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Comparative Study of Influenza Virus Replication in MDCK Cells and in Primary Cells Derived from Adenoids and Airway Epithelium

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Although clinical trials with human subjects are essential for determination of safety, infectivity, and immunogenicity, it is desirable to know in advance the infectiousness of potential candidate live attenuated influenza vaccine strains for human use. We compared the replication kinetics of wild-type and live attenuated influenza viruses, including H1N1, H3N2, H9N2, and B strains, in Madin-Darby canine kidney (MDCK) cells, primary epithelial cells derived from human adenoids, and human bronchial epithelium (NHBE cells). Our data showed that despite the fact that all tissue culture models lack a functional adaptive immune system, differentiated cultures of human epithelium exhibited the greatest restriction for all H1N1, H3N2, and B vaccine viruses studied among three cell types tested and the best correlation with their levels of attenuation seen in clinical trials with humans. In contrast, the data obtained with MDCK cells were the least predictive of restricted viral replication of live attenuated vaccine viruses in humans. We were able to detect a statistically significant difference between the replication abilities of the U.S. (A/Ann Arbor/6/60) and Russian (A/Leningrad/134/17/57) cold-adapted vaccine donor strains in NHBE cultures. Since live attenuated pandemic influenza vaccines may potentially express a hemagglutinin and neuraminidase from a non-human influenza virus, we assessed which of the three cell cultures could be used to optimally evaluate the infectivity and cellular tropism of viruses derived from different hosts. Among the three cell types tested, NHBE cultures most adequately reflected the infectivity and cellular tropism of influenza virus strains with different receptor specificities. NHBE cultures could be considered for use as a screening step for evaluating the restricted replication of influenza vaccine candidates.

Influenza A and B viruses infect 5 to 15% of the global population annually and cause an estimated 250,000 to 500,000 deaths (35, 54). Outbreaks and epidemics of influenza virus regularly cause excess mortality among the elderly and considerable morbidity in all ages during the influenza season (32, 35). Vaccination is the most effective way of preventing disease caused by influenza viruses. Since influenza A and B viruses undergo continuous antigenic change, the influenza vaccine components often need to be updated annually to antigenically match the circulating strains. The two influenza vaccines currently licensed in the United States are the inactivated trivalent influenza vaccine, given by intramuscular injection, and the live attenuated influenza vaccine, administered intranasally (30, 35). It is recognized that live attenuated influenza virus vaccines are more efficacious than inactivated vaccines in young children (1–3, 5, 8, 38) and that both vaccines could afford protection with differing efficacy against drifted strains in adults (4, 8, 27, 33, 36).

Live attenuated influenza virus vaccine contains hemagglutinin (HA) and neuraminidase (NA) gene segments from the three currently circulating influenza strains (H1N1, H3N2, and B) and the six internal protein gene segments (PB1, PB2, PA, NP, M, and NS) from master donor A and B viruses (21, 30). Donor strains were independently developed by sequential passages at lower temperature (25°C) in the United States and the former Soviet Union from virulent H2N2 and B isolates (A/Ann Arbor/6/60 and B/Ann Arbor/1/66, respectively, in the United States and A/Leningrad/134/57 and B/USSR/60/69, respectively, in the former So-

viet Union) (20, 51). Two influenza A virus donor strains were prepared in Russia: A/Leningrad/134/17/57 (H2N2), the “17× passaged” variant of the master strain, for use in adults and A/Leningrad/134/47/57 (H2N2), the “47× passaged” variant of the same parent (which received an extra 30 passages at low temperatures), for use in children (15, 20). Both influenza A and B donor viruses are cold adapted (*ca*) (replicate efficiently at 25°C and 33°C), temperature sensitive (*ts*) (do not replicate at temperatures above 39°C), and attenuated (*att*) (do not produce classic influenza-like illness and are restricted in replication in the lower respiratory tracts of ferrets) (22, 30). These specific phenotypes, mediated by mutations in the internal gene segments (15, 17, 20, 30), lead to limited replication in the respiratory tract of the infected host and stimulate both systemic and cellular immune responses (30, 42, 54). The *ca* U.S. and Russian master donor strains have not been directly compared for infectivity, immunogenicity, and safety in clinical trials with humans.

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As live attenuated influenza vaccines replicate in the nasopharynx of the recipient, infectious vaccine virus can be cultured from upper respiratory tract secretions after vaccination, a phenomenon termed “virus shedding.” Previous studies have estimated the median human infectious dose required for infection with live attenuated seasonal influenza vaccine to be 2.5 to 4.5 log₁₀ 50% tissue culture infective doses (TCID₅₀) in seronegative children and 5.0 to 6.4 log₁₀ TCID₅₀ in seronegative adults (12, 31, 42, 49). There is a direct correlation between the magnitude of shedding of influenza virus and the illness experienced by the host (30). Therefore, for reasons of safety, infectivity, and immunogenicity, it is desirable to know in advance the levels of replication of potential candidate live attenuated vaccine strains for human use.

In addition to yearly outbreaks and epidemics, influenza A viruses cause periodic pandemics, in which viruses containing novel HA and/or NA are introduced into susceptible human populations (54). In preparation for the next influenza pandemic, a number of strategies to develop pandemic vaccines are under way, including the use of live attenuated vaccines. Unfortunately, it is hard to predict the levels of replication in humans of candidate vaccines bearing HA influenza virus subtypes with pandemic potential (H2, H5, H7, and H9 HA subtypes) before performing human clinical trials (45). The replication of such attenuated vaccine strains in mice and ferrets is not predictive of replication of these viruses in humans. For example, H5N1 and H9N2 *ca* vaccine strains replicated minimally in humans but were readily recovered by culture in small-animal models (9, 18, 19, 47). The reasons for this discrepancy are not completely understood, but it may be related to (i) preexisting antibodies to HAs and/or NAs in human serum that cross-react with the avian HAs and/or NAs and decrease virus vaccine replication, (ii) cellular immunity, or (iii) decreased affinity of the avian HAs for sialic acid (SA) receptors in the human upper airways (44). Human influenza virus HAs preferentially bind to cell surface receptors terminating in SA α 2,6-galactose (SA α 2,6Gal), whereas avian influenza viruses preferentially bind to receptors terminating in SA α 2,3Gal (39, 44). Thus, new screening tools or models that predict the infectivity of *ca* influenza viruses in the human host need to be developed.

In the present study, we compared the replication kinetics of wild-type (*wt*) and *ca* influenza viruses, including H1N1, H3N2, H9N2, and B strains, in Madin-Darby canine kidney (MDCK) cells and human epithelial cells derived from adenoids (HAEC cells) and bronchial epithelium (NHBE cells). We also compared the replication abilities of the U.S. (*ca* A/Ann Arbor/6/60 [H2N2]) and three Russian (*ca* A/Leningrad/134/17/57 [H2N2], *ca* A/Leningrad/134/47/57 [H2N2], and *ca* A/Leningrad/134/80/57 [H2N2]) vaccine donor strains side by side in MDCK and NHBE cells. Since live attenuated influenza vaccines could potentially bear HA and NA genes of different origin (human, avian, swine, or equine), we also assessed which of the three cell cultures could optimally evaluate the infectivity and cellular tropism of influenza viruses from different hosts and with different receptor specificities.

MATERIALS AND METHODS

Cells. MDCK cells were obtained from the American Type Culture Collection (Manassas, VA) and were maintained as described elsewhere (16). Primary NHBE cells from human tracheal/bronchial tissues were obtained from Lonza (Walkersville, MD). Cells of passage 2 were grown on membrane supports (6.5-mm Transwell; Corning Inc., Corning, NY) at

the air-liquid interface in serum-free and hormone- and growth factor-supplemented medium as described previously (16, 25). Fully differentiated 4- to 8-week-old cultures were used for all experiments.

Adenoids were obtained at the time of adenoidectomy performed for independently defined clinical indications under a protocol approved by the Vanderbilt Institutional Review Board (Nashville, TN). The isolation and growth of primary epithelial cells from adenoidal tissue (HAEC cells) were previously described (13, 53). Briefly, optimal recovery of viable epithelial cells was obtained by placing the whole tissue in minimal essential medium with 0.1% pronase type 14 (Sigma Chemicals, St. Louis, MO) and antibiotics and rocking overnight at 4°C. The superficial layers of cells were further dispersed by pipetting, and cells were placed in medium containing 10% fetal calf serum to inactivate the pronase. The cells were then centrifuged, resuspended in 50% Ham's F-12 medium (Mediatech Inc., Manassas, VA)–50% Eagle's minimal essential medium with supplements (insulin, 5 μ g/ml; transferrin, 5 μ g/ml; epidermal growth factor, 10 ng/ml; cholera toxin, 10 ng/ml; hydrocortisone, 10⁻⁶ M; bovine hypothalamic extract, 15 μ g/ml; HEPES buffer, 0.015 M; retinol, 10⁻⁷ M; gentamicin, 50 μ g/ml; penicillin G, 15 U/ml; streptomycin, 15 U/ml; and fetal calf serum, 0.5%) and seeded on 24-well tissue culture plates coated with a collagen matrix of Vitrogen 100 (Cohesion, Palo Alto, CA). The cells were incubated at 37°C under 5% CO₂ until they reached 90% confluence.

Virus isolates. The *wt* and *ca* A/California/10/78 (H1N1), A/Alaska/6/77 (H3N2), A/Washington/897/80 (H3N2), and *ca* A/Ann Arbor/6/60 (H2N2) influenza viruses were kindly provided by Kanta Subbarao at the National Institute of Allergy and Infectious Diseases, Bethesda, MD. The *wt* and *ca* A/New Caledonia/20/99 (H1N1), A/Panama/2007/99 (H3N2), A/Wyoming/03/03 (H3N2), B/Hong Kong/330/01, and *ca* A/chicken/HK/G9/97 (H9N2) influenza viruses were obtained from the Influenza Division of the Centers for Disease Control and Prevention, Atlanta, GA. The *wt* A/Leningrad/134/57 (H2N2), *ca* A/Leningrad/134/17/57 (H2N2), *ca* A/Leningrad/134/47/57 (H2N2), and *ca* A/Leningrad/134/80/57 (H2N2) viruses were obtained from the Institute for Experimental Medicine, Russian Academy of Medical Science, St. Petersburg, Russia. Human (A/Tottori/849/94 AL3 [H3N2], A/Tottori/849/94 K4 [H3N2], A/Tottori/872/94 AL3 [H3N2]), avian (A/duck/Ukraine/1/63 [H3N8], A/duck/Hokkaido/8/80 [H3N8]), equine (A/equine/TN/5/86 [H3N8]), and swine (A/swine/Italy/635/87 [H3N2]) strains were kindly provided by Yoshihiro Kawaoka at the University of Wisconsin, Madison, WI. Stock viruses were prepared by one passage in the allantoic cavities of 10-day-old embryonated chicken eggs for 48 h at 37°C (or at 33°C for *ca* and B viruses), and aliquots were stored at -70°C until used. All experimental work was performed in a biosafety level 2 laboratory approved for use with these strains by the U.S. Department of Agriculture and the U.S. Centers for Disease Control and Prevention.

Infectivities of influenza viruses. The infectivities of H1N1, H2N2, H3N2, and H3N8 influenza A and influenza B viruses were determined as PFU/ml in MDCK cells. All the viruses were titrated in MDCK cells due to inability to plaque in other cell culture models despite the fact that the use of MDCK cells for determining infectious titers could be regarded as a potential confounding factor of this study. Briefly, confluent MDCK cells were incubated at 37°C (or at 33°C for *wt* and *ca* reassortant viruses) for 1 h with 10-fold serial dilutions of virus. The cells were then washed and overlaid with minimal essential medium containing 1 μ g/ml L-(tosyl-amido-2-phenyl)ethylchloromethylketone (TPCK)-treated trypsin, 0.3% bovine serum albumin (BSA), and 0.9% Bacto agar. After 3 days of incubation at 37°C (or 33°C), cells were stained with 0.1% crystal violet in 10% formaldehyde solution, and the PFU per milliliter were determined.

The infectivity of *ca* A/chicken/HK/G9/97 (H9N2) virus was defined as log₁₀ 50% tissue culture infective dose (TCID₅₀) as described previously (16), because this H9N2 virus did not produce plaques in MDCK cells. Briefly, confluent monolayers of MDCK cultures growing in 96-well plates were inoculated with serial virus dilutions (each dilution was added to five wells) in the presence of 1 μ g/ml TPCK-treated trypsin. After 3 days, virus was titrated by hemagglutination assay, and virus titers were

expressed as $\log_{10} \text{TCID}_{50}/\text{ml}$ by the endpoint method of Reed and Muench (40).

Replication kinetics. To determine multistep growth curves, HAEC and MDCK cells were infected with viruses at an identical multiplicity of infection (MOI) of 0.01 PFU/cell at 33°C. After 1 h of incubation, the cells were washed and overlaid with infection medium (minimal essential medium with 0.3% BSA); 1 $\mu\text{g}/\text{ml}$ TPCK-treated trypsin was added only in MDCK cells, because HAEC cells support the growth of influenza viruses without exogenous trypsin (13). Supernatants were collected at 1, 24, 48, and 72 h postinfection and stored at -70°C for titration by plaque assay.

To determine multistep growth curves in NHBE cells, triplicate cell cultures growing in 6.5-mm-diameter inserts were washed extensively with sterile phosphate-buffered saline (PBS) to remove mucus secretions on the apical surface prior to infection and then were inoculated via the apical side with each influenza virus at an MOI of 0.01 at 33°C. After 1 h of incubation, the inoculum was removed. Viruses released into the apical compartment of NHBE cells were harvested at the indicated time points by the apical addition and collection of 300 μl of medium allowed to equilibrate for 30 min at 33°C. The virus titers were determined as \log_{10} PFU/ml in MDCK cells.

The area under the curve (AUC) viral load was defined as the area under the multistep growth curve and calculated by the trapezoidal rule, using exact viral titers at 24, 48, and 72 h postinfection as determined by plaque assay in MDCK cells.

Immunostaining and light microscopy. MDCK and NHBE cells were infected with H3 human, avian, swine, and equine viruses at an MOI of 0.01 and fixed for 30 min in 4% formaldehyde at 8 and 24 h postinfection. Fixed cultures were permeabilized with 0.5% Triton X-100, blocked with 3% BSA, and stained with mouse anti-NP IgG diluted in 3% BSA in PBS. After a 1-h incubation, the cells were then incubated with goat horseradish peroxidase (HRP)-labeled anti-mouse IgG (Sigma-Aldrich, St. Louis, MO). For localization of ciliated cells, fixed NHBE cells were costained with anti- β -tubulin IV antibody and HRP-labeled secondary antibody for detection. The cultures were mounted using Crystal Mount (Biomedica, Foster City, CA).

For cell counting, the cultures were observed *en face* by using a Nikon microscope at 40 \times and 100 \times objectives. In microscopic fields, the percentage of infected cells with respect to the total number of cells was calculated. In NHBE cells, the percentage of ciliated infected cells with respect to the total number of infected cells was also calculated. For each sample, 20 fields were analyzed and the results were averaged.

Receptor-binding assay. The binding of H3 influenza viruses to fetuin (containing $\alpha 2,3$ - and $\alpha 2,6$ -linked sialyl receptors) was measured in a direct solid-phase assay using the immobilized virus and horseradish peroxidase-conjugated fetuin, as described previously (14). The affinity of viruses for synthetic 3'- and 6'-sialylglycopolymers obtained by conjugation of a 1-*N*-glycyl derivative of 3'- or 6'-sialyllactose (3'SL or 6'SL) or a 3-aminopropylglycoside of 3'- or 6'-sialyllactosamine (3'SLN or 6'SLN) with poly(4-phenylacrylate) (7) was measured in a competitive assay based on the inhibition of binding to peroxidase-labeled fetuin (24). 3'SLN and 6'SLN-acrylic polymers contain an additional amino group compared to 3'SL or 6'SL and more closely approximate "avian-type" or "human-type" sialyl receptors, respectively (26). Association constant (K_d) values were determined as the sialic acid (Neu5Ac) concentration at the point $A_{\text{max}}/2$ on Scatchard plots.

Statistical analysis. The virus yields, AUCs, mean peak viral titers, and binding to sialyl receptors of influenza A and B viruses were compared either by analysis of variance (ANOVA) or by use of an unpaired *t* test. A probability value of 0.05 was prospectively chosen to indicate that the findings were not the result of chance alone.

RESULTS

Replication kinetics of *wt* and *ca* A/California/10/78 (H1N1), A/Alaska/6/77 (H3N2), and A/Washington/897/80 (H3N2) influenza viruses in HAEC, MDCK, and NHBE cells. To assess

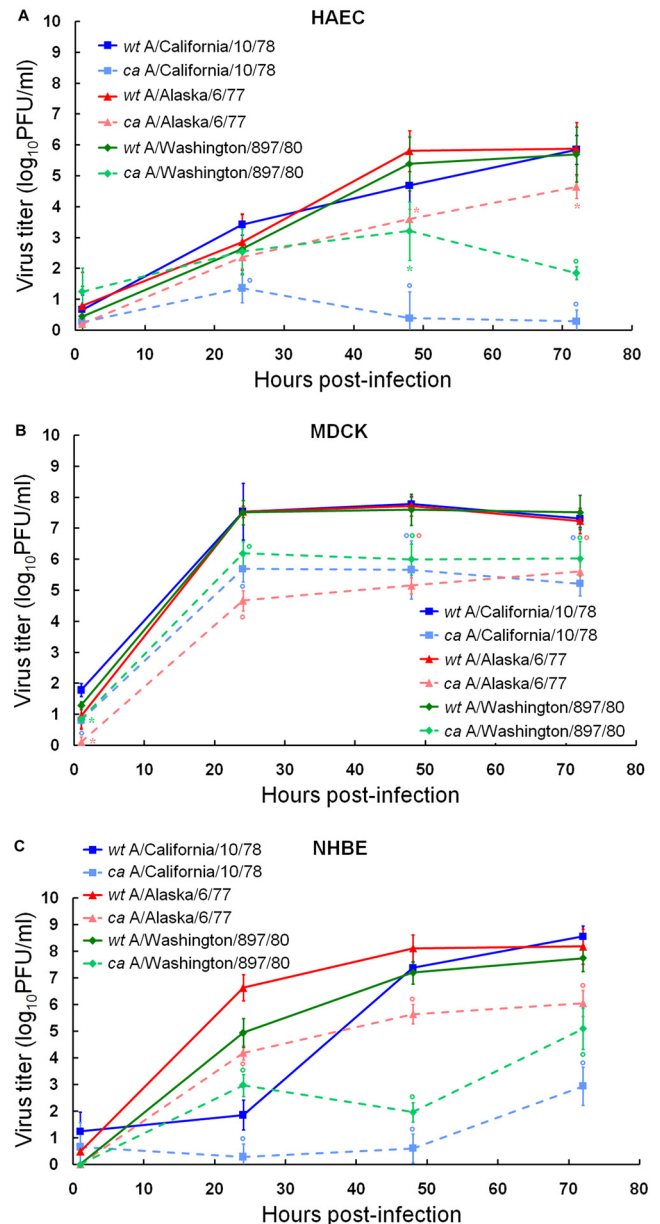


FIG 1 Replication kinetics of *wt* and *ca* A/California/10/78 (H1N1), A/Alaska/6/77 (H3N2), and A/Washington/897/80 (H3N2) influenza viruses in HAEC (A), MDCK (B), and NHBE (C) cell cultures. Cultures were infected with viruses at an MOI of 0.01 PFU/cell at 33°C. Supernatants were collected at 1, 24, 48, and 72 h postinfection, and the virus titers were determined as \log_{10} PFU/ml in MDCK cells. *, $P < 0.05$; °, $P < 0.01$ (compared with the value for the respective *wt* virus, determined with an unpaired *t* test).

which cell culture system could be used to adequately evaluate the viral growth of attenuated vaccine candidates, we first determined the levels of replication of early *ca* live influenza A virus vaccine strains, i.e., *ca* A/California/10/78 (H1N1), *ca* A/Alaska/6/77 (H3N2), and *ca* A/Washington/897/80 (H3N2) reassortants, in comparison with their respective *wt* viruses in HAEC, MDCK, and NHBE cells (Fig. 1; Table 1). Viral replication was compared by inoculating all three cultures with the *wt* and *ca* viruses at an MOI of 0.01 at 33°C and determining yields of the viral progeny at 1, 24,

TABLE 1 Areas under the curve and mean peak viral titers for *wt* and *ca* A/California/10/78, A/Alaska/6/77, and A/Washington/897/80 influenza viruses in HAEC, MDCK, and NHBE cell cultures

| Virus | Subtype | Mean AUC ^a (mean peak titer, log ₁₀ PFU/ml) | | |
|------------------------------------|---------|---|-------------|-------------|
| | | HAEC | MDCK | NHBE |
| A/California/10/78 | | | | |
| <i>wt</i> | H1N1 | 223.6 (5.9) | 364.8 (7.8) | 301.8 (8.6) |
| <i>ca</i> | H1N1 | 28.8 (1.4) | 266.7 (5.7) | 53.0 (2.9) |
| Degree of restriction ^b | | 194.8 (4.5) | 98.1 (2.1) | 248.8 (5.7) |
| A/Alaska/6/77 | | | | |
| <i>wt</i> | H3N2 | 244.0 (5.9) | 362.5 (7.7) | 372.1 (8.2) |
| <i>ca</i> | H3N2 | 170.3 (4.6) | 246.7 (5.6) | 258.2 (6.0) |
| Degree of restriction | | 73.7 (1.3) | 115.8 (2.1) | 113.9 (2.2) |
| A/Washington/897/80 | | | | |
| <i>wt</i> | H3N2 | 229.1 (5.7) | 362.5 (7.6) | 324.6 (7.7) |
| <i>ca</i> | H3N2 | 129.7 (3.2) | 290.5 (6.2) | 143.6 (5.1) |
| Degree of restriction | | 99.4 (2.5) | 72.0 (1.4) | 181.0 (2.6) |

^a The area under the curve (AUC) represents the total viral load at 24, 48, and 72 h postinfection.

^b The degree of restriction of viral replication is expressed as the value for *wt* virus minus the value for the corresponding *ca* virus.

48, and 72 h postinfection by plaque titration in MDCK cells. The growth of influenza viruses was supplemented by the addition of trypsin in MDCK cells, whereas no proteolytic enzymes were added to the epithelial cell systems.

Our data showed that *wt* A/California/10/78 (H1N1), *wt* A/Alaska/6/77 (H3N2), and *wt* A/Washington/897/80 (H3N2) replicated to the same extent in each cell line tested, as seen by similar total amounts of viral load (AUC) and almost equal peak viral titers (Table 1). All *wt* strains grew to significantly higher titers than the respective *ca* viruses at 48 and 72 h after infection in HAEC, MDCK, and NHBE cells (1.2 to 6.8 log units; $P < 0.01$) (Fig. 1). The replication abilities of the *ca* reassortant viruses (i.e., AUCs, peak viral titers, and virus yields at each time point) differed significantly ($P < 0.05$) from each other in HAEC and NHBE cells but not in MDCK cells (Fig. 1; Table 1).

We further performed pairwise comparisons of the cumulative amounts of each virus and peak viral titers in the three cell types (see Table S1 in the supplemental material). The levels of replication of the *wt* and *ca* viruses together with the levels of decrease of replication of the *ca* viruses were almost always significantly different in the three cell culture systems. The most limited growth of both *wt* and *ca* viruses was observed in HAEC cells ($P < 0.05$). Overall, our parallel experiments demonstrated that all three *ca* reassortants, i.e., *ca* A/California/10/78 (H1N1), *ca* A/Alaska/6/77 (H3N2), and *ca* A/Washington/897/80 (H3N2), exhibited the most attenuated growth in NHBE cells (i.e., decreases of viral peak titers were 5.7, 2.2, and 2.6 log units in comparison to the respective *wt* viruses, respectively) (Table 1).

Replication kinetics of *wt* and *ca* A/New Caledonia/20/99 (H1N1), A/Panama/2007/99 (H3N2), and A/Wyoming/03/03 (H3N2) influenza viruses in MDCK and NHBE cells. We evaluated the replication abilities of more contemporary *ca* influenza A virus vaccine strains, i.e., *ca* A/New Caledonia/20/99 (H1N1), *ca* A/Panama/2007/99 (H3N2), and *ca* A/Wyoming/03/03 (H3N2), in comparison with their respective *wt* viruses in MDCK and

NHBE cells (Fig. 2; Table 2). We observed that two *wt* H3N2 viruses, *wt* A/Panama/2007/99 and *wt* A/Wyoming/03/03, replicated similarly (i.e., no significantly different AUCs, peak viral titers, or virus yields were observed) and to significantly higher titers than *wt* A/New Caledonia/20/99 (H1N1) at 24, 48, and 72 h after infection in both cell types (1.6 to 3.4 log units; $P < 0.01$). The replication kinetics of two *ca* H3N2 reassortant viruses, *ca* A/Panama/2007/99 and *ca* A/Wyoming/03/03, did not differ significantly from each other (Fig. 2) or between the two cell systems (see Table S1 in the supplemental material). They replicated to significantly lower titers than the respective *wt* viruses at 24, 48, and 72 h after infection in MDCK and NHBE cells (3.2 to 6.7 log units; $P < 0.01$) (Fig. 2). Notably, *ca* A/New Caledonia/20/99 (H1N1) virus replicated to a significantly higher extent than the respective *wt* virus and the two other *ca* H3N2 reassortants in MDCK cells, as seen by its significantly higher AUCs, peak viral titers, and virus yields in this cell line ($P < 0.01$) (Table 2). In contrast, *ca* A/New Caledonia/20/99 (H1N1) virus exhibited a viral load similar to those of *ca* H3N2 viruses *ca* A/Panama/2007/99 and *ca* A/Wyoming/03/03 and significantly limited growth with a decrease of peak viral titer of 1.9 log units ($P < 0.01$) in comparison to the respective *wt* virus in NHBE cells (Fig. 2; Table 2). Taken together, our experiments clearly showed that differentiated NHBE cultures exhibited the greatest restriction for all stud-

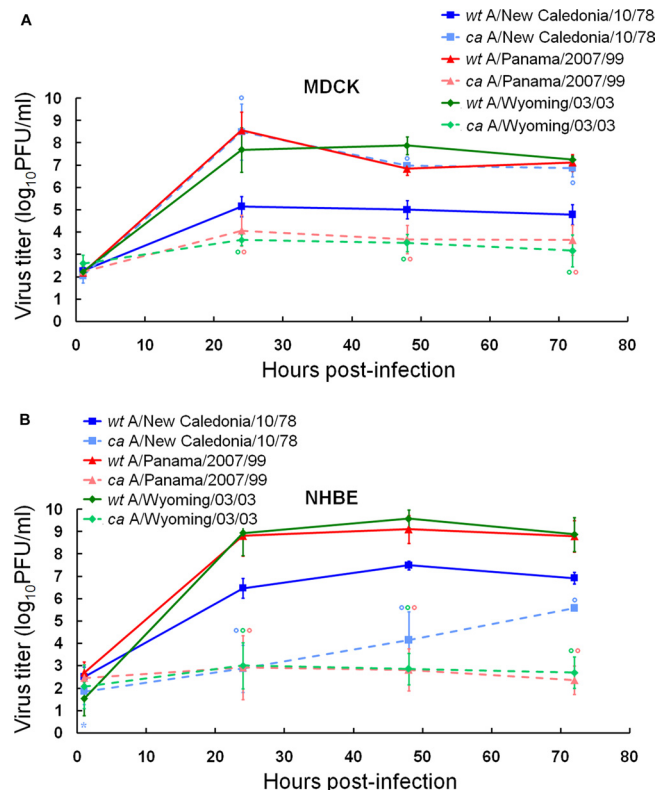


FIG 2 Replication kinetics of *wt* and *ca* A/New Caledonia/20/99 (H1N1), A/Panama/2007/99 (H3N2), and A/Wyoming/03/03 (H3N2) influenza viruses in MDCK (A) and NHBE (B) cell cultures. Cultures were infected with viruses at an MOI of 0.01 PFU/cell at 33°C. Supernatants were collected at 1, 24, 48, and 72 h postinfection, and the virus titers were determined as log₁₀ PFU/ml in MDCK cells. *, $P < 0.05$; °, $P < 0.01$ (compared with the value for the respective *wt* virus, determined with an unpaired *t* test).

TABLE 2 Areas under the curve and mean peak viral titers for *wt* and *ca* A/New Caledonia/20/99, A/Panama/2007/99, and A/Wyoming/03/03 influenza viruses in MDCK and NHBE cell cultures

| Virus | Subtype | Mean AUC (mean peak titer, log ₁₀ PFU/ml) ^a | |
|------------------------------------|---------|---|-------------|
| | | MDCK | NHBE |
| A/New Caledonia/20/99 | | | |
| <i>wt</i> | H1N1 | 239.5 (5.2) | 340.8 (7.5) |
| <i>ca</i> | H1N1 | 351.8 (8.5) | 201.2 (5.6) |
| Degree of restriction ^b | | 112.3 (3.1) | 139.6 (1.9) |
| A/Panama/2007/99 | | | |
| <i>wt</i> | H3N2 | 352.2 (8.6) | 429.9 (9.1) |
| <i>ca</i> | H3N2 | 180.7 (4.1) | 130.9 (2.9) |
| Degree of restriction | | 171.5 (4.5) | 299.0 (6.2) |
| A/Wyoming/03/03 | | | |
| <i>wt</i> | H3N2 | 368.6 (7.9) | 443.8 (9.6) |
| <i>ca</i> | H3N2 | 166.2 (3.7) | 137.1 (3.0) |
| Degree of restriction | | 202.4 (4.3) | 306.7 (6.6) |

^a The area under the curve (AUC) represents the total viral load at 24, 48, and 72 h postinfection. Bold indicates an increase in titer and lack of restricted replication of *ca* influenza vaccine virus.

^b The degree of restriction of viral replication is expressed as the value for *wt* virus minus the value for the corresponding *ca* virus.

ied *ca* H1N1 and H3N2 vaccine viruses among three cell types tested.

Comparison of replication kinetics of U.S. and Russian *ca* influenza vaccine donor strains in MDCK and NHBE cells. Since there is a correlation between the level of replication of influenza virus and its capacity to induce immunity (30), we compared side by side the replication abilities of different *ca* H2N2 vaccine donor strains, i.e., *ca* A/Ann Arbor/6/60, *ca* A/Leningrad/134/17/57, and *ca* A/Leningrad/134/47/57, in MDCK and NHBE cells (Fig. 3; Table 3). *wt* A/Leningrad/134/57 (H2N2) and its “80× passaged” variant *ca* A/Leningrad/134/80/57 (H2N2) were also included in the comparison. In MDCK cells, we observed that although *ca* A/Leningrad/134/17/57 and *ca* A/Leningrad/134/47/57 exhibited significantly higher peak viral titers and virus yields than *wt* A/Leningrad/134/57 and *ca* A/Leningrad/134/80/57 at 24, 48, and 72 h after infection (~0.6 log units; $P < 0.01$) (Fig. 3A), all four strains shed similar amount of virus (Table 3). In NHBE cells, *wt* A/Leningrad/134/57 and *ca* A/Leningrad/134/17/57 replicated similarly, whereas *ca* A/Leningrad/134/47/57 and *ca* A/Leningrad/134/80/57 showed significantly lower peak viral titers and virus yields at 24, 48, and 72 h after infection (0.7 to 2.9 log units; $P < 0.05$) (Fig. 3B). *ca* A/Leningrad/134/80/57 (H2N2) virus replicated to a significantly lower extent, as seen by AUC, than the respective *wt* strain ($P < 0.01$) (Table 3), which was consistent with its higher *in vitro* passage history.

We further evaluated the replication abilities of the Russian influenza A vaccine donor strains in comparison with the U.S. donor strain, *ca* A/Ann Arbor/6/60 (H2N2), in MDCK and NHBE cells (Fig. 3; Table 3). We did not observe significantly different AUCs for all the viruses in MDCK cells; however, *wt* A/Leningrad/134/57 (H2N2) and its “80× passaged” *ca* A/Leningrad/134/80/57 (H2N2) variant exhibited significantly lower peak viral titers and virus yields at 24, 48, and 72 h after infection than *ca* A/Ann Arbor/6/60 in this cell line (~0.7 log units; $P < 0.05$). In NHBE cells, the

replication kinetics of *ca* A/Ann Arbor/6/60 and *ca* A/Leningrad/134/80/57 did not differ from each other (as seen by similar AUCs and peak viral titers) (Table 3). However, *wt* A/Leningrad/134/57, *ca* A/Leningrad/134/17/57, and *ca* A/Leningrad/134/47/57 grew to significantly higher titers at 48 and 72 h after infection than *ca* A/Ann Arbor/6/60 (1.8 to 4.3 log units; $P < 0.01$) (Fig. 3B) and shed significantly larger amounts of virus ($P < 0.01$) (Table 3). Taken together, our data showed statistically significant differences between the replication abilities of the U.S. and Russian *ca* vaccine donor strains in NHBE cells.

Replication kinetics of influenza viruses isolated from different species in HAEC, MDCK, and NHBE cells. In this study, we assessed which of the three cell culture models could reflect the load of human infection based on the source (host) of the virus. For this purpose, we chose seven viruses of the H3 HA subtype that were isolated from different animal species, i.e., humans, birds, pigs, and horses, and assayed their yields after multiple replication cycles in HAEC, MDCK, and NHBE cultures (Fig. 4). Our results demonstrated no statistically significant difference between the replication kinetics of all the strains in HAEC and MDCK cells (Fig. 4A and B). Human, avian, swine, and equine isolates replicated to titers of $10^{4.1}$ to $10^{8.8}$ PFU/ml, suggesting that these two cell lines were totally susceptible to influenza viruses of

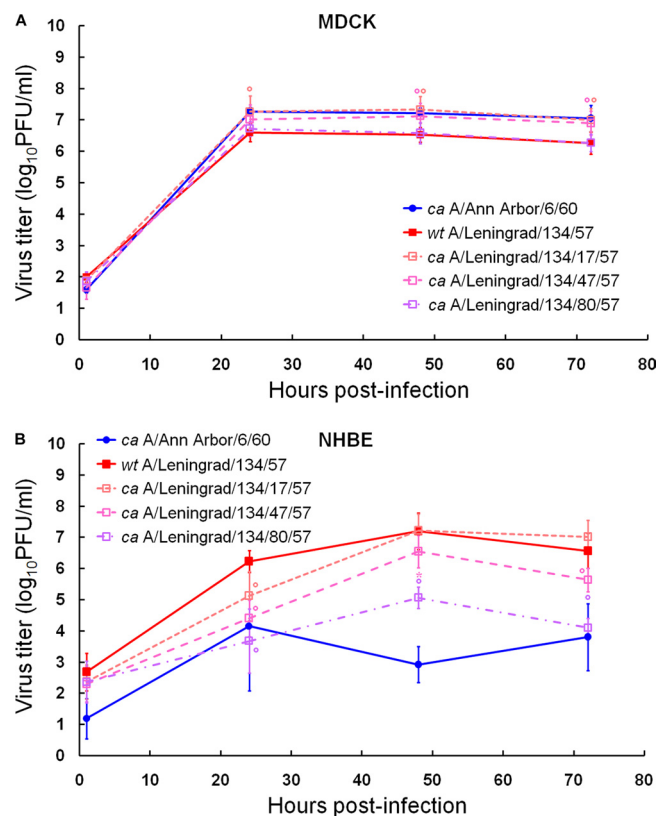


FIG 3 Replication kinetics of *ca* A/Ann Arbor/6/60, *wt* A/Leningrad/134/57, *ca* A/Leningrad/134/17/57, *ca* A/Leningrad/134/47/57, and *ca* A/Leningrad/134/80/57 influenza viruses in MDCK (A) and NHBE (B) cell cultures. Cultures were infected with viruses at an MOI of 0.01 PFU/cell at 33°C. Supernatants were collected at 1, 24, 48, and 72 h postinfection, and the virus titers were determined as log₁₀ PFU/ml in MDCK cells. *, $P < 0.05$; °, $P < 0.01$ (compared with the value for *wt* A/Leningrad/134/57 virus, determined by one-way ANOVA for Russian strains only).

TABLE 3 Areas under the curve and mean peak viral titers for *ca* A/Ann Arbor/6/60, *wt* A/Leningrad/134/57, *ca* A/Leningrad/134/17/57, *ca* A/Leningrad/134/47/57, and *ca* A/Leningrad/134/80/57 influenza viruses in MDCK and NHBE cell cultures

| Virus | Subtype | Mean AUC (mean peak titer, log ₁₀ PFU/ml) ^a | |
|---------------------------------|---------|---|--|
| | | MDCK | NHBE |
| <i>ca</i> A/Ann Arbor/6/60 | H2N2 | 344.7 (7.3) | 165.5 (4.2) |
| <i>wt</i> A/Leningrad/134/57 | H2N2 | 310.8 (6.6 ^c) | 326.2 ^c (7.2 ^c) |
| <i>ca</i> A/Leningrad/134/17/57 | H2N2 | 346.8 (7.3) | 318.9 ^c (7.2 ^c) |
| | | 36.0 (0.7) | 7.3 (0) |
| <i>ca</i> A/Leningrad/134/47/57 | H2N2 | 337.7 (7.1) | 277.6 ^c (6.6 ^c) |
| | | 26.9 (0.5) | 48.6 (0.6) |
| <i>ca</i> A/Leningrad/134/80/57 | H2N2 | 313.8 (6.7 ^c) | 214.7 (5.1) |
| | | 3.0 (0.1) | 111.5 (2.1) |

^a The area under the curve (AUC) represents the total viral load at 24, 48, and 72 h postinfection. Bold indicates an increase in titer and lack of restricted replication of *ca* influenza vaccine virus.

^b The degree of restriction of viral replication is expressed as the value for *wt* virus minus the value for the corresponding *ca* virus.

^c $P < 0.01$ compared with the value for *ca* A/Ann Arbor/6/60 (H2N2) virus, by one-way ANOVA.

different origin and, therefore, could not predict the risk of human infection (Table 4). In contrast, we observed three different patterns of replication corresponding to the origin of influenza virus in NHBE cells (Fig. 4C). (i) Three human isolates, A/Tottori/849/94 (H3N2) AL3, A/Tottori/849/94 (H3N2) K4, and A/Tottori/872/94 (H3N2) AL3, grew to significantly higher titers (1.0 to 8.0 log units higher; $P < 0.05$) than the rest of the viruses. (ii) The replication abilities of the avian viruses, A/duck/Ukraine/1/63 (H3N8) and A/duck/Hokkaido/8/80 (H3N8), did not differ from that of the swine A/swine/Italy/635/87 (H3N2) virus, and the yields of avian and swine isolates were approximately 66% of those of the human viruses at 24, 48, and 72 h after infection. (iii) The equine A/equine/TN/5/86 (H3N8) isolate exhibited the least replication in NHBE cells (mean peak titer of 1.2 log₁₀ PFU/ml) (Fig. 4C; Table 4), indicating that this virus possessed minimal infectivity compared to the other viruses tested. Statistically significant differences between cumulative amounts of viral load and mean peak viral titers of human versus equine versus avian and swine isolates were observed in NHBE but not in HAEC or MDCK cells ($P < 0.01$; Table 4).

Cellular tropism and virus spread of influenza viruses isolated from different species in HAEC, MDCK, and NHBE cells. To determine why NHBE cultures are capable of reflecting the capacity of influenza strains to infect humans, we further assessed cell-specific tropism of influenza viruses of different origin in NHBE and MDCK cells. We infected two cell lines with either virus at an MOI of 0.01, fixed the cells at 8 h postinfection (i.e., after the first cycle of viral replication), and then immunostained the cultures for viral antigen (Table 4). The patterns of infection with H3 influenza virus strains of different origin were strikingly different between cell lines. All viruses were able to infect MDCK cells in the presence of trypsin, with significantly different capacities ($P < 0.05$). These data suggested that human, avian, swine, and equine viruses possessed no host range limitation in this cell

line. In contrast, in NHBE cells at 8 h postinfection, only three human isolates, A/Tottori/849/94 (H3N2) AL3, A/Tottori/849/94 (H3N2) K4, and A/Tottori/872/94 (H3N2) AL3, showed replication (Table 4), suggesting that these viruses from the human host possess better tropism for NHBE cells than avian, swine, and equine isolates.

We further compared patterns of virus spread by H3 influenza viruses of different origin at 24 h after viral inoculation. Human, avian, and swine strains infected all cells in MDCK monolayers, and equine isolate A/equine/TN/5/86 (H3N8) infected ~53% of

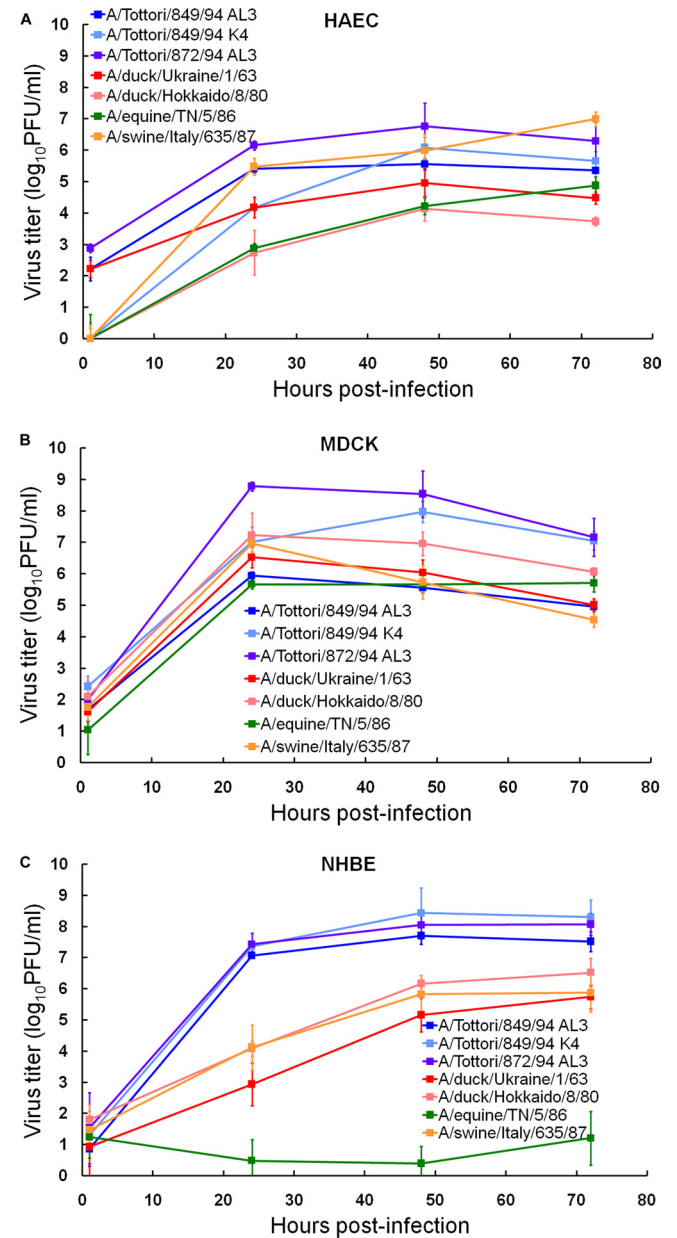


FIG 4 Replication kinetics of H3 human, avian, swine, and equine influenza A viruses in HAEC (A), MDCK (B), and NHBE (C) cell cultures. Cultures were infected with viruses at an MOI of 0.01 PFU/cell. Supernatants were collected at the indicated time points and titrated in MDCK cells by plaque assay. Representative results expressed as log₁₀ PFU/ml from 3 independent experiments are shown.

TABLE 4 Areas under the curve, mean peak viral titers, and cell tropism of H3 influenza viruses isolated from different hosts in HAEC, MDCK, and NHBE cell cultures

| Virus | Subtype | AUC, ^a mean \pm SD (mean peak viral titer, log ₁₀ PFU/ml) | | | % of infected cells at 8 h (24 h) postinfection, mean \pm SD ^b | |
|----------------------|---------|---|------------------------|------------------------|---|-------------------------------|
| | | HAEC | MDCK | NHBE | MDCK | NHBE |
| A/Tottori/849/94 AL3 | H3N2 | 262.4 \pm 6.2 (5.6) | 263.9 \pm 6.2 (5.9) | 359.8 \pm 11.0 (7.7) | 9.5 \pm 2.3 (100) | 0.9 \pm 0.4 (8.1 \pm 2.6) |
| A/Tottori/849/94 K4 | H3N2 | 263.9 \pm 13.0 (6.1) | 360.0 \pm 17.2 (8.0) | 390.2 \pm 27.2 (8.4) | 3.5 \pm 0.1 (100) | 0.5 \pm 0.2 (9.3 \pm 3.4) |
| A/Tottori/872/94 AL3 | H3N2 | 311.5 \pm 26.6 (6.8) | 396.2 \pm 26.6 (8.8) | 379.1 \pm 19.4 (8.1) | 3.9 \pm 1.3 (100) | 0.6 \pm 0.3 (7.3 \pm 5.5) |
| A/duck/Ukraine/1/63 | H3N8 | 222.7 \pm 16.1 (5.0) | 283.2 \pm 16.1 (6.5) | 227.7 \pm 25.7 (5.7) | 2.3 \pm 0.4 (100) | \leq 0.01 (1.3 \pm 0.7) |
| A/duck/Hokkaido/8/80 | H3N8 | 176.7 \pm 19.2 (4.1) | 326.4 \pm 19.2 (7.2) | 275.2 \pm 11.4 (6.5) | 6.0 \pm 0.7 (100) | \leq 0.01 (2.6 \pm 0.8) |
| A/equine/TN/5/86 | H3N8 | 194.2 \pm 11.6 (4.9) | 272.3 \pm 11.6 (5.7) | 29.5 \pm 31.6 (1.2) | 1.1 \pm 0.4 (53.0 \pm 19.9) | \leq 0.01 (\sim 0.07) |
| A/swine/Italy/635/87 | H3N2 | 293.2 \pm 18.6 (7.0) | 275.5 \pm 18.6 (7.0) | 259.5 \pm 30.7 (5.9) | 3.7 \pm 0.9 (100) | \leq 0.01 (0.7 \pm 0.5) |

^a The area under the curve (AUC) represents the total viral load at 24, 48, and 72 h postinfection.

^b MDCK, HAEC, and NHBE cells were infected with H3 influenza viruses of different origins at an MOI of 0.01. Due to similar results being observed in MDCK and HAEC cells, data for HAEC cells are not shown.

all the cells (Table 4). In contrast, limited growth and the focal nature of the influenza infection were observed in NHBE cultures. After infection (24 h), human viruses A/Tottori/849/94 (H3N2) AL3, A/Tottori/849/94 (H3N2) K4, and A/Tottori/872/94 (H3N2) AL3 infected \sim 7-fold more cells than avian and swine strains (Table 4). In addition, human isolates produced continuous foci of infected cells, which included both nonciliated and ciliated (\sim 46%) (data not shown) cells. Avian viruses A/duck/Ukraine/1/63 (H3N8) and A/duck/Hokkaido/8/80 (H3N8) infected the same amount of cells as the swine A/swine/Italy/635/87 (H3N2) virus (\sim 1.5%) (Table 3), and most of the infected cells were ciliated (65%) (data not shown). The equine A/equine/TN/5/86 (H3N8) isolate exhibited negligible infection in NHBE cells (Table 4). Taken together, our results suggested that NHBE cell cultures could approximate the cellular tropism of influenza viruses isolated from different species to the human respiratory tract.

Receptor specificity of H3 influenza A viruses isolated from different species. To examine whether the observed cellular tropism and virus spread of influenza viruses of different origin in NHBE cells were mediated by the receptor specificity of the viral HA, we measured the receptor specificities of the H3 influenza virus isolates to synthetic sialic substrates (3'SL/N and 6'SL/N) (Fig. 5). As shown by the K_a values, human viruses A/Tottori/

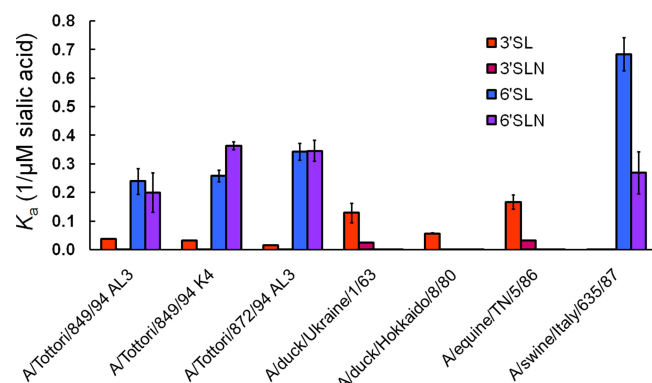


FIG 5 Receptor specificities of H3 human, avian, swine, and equine influenza A viruses. Association constants (K_a) of virus complexes with synthetic sialylglycopolymers conjugated to 3'SL(N) and 6'SL(N) are shown. Higher K_a values indicate stronger binding. Values are the means \pm standard deviations (SD) from 4 independent experiments.

849/94 (H3N2) AL3, A/Tottori/849/94 K4, and A/Tottori/872/94 (H3N2) AL3 exhibited increased affinity for 6'SL/N (synthetic sialosaccharides with the "human-type" SA α 2,6Gal linkage), whereas the binding to the "avian-type" 3' substrates 3'SL/N was negligible. A similar pattern was observed for the swine A/swine/Italy/635/87 (H3N2) virus. Two avian H3 viruses, A/duck/Ukraine/1/63 (H3N8) and A/duck/Hokkaido/8/80 (H3N8), and one equine isolate, A/equine/TN/5/86 (H3N8), bound strongly to 3'SL only (Fig. 5). Therefore, our experiments showed that differential cell tropism and virus spread of H3 influenza viruses isolated from different species were dependent, but only partially, on HA receptor specificity in NHBE cells.

Replication kinetics of *ca* A/chicken/HK/G9/97 (H9N2) and *wt* and *ca* B/Hong Kong/330/01 influenza viruses in MDCK and NHBE cells. Live attenuated A/Ann Arbor/6/60 *ca* influenza vaccines have been made with HA influenza subtypes with pandemic potential (H2, H5, H7, and H9 HA subtypes). The replication of such vaccine strains in mice and ferrets has not been predictive of replication of these viruses in humans (18, 19, 45, 46). Therefore, we assessed the growth capacity of the *ca* H9N2 pandemic vaccine strain, *ca* A/chicken/HK/G9/97, in MDCK and NHBE cells. We observed that the level of replication of *ca* A/chicken/HK/G9/97 (H9N2) was significantly different in the two cell culture systems (Table 5; see Table S1 in the supplemental material). In MDCK cells, *ca* H9N2 virus replicated to a significantly higher extent than other *ca* H1N1 and H3N2 reassortants studied, as seen by its significantly higher AUC ($P < 0.01$) (Tables 1, 2, and 5). In contrast, *ca* A/chicken/HK/G9/97 (H9N2) exhibited a viral load similar to those of other *ca* viruses, except *ca* A/California/10/78 (H1N1) ($P < 0.01$) (Table 1) and *ca* A/Panama/2007/99 (H3N2) ($P < 0.05$) (Table 2), in NHBE cultures. We observed significantly different peak viral titers of all studied *ca* vaccine strains in MDCK cells ($P < 0.05$). Conversely, no statistically significant difference was found between peak viral titers of *ca* reassortants, except *ca* A/Alaska/6/77 (H3N2), in NHBE cells ($P < 0.05$) (Tables 1, 2, and 5).

Finally, we assessed whether the NHBE culture model could adequately reflect the restriction of replication of the *ca* live influenza B virus vaccine strain, *ca* B/Hong Kong/330/01, in comparison with the respective *wt* B/Hong Kong/330/01 virus. Both isolates replicated to titers of $\sim 10^{3.5}$ PFU/ml in MDCK cells, showing no statistically significant difference between their replication kinetics in this cell line (Table 5). In contrast, *wt* and *ca* B viruses

TABLE 5 Areas under the curve and mean peak viral titers of *ca* A/Chicken/HK/G9/97 and *wt* and *ca* B/Hong Kong/330/01 influenza viruses in MDCK and NHBE cell cultures

| Virus | Subtype | Mean AUC (Mean peak titer, log ₁₀ PFU/ml) ^a | |
|------------------------------------|---------|---|--|
| | | MDCK | NHBE |
| <i>ca</i> A/chicken/HK/G9/97 | H9N2 | 443.2 (7.1) | 216.6 (3.8) |
| B/Hong Kong/330/01 | | | |
| <i>wt</i> | | 218.2 (3.3) | 342.8 (5.4) |
| <i>ca</i> | | 235.0 (3.7) | 217.1 ^c (3.5 ^d) |
| Degree of restriction ^b | | 16.8 (0.4) | 125.7 (1.9) |

^a The area under the curve (AUC) represents total viral load at 24, 48, and 72 h postinfection. *ca* A/chicken/HK/G9/97 (H9N2) influenza virus did not produce plaques in MDCK cells; these values are defined as log₁₀ TCID₅₀/ml ± SD determined in MDCK cells after 3 days of incubation at 33°C with 10-fold serially diluted virus. Bold indicates an increase in titer and lack of restricted replication of *ca* influenza vaccine virus.

^b The degree of restriction of viral replication was expressed as the value for *wt* virus minus the value for the corresponding *ca* virus.

^c $P < 0.05$ compared with the value for *wt* B/Hong Kong/330/01 virus, determined with an unpaired *t* test.

^d $P < 0.01$ compared with the value for *wt* B/Hong Kong/330/01 virus, determined with an unpaired *t* test.

replicated to significantly different titers at 24, 48, and 72 h after infection in NHBE cells (2.0-log-unit difference; $P < 0.01$) (data not shown). The *ca* B/Hong Kong/330/01 strain exhibited significantly limited growth, with a decrease of peak viral titer of 1.9 log unit ($P < 0.01$) compared to that of the *wt* virus in the epithelial cells (Table 5). Therefore, our experiments showed that differentiated NHBE cultures could reflect the restricted replication of *ca* influenza B viruses in humans.

DISCUSSION

We compared the replication kinetics of *ca* live attenuated vaccine candidates, including H1N1, H3N2, and B strains, in comparison with their respective *wt* viruses in MDCK cells, human adenoid epithelial cells, and bronchial airway epithelium to assess which cell culture model could more consistently and accurately reflect their infectivities in humans. Since the levels of replication of several *wt* and *ca* influenza A virus strains used in this study have been previously evaluated in clinical trials with adult and seronegative pediatric volunteers (10, 11, 28, 29), we were able to compare their viral growth in humans and three tissue culture systems. Previous studies demonstrated that three *ca* reassortant viruses, *ca* A/California/10/78 (H1N1), *ca* A/Alaska/6/77 (H3N2), and *ca* A/Washington/897/80 (H3N2), exhibited different degrees of attenuation (>3.9, >4.5, and ~2.8 log units, respectively), as measured by comparison of mean peak viral titers (log₁₀ TCID₅₀/ml of nasopharyngeal wash sample) between the *wt* and *ca* viruses administered at similar doses to humans (10, 28, 29). Unfortunately, we were unable to determine any significant correlation by Spearman's rank correlation analysis ($P > 0.05$) between degrees of restriction of *ca* A/California/10/78 (H1N1), *ca* A/Alaska/6/77 (H3N2), and *ca* A/Washington/897/80 (H3N2) strains in humans and those in any of the cell culture systems tested. However, our data demonstrated that the levels of attenuation of these three *ca* viruses in human subjects were consistently higher (~2.8 logs) (10, 28–30) than those in MDCK cells. Furthermore, the *ca* A/New Caledonia/20/99 (H1N1) reassortant replicated to significantly

higher viral titers than the respective *wt* H1N1 virus ($P < 0.01$), and no statistically significant difference was observed between the replication abilities of *wt* and *ca* B/Hong Kong/330/01 viruses in MDCK cells. Taking these findings together, we can speculate that data obtained in MDCK cells could be the least predictive of restricted viral replication of *ca* live attenuated vaccine viruses in humans.

In this study, we observed that limited growth of *ca* vaccine viruses was reflected in HAEC and NHBE cultures. However, HAEC cells remain very difficult to grow and could vary between different donors, whereas the two other cell types studied are commercially available and therefore could be used from the same source/donor for different experiments. Our experiments showed that differentiated NHBE cultures exhibited a consistently greater restriction for all studied *ca* H1N1, H3N2, and B vaccine viruses among the three cell lines tested. A similar pattern was observed for *ca* A/California/10/78 (H1N1) virus in NHBE cells isolated from a different human donor (data not shown). Despite the fact that all tissue culture models lack a functional adaptive immune system, which would obviously play a role in virus replication in the host, NHBE cells exhibited the best correlation between the degrees of restriction of viral replication of *ca* A/California/10/78 (H1N1), *ca* A/Alaska/6/77 (H3N2), and *ca* A/Washington/897/80 (H3N2) and those seen in clinical trials with humans (10, 28–30). *ca* A/New Caledonia/20/99 (H1N1), *ca* A/Panama/2007/99 (H3N2), and *ca* A/Wyoming/03/03 (H3N2) showed the lowest virus yields in NHBE cells, which were in good correlation with recently published data (6, 23). Therefore, NHBE cells may be considered for use as the screening step for evaluating the restricted replication phenotype of potential influenza virus vaccine candidates in humans.

There has been a long-standing parallel development in Russia of potential live attenuated vaccines (20, 51). To date, there are no reports of side-by-side comparisons of the U.S. and Russian *ca* H2N2 vaccine donor strains, *ca* A/Ann Arbor/6/60, *ca* A/Leningrad/134/17/57, and *ca* A/Leningrad/134/47/57, in cell culture models or in humans. So far, in only one study were immune responses to these donor strains in the lungs and mediastinal lymph nodes of mice compared (52). Overall, combined data for viral clearance, antibody-secreting cells, and cytokine responses suggested that *ca* A/Leningrad/134/17/57 is a superior immunogen to *ca* A/Leningrad/134/47/57, which in turn is superior to *ca* A/Ann Arbor/6/60 (52). In addition, two reassortant vaccines prepared from the *ca* A/Ann Arbor/6/60 and *ca* A/Leningrad/134/17/57 donor strains with the surface antigens of A/Korea/1/82 (H3N2) were compared in rats, ferrets, and humans (34). A reassortant prepared from the Russian strain induced slightly better rates of seroconversion, but conclusions as to their relative immunogenicities could not be made because of differences in the number of internal genes present in each reassortant. Here, for the first time, we compared side by side the replication abilities of the U.S. and Russian *ca* H2N2 vaccine donor strains in two cell lines, MDCK and NHBE cells. We did not observe statistically significant differences in replication kinetics of these viruses (as seen by similar AUCs, viral peak titers, and virus yields at all time points) in MDCK cells. In contrast, a statistically significant difference between the replication abilities of the U.S. and Russian *ca* vaccine donor strains was detected in NHBE cultures ($P < 0.05$). *ca* A/Leningrad/134/17/57, which has become the main vaccine donor strain in Russia, replicated to significantly higher titers (~1

log unit; $P < 0.05$) than *ca* A/Leningrad/134/47/57, which in turn replicated to significantly higher titers (~ 2.7 log units; $P < 0.01$) than *ca* A/Ann Arbor/6/60 at 48 and 72 h after infection in human airway epithelium. Therefore, we observed that NHBE cultures were able to rank the levels of replication of various *ca* live attenuated influenza vaccines compared to each other. This property can assist in choosing the *ca* vaccine donor strain with the appropriate balance between replicative capacity and lack of reactogenicity.

For use in pandemic preparedness, live attenuated influenza vaccines will likely need to represent novel HA and NA genes from different species (45, 46). In this study, we assessed whether NHBE cultures could reflect the ability of an animal influenza virus to infect humans. Taking into account that previous studies demonstrated transmission of avian and swine viruses to humans (41) but that horse-to-human transmission remains to be reported (48), we can speculate that the pattern of virus infectivity of the studied H3 influenza viruses from different species of origin (i.e., human, avian, swine, and equine) in NHBE cells could parallel the pattern of infectivity of these strains in humans.

Our results also showed that NHBE cell cultures most adequately approximated the cellular tropism of influenza viruses isolated from different species to the human respiratory tract. Previous studies have suggested that airway epithelial cell cultures contain ciliated, nonciliated, and goblet cells. Although both types of receptors (SA α 2,6 and SA α 2,3) are present on the cell surface in NHBE cultures (as they are in HAEC cultures [43]), NHBE cultures express abundantly more SA α 2,6, while SA α 2,3 is expressed at a lower level (25, 37). It was shown that SA α 2,6 receptor moieties were abundant on the apical surface of nonciliated cells and were particularly concentrated on the microvilli. However, a lower level of SA α 2,6 was also observed on the apical surface of some ciliated and goblet cells (37, 50), suggesting that α 2,6-linked SA is distributed across all cell types in NHBE cultures. "Avian-type" SA α 2,3 receptors were shown to be predominant on the apical surface of most, but not all ciliated cells at the base of the cilia in the region of microvilli and were also found to a much lesser degree on some nonciliated cells (25, 50). Taken together, our experiments showed that differential cell tropism and virus spread of human and avian influenza isolates were dependent, but only partially, on HA receptor specificity. However, our data also demonstrated that despite the fact that both human and swine viruses exhibited human-like virus receptor specificity, only human viruses replicated to a higher extent in NHBE cells. Among H3 viruses of different origin with avian-like receptor specificity, only avian and not equine strains were able to establish some limited infection in human airway epithelium. Overall, possible explanations for the more limited growth of the avian, swine, and equine strains compared to the human viruses could be innate immunity and some unknown host range mechanisms that are present in NHBE cells but not in MDCK or HAEC cells.

Finally, in this study we assessed the replication ability of the *ca* A/chicken/HK/G9/97 (H9N2) pandemic vaccine strain, which contained avian surface glycoproteins and was tested in a clinical trial in humans (18), in MDCK and NHBE cells. We compared its replication with those of human *ca* H1N1 and H3N2 vaccine strains in an attempt to explain the robust difference seen in replication between seasonal and avian *ca* influenza vaccines in healthy adult volunteers (9, 18, 19, 45, 46). The *ca* H9N2 virus replicated similarly to other classical *ca* reassortants and exhibited

similar viral titers in NHBE cultures. However, the degree of restriction differed from that observed in the clinical trial in humans (18). We can speculate that although NHBE cultures are able to reflect the restricted phenotype of *ca* influenza vaccine viruses, the lack of replication of the avian *ca* H9N2 vaccine virus seen in adults (18), but not in the NHBE cell model, suggests the presence of host immune factors in humans that induce innate and heterosubtypic protection against infection with avian *ca* vaccines. Such heterosubtypic immunity could be conferred by previous infection with influenza viruses belonging to another HA subtypes and appears to be enough to restrict to some extent the replication of human *ca* vaccines as well (10, 28, 29).

In conclusion, in the present study we compared three tissue culture systems, i.e., HAEC, MDCK, and NHBE cells, for their ability to model the levels of restriction of *ca* influenza vaccine viruses and the risk of infection and cellular tropism of influenza strains of different origin with different receptor specificities. Our data showed that differentiated cultures of human airway epithelium exhibited the best approximation to the events in humans. Although clinical trials with human subjects are essential, NHBE cultures could be considered for use as a screening step for evaluating and possibly ranking the restricted replication of influenza vaccine candidates having HA proteins of different origin.

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