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In Vitro Assessment of Attachment Pattern and Replication Efficiency of H5N1 Influenza A Viruses with Altered Receptor Specificity▽

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The continuous circulation of the highly pathogenic avian influenza (HPAI) H5N1 virus has been a cause of great concern. The possibility of this virus acquiring specificity for the human influenza A virus receptor, α2,6-linked sialic acids (SA), and being able to transmit efficiently among humans is a constant threat to human health. Different studies have described amino acid substitutions in hemagglutinin (HA) of clinical HPAI H5N1 isolates or that were introduced experimentally that resulted in an increased, but not exclusive, binding of these virus strains to α2,6-linked SA. We introduced all previously described amino acid substitutions and combinations thereof into a single genetic background, influenza virus A/Indonesia/5/05 HA, and tested the receptor specificity of these 27 mutant viruses. The attachment pattern to ferret and human tissues of the upper and lower respiratory tract of viruses with α2,6-linked SA receptor preference was then determined and compared to the attachment pattern of a human influenza A virus (H3N2). At least three mutant viruses showed an attachment pattern to the human respiratory tract similar to that of the human H3N2 virus. Next, the replication efficiencies of these mutant viruses and the effects of three different neuraminidases on virus replication were determined. These data show that influenza virus A/Indonesia/5/05 potentially requires only a single amino acid substitution to acquire human receptor specificity, while at the same time remaining replication competent, thus suggesting that the pandemic threat posed by HPAI H5N1 is far from diminished.

Influenza A virus is a negative-strand RNA virus with a segmented genome within the family of *Orthomyxoviridae*. Influenza A viruses are divided into subtypes based on the surface glycoproteins hemagglutinin (HA) and neuraminidase (NA). Currently, 16 subtypes of HA and 9 subtypes of NA have been identified in the natural reservoir of all influenza A viruses, wild aquatic birds (24). Occasionally, viruses from this reservoir cross the species barrier into mammals, including humans. When animal influenza viruses are introduced in humans, the spread of the virus is generally limited but may on occasion result in sustained human-to-human transmission. Three influenza A virus subtypes originating from the wild bird reservoir—H1, H2, and H3—have formed stable lineages in humans, starting off with a pandemic and subsequently causing yearly influenza epidemics. In the 20th century, three such pandemics have occurred, in 1918 (H1N1), 1957 (H2N2), and 1968 (H3N2). In 2009, the swine-origin H1N1 virus caused the first influenza pandemic of the 21st century (23).

Efficient human-to-human transmission is a prerequisite for any influenza A virus to become pandemic. Currently, the determinants of efficient human-to-human transmission are not completely understood. However, it is believed that a switch of receptor specificity from α2,3-linked sialic acids (SA), used by avian influenza A viruses, to α2,6-linked SA, used by human influenza viruses, is essential (6, 17, 31). It has been shown that the difference in receptor use between avian and human influenza A viruses combined with the distribution of the avian and human virus receptors in the human respiratory tract results in a different localization of virus attachment (26, 33–35). Human viruses attach more abundantly to the upper respiratory tract and trachea, whereas avian viruses predominantly attach to the lower respiratory tract (5, 33–35). Theoretically, the increased presence of virus in the upper respiratory tract, due to the specificity of human influenza A viruses for α2,6-linked SA, could facilitate efficient transmission.

Since 1997, highly pathogenic avian influenza (HPAI) H5N1 virus has been circulating in Southeast Asia and has spread westward to Europe, the Middle East, and Africa, resulting in outbreaks of HPAI H5N1 virus in poultry and wild birds and sporadic human cases of infection in 15 different countries (38). The widespread, continuous circulation of the HPAI H5N1 strain has spiked fears that it may acquire specificity for α2,6-linked SA, potentially resulting in a pandemic. Given the currently high case fatality rate of HPAI H5N1 virus infection in humans of ca. 60%, the effect of such a pandemic on the human population could be devastating. In recent years, several amino acid substitutions in HA of HPAI H5N1 viruses have been described, either in virus isolates from patients or introduced experimentally, that increased the binding of the HPAI H5N1 HA to α2,6-linked SA (1, 2, 10, 14, 16, 29, 39, 40). However, none of the described substitutions conferred a full switch of receptor specificity from α2,3-linked SA to α2,6-linked SA and the substitutions were described in virus strains

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of different geographical origins. Furthermore, it is unknown whether these substitutions led to increased attachment of the virus to cells of the upper respiratory tract, the primary site of replication of human influenza A viruses.

Here, we have introduced all of the 21 previously described amino acid substitutions or combinations thereof that changed the receptor specificity of HPAI H5N1 virus strains and six additional combinations not previously described, into HA of influenza virus A/Indonesia/5/05 (IND05). Indonesia is the country that has the highest cumulative number of human cases of HPAI H5N1 virus infection (38). The receptor specificity of 27 mutant H5N1 viruses was determined and the attachment pattern of a subset of these viruses to tissues of the respiratory tract of ferret and human was determined and compared to the attachment pattern of human influenza A virus (H3N2). Subsequently, the role of NA in efficient replication of these mutant viruses was investigated. The data presented here show that receptor specificity of HA of the IND05 virus can be changed by introducing a single amino acid substitution in the receptor-binding domain, resulting in replication competent viruses that attach abundantly to the human upper respiratory tract.

MATERIALS AND METHODS

Cells. Madin-Darby canine kidney (MDCK) cells were cultured in Eagle minimal essential medium (EMEM; Lonza, Breda, Netherlands) supplemented with 10% fetal calf serum (FCS), 100 IU of penicillin/ml, 100 μg of streptomycin/ml, 2 mM glutamine, 1.5 mg of sodium bicarbonate/ml, 10 mM HEPES, and nonessential amino acids. 293T cells were cultured in Dulbecco modified Eagle medium (Lonza) supplemented with 10% FCS, 100 IU of penicillin/ml, 100 μg of streptomycin/ml, 2 mM glutamine, 1 mM sodium pyruvate, and nonessential amino acids. A549 cells were cultured in HAM F-12 medium (Lonza) supplemented with 10% FCS (HyClone), 100 IU of penicillin/ml, 100 μg of streptomycin/ml, and 2 mM glutamine.

Viruses. Seasonal influenza viruses A/Netherlands/213/03 (NL03; H3N2) and A/Netherlands/26/07 (NL07; H1N1) were isolated from human cases of influenza and subsequently passaged in MDCK cells. Influenza virus A/Indonesia/5/05 (IND05; H5N1) and A/Vietnam/1194/04 (VN04; H5N1) were isolated from human cases of HPAI virus infection and passaged once in embryonated chicken eggs and once in MDCK cells. Reassortant and mutant viruses consisting of six or seven gene segments of influenza virus A/PR/8/34 (PR8) and HA and/or NA of influenza virus A/Indonesia/5/05 were produced by using reverse genetics techniques as described previously (9). The genotypes of recombinant viruses were confirmed by sequencing prior to use.

Plasmids. HA and/or NA of influenza viruses NL03, NL07, VN04, and IND05 were amplified by reverse transcription-PCR and cloned in the BsmBI site of a modified version of plasmid pHW2000 (9). Twelve nucleotides encoding four amino acid residues (underlined) making up the multibasic cleavage site PQRER/SRRKKRG in the HA gene of influenza virus VN04 and IND05 were removed as described previously (37), so viruses containing these HA genes were no longer considered highly pathogenic. For the construction of 27 plasmids containing HA of influenza virus IND05 with one or several nucleotide substitutions, a QuikChange multi-site-directed mutagenesis kit (Qiagen, Venlo, Netherlands) was used according to instructions of the manufacturer.

For insertion of 20 amino acids in the stalk region of NA of influenza virus IND05, a 60-nucleotide fragment encoding the NA stalk of HPAI H5N1 virus A/HongKong/213/03 (CNQSIITYENNTWVNQTYVN) was amplified and inserted at nucleotide position 165 of IND05 NA. All plasmids were sequenced by using a BigDye Terminator v3.1 Cycle sequencing kit (Applied Biosystems, Nieuwerkerk a/d Ijssel, Netherlands) and a 3130XL genetic analyzer (Applied Biosystems), according to the instructions of the manufacturer. All primer sequences are available upon request.

Transfection and infection of cells. Transfection of 293T cells and production of recombinant viruses was performed by transient calcium phosphate-mediated transfection as previously described (9). The supernatant of the transfected cells was harvested 48 h after transfection and was used to inoculate MDCK cells. At 1 h after inoculation, MDCK cells were washed once with phosphate-buffered saline (PBS) and cultured in infection medium consisting of EMEM (Lonza) supplemented with 100 IU of penicillin/ml, 100 μg of streptomycin/ml, 2 mM glutamine, 1.5 mg of sodium bicarbonate/ml, 10 mM HEPES, nonessential amino acids, and 17.5 μg of trypsin (Lonza)/ml.

Modified TRBC hemagglutination assay. Modified turkey red blood cells (TRBC) were prepared with modifications as described previously (22). Briefly, all α2,3-, α2,6-, α2,8-, and α2,9-linked SA were removed from the surface of TRBC by incubating 62.5 μl of 20% TRBC in PBS with 50 mU *Vibrio cholerae* NA (VCNA; Roche, Almere, Netherlands) in 8 mM calcium chloride at 37°C for 1 h. Removal of sialic acids was confirmed by a complete loss of hemagglutination of the TRBC by control influenza A viruses. Subsequently, resialylation was performed using 0.5 mU of α2,3-(*N*)-sialyltransferase (Calbiochem, San Diego, CA) or 2 mU of α2,6-(*N*)-sialyltransferase (Japan Tobacco, Inc., Shizuoka, Japan) and 1.5 mM CMP-sialic acid (Sigma-Aldrich, Zwijndrecht, Netherlands) at 37°C in 75 μl for 2 h to produce α2,3-TRBC and α2,6-TRBC, respectively. After a washing step, the TRBC were resuspended in PBS containing 1% bovine serum albumin to a final concentration of 0.5% TRBC. Resialylation was confirmed by hemagglutination of viruses with known receptor specificity. The receptor specificity of mutant viruses was tested by performing a standard HA assay with the modified TRBC. In brief, serial 2-fold dilutions of virus in PBS were made in a 50-μl volume; 50 μl of 0.5% TRBC was added, followed by incubation for 1 h at 4°C before determining the hemagglutination titer.

Virus purification and labeling. Viruses were purified and labeled with fluorescein isothiocyanate (FITC; Sigma-Aldrich) as described previously (34). Briefly, virus stocks prepared in MDCK cells were concentrated and purified using sucrose gradients, inactivated by dialysis against 0.1% formalin, and labeled with an equal volume of 0.1 mg of FITC/ml.

Respiratory tract tissues. The paraffin-embedded human respiratory tract tissue sections were obtained from the Department of Pathology, Erasmus Medical Center. The paraffin-embedded ferret respiratory tract tissue sections were obtained from the Department of Virology, Erasmus Medical Center. All of the selected tissues were without histological lesions or evidence of respiratory tract infection. Tissues from three individuals of each species were analyzed.

Virus histochemistry on tissue sections. Virus histochemistry was performed as described previously (7, 34). Briefly, formalin-fixed paraffin-embedded tissues were deparaffinized with xylene and rehydrated with graded alcohol. FITC-labeled influenza viruses (50 to 100 hemagglutination units) were incubated with tissues overnight at 4°C. The FITC label was detected with a peroxidase-labeled rabbit anti-FITC antibody (Dako, Heverlee, Belgium), and the signal was amplified with a tyramide signal amplification system (Perkin-Elmer, Groningen, Netherlands) according to the manufacturer's instructions. Peroxidase was revealed with 3-amino-9-ethyl-carbazole (Sigma-Aldrich), and tissues were counterstained with hematoxylin and embedded in glycerol-gelatin (Merck, Darmstadt, Germany). Attachment of influenza virus to tissues was visible as granular to diffuse red staining on the apical surface of epithelial cells.

Virus titrations. Viruses were titrated by endpoint dilution in MDCK cells as described previously (9). Briefly, MDCK cells were inoculated with 10-fold serial dilutions of culture supernatants. At 1 h after inoculation, the cells were washed with PBS and supplemented with infection medium. Three days after inoculation, the supernatants of infected cell cultures were tested for agglutination activity using TRBC as an indicator of infection of the cells. Infectious titers were calculated from five replicates according to the method of Spearman-Karber.

Replication kinetics. Multistep replication kinetics were determined by inoculating MDCK or A549 cells at a multiplicity of infection (MOI) of 0.01 50% tissue culture infective dose(s) ($TCID_{50}$) per cell. One hour after inoculation, at time point zero, cells were washed once with PBS, and fresh infection media was added. Supernatants were sampled at 6, 12, 24, and 48 h after inoculation, and the virus titers in these supernatants were determined.

RESULTS

Production of mutant H5 viruses. Over the past several years, there have been several reports of HPAI H5N1 viruses with increased affinity for the human influenza A virus receptor, α2,6-linked SA, in combination with residual affinity for α2,3-linked SA (1, 2, 10, 14, 16, 29, 39, 40). These amino acid substitutions resulting in increased affinity for α2,6-linked SA were described in different HPAI H5N1 viruses and using different experimental assays. The 21 described amino acid substitutions or combinations thereof and 6 additional combi-

TABLE 1. Overview of the 27 mutant A/Indonesia/5/05 HA molecules used in this study

Amino acid substitution(s)[a]	Amino acid position based on H3 numbering	Virus rescue	Source or reference
S129V*	133	+	1
A134V	138	+	1
S133A†	137	+	40
G139R	143	+	39
N154S†	158	+	14
N182K	186	+	39
E186D†	190	+	29
T188I†	192	+	40
Q192R	196	+	39
N193K	197	+	39
K218E†	222	+	16
G221D†	225	+	29
Q222L†	226	+	29
S223N†	227	+	10, 39
G224S†	228	+	29
E186D G221D†		+[b]	29
Q192R S223N		+	39
S133A T188I†		+	40
G139R N182K		+	39
S129V* A134V		+	1
Q222L G224S†		+	29
N182K Q222L G224S		+	This study
Q192R Q222L G224S		+	This study
Q222L S223N G224S		+	This study
N182K Q222L S223N G224S		+	This study
Q192R Q222L S223N G224S		+	This study
N182K Q192R Q222L S223N G224S		+	This study

[a] The amino acid positions are based on H5 numbering. *, L129V (1). †, H3 amino acid numbering used in the indicated reference.
[b] Virus rescue positive (but the hemagglutination titer was too low for further analysis).

TABLE 2. Receptor specificity of the different viruses as determined by a modified TRBC hemagglutination assay

Group	Mutant[a]	HA titer (HAU[b]/50 μl)		
		TRBC	α2,3-TRBC	α2,6-TRBC
I	A134V	16	0	0
	E186D	16	0	0
II	VN04 (H5N1)	128	128	0
	IND05	64	64	0
	S129V	64	64	0
	S133A	64	64	0
	G139R	64	64	0
	T188I	64	64	0
	Q192R	64	64	0
	N193K	64	32	0
	K218E	16	1	0
	G221D	64	4	0
	S129V A134V	8	1	0
	S133A T188I	128	128	0
III	NL03 (H3N2)	128	0	128
	N182K	64	0	16
	Q222L	32	0	8
	S223N	64	0	2
	G224S	64	0	32
	Q192R S223N	64	0	8
	Q222L G224S	32	0	32
	N182KQ222L G224S	64	0	64
	Q192R Q222L G224S	64	0	4
	Q222L S223N G224S	64	0	2
	N182K Q222L S223N G224S	64	0	32
	Q192R Q222L S223N G224S	64	0	1
IV	N154S	32	32	1
	G139R N182K	64	1	2
	N182K Q192R Q222L S223N G224S	64	2	64

[a] All amino acid substitutions are indicated based on H5 numbering.
[b] HAU, hemagglutination units.

nations not previously described (Table 1) were introduced in the HA gene of influenza virus A/Indonesia/5/05 (IND05) from which the multibasic cleavage site was deleted, so that viruses containing these HAs were no longer highly pathogenic. Using reverse genetics, rescue of these mutant HAs in combination with seven gene segments encoding influenza virus A/PR/8/34 was attempted. All of the 27 mutant viruses could be rescued (Table 1). The virus containing IND05-$HA_{E186D,G221D}$ was excluded from further experiments because it had a low HA titer as determined by a standard HA assay and its receptor specificity could not be determined.

Receptor specificity of the mutant H5 viruses. A hemagglutination assay using modified TRBC that contained either α2,3-linked SA (α2,3-TRBC) or α2,6-linked SA (α2,6-TRBC) exclusively on their cell surface was used to determine the receptor specificity of the viruses with mutant HAs. NL03 (H3N2) and VN04 (H5N1) were used as a prototype human and avian influenza A virus, respectively, since their attachment pattern to the human respiratory tract was characterized previously (33–35). As shown in Table 2, NL03 exclusively showed attachment to α2,6-linked SA, whereas VN04 exclusively bound α2,3-linked SA. These control viruses also showed that modified TRBC contained amounts of SA that yielded equivalent HA titers to the titers obtained with regular TRBC. Next, the receptor specificity of the 26 viruses with mutant HAs was tested. Based on the results, the viruses were divided into four groups (Table 2). Group I contained viruses that agglutinated unmodified TRBC but not the modified TRBC containing either α2,3- or α2,6-linked SA. The 10 mutant viruses in group II agglutinated α2,3-TRBC but not α2,6-TRBC, despite the introduced amino acid substitutions. In group III, the mutants IND05-HA_{N182K}, IND05-HA_{Q222L}, IND05-HA_{S223N}, IND05-HA_{G224S}, IND05-$HA_{Q192R,S223N}$, and IND05-$HA_{Q222L,G224S}$ agglutinated only α2,6-TRBC and not α2,3-TRBC, despite the fact that these amino acid substitutions or combinations thereof were described to have dual receptor binding in their original background (29, 39). This prompted us to create additional combinations of mutations, resulting in six additional mutants (Table 1). Of these additional mutants, only the IND05-$HA_{N182K,Q222L,G224S}$ virus agglutinated α2,6-TRBC, as well as the single and double mutants, whereas the other combinations resulted in lower affinity for α2,6-TRBC (Table 2). In fact, the IND05-$HA_{N182K,Q192R,Q222L,S223N,G224S}$ virus agglutinated α2,6-TRBC but also retained affinity for α2,3-TRBC (Table 2), placing them in group IV together with two other viruses.

Attachment of selected H5 mutants to the ferret respiratory tract. Next, the attachment pattern of virus with IND05-HA and the IND05-HA_{N182K}, IND05-HA_{E186D}, IND05-

TABLE 3. Attachment of selected mutant virus to upper and lower respiratory tract tissues of ferret and human origin

Virus	Tissue origin	Attachment[a] to:									
		Nasal turbinates		Trachea		Bronchus		Bronchiole		Alveoli	
		Score	PCT	Score	PCT	Score	PCT	Score	PCT	Score	PCT
NL03	Ferret	++*†	Cil	++*	Cil	+*†	Cil	+/–		++	I
	Human	++*†	Cil	++*†	Cil	++*†	Cil	++	Cil	++	I
IND05	Ferret	–		–		–		+/–		++	II
	Human	–		–		–		+	Noncil	+	II
IND05-HA_{E186D}	Ferret	–		–		–		–		+	II
	Human	ND		ND		ND		ND		ND	
IND05-$HA_{G139R,N182K}$	Ferret	–*		–		–		–		++	II
	Human	ND		ND		ND		ND		ND	
IND05-HA_{N182K}	Ferret	++*†	Cil	+*	Cil	+*†	Cil	–		+/–	
	Human	++*†	Cil	+*†	Cil	++*†	Cil	+†	Cil	+	I
IND05-HA_{Q222L}	Ferret	+	Cil	–		+/–*		+	Noncil	+	II
	Human	ND		ND		ND		ND		ND	
IND05-HA_{G224S}	Ferret	+/–		–		–		–		+	II
	Human	ND		ND		ND		ND		ND	
IND05-$HA_{Q222L,G224S}$	Ferret	++*†	Cil	+/–		+/–*†		+	Noncil/Cil	+	I/II
	Human	++*†	Cil	+*†	Cil	++*†	Cil	++	Cil	++	I
IND05-$HA_{N182K,Q222L,G224S}$	Ferret	++*†	Cil	+/–*		+*	Cil	+/–		+/–	
	Human	++*†	Cil	+/–*		++*†	Cil	+	Cil	++	I

[a] The mean abundance of cells to which virus attached was scored as follows: –, no attachment; +/–, attachment to rare or few cells; +, attachment to a moderate number of cells; ++, attachment to many cells. The scores are median scores of three individual tissues. Where possible, the predominant cell type (PCT) to which the virus attached is indicated as follows: Cil, ciliated cells; Noncil, nonciliated cuboidal cell; I, type I pneumocytes; II, type II pneumocytes. ND, not determined. *, Submucosal glands positive; †, goblet cells occasionally positive.

HA_{Q222L}, IND05-HA_{G224S}, IND05-$HA_{G139R,N182K}$, IND05-$HA_{Q222L,G224S}$, and IND05-$HA_{N182K,Q222L,G224S}$ mutants to fixed tissues of the upper and lower respiratory tract, including the nasal turbinates, trachea, bronchus, bronchiole, and alveoli, was determined by using virus histochemistry. Because of the limited availability of human tissues for such studies, the initial screening was performed with ferret tissues. Ferrets are a widely accepted animal model for influenza A virus infections and attachment of avian and human influenza A viruses to the ferret lung was shown to be comparable to the attachment pattern to human respiratory tract (19, 35). The IND05-HA_{E186D} and IND05-$HA_{G139R,N182K}$ viruses were selected because of their ability to agglutinate unmodified TRBC, but not, or to a much lesser extent, α2,3- or α2,6-TRBC. Both viruses showed an attachment pattern similar to that of IND05, with some small differences (Table 3). Neither of the viruses attached to the nasal turbinates, trachea, or bronchus. In the bronchioles, IND05-HA virus attached rarely, but the IND05-HA_{E186D} and IND05-$HA_{G139R,N182K}$ viruses did not; in the alveoli all three viruses attached to type II pneumocytes.

The remaining mutants were from group III and thus agglutinated α2,6-TRBC exclusively. In order to determine which of these mutants exhibited an attachment pattern similar to that of a human influenza A virus, the attachment pattern of the mutant viruses was compared to that of the human influenza virus NL03 (H3N2) and to IND05 as a control. The IND05 virus predominantly attached to type II pneumocytes in the alveoli, whereas the NL03 virus attached abundantly to ciliated epithelial cells in the nasal turbinates, trachea, and bronchi and to type I pneumocytes in the alveoli (Table 3 and Fig. 1). The attachment pattern of the IND05-HA_{G224S} was least comparable to NL03, with rare attachment in the nasal cavity, no demonstrable attachment in trachea, bronchus, or bronchioles, and moderate attachment to type II pneumocytes in the alveoli (Table 3). The attachment pattern of the IND05-HA_{Q222L} mutant was also dissimilar from that of NL03, with moderate attachment in nasal turbinates, no demonstrable attachment in the trachea, rare attachment in the bronchus, and moderate attachment in the bronchiole to nonciliated epithelial cells and to type II pneumocytes in the alveoli (Table 3). The remaining mutants—IND05-HA_{N182K}, IND05-$HA_{Q222L,G224S}$, and IND05-$HA_{N182K,Q222L,G224S}$—all showed abundant attachment to ciliated epithelial cells in the nasal turbinates, like the human NL03 virus (Table 3 and Fig. 1), but did not show similarly abundant attachment to the trachea or type I pneumocytes in the alveoli.

Attachment of three mutant H5 viruses to the human respiratory tract. Since the IND05-HA_{N182K}, IND05-$HA_{Q222L,G224S}$, and IND05-$HA_{N182K,Q222L,G224S}$ viruses had an attachment pattern to ferret respiratory tract most similar to that of the human NL03 virus, the attachment pattern of these viruses to tissues of the human upper and lower respiratory tract, including the nasal turbinates, trachea, bronchus, bronchiole, and alveoli, was determined. Like the human NL03 virus, all three mutant viruses showed abundant attachment to

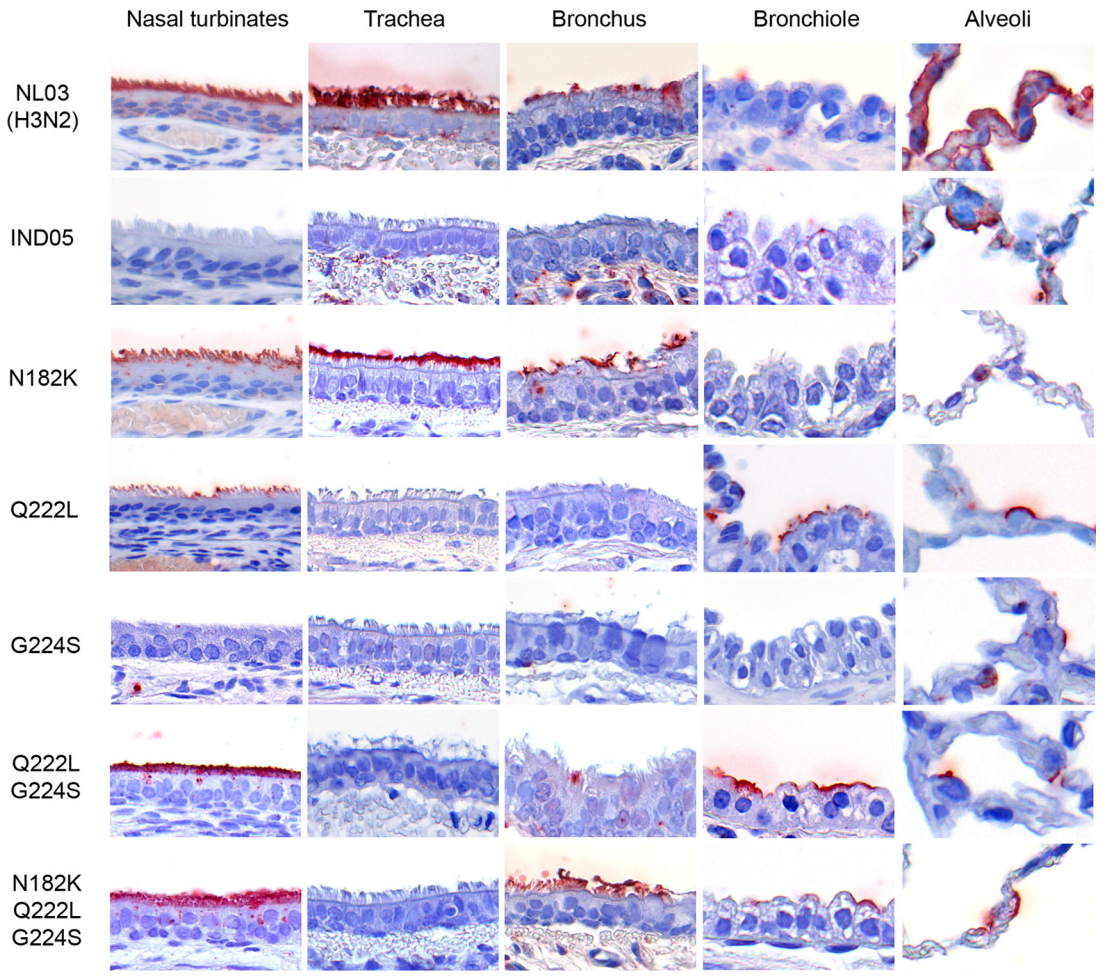

FIG. 1. Attachment of viruses with altered receptor specificity to tissues of the ferret upper and lower respiratory tracts. The attachment pattern of viruses NL03 (H3N2) and IND05 (H5N1) and the five mutant viruses IND05-HA_{N182K}, IND05-HA_{Q222L}, IND05-HA_{G224S}, IND05-$HA_{Q222L,G224S}$, and IND05-$HA_{N182K,Q222L,G224S}$ to ferret nasal turbinates, trachea, bronchus, bronchiole, and alveoli was determined. Virus attachment is shown in red. The panels were chosen to reflect the attachment pattern in the whole tissue section as much as possible, but small differences between the single panels and overall view may exist.

the ciliated epithelial cells of the nasal turbinates and bronchus, but attachment to the trachea and lower respiratory tract differed (Table 3 and Fig. 2). The mutant viruses attached less abundantly than the NL03 virus to the ciliated epithelial cells of the trachea, with attachment of the IND05-$HA_{N182K,Q222L,G224S}$ mutant being only rare. In the bronchiole, the IND05-$HA_{Q222L,G224S}$ mutant attached as abundantly as the NL03 virus, but attachment of the other two mutants to this tissue was less abundant. In the alveoli, all three mutant viruses attached to type I pneumocytes, a finding in agreement with attachment of the NL03 virus and in contrast with the attachment of the IND05 virus to type II pneumocytes (Table 3 and Fig. 2).

H5 viruses with altered receptor specificity are replication competent. Besides the receptor specificity and attachment pattern of the mutant H5 viruses, which showed similarity to that of a human influenza A virus, we determined the replication efficiency of these viruses, since efficient replication is a prerequisite for the emergence of a mutant strain. The functionality of the mutant HAs was investigated by determining the replication kinetics of viruses consisting of IND05-HA, IND05-HA_{N182K}, IND05-$HA_{Q222L,G224S}$, or IND05-$HA_{N182K,Q222L,G224S}$ and seven PR8 gene segments in MDCK and A549 cells. Although all viruses replicated to low titers in A549 cells, the three viruses with the mutant HAs replicated to virus titers similar or slightly higher than those of the virus with IND05-HA in both MDCK and A549 cells (Fig. 3A and E), indicating that the amino acid substitutions in HA did not have a negative effect on replication kinetics *in vitro*.

Effect of NA on replication efficiency of mutant H5. The receptor binding properties of HA have to be balanced with the NA activity of NA (18, 36). Since the viruses with IND05-HA_{N182K}, IND05-$HA_{Q222L,G224S}$, and IND05-$HA_{N182K,Q222L,G224S}$ substitutions had an altered receptor specificity, this raised the question whether viruses with these HA molecules could still replicate efficiently in combination with IND05-NA. Thus, recombinant viruses consisting of six gene segments of influenza virus PR8, IND05-NA, and one of IND05-HA, IND05-HA_{N182K},

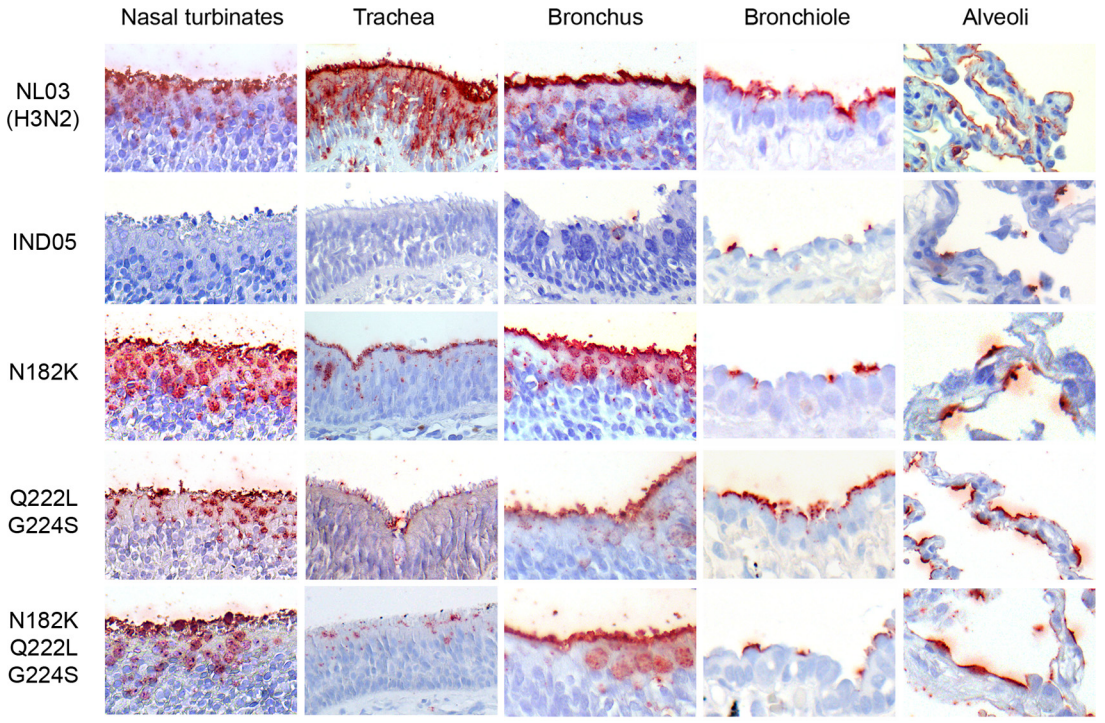

FIG. 2. Attachment of viruses with altered receptor specificity to tissues of the human upper and lower respiratory tract. The attachment pattern of viruses NL03 (H3N2) and IND05 (H5N1) and the three mutant viruses IND05-HA_{N182K}, IND05-$HA_{Q222L,G224S}$, and IND05-$HA_{N182K,Q222L,G224S}$ to human nasal turbinates, trachea, bronchus, bronchiole, and alveoli was determined. Virus attachment is shown in red. The panels were chosen to reflect the attachment pattern in the whole tissue section as much as possible, but small differences between the single panels and overall view may exist.

IND05-$HA_{Q222L,G224S}$, or IND05-$HA_{N182K,Q222L,G224S}$ were produced. The virus with IND05-$HA_{N182K,Q222L,G224S}$ and IND05-NA could not be rescued using a standard protocol. Upon addition of exogenous VCNA to the supernatant of transfected 293T cells and inoculated MDCK cells, virus was rescued with a low hemagglutination titer. However, in each of three attempts, the rescued virus contained either a K153E substitution or a K153 deletion in HA. Because of this mutation, replication efficiency of this virus was not determined. Of note, when these virus stocks were tested in a HA assay with modified TRBC, it was shown that the substitution/deletion at position 153 of HA resulted in decreased receptor binding but not in a shift in receptor specificity from α2,6-linked SA to α2,3-linked SA.

The replication efficiency of viruses with the IND05-HA_{N182K} or IND05-$HA_{Q222L,G224S}$ and IND05-NA was determined in MDCK cells and compared to that of a virus with IND05-HA and IND05-NA. As shown in Fig. 3B, the IND05-HA_{N182K} or IND05-$HA_{Q222L,G224S}$ viruses replicated to a more than 100-fold lower titer with IND05-NA than with PR8-NA. However, this was also the case with IND05-HA and is thus probably not related to the receptor specificity of HA.

Upon replication of avian influenza viruses in poultry, a deletion can occur in the stalk region of NA, as has been the case with HPAI H5N1 virus (3). Hypothesizing that this adaptation to replication in poultry may hamper efficient replication in mammalian cells, especially in combination with a HA with α2,6-linked SA specificity, the stalk fragment of IND05-NA was replaced by that of A/HongKong/213/03 NA, resulting in IND05-NA_{stalk}. The effect of this insertion in NA on the replication efficiency of viruses with IND05-HA, IND05-HA_{N182K}, IND05-$HA_{Q222L,G224S}$, or IND05-$HA_{N182K,Q222L,G224S}$ was determined in MDCK cells. The insertion of the stalk in IND05-NA did not have an effect on the replication of virus with IND05-HA or on IND05-HA_{N182K} (Fig. 3C). However, the IND05-$HA_{Q222L,G224S}NA_{stalk}$ mutant replicated to a 10-fold-higher virus titer than the IND05-$HA_{Q222L,G224S}$NA, and the IND05-$HA_{N182K,Q222L,G224S}NA_{stalk}$ mutant could be rescued efficiently using a standard protocol, indicating that the lack of virus production and generation of mutations in HA was a result of incompatibility of the $HA_{N182K,Q222L,G224S}$ variant with IND05 NA.

Next, the mutant HAs were combined with N1 of influenza virus A/NL/26/07 (NL07-NA), a recent seasonal H1N1 isolate. The virus with IND05-HA and NL07-NA replicated to slightly higher titers than IND05-HANA (Fig. 3D). All viruses with mutant HA also replicated to higher titers with NL07-NA than with IND05-NA, with the smallest effect seen in combination with IND05-HA_{N182K} (~6-fold increase) and the largest effect, an ~50-fold increase in virus titer, with IND05-$HA_{Q222L,G224S}$ (Fig. 3D).

When these experiments were repeated in A549 cells, the effect of changing NA on the replication efficiency of the viruses was not as pronounced. Only the virus with IND05-HA_{N182K} and NL07-NA replicated to a 10-fold-higher virus titer than the same virus with IND05-NA (Fig. 3F to H).

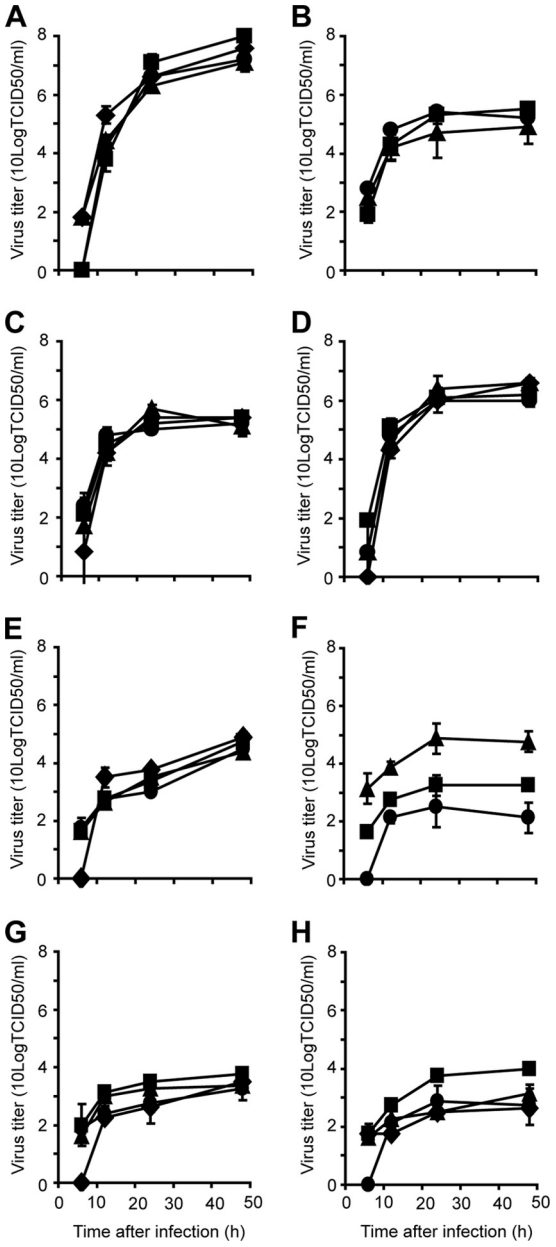

FIG. 3. Replication kinetics of viruses with IND05-HA with altered receptor specificity and different NA genes in MDCK (A to D) and A549 (E to H) cells. Cells were inoculated at an MOI of 0.01 $TCID_{50}$ of virus/cell with the IND05-HA (●), IND05-HA_{N182K} (■), IND05-$HA_{Q222L,G224S}$ (▲), or IND05-$HA_{N182K,Q222L,G224S}$ (◆) in combination with NA of influenza virus PR8 (A and E), NA of influenza virus IND05 (B and F), NA of influenza virus IND05 with a stalk insertion (NA_{stalk}) (C and G), or NA of influenza virus NL07 (H1N1) (D and H). Remaining gene segments originated from influenza virus PR8. Supernatants were harvested at 6, 12, 24, and 48 h after inoculation and titrated by endpoint dilution in MDCK cells. Geometric mean titers were calculated from two independent experiments; error bars indicate the standard deviation.

DISCUSSION

Although the current H1N1 pandemic may have distracted the attention from the continuing circulation of HPAI H5N1 virus in large parts of the world, its pandemic threat has not decreased. We show here that amino acid substitutions in HA of the H5N1 IND05 virus may increase its pandemic potential. Not only do these substitutions change the receptor specificity of these viruses to α2,6-linked SA, these viruses also have an attachment pattern in the human respiratory tract that resembles that of human influenza A viruses.

Despite reports of isolated clusters of human-to-human transmission (32), the HPAI H5N1 virus thus far has not been capable of efficient human-to-human transmission, an absolute requirement for an influenza A virus to become pandemic. It is generally accepted that a switch in receptor specificity from α2,3-linked SA to α2,6-linked SA and a resultant shift to replication in the upper respiratory tract is necessary for any respiratory transmission of influenza A viruses to occur (31). The HPAI H5N1 virus has continuously circulated in poultry in Indonesia since 2004 and has caused 163 human cases there since 2005, identifying Indonesia as a hot spot for the human-animal interface of the HPAI H5N1 virus, together with Egypt, where the HPAI H5N1 virus has also caused a relatively large number of human cases (38). When comparing the effect of the amino acid substitutions in IND05-HA on the α2,3-/α2,6-linked SA binding specificities as determined in the present study to those observed in the original studies, it is clear that the substitutions in the IND05 background result in a much more pronounced effect or the absence thereof (Table 2). The original studies described residual α2,3-linked SA binding in combination with the newly acquired α2,6-linked SA binding, whereas only 3 of 27 IND05-HA mutants showed this dual receptor specificity. Of the remaining mutants, 10 bound α2,3-linked SA exclusively and 11 resulted in exclusive α2,6-linked SA binding. For two substitutions, A134V and E186D, we could not detect binding to either α2,3-linked SA or α2,6-linked SA. This could be due to the fact that these HAs have a very low receptor affinity or to the fact that VCNA is able to remove all α2,3- and α2,6-linked SA from the TRBC, but not all conformations of SA can be restored by the sialyltransferases used to resialylate these TRBC. Our results suggest that IND05-like viruses are more predisposed to acquiring solely α2,6-linked SA receptor preference. With several mutant viruses, large differences in receptor specificity were observed between the original and the present study. The IND05-$HA_{S129V,A134V}$, IND05-HA_{Q192R}, and IND05-HA_{G139R} viruses showed less α2,6-linked SA binding in the IND05-HA background, whereas the IND05-HA_{N182K}, IND05-HA_{Q222L}, IND05-HA_{G224S}, and IND05-$HA_{Q222L,G224S}$ viruses bound more efficiently to α2,6-linked SA than the same amino acid substitutions in their original background. This seems to be due to the genetic context in which these substitutions were introduced (IND05-HA) rather than the assay used to determine receptor specificity, since both less- and more-abundant α2,6-linked SA binding were observed here with amino acid substitutions originating from the same study. For instance, the N182K substitution described by Yamada et al. resulted in limited α2,6-linked SA binding in the original study, but abundant α2,6-linked SA binding in the IND05-HA background, whereas the Q192R substitution described by the same authors resulted in α2,6-linked SA binding in the original study but did not lead to a change in receptor preference in the IND05-HA background (39).

Although the modified TRBC assay provides a simple and easy-to-interpret test to quickly determine the receptor speci-

ficity of a certain virus isolate or laboratory-created mutant, it does not distinguish between different glycan topologies that may be important for attachment to and replication in the upper respiratory tract (5, 27). Therefore, we used virus histochemistry to determine the attachment pattern to upper and lower respiratory tract tissues of the mutant H5 viruses with a preference for α2,6-linked SA. In contrast to glycan arrays, virus histochemistry is a qualitative assay in which it is very difficult to quantify the observed attachment. On top of that, it only shows the type of cells in the respiratory tract to which a virus attaches, but not through which exact receptor. However, an advantage of virus histochemistry over glycan arrays is that it shows biologically relevant attachment, whereas the localization of glycans used in the arrays in the human respiratory tract is currently unknown. Three mutant viruses IND05-HA_{N182K}, IND05-$HA_{Q222L,G224S}$, and IND05-$HA_{N182K,Q222L,G224S}$ showed an attachment pattern to the human upper and lower respiratory tract that resembled that of human influenza viruses rather than avian viruses (Fig. 2). Nevertheless, small differences were observed between these mutants and human influenza A viruses. For instance, the three mutant viruses showed less-abundant attachment to the trachea than the H3N2 virus, albeit more abundant than the IND05 virus. Why the attachment to the trachea was less abundant despite the fact that attachment to the nasal turbinates was in the same range as that of H3N2 is unclear. One possible explanation is that the mutant viruses have a preference for a slightly different glycan topology than the H3N2 virus and the distribution of the different glycan topologies expressed in the nasal turbinates and trachea is not identical. Because of these small differences in attachment pattern, it is impossible to predict which, if any, of the three receptor binding mutants is most likely to transmit efficiently via contact, aerosols, or respiratory droplets. Testing these viruses in an animal model for transmission is required to demonstrate whether the changes in receptor specificity observed in the modified TRBC and virus histochemistry assay are sufficient for efficient human-to-human transmission.

Increased virus titers were detected in MDCK cells when the viruses with a preference for the human receptor were combined with NA originating from a human influenza A virus (Fig. 3). Although this effect was seen with only one of three mutants in A549 cells, this may be due to the fact that virus replication in A549 cells was very inefficient. The results in MDCK cells suggest that, due to the need for a balance between receptor binding of HA and sialic acid removal by NA (18, 36), amino acid substitutions or reassortment of NA will likely enhance the ability of the HPAI H5N1 virus to replicate efficiently in humans. This fits with observations that the substrate specificity of NA of the pandemic H2N2 1957 virus acquired increased affinity for α2,6-linked SA over time through amino acid substitutions (4, 15).

Likewise, several adaptations in the internal genes of avian influenza viruses to replication in humans have been described (reviewed in reference 8). The majority of these substitutions are in the influenza virus polymerase complex, indicating its importance in adaptation to a new host species. One of the well-described substitutions is the E627K substitution in PB2, which has been described as a host range determinant and major pathogenicity factor (12, 20, 21, 25, 30). Moreover, the aerosol transmission efficiency of a human H3N2 influenza A virus was directly related to the presence of a 627K or 701N residue in PB2 (28), and contact transmission of A/duck/Guangxi/35/01 (H5N1) was dependent on a combination of the ability of HA to bind α2,6-linked SA and the presence of a 701N residue in PB2 (11).

A likely effect of the change in receptor specificity from α2,3- to α2,6-linked SA is changing the site of primary virus replication of H5N1 viruses from the lower to the upper respiratory tract. This could result in a less pathogenic virus, with severe pneumonia being a less frequent complication as observed in seasonal influenza A virus cases. Indeed, a HPAI H5N1 virus with increased affinity for α2,6-linked SA showed a reduced pathogenicity and limited systemic spread in a mouse model (41). These results may, however, have been affected by the virtual absence of α2,6-linked SA from the mouse lung (13) and reduced binding of human influenza viruses to mouse alveolar epithelium compared to avian influenza viruses (35). Thus, it would be of interest to determine the effect of the substitutions in HA described here on the pathogenicity of the HPAI H5N1 virus in a ferret model, since it has been shown that ferrets have one of the most similar attachment patterns to humans with respect to both avian and human influenza A viruses (35).

The *in vitro* data described here have to be confirmed by *in vivo* experiments using viruses with HA with the basic cleavage site and the internal genes of A/Indonesia/5/05. Of note, such viruses with the N182K, Q222L G224S, or N182K Q222L G224S substitutions in HA showed sole α2,6-linked SA receptor binding preference in the modified TRBC assay (data not shown). However, the results presented here suggest that one or a few amino acid substitutions in HA, possibly in combination with changes in NA, could result in a virus capable of efficient replication in humans and potentially sustained human-to-human transmission, thereby stressing the need for continuous surveillance of circulating HPAI H5N1 viruses.

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