Lack of transmission of a human influenza virus with avian receptor specificity between ferrets is not due to decreased virus shedding but rather a lower infectivity \textit{in vivo}

Kim L. Roberts,\textsuperscript{1} Holly Shelton,\textsuperscript{1} Margaret Scull,\textsuperscript{2,3,4,5} Raymond Pickles\textsuperscript{2,3} and Wendy S. Barclay\textsuperscript{1}

\textsuperscript{1}Department of Virology, Division of Infectious Disease, Imperial College London, St Mary’s Campus, Norfolk Place, London W2 1PG, UK
\textsuperscript{2}Department of Microbiology and Immunology, University of North Carolina at Chapel Hill, NC 27599, USA
\textsuperscript{3}Cystic Fibrosis Center, University of North Carolina at Chapel Hill, NC 27599, USA
\textsuperscript{4}Laboratory of Virology and Infectious Disease, The Rockefeller University, New York, NY 10065, USA
\textsuperscript{5}Center for the Study of Hepatitis C, The Rockefeller Univertity, New York, NY 10065, USA

Influenza virus attatches to host cells by sialic acid (SA). Human influenza viruses show preferential affinity for \(\alpha_2,6\)-linked SA, whereas avian influenza viruses bind \(\alpha_2,3\)-linked SA. In this study, mutation of the haemagglutinin receptor-binding site of a human H3N2 influenza A virus to switch binding to \(\alpha_2,3\)-linked SA did not eliminate infection of ferrets but prevented transmission, even in a co-housed model. The mutant virus was shed from the noses of ferrets directly inoculated with virus in the same amounts and for the same length of time as wild-type virus. Mutant virus infection was localized to the same anatomical regions of the upper respiratory tract of directly inoculated animals. Interestingly, wild-type virus was more readily neutralized than the mutant virus \textit{in vitro} by ferret nasal washes containing mucus. Moreover after inoculation of equal doses, the mutant virus grew poorly \textit{ex vivo} ferret nasal turbinate tissue compared with wild-type virus. The dose of mutant virus required to establish infection in the directly inoculated ferrets was 40-fold higher than for wild-type virus. It was concluded that minimum infectious dose is a predictor of virus transmissibility and it is suggested that, as virus passes from one host to another through stringent environmental conditions, viruses with a preference for \(\alpha_2,3\)-linked SA are unlikely to inoculate a new mammalian host in sufficient quantities to initiate a productive infection.

\textbf{INTRODUCTION}

Influenza is caused by an acute infection of humans with a small enveloped RNA virus. The virus is labile in the environment (Thomas \textit{et al.}, 2008) and requires an efficient transmission route from one host to another for its evolutionary success. Influenza virus particles are transmitted by direct contact and through the air in large droplets or as aerosols (Tellier, 2009). Influenza viruses emerge sporadically from animal reservoirs to infect people (Taubenberger & Morens, 2010). The ability of zoonotic agents to achieve transmission between human hosts determines their potential to cause a pandemic. This is lucidly illustrated by the lack of transmission of the highly pathogenic avian influenza virus H5N1, despite its unprecedented distribution in birds, in contrast with the sudden emergence of a novel swine-origin H1N1 influenza strain as the first pandemic influenza virus of the 21st century. Understanding why some viruses are transmitted between people whereas others are not is key for assessing the pandemic threat posed by novel influenza virus strains.

The ferret is currently accepted as the best animal model for studies of transmission and virulence of human influenza virus (Matsuoka \textit{et al.}, 2009). Ferrets are susceptible to infection with human influenza strains without adaptation of the virus and, as a consequence of infection, display symptoms typical of human influenza including fever, lethargy and sneezing. This is partly due to the fact that their anatomical expression of sialic acid (SA), the receptor by which influenza virus attacks to cells, is similar to that of humans. Thus, in the upper respiratory tract of both species, SA is predominantly linked to glycoproteins and glycolipids with an \(\alpha_2,6\) glycosidic bond, whereas in the lungs, \(\alpha_2,3\)-linked SA is detected on cells of ferret bronchioles and human pneumocytes (Kirkeby \textit{et al.}, 2009; Leigh \textit{et al.}, 1995; Shinya \textit{et al.}, 2006).
Highly pathogenic H5N1 avian influenza virus can infect ferrets but is not transmitted between them (Maines et al., 2006). Similarly, other avian strains including H7 and H9 can establish infection in directly inoculated animals but are rarely transmitted (Belser et al., 2008; Song et al., 2009; Sorrell et al., 2009; Wan et al., 2008). Those avian influenza viruses that do show some level of transmission possess haemagglutinin (HA) proteins with some affinity for viruses that do show some level of transmission possess haemagglutinin (HA) proteins with some affinity for s2,6-linked SA (Belser et al., 2008; Wan et al., 2008). In seminal studies with reconstructed 1918 H1N1 influenza virus, Tumpey et al. (2007) showed that two amino acids at the receptor-binding site of the HA protein determined the SA-binding preference and also the transmissibility of the virus in the ferret. For the H3 HA subtype, the equivalent amino acid residues that differ between human-adapted and avian H3 viruses are located at positions 226 and 228 in HA (Matrosovich et al., 2000), and the nature of these residues profoundly affects the shape of the receptor-binding pocket (Ha et al., 2003). H3N2 influenza viruses that have leucine at residue 226 and serine at position 228 bind to s2,6-linked SA, and this change from the avian-signature glutamic acid at 226 and glycine at 228 was observed in viruses isolated shortly after the emergence of H3N2 as a pandemic strain that was transmitted between humans in 1968 (Matrosovich et al., 1997; Naeve et al., 1984). Thus, both in the ferret model and in humans, it is already well established that receptor-binding preference has a profound effect on virus transmission, although the cellular and molecular explanation for this observation is still not completely clear.

Using human H3N2 influenza virus as a model system, we sought here to identify the biological correlates of receptor switching that might explain why receptor preference is such an important factor in determining influenza transmissibility.

We previously described a pair of recombinant influenza viruses based on the human H3N2 virus strain A/Victoria/3/75 that differed in only two amino acids within the HA receptor-binding site at residues 226 and 228 (Scull et al., 2009). Both viruses grew to similar titres in differentiated human airway epithelium (HAE) cultures by 48 h post-infection (p.i.) when incubated at 37 °C. However, at 32 °C, a temperature close to that in the proximal human airway, the replication of the virus with an avian virus-like receptor-binding site, Vic-226-228HA, was reduced by 2 logs and spread within the cultures was less efficient. The wild-type human virus A/Victoria/3/75 infected both ciliated and non-ciliated cells in this complex airway culture system, whilst the virus with avian virus-like receptor-binding specificity exclusively infected ciliated cells, reflecting the tropism that we and others have reported previously for wild-type human and avian viruses (Matrosovich & Klenk, 2003; Matrosovich et al., 2004; Shelton et al., 2011; Thompson et al., 2006).

We describe here a detailed comparison of the replication and transmission of these two viruses in the ferret. We tested the hypothesis that altering receptor binding would affect shedding of the virus in the nasal wash and the anatomical distribution of the virus throughout the respiratory tract, as both of these parameters might be expected to affect transmissibility.

RESULTS

A human H3N2 influenza virus with avian virus receptor specificity

An isogenic pair of recombinant influenza A viruses based on the H3N2 strain A/Victoria/3/75 were constructed previously and differed only in two amino acid residues in the HA gene, L226Q and S228G (Scull et al., 2009).

This change in receptor-binding site made no difference to the replication of the virus in Madin–Darby canine kidney (MDCK) cells at either 32 or 37 °C (Fig. 1). In contrast, we showed previously (Scull et al., 2009) that the virus with avian-like receptor-binding specificity was restricted to the ciliated cell type in HAE cell cultures and was in this system compromised in cell-to-cell spread at the cooler temperature, which represents the natural environment of the human upper respiratory tract.

Transmissibility of the wild-type or the HA mutant recombinant virus with avian virus-like receptor specificity

To determine whether the recombinant influenza viruses were transmitted between ferrets, a co-housing model for transmission was established. This model was chosen because it is inclusive of all routes of transmission (fomite, contact, droplet and aerosol), and we reasoned that, under different environmental or sociological conditions, any of these methods might be relevant for human influenza outbreaks. Two cages, each capable of housing four ferrets, were set up. Two ferrets per virus were inoculated with

**Fig. 1.** Multicycle replication of the wild-type A/Victoria/3/75 virus and its receptor-binding mutant, Vic-226-228HA, in MDCK cells. Recombinant influenza virus A/Victoria/3/75 (H3N2) (solid lines) or its receptor-binding mutant, Vic-226-228HA (dotted lines), was used to infect MDCK monolayers at an m.o.i. of 0.001. Experiments were carried out in triplicate and results are shown as means ± SD. Infected cells were incubated at 32 or 37 °C and the virus released was titrated by plaque assay at regular time points. Student’s t-test was used to compare the viral titres of the two viruses at each time point and revealed no statistically significant differences.
1 × 10⁴ p.f.u. A/Victoria/3/75 or 1 × 10⁵ p.f.u. Vic-226-228HA. We employed a slightly higher inoculum dose of the mutant virus in these experiments because our work in human airway cultures had suggested that the HA mutation results in an approximately 1 log attenuation of replication in the airway epithelium at 32 °C (Scull et al. 2009). On day 1 p.i., three naïve sentinel ferrets were co-housed with each inoculated donor. Each day, nasal washes collected from the animals were tested for the presence of virus.

Both of the donors inoculated with A/Victoria/3/75 became positive for viral antigen in their nasal wash on day 1 p.i. and five of the six sentinels contracted infection (Table 1). Three sentinels became positive on day 2 post-exposure, whilst two others became positive on days 3 and 4 post-exposure, respectively. One sentinel did not become positive for antigen in the nasal wash but did seroconvert (HA titre of 320).

Both donors inoculated with Vic-226-228HA shed virus antigen in the nasal wash from day 1 p.i. However, none of the co-housed sentinels became positive for virus antigen in their nasal wash, and only one showed seroconversion by HAI (HA titre of 2560) (Table 1).

**Infection of ferrets with different doses of wild-type virus or HA mutant virus**

We next compared how readily ferrets became infected by lower doses of A/Victoria/3/75 and Vic-226-228HA viruses, and how the inoculation dose affected the profile of virus shedding. We noted a relationship between the dose used for the intranasal inoculation and the kinetics of virus shedding (Fig. 2). As the dose of inoculum decreased, the day that shedding started and the day of peak shedding were delayed. However, the amount of virus shed on the peak day did not change. Importantly, a higher dose of Vic-226-228HA virus was required to establish infection than for the wild-type virus. By inoculating groups of ferrets with different doses of either virus and applying the Spearman–Karber formula, we calculated that the 50 % ferret infectious dose for wild-type virus was 10⁰.⁶⁶ (5 p.f.u.) whereas for mutant virus it was 10³.²⁵ (180 p.f.u.), around 40-fold higher. Nevertheless, as the dose of Vic-226-228HA virus inoculum was increased, shedding began earlier and the titres shed increased such that, with an inoculum of 1 × 10⁴ p.f.u. (as used in the transmission experiment above), robust infection was established and the titres shed at day 3 were around 9 × 10⁵ p.f.u. ml⁻¹, comparable to those observed after inoculation of the lower doses (10⁴ down to 10 p.f.u.) of wild-type virus (Table 1 and Fig. 2).

**Transmissibility of the wild-type virus at a low inoculation dose**

Although donor animals infected with 1 × 10⁵ p.f.u. Vic-226-228HA virus shed high titres, the peak of shedding was not until day 3 p.i. (Fig. 2g). To test the hypothesis that delayed replication kinetics in the donor may reduce transmissibility, we repeated the co-housed transmission experiment with donor animals who received a low inoculum (10 p.f.u.) of A/Victoria/3/75. At this dose, the virus shedding profile was very similar to that observed after inoculation of 10⁵ p.f.u. Vic-226-228HA mutant virus (compare Fig. 2a and g).

Both of the donor ferrets infected with 10 p.f.u. wild-type A/Victoria/3/75 virus shed virus from day 2 p.i. (Fig. 3a). All six sentinels became positive for infectious virus shed in the nasal wash during the co-housing period (Fig. 3b and Table 1). One sentinel in each cage did not shed virus in the nasal wash until late in the experiment. Because infected sentinels were not removed from the cage during this experiment, it is impossible to say whether transmission occurred from the donors or the other infected sentinels. Nevertheless, these data show that wild-type A/ Victoria/3/75 virus was still transmitted efficiently after a very low inoculum dose to the donor animals.

**Characterization of the anatomical site of virus replication**

It has been suggested that transmission of avian influenza virus is limited because the anatomical site where virus replicates is too distal in the human respiratory tract for shed virus to accumulate adequately in proximal respiratory

---

**Table 1. Transmission of A/Victoria/3/75 HA but not Vic-226-228HA virus to co-housed sentinel ferrets**

Two ferrets were inoculated with 1 × 10⁴ p.f.u. A/Victoria/3/75 or 1 × 10⁵ p.f.u. Vic-226-228HA virus. On day 1 p.i., three naïve sentinel animals were introduced to each cage. Daily nasal washes were collected from conscious animals and viral antigen was detected using a Flu A Directigen kit (BD Diagnostics). Serum was collected 21 days after exposure from animals that did not test positive for antigen in the nasal wash and seroconversion was detected by haemagglutinin inhibition (HAI) assay. For experiments with a low dose of A/Victoria/3/75 virus, two ferrets were inoculated with 10 p.f.u., and on day 1 p.i., three naïve sentinels were introduced to each cage. Daily nasal washes were titrated by plaque assay and seroconversion was detected by HAI assay in serum collected 21 days after infection of donors.

<table>
<thead>
<tr>
<th>Virus</th>
<th>Inoculum dose (p.f.u.)</th>
<th>Peak titre of virus in nasal wash (day of peak shedding)</th>
<th>Transmission (antigen detection)</th>
<th>Transmission (serology)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/Victoria/3/75</td>
<td>10⁴</td>
<td>10⁵ (day 2)</td>
<td>5/6</td>
<td>1/1</td>
</tr>
<tr>
<td>Vic-226-228HA</td>
<td>10⁵</td>
<td>10⁵ (day 3)</td>
<td>0/6</td>
<td>1/6</td>
</tr>
<tr>
<td>A/Victoria/3/75</td>
<td>10</td>
<td>10⁵ (day 3)</td>
<td>6/6</td>
<td>6/6</td>
</tr>
</tbody>
</table>
secretions (Shinya et al., 2006; van Riel et al., 2006, 2007). To address this possible explanation for the lack of transmission of Vic-226-228HA mutant virus, infected animals were culled on day 1 or 3 p.i. and dissected to determine the location of virus replication throughout the respiratory tract (Fig. 4a). Virus was found in all of the nasal turbinate samples tested, regardless of whether the animals were infected with A/Victoria/3/75 or Vic-226-228HA (Fig. 4b). Titres of infectious virus present in other upper respiratory tract sites were similar, regardless of the inoculum used to infect the animals or the nature of the virus HA receptor-binding specificity. Virus was not recovered in any of the tracheal or lung samples (data not shown), suggesting that neither virus penetrated efficiently to infect the lower respiratory tract during these experiments.

Infection of ex vivo ferret nasal turbinate tissue

To determine whether both viruses were equally capable of infecting tissue from the site of inoculation, ex vivo nasal turbinate tissue was harvested from a naïve ferret and infected with $10^4$ p.f.u. A/Victoria/3/75 or Vic-226-228HA virus. No virus was detected in either tissue at 48 h p.i. (data not shown) or 66 h p.i. (Fig. 5a). Virus was detected from one sample of each group at 90 h p.i. and the titre of the wild-type virus was 100 times higher than that of the receptor-binding mutant. By 116 h p.i., a second sample inoculated with A/Victoria/3/75 had released replicated virus. Although these tissue samples were clearly somewhat refractory to infection and showed some variability, the data suggested that the wild-type virus was more efficient at infecting nasal turbinate tissue, the site of intranasal inoculation, than the receptor mutant.

To attempt to explain these observations, we probed the fixed turbinate tissue with lectins specific for α2,3-linked or α2,6-linked SA [Maackia amuensis (MAA) lectin and Sambucus nigra (SNA) lectin, respectively]. There was abundant α2,6-linked SA expression at the apical surface of the turbinate tissue. In contrast, α2,3-linked SA expression was sparse and sporadic (Fig. 5b).

**Fig. 2.** Infectivity of influenza virus in directly inoculated ferrets. Pairs of ferrets were inoculated intranasally under light anaesthetic with different doses of A/Victoria/3/75 virus (a–c) or its receptor-binding mutant, Vic-226-228HA (d–g). Animals were nasally washed every day thereafter and the amount of virus shed in the nasal wash was detected by plaque assay.
It has been suggested that viruses that bind to α2,3-linked SA might be affected by mucus-containing secretions that lie over the respiratory epithelium (Matrosovich & Klenk, 2003). To test this hypothesis, we performed an infectivity reduction assay in which virus was pre-incubated with airway secretions collected as washings from the nasal cavities of uninfected ferrets. Mucus-containing secretions were not frozen before exposure to virus, as this has been reported to destroy the integrity of the mucoid structure (Lai et al., 2009). Following pre-incubation of mucus-containing secretions and virus, the remaining virus infectivity was titrated on MDCK cells.

For the wild-type virus, infectivity following incubation with airway secretions was significantly inhibited in a dose-dependent manner. Indeed, the infectivity of the wild-type virus was reduced to 30% at the maximal concentration of nasal wash (P<0.05) (Fig. 6a). Surprisingly, under the same conditions, the infectivity of the receptor mutant virus was not affected. It has been reported that human respiratory mucus predominantly contains α2,3-linked SA (Couceiro et al., 1999). To test the content of the preparations we used, we probed the ferret nasal washes with SNA and MAA lectins. Both lectins bound to components in the nasal wash, demonstrating that soluble forms of α2,6-linked SA were abundant in these secretions (Fig. 6b).

**DISCUSSION**

It is clear that the binding specificity of the influenza virus HA protein for different linkages of SA receptor determines transmissibility in the ferret model, and probably also in humans. In the studies reported here, just two point mutations in the H3 HA receptor-binding pocket prevented virus transmission between animals in direct contact with each other. Droplet transmission is an even more stringent measure of transmissibility (Bouvier et al., 2008; Duan et al., 2010; Van Hoeven et al., 2009) so, although we did not test it here, there is no doubt that the receptor-binding mutation we generated would prevent all forms of virus transmission. Thus, our data concur with the notion that efficient α2,6-linked SA receptor binding is absolutely necessary (although it may not be sufficient) for transmission (Pappas et al., 2010; Tumpey et al., 2007).

The reason for the lack of transmission of a virus with preference for the avian type α2,3-linked SA receptor has not been assessed previously in detail. One explanation would be that less virus is shed from donor animals. This was inferred by studies performed in the guinea pig, where a human H1N1 virus (Tx/91) that was transmitted less efficiently than a human H3N2 strain (Pan/99) showed reduced virus shedding, and a lower titre of infectious virus was detected in the air (Mubareka et al., 2009). In addition, interferon treatment of donor guinea pigs infected with pandemic H1N1 2009 virus resulted in decreased titre of shed virus, and this eliminated onward transmission to untreated donors (Steel et al., 2010). In contrast, the K627E PB2 host range mutation introduced to a recombinant 1918 H1N1 influenza virus did not compromise the peak titre shed in the nasal wash of directly inoculated ferrets but did eliminate aerosol transmission (Van Hoeven et al., 2009). Likewise, there was little difference in the titres of wild-type virus and oseltamivir-resistant mutants shed from directly inoculated guinea pigs, but the mutants showed lower transmissibility (Bouvier et al., 2008). Similarly, ferrets and guinea pigs infected with H5N1 viruses tend to shed very high titres in infected nasal washes and in the air (Mubareka et al., 2009), as do infected humans (de Jong et al., 2006), yet these viruses have rarely been shown to transmit between mammals. The data we have presented here illustrate that there was no compromise in the titres of the receptor-binding mutant virus shed from donor animals.

In our experimental set-up, conscious nasal washing was performed daily, allowing a more detailed profile of the...
kinetics of virus shedding than has been reported in several other studies. We noted that the day of peak titre shedding shifted according to the inoculum dose, and the shed virus curve also changed shape, with high peaks and low troughs at higher inocula versus a smoother shape with several peaks at lower inocula. We suspect that this reflects the detailed interplay between the virus and the host innate response. It is interesting to note that the shape of the virus shedding curves in Fig. 3(b) and (c) from sentinel animals differed from that obtained from the donor animals that were directly inoculated with 10 p.f.u. (Fig. 3a). This might suggest that the dose the sentinels received was lower. However, establishing the dose that results in transmission will require more careful analysis.

The transmission-negative mutant we employed for our studies was based on a human strain of H3N2 influenza A virus, A/Victoria/3/75, with two mutations in the HA receptor-binding region, at residues 226 and 228 (Scull et al., 2009). Similar mutants have been described by others in the literature with characteristics akin to those we reported here. Matrosovich et al. (2007) showed that a 226/228 HA mutant H3N2 virus based on the 1968 Hong Kong pandemic strain was impeded in its spread through human airway cell cultures (Matrosovich et al., 2007), especially at low infectious doses, a scenario that may mimic the transmission bottleneck. Leigh et al. (1995) found that virus titres shed in the nasal wash of ferrets directly inoculated with H3N2 Q226L mutant virus were as high as those from animals infected with wild-type virus. In addition, the mutant was not (re)targeted to ferret lung (Leigh et al., 1995), despite predictions based on the anatomical distribution of SA in the ferret and human lung that viruses with avian virus-like receptor-binding profiles might be restricted to the distal respiratory tract (Shinya et al., 2006; van Riel et al., 2006). This is in accordance with
the data presented here, where, although only small numbers of animals were analysed, there was no difference in the anatomical location of infectious virus between wild-type or receptor mutant. Indeed, where lung infection has been detected, viruses that transmit between ferrets replicated to higher titres in the lung, where a 2,6-linked SA is in fact abundant, than those that did not (Sorrell et al., 2009; Xu et al., 2010). The lack of distal respiratory tract infection by wild-type or mutant virus in our experiments may be accounted for by the lower inoculum doses (10^5 p.f.u. or less), administered in a small volume (total 200 μl vs 1–3 ml used by other groups). This resulted in a localized infection of the nasal cavity that did not extend past the larynx, regardless of the receptor-binding properties of the virus.

The concept that transmission depends on sneezing has been mooted. Both groups of animals sneezed during the experiments, particularly after the nasal wash procedure. Moreover, we sent blinded sections of ferret nasal turbinate, trachea and lung for independent histopathological examination. Although the pathologist was able to identify which sections were from infected animals, there was no difference in the extent of pathology between animals infected with wild-type or mutant virus (data not shown). Thus, it is not clear from our studies that there is any relationship between influenza-like symptoms or pathogenesis and transmission of virus, although this requires further study.

Having shown that adequate virus was present in the noses of infected animals, we considered three barriers that might explain the lack of transmission of the Vic-226-228HA receptor-binding mutant. The first was the presence of respiratory secretions including mucus that lines the airways. We hypothesized that airway secretions might neutralize the infectivity of the mutant virus more efficiently than that of the wild-type. Indeed, seminal studies by Couceiro et al. (1993) showed that expectorated human mucus was better able to inhibit the haemagglutination of

**Fig. 5.** Susceptibility of nasal turbinate tissue to influenza virus infection. (a) A/Victoria/3/75 or Vic-226-228HA virus was used to infect ex vivo nasal turbinate tissue from a naïve ferret at 10^4 p.f.u. in 100 μl, in triplicate. Infected tissue was incubated at 37 °C and the virus released was titrated by plaque assay at regular time points. (b) Histological sections of naïve ferret nasal turbinate probed with biotin-conjugated MAA (to detect α2,3-linked SA) or SNA (to detect α2,6-linked SA) followed by streptavidin–Alexa Fluor 488 (Invitrogen) (green fluorescence) and counterstained with DAPI (blue fluorescence). Bars, 20 μm. The lower panels show phase-contrast images of the tissue.

**Fig. 6.** Inhibition of virus infectivity by respiratory mucus. (a) Wild-type A/Victoria/3/75 or Vic-226-228HA mutant virus was incubated with PBS or nasal wash from uninfected ferrets and titrated on MDCK cells. The remaining infectivity of nasal wash samples was compared with treatment with PBS alone and calculated as a percentage. Student’s t-test was used to assess the significance of the difference in infectivity remaining after incubation with nasal wash compared with incubation with PBS. The data shown represent four independent experiments. Results are shown as means ± SD. *, P<0.05. (b) Nasal wash from uninfected ferrets was probed for SA composition using the lectins SNA (to detect α2,6-linked SA) and MAA (to detect α2,3-linked SA). The blots were developed with streptavidin–HRP conjugate and ECL substrate.
and 2,6-resialated red blood cells by a receptor-binding mutant than by the wild-type parental virus. These results have been interpreted over the years to imply that human mucus contains predominantly 2,3-linked SA. However, we detected abundant SNA lectin-reactive material in ferret nasal washes and also in preparations of secretions harvested from human airway cells (data not shown). This suggests that components of airway secretions have high levels of 2,6-linked SA. Quantitative comparison of the levels of soluble 2,3, 2,6-linked and 2,6-linked SAs in these preparations was not possible because the degree of specific labelling of the lectins and their relative affinities are not known. An alternative explanation for the results of Couceiro et al. (1993) is that the rather poor affinity of their mutant virus for 2,6-resialated red blood cells was more readily inhibited by the SA in mucus than was the robust binding of the wild-type virus. Alternatively, the expectorated mucus preparations they utilized may have contained a higher amount of 2,3-linked SA than our preparations. Intriguingly, our experimental data showed that wild-type A/Victoria/3/75 virus was more efficiently blocked from entering cells after pre-incubation with ferret nasal wash secretions than was the Vic-226-228HA mutant virus. It has been described that human airway cultures secrete exosomes, small viral-sized membranous structures that are rich in 2,6-linked SA (Kesimer et al., 2009). Pre-incubation of A/Victoria/3/75 virus with exosomes efficiently neutralized virus infectivity in MDCK cells (Kesimer et al., 2009). We raise the possibility that exosomes account for a large proportion of the virus-inhibitory activity in the respiratory secretions tested here. The role of exosomes in influenza virus transmission remains to be explored. In addition, we speculate that adherence of wild-type virus to the 2,6-linked SA-containing mucus layer overlying the ciliated epithelium may prevent virus from being expelled by the ciliary beat of the respiratory escalator.

The second barrier that may limit the transmission of receptor-binding mutant virus between ferrets is the relative paucity of 2,3-linked SA receptors on target tissue in the ferret upper airway. We found that MAA lectin reacted with fewer cells in ferret nasal turbinate, a major early anatomical site for virus amplification, than did SNA, which practically coated the apical epithelium surface. Others have found much greater expression of 2,6-linked SA than of 2,3-linked SA on ferret trachea (Kirkeby et al., 2009; Leigh et al., 1995). Therefore, the chances of a virion with HA that binds well to 2,3-linked SA encountering a cell that is readily permissive to infection in the ferret airway are low. Indeed, direct inoculation of ferret nasal turbinate ex vivo indicated that the mutant virus was not readily amplified in this tissue.

Thirdly, we have shown previously that the ability of the mutant with avian virus-like receptor specificity to spread through human airway cells at low temperature is compromised in comparison with wild-type virus (Scull et al., 2009). Thus, during transmission events in the nose, successful initiation and then colonization may not ensue unless receptor binding is optimal. The mutant virus required a much higher dose to establish infection in directly inoculated animals than did the wild-type virus, which established infection at a dose of 5 p.f.u. Likewise, both Bouvier et al. (2008) and Steel et al. (2009) have suggested previously that ID50 correlates with transmissibility in the guinea pig model. Using our pair of viruses, the difference in ID50 was around 40-fold. We propose that the physical hurdles that virions encounter as they pass through the environment are so extreme that only a handful of particles reach susceptible target cells intact. Therefore, a virus that cannot establish infection at doses of less than 10 p.f.u. is unlikely to be transmitted efficiently, and minimum infectious dose may be a good indicator of transmissibility.

**METHODS**

**Cells.** MDCK cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Gibco, Invitrogen) supplemented with 10 % FBS and 1 % penicillin/streptomycin (Sigma-Aldrich).

**Viruses.** The recombinant viruses wild-type A/Victoria/3/75 (H3N2) and mutant Vic-226-228HA were generated by reverse genetics, as described previously (Elleman & Barclay, 2004; Neumann et al., 1999). Vic-226-228HA was constructed by substituting two amino acids in the HA gene (L226Q and S228G) that conferred an avian-like receptor-binding preference (Connor et al., 1994; Matrosovich et al., 1997).

**Plaque assay.** Plaque assays were performed using MDCK cells. Cell layers inoculated with 100 µl serially diluted samples were overlaid with 0.6 % agarose (Oxoid) in supplemented DMEM with 2 µg trypsin (Worthington) ml⁻¹ and incubated at 37 °C for 3 days.

**Animal studies.** Female ferrets (14–16 weeks old) weighing 400–650 g were used. After acclimatization, sera were obtained and tested by HAI assay for antibodies against A/Victoria/3/75 (H3N2) and by virus neutralization assay for antibodies against A/Brisbane/10/07, a recent H3N2 influenza A virus, or A/England/313/08, a recent H1N1 seasonal influenza virus. These experiments were performed before the emergence of the pandemic H1N1 2009 virus. All ferrets were negative for influenza antibodies at the start of the experiments. Ferrets were implanted subcutaneously with IPTT-300 transponders (BioMedic Data Systems) for identification and daily body temperature monitoring. Body weight was measured daily. Strict procedures were followed to prevent aberrant cross-contamination between animals. Sentinel animals were handled before inoculated animals, and work surfaces and handlers’ gloves were decontaminated between animals. For inoculation, ferrets were lightly anaesthetized with ketamine (22 mg kg⁻¹) and xylazine (0.9 mg kg⁻¹) and inoculated intranasally with virus diluted in PBS (0.1 ml per nostril). For transmission experiments, the basic study consisted of two inoculated donor ferrets, each co-housed for 24 h p.i. with three naïve sentinel animals. All animals were nasal washed daily, while conscious, by instilling 2 ml PBS into the nostrils, and the expectorate was collected in modified 250 ml centrifuge tubes. Antigen was detected in nasal wash samples using a Flu A Directigen kit (BD Diagnostics). BSA (0.3 %) was added to the nasal wash expectorate before infectious virus was titrated by plaque assay. The limit of virus detection in the plaque assays was 10 p.f.u. ml⁻¹. To collect tissue samples, inoculated animals were culled by intramuscular injection of ketamine (25 mg kg⁻¹) and xylazine (1.5 mg kg⁻¹), followed by intraperitoneal injection of a lethal overdose of sodium pentobarbital. Extracted
tissues were homogenized in PBS and the supernatants titrated by plaque assay.

**Ex vivo nasal turbinate infection.** Nasal turbinate tissue was extracted aseptically from a naïve ferret into serum-free DMEM containing 1% penicillin/streptomycin. Tissue was separated into equal-sized pieces (~3 mm³) and inoculated in triplicate with 10⁹ p.f.u. A/Victoria/3/75 or Vic-226-228HA virus in 100 µl and incubated at 37 °C for 2 h. The inoculum was then removed and the tissue sections were washed in fresh medium and returned to the incubator in 200 µl DMEM. At various time points, the medium was collected and replaced. The recovered virus was titrated by plaque assay.

**Lectin staining of ferret nasal turbinate.** Nasal turbinate tissue from a naïve ferret was extracted aseptically, fixed with buffered formalin and embedded in paraffin (ProPath). Cut sections were deparaffinized and rehydrated. Non-specific binding was blocked using 3% BSA in PBS for 1 h at room temperature. Sections were then probed with biotin-conjugated MAA lectin (EY Laboratories), which detects s2,3-linked SA, or biotin-conjugated SNA lectin (Vector Laboratories), which detects s2,6-linked SA, for 1 h, washed in blocking buffer and then incubated with streptavidin–Alexa Fluor 488 (Invitrogen). Sections were counterstained with DAPI and images were acquired using a Zeiss 510 Meta confocal microscope.

**Infectivity reduction assay.** Nasal wash was collected from uninfected ferrets and 0.3% BSA was added. Virus was diluted to 50–100 p.f.u. in 200 µl in nasal wash or in PBS containing 0.3% BSA and incubated for 1 h on ice. In triplicate, MDCK cells in six-well plates were infected with 200 µl per well of either mixture for 1 h at 37 °C. The monolayers were washed with PBS and overlaid as described for plaque assays. The percentage infectivity remaining compared with incubation with PBS alone was calculated.

**Immunoblotting with lectins.** Nasal wash from uninfected ferrets was applied to a nitrocellulose membrane (Amersham Biosciences) and allowed to dry. Non-specific binding was blocked using 3% BSA and 0.1% Tween 20 in PBS for 2 h at room temperature. The membranes were probed with biotin-conjugated SNA or MAA diluted in blocking buffer and incubated overnight at 4 °C. The monolayers were washed with PBS and overlaid as described for plaque assays. The percentage infectivity remaining compared with incubation with PBS alone was calculated.

**ACKNOWLEDGEMENTS**

We are grateful to Peter Stilwell for dedicated technical assistance and to the staff at Imperial College Central Biological Services for expert assistance with animal handling. This work was funded by MRC project grant G0600504.

**REFERENCES**


