosovich et al., 2000; Kobasa et al., 2004; Glaser et al., 2005). By contrast, H5N1 chicken influenza viruses that cause sporadic cases of severe disease in humans since 1997 typically have an avian-virus-like receptor specificity (Matrosovich et al., 1999; Gambaryan et al., 2006) and are unable to transmit efficiently from human to human. It is generally

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believed, therefore, that alteration of the receptor specificity is a prerequisite for the highly effective replication and human-to-human transmission which characterize pandemic virus strains (reviewed by Cox and Subbarao, 2000; Horimoto and Kawaoka, 2001; Baigent and McCauley, 2003; Matrosovich et al., 2006). However, this theory has never been formally proven, and neither magnitude of receptor-dependent restriction on avian virus replication in humans, nor restriction mechanisms have been defined.

To address these questions, we employ differentiated cultures of human tracheo-bronchial epithelial cells (HTBE) (Gray et al., 1996). These cultures are pseudostratified and polarized, contain ciliated, secretory, and basal cells, and resemble human airway epithelium *in vivo* both morphologically and functionally (Gray et al., 1996; Matrosovich et al., 2004; Thompson et al., 2006; Ibricevic et al., 2006). Using this experimental system, we found that human and avian viruses target different types of airway epithelial cells in correlation with the expression levels of the corresponding types of sialic acid receptors (Matrosovich et al., 2004). Namely, at the initial stages of infection, human viruses mainly infected non-ciliated cells whereas avian viruses predominantly infected ciliated cells. After several cycles of replication, the human virus produced large continuous foci of infected cells that included both ciliated and non-ciliated cells. By contrast, the avian virus formed less prominent foci remaining mainly associated with ciliated cells (Matrosovich et al., 2004). Thompson et al. (2006) used a similar in vitro model of human airway epithelium and confirmed our findings. They noticed also that avian viruses appeared to infect airway epithelial cells more slowly than human viruses as avian virus antigen was not detected at early times (6 h) post infection. Shinya et al. (2006) reported that avian viruses in contrast to human viruses did not infect pieces of surgically removed human airway epithelium. These limited observations suggest that the replication of the avian viruses in humans is restricted due to inefficient viral entry and/or spread in the airway epithelium. However, as natural virus isolates were used in these experiments, one could not discriminate whether the observed differences in cell tropism and replication were determined solely by different receptor specificity of the viruses or by mechanisms that were dependent on viral genes other than the HA gene.

In this study, we wished to overcome this problem and to specify the level of receptor-dependent restriction on avian virus replication in human airway epithelium. To this end, we used reverse genetics to generate a pair of viruses that differed solely by two amino acids in the HA receptor-binding site and had either human-virus-like or avian-virus-like receptor specificity. We next compared these viruses for the efficiency of their replication in HTBE cultures.

Results and discussion

Generation of recombinant viruses with different receptor specificity

To prepare recombinant viruses with either human-virus-like or avian-virus-like receptor specificity, we used the eight-plasmid reverse genetics system described by Hoffmann et al. (2000). Initially, we generated two viruses, R1 and R2, that shared six genes of internal viral proteins from laboratory virus strain A/WSN/33 (H1N1) and harbored the HA and NA genes from the pandemic virus A/Hong Kong/1/68 (H3N2) (HK/68). R1 contained original HA of the HK/68 virus; R2 differed from R1 solely by mutations at HA codons 226 and 228 that reverted the HA amino acid sequence (226L/228S) back to the consensus sequence of the avian virus HA (226Q/228G). The choice of these mutations was made based on the known essential role of amino acids in position 226 and 228 in the receptor specificity of H3 viruses and in the emergence of human pandemic virus from an avian precursor (see Matrosovich et al., 2000 and references therein).

After rescuing recombinant viruses and confirming the specific identity of their HAs and NAs by sequencing, we tested R1 and R2 for their binding to a pair of biotinylated sialylglycopolymers, 3SL-PAA and 6SLN-PAA (Fig. 1A). Three natural viruses were included in these experiments for a comparison, namely, original HK/68 strain and two viruses used in our previous study (Matrosovich et al., 2004), A/Mallard/Alberta/119/98 (H1N1) (Dk/98) and A/Memphis/14/96-M (H1N1) (Mem/96-M). The binding pattern of R1 was identical, within experimental error, to that of the wild type HK/68, with either virus displaying a typical human-virus-like preference for Neu5Ac(α2–6)Gal-containing receptor 6SLN-PAA. Interestingly, both HK/68 and R1 were able to bind to Neu5Ac(α2–3)Gal-containing glycopolymer 3SL-

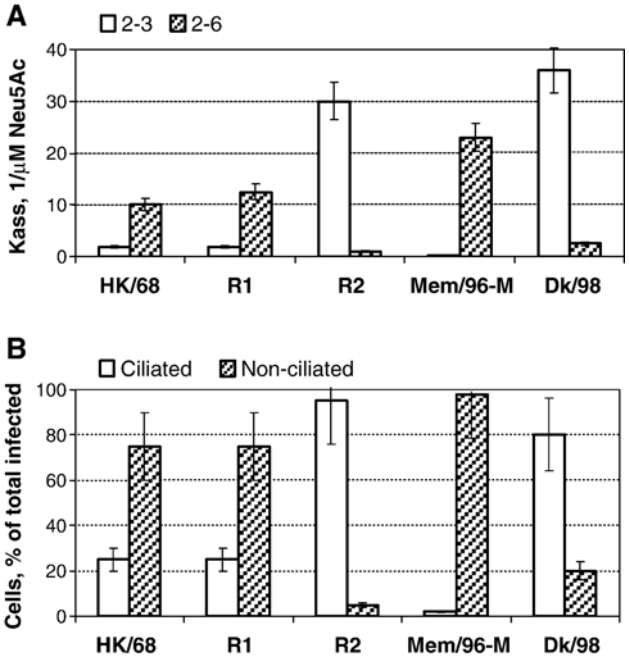

Fig. 1. Receptor specificity (A) and cell tropism (B) of influenza viruses. (A) Association constants (K_{ass}) of virus complexes with sialylglycopolymers 3SL-PAA and 6SLN-PAA (open and hatched bars, respectively). Higher K_{ass} values indicate stronger binding. (B) HTBE cultures were infected at multiplicity of infection 0.2, fixed 8 h post infection and double-immunostained for viral antigen and cilia of ciliated cells. Percentages of infected ciliated cells (open bars) and infected non-ciliated cells (hatched bars) with respect to the total amount of infected cells were determined as described in Materials and methods. The abbreviations for the virus names are explained in the text.

PAA, although substantially weaker than they are bound to the optimal receptor, 6SLN-PAA. This feature clearly distinguished either virus from recent epidemic H1N1 virus Mem/96-M that did not appreciably bind to 3SL-PAA. As expected, R2 displayed a binding pattern that was similar to that of the duck virus Dk/98. Namely, both viruses preferentially bound to 3SL-PAA and displayed a much lower affinity for the 6-linked receptor analog 6SLN-PAA. This result agreed with the presence of the avian-virus-like amino acids at the key positions 226 and 228 of the R2 HA. Thus, recombinant viruses R1 and R2 represented receptor specificity of a human pandemic virus and of an aquatic bird virus, respectively, in the otherwise identical genetic background.

Cell tropism of R1 and R2

To determine the type of cells targeted by R1 and R2 during initial entry of the viruses into human airway epithelium, we fixed HTBE cultures 8 h post infection, that is, after the first cycle of viral replication. We immunostained fixed cultures for cilia of ciliated cells and for viral antigen and determined proportion of infected ciliated and non-ciliated cells (Fig. 1B). The patterns of viral tropism to non-ciliated and ciliated cells closely mirrored the patterns of viral binding to 6SLN-PAA and 3SL-PAA (compare Figs. 1A and B). Thus, three human viruses, R1, HK/68, and Mem/96-M, which showed strong binding to 6-linked receptor, preferentially infected non-ciliated cells. R2 and Dk/98 preferentially infected ciliated cells, in a correlation with strong binding of these viruses to 3SL-PAA and much weaker binding to 6SLN-PAA.

Interestingly, R1 and HK/68 infected a higher percentage of ciliated cells than did the recent human virus Mem/96-M. This effect correlated with a higher affinity of HK/68 for 3-linked receptor and with its lower affinity for 6-linked receptor as compared to Mem/96-M. A similar phenomenon was observed by Thompson et al. (2006), who reported that human H3N2/1969 virus infected a higher proportion of ciliated cells in airway epithelial cultures than did two human H3N2 viruses from 1999 and 2000. We agree with these authors that a reduced tropism of H3N2 virus isolates from 1968 and 1969 to non-ciliated cells could reflect non-complete adaptation of these pandemic virus strains to humans.

We next compared patterns of infection by R1 and R2 24 h after viral inoculation. In the cultures infected with R1, we observed formation of continuous foci of infected cells, which included both non-ciliated and ciliated cells (Figs. 2A, C). By contrast, R2 did not form continuous foci and was predominantly found in ciliated cells (Figs. 2B, D). These results suggested that R1 spread in the cultures via infection of any encountered cell irrespective of its type, whereas R2 spread less efficiently and mainly via ciliated cells.

It has to be emphasized that neither R1 nor R2 had all-or-none preference for a specific cell type. However, the avian-like virus displayed a more restricted tropism than did the human virus (see Fig. 2), the same pattern was observed previously in

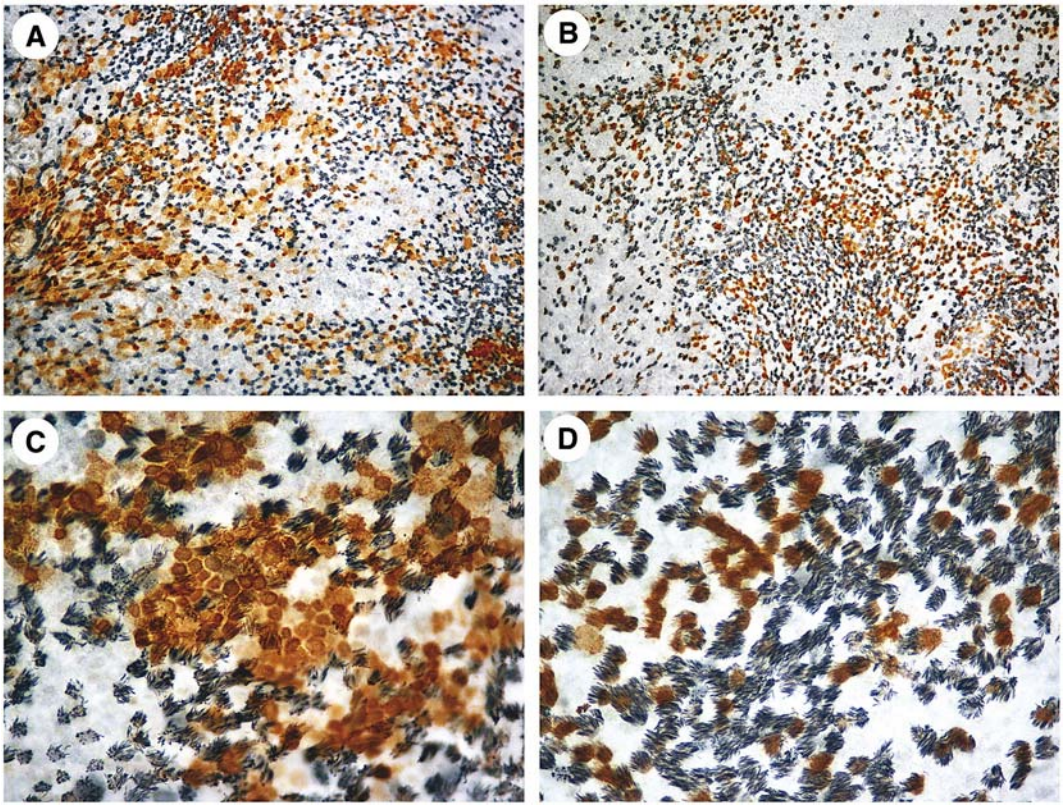

Fig. 2. Patterns of infection by recombinant viruses R1 (A, C) and R2 (B, D). HTBE cultures were infected at a multiplicity of infection 0.04, fixed 24 h post infection, and double immunostained for virus (brown) and cilia of ciliated cells (gray). Objectives, 10× (A, B) and 40× (C, D).

experiments with natural human and avian viruses (Matrosovich et al., 2004; Thompson et al., 2006). This correlation confirms that distinctions in the cell tropism of natural viruses were determined, at least in part, by the differences in the viral receptor specificity.

Replication efficiency

To compare replication of R1 and R2, we inoculated HTBE cultures with these viruses at identical multiplicity of infection (MOI) and determined yields of the viral progeny in the apical washings at different times post infection. In the first experiment, we infected cultures at MOI 0.04 and followed them up for 72 h. At 48 h, and 72 h, R2 grew to 2.2 and 4.8 lower titers, respectively, than did R1 (data not shown).

In the second experiment, we tested lower MOIs and extended observation time to 120 h. In the cultures infected with either 4000 or 400 plaque forming units of the virus (Figs. 3A, B), the yield of R1 in the apical fluid progressively increased during the whole observation period and finally reached 10^7 infectious units (IU) of the virus per 0.1 ml (about 3×10^7 IU per culture). By contrast, the titers of R2 reached a plateau of about 10^6 IU/0.1 ml at 48 to 72 h post infection (PI). As a result, the yield of R2 was about 10 times lower than that of R1 at 96 and 120 h PI. Because virus titers varied substantially in replicate cultures infected with the lowest virus dose (40 PFU/culture; MOI 0.00004), Fig. 3C shows results for individual replicates. Among four R1-infected cultures, two cultures produced less virus than the other two cultures during the first 4 days of infection; however; at 120 h PI, the yield in all four cultures was comparable (10^7 IU/0.1 ml). By this time, only two of four R2-infected cultures yielded reasonably high amounts of virus ($\sim10^6$ IU/0.1 ml) which were still lower than those in R1-infected cultures. In two other R2-infected cultures, no virus could be detected during the first 3 days of infection, and the titers were below 10^4 IU/0.1 ml at the end of the observation period. To test whether a relatively high yield of R2 in two infected cultures could be associated with selection of receptor-binding mutants, we sequenced the complete HA genes of all viruses harvested from R2-infected cultures 120 h PI. The sequences were identical to that of the original R2 HA gene ruling out our hypothesis about the selection of HA mutants. It remains obscure, whether mutations in the neuraminidase and/or other viral proteins could be responsible for a variable yield of R2 in Fig. 3C. Although replicate cultures typically contained similar average amounts of ciliated cells (as judged by immunostaining for beta-tubulin), differentiated HTBE cultures are inherently more heterogeneous than non-differentiated laboratory cell lines. It seems likely, therefore, that at very low MOI even minor variations of the cell composition and mucus secretion in replicate cultures could be amplified into significant variations in the viral yield. To corroborate the data obtained with R1 and R2, which harbored 6 internal genes from WSN/33, we generated a new pair of recombinant viruses, R1-HK and R2-HK, with all their genes derived from HK/68. R1-HK thus represented an exact recombinant replica of the human pandemic virus, whereas R2-HK modeled the putative progenitor of this virus with an avian-virus-like receptor specificity. We compared yields of R1-HK and R2-HK in HTBE cultures 72 h post infection (Table 1). In the first two experiments, R2-HK produced about 4 to 6 times less infectious progeny than did R1-HK. These results agreed with the data obtained for the R1–R2(WSN) pair of viruses (see Fig. 3). In the third experiment, we wished to model more closely conditions of the in vivo infection in the airway epithelium by preserving the integrity of the mucus blanket. To minimize disturbance and dilution of the mucus, we neither washed the cultures before virus inoculation, nor removed the inoculum after viral adsorption. Also, we reduced the size of inoculum to 20 μl and incubated infected cultures at air–liquid interface throughout the whole experiment. Under these conditions, the yield of R1-HK was less than 10% of its yield in the previous two experiments demonstrating substantial interference of the mucus blanket with the viral infection. For each of the three infection doses used, R2-HK replicated to lower titers than did R1-HK. In the case of two higher doses (1000 and 100 PFU per culture), the difference

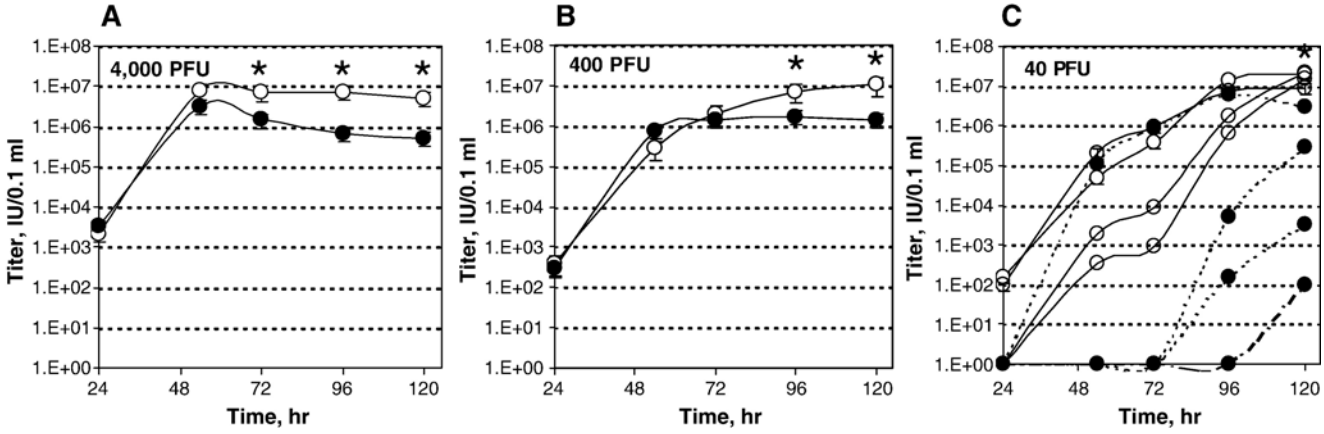

Fig. 3. Replication kinetics of R1 (open circles) and R2 (closed circles) in HTBE cultures. (A and B) Cultures were infected with 4000 PFU (MOI 0.004) and 400 PFU (MOI 0.0004) of the viruses, respectively. Each data point represents the mean ± SD from 4 cultures. (C) Cultures were infected with 40 PFU (MOI 0.00004). Data for individual replicate cultures are shown. Star next to data point indicates that the difference between titers of R1 and R2 is statistically significant ($p<0.05$).

Table 1
Yield of recombinant viruses R1-HK and R2-HK at the apical side of HTBE cultures 72 h post infection

Experiment[a]	MOI, PFU per culture	Yield per culture (SD), 10^6 IU		Ratio of yields, R1-HK/R2-HK
		R1-HK	R2-HK	
1	200	170 (46)	39 (5.4)	4.4[b]
2	2000	48 (6.4)	7.6 (2.2)	6.3[c]
	200	66 (3.6)	10.8 (3.1)	6.1[c]
3	1000	5.7 (2.5)	2.4 (0.62)	2.4[d]
	100	4.7 (1.8)	3.0 (2.7)	1.6
	10	2.5[e] (3.9)	0.0058[f] (0.0017)	430

[a]Each of the 3 experiments was performed on a different day using 4 replicate cultures for each MOI. The first two experiments were done as described in Materials and methods. Experiment No. 3 was done with the following modifications: we did not wash the cultures before infection, inoculated them with 20 μl of viral suspensions, and did not remove the inoculum.
[b,c,d]$p<0.002$ ([b]), $p<0.0001$ ([c]), and $p<0.05$ ([d]) (p-value for comparison between yields of R1-HK and R2-HK, unpaired t-test).
[e,f]The yields in individual replicate cultures were ([e]) 0.03, 0.051, 1.7, 8.2 and ([f]) 0.0044, 0.0045, 0.0063, 0.008.

between R1-HK and R2-HK was rather low. The marked distinction between these viruses was observed in the cultures infected with 10 PFU. The yield of R1-HK varied significantly in individual infected cultures (from 3×10^4 to 8×10^6 IU); however, the averaged yield was comparable to those in cultures infected at higher MOI. By contrast, although all 4 cultures inoculated with 10 PFU of R2-HK became infected, the yield of viral progeny was decreased more than 100-fold compared to cultures infected with 100 and 1000 PFU.

Taken together, these experiments demonstrate that recombinant viruses with the avian-virus-like receptor specificity replicate in human airway epithelial cultures to lower titers than do their human-virus-like counterparts. Furthermore, at low multiplicity (less than 100 PFU per culture), the avian-like viruses failed to establish efficient infection. The mechanisms of these effects remain to be elucidated. The most obvious differences between R1 and R2 in these cultures observed so far were distinctive cell tropism and spread (Figs. 1 and 2). It is possible, therefore, that R2 replicates to lower titers than does R1 because R2 cannot infect a substantial fraction of airway epithelial cells, non-ciliated cells, which are susceptible to R1. Other potential mechanisms could be a lower virus production in ciliated cells as compared to non-ciliated cells, a slower viral entry into airway epithelium (Thompson et al., 2006), or a poor release of virus progeny from infected cells.

Our results on viral replication in HTBE cultures predict that under conditions of natural human infection the virus with avian-virus-like receptor specificity will be shed in lower titers and in addition will be less contagious than the virus with human-virus-like specificity. Although the differences between R1 and R2 in yield and infectivity were not striking, they appear to be significant enough to hamper or even prevent the human-to-human transmission of the virus with the avian-like HA. Thus, our data agree with the theory that alteration of receptor specificity will facilitate the emergence of a new pandemic virus.

On the other hand, the fact that replication of R2 and R2-HK reassortant viruses was only moderately impaired in HTBE cultures argues against a popular opinion about inability of avian viruses to replicate in the human upper respiratory tract due to a lack of corresponding receptors. Clearly, receptor specificity is not the only factor responsible for the relatively rare cases of human infections with avian viruses.

Materials and methods

Wild-type and recombinant viruses

A/Hong Kong/1/68 (H3N2) (HK/68) (Brown et al., 2001) was provided by Earl Brown at University of Ottawa, Ottawa, Canada. Robert Webster at St. Jude Children's Research Hospital, Memphis, TN provided the clinical human isolate in MDCK cells A/Memphis/14/96-M (H1N1) (Mem/96-M), the duck virus A/Mallard/Alberta/119/98 (H1N1) (Dk/98), and antisera against whole H3N2 and H1N1 viruses. We prepared and aliquoted virus stocks after making one passage of the human viruses in MDCK cells and of the duck virus in embryonated hen's eggs.

Eric Hoffmann and Robert Webster at St. Jude Children's Research Hospital, Memphis, TN, kindly provided Pol I/Pol II expression vector pHW2000 (Hoffmann et al., 2000) and eight pHW2000-based plasmids for the rescue of A/WSN/33 (H1N1) virus.

We amplified each gene segment of HK/68 virus by RT-PCR from isolated RNA using a set of universal primers (Hoffmann et al., 2001) and ligated the products into the pHW2000 plasmid as described previously (Hoffmann et al., 2000, 2001). To generate amino acid substitutions L226Q and S228G in the HA, we used site-directed mutagenesis kit (QuikChange, Stratagene). Double-base mutations at either codon of the HA gene were introduced to prevent reversion of the sequence. We confirmed the identity of all plasmids by sequencing.

We generated recombinant viruses using previously described protocol of the eight plasmid reverse genetics system (Hoffmann et al., 2000). In brief, we transfected 293T cells with a mixture of eight plasmids encoding each viral gene segment with Lipofectamine 2000 (Invitrogen) as recommended by the manufacturer. Six hours later, we replaced the transfection medium with DMEM containing 0.1% BSA. After 2 days of incubation, we treated supernatants collected from transfected 293T cells with TPCK-trypsin and amplified rescued viruses in MDCK cells.

We prepared four recombinant viruses. Two of them, R1 and R2, contained the HA and NA genes from the A/Hong Kong/1/68 virus and the remaining gene segments from A/WSN/33. The other two viruses, R1-HK and R2-HK, contained all 8 genes from the HK/68 virus. For either pair of viruses, the R1 counterpart contained the original H3 HA, and the R2 counterpart contained the HA with the L226Q/S228G mutations. We aliquoted the R1 and R2 viruses directly after rescue without plaque purification. We plaque purified R1-HK and R2-HK twice in MDCK cells before preparing aliquots. The infectivity of all aliquoted viruses was characterized by plaque

titration in MDCK cells, and the specific identity of HAs and NAs was confirmed by sequencing.

Receptor-binding assay

Synthetic biotinylated sialylglycopolymers, 3SL-PAA and 6SLN-PAA (carrying the Neu5Ac(α2–3)Gal(β1–4)Glc and Neu5Ac(α2–6)Gal(β1–4)GlcNAc moieties, respectively) were kindly provided by Alexander Tuzikov and Nikolai Bovin at Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry, Moscow, Russia. We determined the binding of these polymers to the viruses using a solid-phase assay and calculated association constants of virus–polymer complexes as previously described (Matrosovich et al., 2000).

Airway epithelial cultures

Differentiated HTBE cultures were prepared as described before (Gray et al., 1996; Matrosovich et al., 2004). In brief, we purchased primary human tracheo-bronchial cells from Clonetics (Cambrex), expanded them on plastic, and stored in aliquots. We propagated these passage 1 cells on membrane supports (12-mm Transwell-Clear, Corning Inc.) at an air–liquid interface (ALI) in serum-free, hormone- and growth factor-supplemented growth medium (GM) (Gray et al., 1996). Fully differentiated 5- to 7-week-old cultures prepared from the same lot of commercial primary cells isolated from a single donor were used for all experiments.

Viral infections in HTBE cultures

We studied cell tropism and virus spread as previously described (Matrosovich et al., 2004). Briefly, we washed the cultures 10 times with the DMEM to remove accumulated mucins and incubated the apical sides of the cultures with 0.2 ml of viral dilutions in the complete growth medium. After 1 h of incubation, we removed the viruses and incubated cultures at 35° under ALI conditions for either 7 h or 23 h. We next fixed the cultures with 4% paraformaldehyde for 30 min at 4° and double-immunostained them for cilia of ciliated cells and for viral antigen using antibodies against beta-tubulin and whole viruses, respectively, and peroxidase-labeled secondary antibodies. Stained cultures were mounted and observed en face using a Nikon Optiphot-2 microscope equipped with a CCD camera. For cell counting, we used a 100× objective with oil immersion. In microscopic fields containing between 5 and 20% ciliated cells with respect to the total amount of superficial cells, each infected cell was classified as either ciliated, non-ciliated, or as undefined. Percentages of ciliated and non-ciliated infected cells with respect to the total amount of infected cells were calculated. Twenty-five to thirty-five fields were analyzed per sample, and the results were averaged.

To characterize replication kinetics of R1 and R2 (i.e., recombinant viruses with internal genes from WSN/33 virus), we infected washed cultures with 40 to 40,000 plaque-forming units of the viruses in 0.2 ml GM. We removed the inoculum following 1-h incubation, overlaid apical sides of infected cultures with 0.1 ml fresh GM, and incubated them for 72 to 120 h. We collected aliquots of the apical medium at 24 h intervals by adding 0.2 ml fresh GM to the apical compartment, incubating the cultures for 15 min, and removing 0.2 ml aliquots. We stored the aliquots at −80 ° until the end of experiment and analyzed them simultaneously for infectious virus yields by titration in MDCK cells as described below.

We compared yields of R1-HK and R2-HK 72 h post infection. In two of the three experiments performed on different days, we infected washed cultures with 0.2 ml viral suspensions, removed the inoculum 1 h later, and incubated the cultures without adding the growth medium to the apical compartment (ALI conditions). Following 72-h incubation, we added 0.2 ml fresh GM to the apical site and collected the virus material 1 h later. The third experiment differed from the first two experiments as follows. We did not wash the cultures before infection. We applied 20 μl per culture of viral suspensions and incubated the cultures at ALI for 3 days without removing the inoculum.

Virus titration in MDCK cells

MDCK cells grown in 96-well plates (Costar) were washed 3 times with minimal essential medium and inoculated with 0.1 ml of 10-fold dilutions of the viruses in DMEM supplemented with 0.1% BSA (4 replicates per dilution). Eight to sixteen hours post-inoculation, the cells were fixed with 4% paraformaldehyde for 30 min at 4°. Fixed cultures were immunostained for the expression of influenza virus nucleoprotein (NP) by incubating for 1 h with anti-NP monoclonal antibodies (kindly provided by Alexander Klimov at Centers for Disease Control, USA) followed by 1 h of incubation with peroxidase-labeled anti-mouse antibodies (Sigma) and 30 min of incubation with True Blue substrate (KPL). Ten-percent horse serum plus 0.05% Tween 80 in PBS was used for the preparation of working dilutions of immuno-reagents. Numbers of infected cells per well were counted for the virus dilution that produced from 30 to 300 infected cells per well and recalculated into numbers of infectious virus units (IU) per 0.1 ml of the original undiluted virus suspensions.

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