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Influenza virus carrying an R292K mutation in the neuraminidase gene is not transmitted in ferrets

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Abstract

A model of influenza transmission has been established in ferrets in which wild-type influenza infection in a donor ferret can be transmitted sequentially to other ferrets. We have studied the transmission in ferrets of a clinical isolate of A/Sydney/5/97 (H3N2) carrying the neuraminidase 292K mutation compared with the corresponding wild-type virus from the same subject. Donor ferrets (n = four per group) were inoculated intranasally with mutant or wild-type virus and each housed with three naïve contact ferrets. All donor ferrets inoculated with wildtype virus were productively infected and transmitted virus to all 12 contacts, who in turn had high viral titres in their nasal washes. In contrast, only two of the donor ferrets inoculated with mutant virus were productively infected. There was little or no evidence that the two infected donor animals transmitted mutant virus to their contact animals. This ferret model has demonstrated that the mutant influenza virus with lysine at position 292 of the neuraminidase is of reduced infectivity and does not transmit under conditions in which the wild-type virus with arginine at position 292 readily transmits.

Keywords: Influenza; Ferret; Transmission; Neuraminidase; Resistance

1. Introduction

The ferret model of influenza infection is widely recognized as being the most relevant small animal model of influenza infection used in influenza research (Smith and Sweet, 1988; Toms et al., 1976; Reuman et al., 1989). The course of infection and resultant disease symptoms displayed by infected ferrets closely resemble the manifestation of influenza illness in man. The ferret model has been used to investigate influenza pathology (Alluwaimi et al., 1994) and to evaluate vaccine efficacy (Li et al., 1999) and more recently the efficacy of the influenza neuraminidase inhibitors (NAI), oseltamivir phosphate (Mendel et al., 1998) and zanamivir (Waghorn and Goa 1998; Ryan et al., 1995). The ferret model of influenza infection has also been used to test the potential

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of a drug to generate resistant virus (Herlocher et al., 2001a,b) and to assess the fitness of any drug-resistant viruses that have arisen (Blick et al., 1998; Sweet et al., 1991; Ives et al., 2000a,b; Carr et al., 2000).

The predictive value of such experiments in ferrets to the human situation is high, given previous experience with generating influenza resistance to amantadine (Herlocher et al., 2001a,b; Truscon et al., 2001) and with assessment of the relative fitness of rimantadine resistant clinical isolates. Virus isolates which carried mutations in the M2 channel that conferred rimantadine resistance were shown to be equivalent to wild-type virus in terms of fitness and pathogenicity (Sweet et al., 1991). This finding suggested that rimantadine resistant viruses would be capable of transmission from man to man, and transmission of the rimantadine resistant viruses in the family setting is a significant clinical problem (Hayden et al., 1989). It is the rapid and frequent generation of resistant virus that remains fully fit and transmissible which has limited the usefulness of amantadine and rimantadine in treatment of influenza illness. Additionally, the agents are not active against influenza B. NAIs, which represent a new class of anti-influenza agent, are specific and potent inhibitors of neuraminidase of all influenza strains and subtypes tested (Kati, 1998; Woods, 1993).

As with other antiviral agents, with increasing use of the NAIs there exists the potential for the emergence of virus with decreased sensitivity to the treatment drug. However, unlike other chronic virus infections, in which viruses resistant to drug have become a serious problem (HIV, HBV, HSV, CMV) influenza is an acute self-limiting illness in which viral clearance is the norm. Therefore, emergence of drug resistant influenza virus would be expected to be of little clinical consequence to the individual, but may be of epidemiological consequence should such viruses transmit.

Oseltamivir phosphate resistant post treatment virus samples were plaque purified following isolation from subjects taking part in clinical trials. The ferret model has been used to assess the virulence of virus isolates carrying mutations in the NA gene (E119V, R292K, and H274Y; Ives et al., 2000a,b; Carr et al., 2000). All three mutations significantly compromised whole virus infectivity and pathogenicity compared with the respective wild-type counterparts. These findings led to the prediction that transmission of these resistant viruses would be unlikely to occur in a clinical setting.

Classical experimentation by Andrewes and Glover has determined that influenza virus may transmit from infected ferret to uninfected ferret by the airborne route (Andrewes and Glover, 1941). There are other early documented incidences of cross-infection occurring between ferrets housed in close contact (Squires and Belyavin, 1975). These early experiments employed a rise in rectal temperature and observation of some nasal symptoms to detect infection. More recently sequential transmission through five successive in vivo passages has been reported in a study which assessed mutation of viral HA as an immune evasion strategy (Herlocher et al., 2001b).

Infection in a donor ferret can be transmitted sequentially from ferret to ferret with a transmission and replication rate of 100%. This ferret model of influenza virus transmission was employed in this study to further investigate the transmission potential of the R292K mutant virus. Here we have demonstrated that R292K NA virus transmission does not occur under conditions in which wild-type virus transmits readily.

2. Materials and methods

2.1. Overview of experimental design

Influenza virus subtype A/Sydney/5/97 (H3N2) was isolated from pre and last post treatment virus positive (day 4) nasal swabs from a patient on the treatment arm of a Phase III efficacy study for oseltamivir phosphate. The virus was expanded in Monkey Kidney (MK) primary cell culture before undergoing further rounds of expansion and plaque purification in MDCK cells, to provide the stocks of virus used in these experiments. The predicted amino acid sequence of NA protein in the purified and expanded virus from

the pre-treatment sample retained Arg at position 292 of the NA gene, and viral NA from the post-treatment sample contained the Lys for Arg substitution at position 292.

All ferrets were seronegative for influenza A/Wuhan/395/95 (H3N2) at the start of the experiment. Groups of four ferrets were infected intranasally with equal challenge titers of either influenza A/Sydney/5/95 (H3N2) wild type or mutant virus (equivalent to 2.3 $\log_{10}$ $TCID_{50}$). Three naïve ferrets (recipients) were then housed with each infected ferret (donor) per cage immediately following infection (32 ferrets used in total). Wild-type and mutant virus infected animals were kept in separate rooms. Naïve ferrets which did not become infected from donors were co-housed with donors for the duration of the 'in-life' phase of this experiment (17 days). The Directigen Flu A test (Becton Dickinson Microbiological Systems) was used for early detection of infection in the recipient animals, and once a naïve ferret became infected it was removed from the cage to prevent lateral transmission occurring. The full course of infection in both donor and recipient animals was monitored by titration of virus on MDCK cells from daily nasal washes. The seroconversion status of all ferrets was assessed 28 days after the end of the in-life phase, by standard hemagglutinin inhibition (HAI) assay. The viruses isolated from ferret nasal washes were confirmed either mutant, wild-type or mixed population by sequence analysis of the NA gene, performed by Dr Karin Soderbarg at Professional Genetics Laboratories, Uppsala, Sweden.

2.2. Ferret supply and immune status assessment

Male ferrets at 6 weeks of age were supplied by Marshall Farms, North Rose, NY, USA and weighed approximately 0.5 kg. All ferrets were confirmed seronegative for influenza H3N2. Animals were anesthetized and cardiac bled at Marshall Farms. Approximately 5 ml blood were collected and centrifuged to collect sera. All serum was inactivated using receptor destroying enzyme (RDE); preinfection serum was tested using A/Wuhan/359/95 as antigen. About 50 μl inactivated serum were added to well 1 of a 96 well plate and were serially diluted 1:2 through 11 wells. Twenty five microliter antigen with 4 HA U were added to wells 1 through 11 starting with well 11 and allowed to incubate at room temperature for 30 min. Fifty microliter of 0.50% chicken red blood cells (CRBC's) were added to each well starting with well 12 and working backwards. Plates were read for agglutination after 12–20 min. Only animals with titers of <2.5 or 5 were accepted for use in the study.

The animals were then treated once daily for 3 days with 0.1 ml Durapen (300 000 U combination Penicillin) before the day of infection with challenge virus, to eradicate any bacterial infection they may have been carrying.

2.3. Infection of ferrets with wild-type or mutant R292K NA influenza A/Sydney/5/97

Ferrets were lightly anesthetized with 10 mg/kg-ketamine containing 1 mg/kg rompun. They were inoculated intranasally with 0.5 ml (0.25 ml into each nostril) volume of wild-type or 292K NA influenza virus A/Sydney. Both wild-type and mutant virus challenge inoculum contained equal titers of infectious virus (2.3 $\log_{10}$ $TCID_{50}$/0.5 ml).

2.4. Ferret housing

Three naïve ferrets (recipients) were housed with each infected ferret (donor) per cage immediately following infection of donor. Wild-type and mutant virus infected animals were kept in separate rooms. Naïve ferrets that did not become infected from donors were co-housed with donors for the duration of the 'in-life' phase of the experiment (17 days). The Directigen Flu A test (Becton Dickinson Microbiological Systems) was used for early detection of infection in the recipient animals, and once a naïve ferret became infected it was removed from the cage to prevent lateral transmission from occurring.

2.5. Ferret temperatures

Ferret temperatures were recorded twice daily beginning the afternoon after infection and then at 09:00 and 15:00 h on each consecutive day up

until day 14 post infection for the donor ferrets and day 17 post infection for the recipient ferrets. All temperatures were analyzed for significant fever by calculating the number of standard deviations (S.D.) from a normal average temperature of 101.5 °F (obtained by averaging 72 normal ferret temperatures). A temperature of 102.8 °F was three S.D.'s from the normal average temperature and was considered fever.

2.6. Nasal wash collection from donor and recipient ferrets

Nasal washes were collected daily at the same time each day from all ferrets, beginning on day 1 post infection of donor animals. Nasal wash was collected for 14 days from donors and for 17 days from recipients. Three milliliter sterile PBS was administered intranasally using tom kat catheters, and the sneezed wash was collected and volume recorded. Directigen A positivity, viral titers and viral NA and HA sequences were determined from these wash samples.

2.7. Viral titer determination from ferret nasal wash samples

Nasal wash samples were used to infect confluent MDCK cells in a 96-well format. Briefly, the cells were rinsed free of culture media containing 10% FCS with PBS, and the culture media was replaced with trypsin-containing media to support virus replication (MEM containing 25 mM Hepes, 200 mM Penicillin/Streptomycin and 1.25 g/ml TPCK trypsin (trypsin treated with L(tosylamido-1-phenyl) ethyl chloromethyl ketone). Nasal wash sample, 20 μl, was added to 180 μl media in the first row of wells in quadruplicate and serial ten-fold dilutions were made from each well containing sample across the plate. Adsorption took place over a 2 h incubation at 340 °C/5%CO_2. Excess virus was removed from the plate and fresh media was reapplied. The plates were incubated for a further 7 days, and aliquots of the culture supernatants were tested for HA positivity on day 7. Virus titers for each time point were calculated using the Spearman–Karber equation (Finney, 1952).

2.8. Influenza virus NA gene sequence analysis

A novel method for the sequencing of NA genes (residues 100 to the C terminus) was developed and carried out by Professional Genetics Laboratory, Uppsala, Sweden. In brief the methods used were, viral RNA isolation from ferret nasal wash followed by reverse transcription polymerase chain reaction (RT-PCR) to obtain cDNA. The NA gene is then amplified by nested PCR as two overlapping fragments, using a specific primer set designed for the NA subtype N2. The DNA-sequencing reactions use T7 DNA-polymerase and solid phase technique in a Pharmacia ALF Express autosequencer.

2.9. Seroconversion on day 28 post infection of donor ferrets

All ferrets, donors and recipients, were bled on day 28 post infection of the donor animals, in order to assess seroconversion status. HAIs were carried out on pre- and post-infection sera as described in Section 2. A four-fold rise in antibody titer as determined by this means was considered a positive seroconversion.

2.10. Statistical analysis

Virus titers were compared by Student's *t*-test analysis of AUC (area under the curve) using days 1 through 14 post infection.

3. Results

3.1. Infectivity of wild-type and R292K NA influenza A/Sydney/5/97

All four donor animals infected directly with wild-type virus via intranasal administration became virus positive on day 3 post exposure as determined by the Directigen A test, and in fact were virus positive earlier (on day 2) as subsequently determined by the more sensitive titration of nasal wash sample on MDCK cells. By contrast, just two from four donor animals exposed to an equivalent challenge dose of mutant virus

became virus positive on day 3 using the Directigen A test, and on days 1 and 2 post exposure by MDCK cell culture of wash sample. All animals that tested positive by Directigen A and subsequent MDCK cell culture were positive for 2 or more days. The day 12 through day 14 samples were negative by both assay means.

3.2. Transmissibility of wild-type and R292K NA influenza A/Sydney/5/97

All 12 of the recipients of the four wild-type infected donors tested positive for wildtype virus (Table 1, Fig. 1a–d). There were no unequivocal cases in which recipient animals co-housed with mutant virus infected donors were found to carry mutant virus (Table 1, Fig. 2a–b). The average time taken for wild-type virus to transmit (i.e. between first day positive wildtype infected donors and first day positive wild-type recipients) was 2 days ± 0.5. The 2-day delay in transmission of virus from infected donors to uninfected recipients was consistent with other reports on transmission (Andrewes and Glover, 1941; Squires and Belyavin, 1975; Herlocher et al., 2001b). Since only two of the donor animals exposed to mutant virus became productively infected there were just two donor animals (donors five and six) and, therefore, six recipient animals that were valid for assessment of transmissibility of mutant virus. Of these six recipient animals, all three housed with donor five became virus positive by MDCK cell culture (Fig. 2a), but subsequent sequence analysis of the NA gene in samples from these animals revealed that they were in fact carrying wild-type virus (Table 3). NA gene sequence analysis of the virus shed from each time-point by donor five determined that a switch in genotype from mutant NA to wild-type had occurred in the donor animal (Table 3) and thus it was wild-type virus that had transmitted. For the remaining three recipients of the other mutant virus infected donor, one animal only gave a virus positive sample on day 9 post infection, as determined by MDCK cell culture (Fig. 2b). The quantity of virus measured following culture expansion in this way was only very slightly above the limit of detection and titer was detected only on day 9. The same nasal wash sample was virus negative by both Directigen Flu A test and PCR assay, and, therefore, it was not possible to confirm the NA sequence carried by this virus (Table 3). One sequence analysis of virus carried by donor six revealed that this animal also carried a wild-type phenotype, (probably as a mixed population) on at least 1 day post infection with mutant; the second sequence analysis indicated that this animal carried virus with the mutant genotype on each day tested. Since recipient ferret one exposed to mutant donor six did not seroconvert and produced no PCR result, the low titer observed on day 9 was considered a spurious result.

Table 1
Frequency of virus positivity in donor and recipient ferrets (on any day post infection) by MDCK culture

	Donors	Recipients
	%Infectivity	%Transmissibility
WT	100% (4/4)	100% (12/12)
Mutant	50% (2/4)	0% (3*/6)

*, The three positive recipients co-housed with an R292K virus infected donor were subsequently confirmed to be carrying wild-type virus, transmitted from the donor animal following a reversion event on day 6. The other virus positive recipient exposed to mutant virus had one very low $TCID_{50}$ on day 9 only, could not be verified by PCR analysis and did not seroconvert; thus it is not included in the 3/6 listed above.

3.3. Comparison of viral titers shed from donor and recipient ferrets infected with or exposed to wild-type or mutant influenza A/Sydney/5/97

High viral titers were recovered from nasal wash of all four wild-type virus infected donor ferrets (Fig. 1a), consistent with other published data on influenza infection in ferrets (Ryan et al., 1995). For three of the four donor animals, peak virus titers were reached rapidly following infection. For the fourth donor animal (donor four) the first positive sample (containing the highest viral titer) was not obtained until day 9 post infection. Since this same animal had tested positive for influenza virus by Directigen test on day 3 post infection, and because the recipient ferrets housed with donor four also became positive for

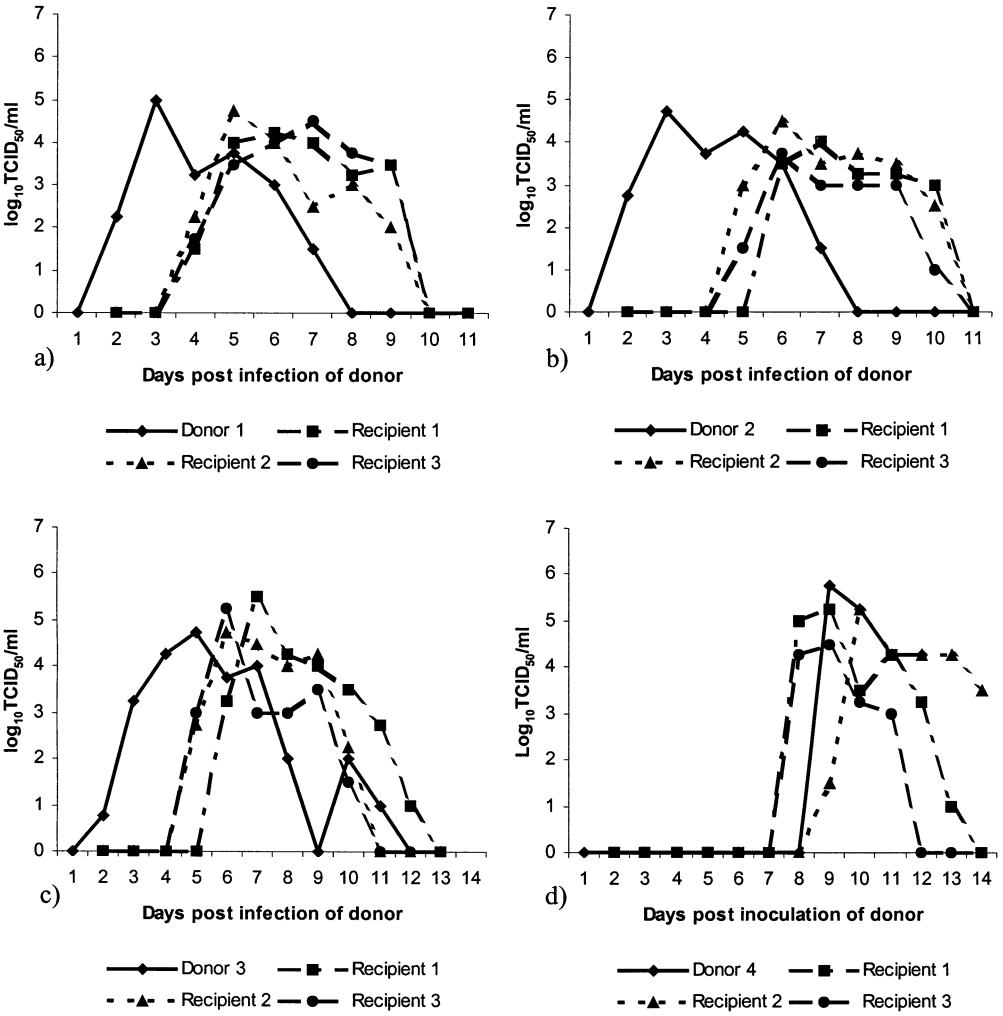

Fig. 1. Virus titers from nasal washes, time course of wild-type infection and transmission. In (a), (b), (c), and (d) all virus recovered from the nasal washes was WT virus.

influenza virus at time points ahead of day 9, it is most likely that this observation resulted from an MDCK assay failure to detect virus from this animal at earlier time-points.

Analysis of nasal washes from the twelve recipient ferrets housed with the wild-type infected donors gave similar profiles of virus recovery over time (Fig. 1a), and there was no difference between the average peak virus titer recovered from donors and recipients as determined by Student's *t*-test analysis of AUCs (Table 2).

The amount of virus recovered from nasal wash of both donor ferrets productively infected with mutant virus was significantly less than that recovered from the wild-type donors (Table 2, $P = 0.02$). This finding was based on a very conservative analysis of AUCs of donors five and six which allowed inclusion of viral titers from all time-points from donor five, even though this animal was subsequently shown to be carrying wild-type virus on day 6, the first time-point that significant quantities of virus were recovered (Fig. 2a). There was little similarity between the profile of virus recovery from mutant virus infected

donors and recipients. Only one recipient animal housed with donor six gave a single virus positive sample as determined by MDCK cell expansion assay, however, the genotype of this virus was unknown and the animal didnot seroconvert.

3.4. Comparison of serology on donor and recipient ferrets infected with or exposed to wildtype or mutant influenza A/Sydney/5/97

There was complete concordance between the virological analysis and the seroconversion status on day 28 post infection of all wild-type virus infected donor and recipient ferrets (Table 4). Although the same was not true for the ferrets challenged with mutant virus, rise in antibody titer for the ferrets exposed to wild type virus averaged 38, whereas rise of antibody titer for the ferrets exposed to mutant virus averaged 1.3. A rise in titer of four is the lowest rise considered positive and the observed titer may vary by one well in successive tests; therefore, the positive rise in titer of recipient three exposed to mutant eight could well be spurious. Three from the four donor animals infected directly with mutant virus seroconverted, whereas only two of these animals had a productive infection, such that virus was recovered from the nasal washes. Three from a possible six recipient ferrets seroconverted following 17 days co-housed with the donor animals challenged with mutant virus. Two of these ferrets had productive wild-type infections and were housed with the donor ferret who reverted to wild-type. The third was not positive for productive infection and was housed with donor ferret six which shed mixed wild type and mutant virus, evidenced by the NA sequence analysis presented in Table 3. The ferret that did exhibit a 1 day low viral titer did not seroconvert. The antibody titers in these animals do not indicate whether wild-type or mutant virus was the transmitting agent.

3.5. NA gene sequence analysis of influenza A/Sydney/5/97 in donor and recipient ferret nasal washes

The sequence of NA genes from virus carried by all ferrets (donor or recipient) infected with wild-type virus was determined for time-points late during the course of infection. In order to facilitate successful PCR analysis, samples known to be of high viral titer from MDCK cell culture were selected as late during the infection period as possible. For wild-type virus infected donor animals this ranged from day 6 to 12 post infection. For wild-type recipient ferrets, the last day high

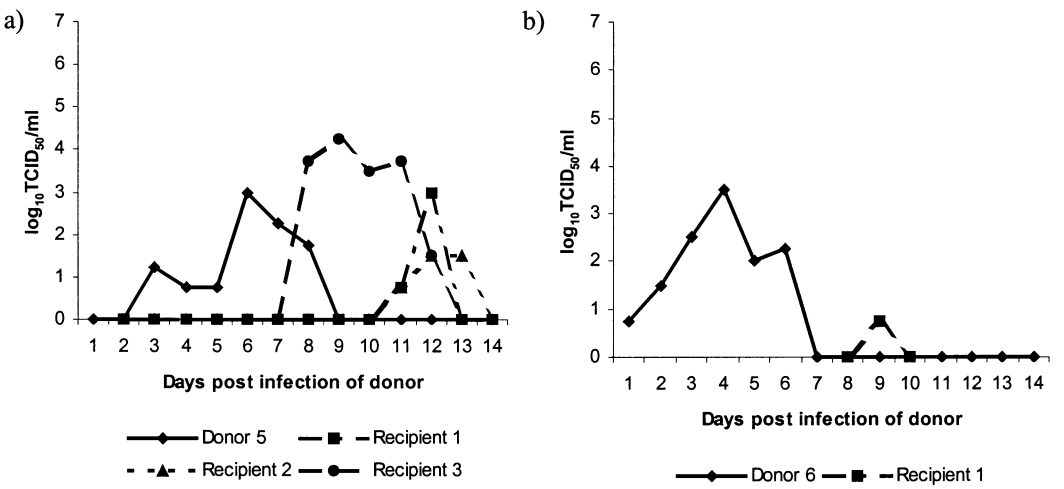

Fig. 2. Virus titers from nasal washes, time course of mutant infection and transmission. In (a), all virus recovered from the recipients was WT virus. In (b) the viral titer retrieved on day 9 was too low to do genetic sequencing. Ferret donors seven and eight did not shed virus either by Directigen testing or by $TCID_{50}$ titer.

Table 2
Mean AUC values

	N	Peak virus titer ($\log_{10}$ $TCID_{50}$/ml)	Mean AUC	*P*-values
Wild type donors	4	4.75 ± 0.41	21.13 ± 1.58	
Wild type recipients	12	4.69 ± 0.16	19.94 ± 0.87	WT donors vs. WT recipients $P = 0.5$ (NS)
Mutant donors	2	3.25 ± 0.25	11.44 ± 1.69	WT donors vs. mutant donors $P = 0.02$
Mutant recipients	1	0.75 ± 0.00	0.75 ± 0.00	Mutant donors vs. mutant recipients $P < 0.001$

AUC calculated from viral titers obtained day 1 through day 14 post infection of donors.

Table 3
Sequencing of mutant nasal washes

Status	Day	Sequence 1	Sequence 2
Mutant donor 5	3	292Lys (Mutant)	292Lys (Mutant)
	4	292Lys (Mutant)	292Lys (Mutant)
	5	292Lys (Mutant)	292Lys (Mutant)
	6	Arg292 (WT)	292Lys (Mutant)
	7	Arg292Lys (mixed)	292Lys (Mutant)
	8	Arg292Lys (mixed)	Arg292 (WT)
Recipient 1	12	Arg292 (WT)	Arg292 (WT)
Recipient 2	11	PCR Neg	PCR Neg
	12	Arg292 (WT)	Arg292 (WT)
	13	PCR Neg	PCR Neg
Recipient 3	8	Arg292 (WT)	Arg292 (WT)
	9	Arg 292 (WT)	Arg292 (WT)
	10	Arg292 (WT)	Arg292 (WT)
	11	Arg292 (WT)	Arg292 (WT)
	12	Arg292 (WT)	Arg292 (WT)
Mutant Donor 6	1	Arg292 (WT)*	PCR Neg
	2	PCR Neg*	292Lys (Mutant)
	3	292Lys (Mutant)	292Lys (Mutant)
	4	292Lys (Mutant)	PCR Neg
	5	292Lys (Mutant)	292Lys (Mutant)
	6	292Lys (Mutant)	292Lys (Mutant)
Recipient 1	9	PCR Neg	PCR Neg

*, Second aliquots of the same nasal wash samples were re-sequenced, and on the second attempt, the mutant donor six day 1 sample was PCR negative, and the day 2 sample gave a mutant virus NA sequence. All other timepoints gave mutant virus NA sequences.

titer samples sequenced ranged from day 9 to 14 post infection. All ferrets that were infected with or exposed to wild-type virus were shown to have shed virus that retained arginine at position 292 on the NA gene (donor animals tested between days 6 and 12 post infection, and recipient animals tested between days 9 and 14 post donor infection). This was an expected finding, given the conserved nature of this residue, but nonetheless confirmed that the wild-type NA genotype was stable during passage in ferrets, consistent with previously reported data (Ives et al., 2000a).

The NA gene in all virus positive samples from donors and recipients infected with or exposed to

mutant virus were sequenced twice and results are presented in Table 3. Donor five carried mutant virus until day 6 (first sequencing) or day 8 (second sequencing) at which time wild-type NA was detected apparently as the only species. However, NA analysis of time points subsequent to day 6 (first sequencing) showed that mixed populations of both mutant and wild-type NA were present, whereas the second sequencing detected only the mutant genotype until day 8. On day 8 the sequence reverted to wild-type sequence. Importantly, none of the recipients of donor five carried mutant virus at any time-point postinfection, as determined by NA sequence analysis, supporting the conclusion that only wild-type virus had transmitted. The first sequen-

Table 4
Seroconversion of ferrets 28 days post infection of donors

Group status	Pre-titer	Post-titer	Rise in titer	Seroconversion
WT Donor 1	<5	80	32	+
Recipient 1	5	40	8	+
Recipient 2	<5	160	64	+
Recipient 3	<5	80	32	+
WT Donor 2	<5	80	32	+
Recipient 1	5	80	16	+
Recipient 2	<5	80	32	+
Recipient 3	5	80	16	+
WT Donor 3	5	80	16	+
Recipient 1	<5	160	64	+
Recipient 2	<5	160	64	+
Recipient 3	<5	80	32	+
WT Donor 4	10	160	16	+
Recipient 1	<5	320	128	+
Recipient 2	5	160	32	+
Recipient 3	5	80	16	+
292K Mutant				
Donor 5	<5	20	8	+
Recipient 1	<5	10	4	+
Recipient 2	<5	<5	0	–
Recipient 3	<5	160	64	+
292K Mutant				
Donor 6	<5	10	4	+
Recipient 1	<5	<5	0	–
Recipient 2	<5	10	4	+
Recipient 3	<5	<5	0	–
292K Mutant				
Donor 7	<5	<5	0	–
Recipient 1	<5	<5	0	–
Recipient 2	<5	<5	0	–
Recipient 3	<5	<5	0	–
292K Mutant				
Donor 8	5	20	4	+
Recipient 1	<5	<5	0	–
Recipient 2	<5	<5	0	–
Recipient 3	5	20	4	+

Pre and post-infection serum antibody titers against A/N2 were measured by HA inhibition assay and a four-fold rise in titer was considered a positive seroconversion; <5 is given the value of 2.5 for the purposes of calculation.

cing detected wild-type rather than mutant virus following amplification on day 1 post infection from donor ferret six. The sequencing reaction detected mutant viral NA as the only population at the time points subsequent to this. The reason why wild-type virus should be detected at a time-point early in infection only is not clear; the second sequencing attempt failed to detect any viral NA at all in the first time-point sample and detected only mutant NA thereafter. It may be that very low (non-detectable) levels of wild-type virus were present at all time-points in this donor animal. Day 9 nasal wash sample from the recipient ferret with low titer on that single day (housed with donor six) tested negative by PCR.

3.6. Rectal temperatures of donor and recipient animals infected with wild-type and mutant virus

Influenza infection in ferrets is characterized by a rectal temperature rise from around 100 to 105 °F (Squires and Belyavin, 1975), or from around 37–39 °C (Reuman et al., 1989). Although the temperature spike is routinely assessed in studies of experimental influenza infection in ferrets, it is acknowledged that it is difficult to obtain reliable data by this means (Matsuyama et al., 1980). Additionally the ability of a given strain of influenza to induce a temperature spike in ferrets, (and indeed man) varies from strain to strain (Maassab et al., 1982). Although rectal temperatures of all donor and recipient ferrets were recorded twice daily during these experiments, there were no clear temperature spikes observed for either wild-type or mutant virus infected animals. Therefore, it was not possible to draw any conclusions regarding comparative viral fitness and pathogenicity of mutant and wild type virus from these data (not shown).

4. Discussion

The ferret model of transmission of influenza virus has been used to assess the transmissibility of R292K NA influenza virus. The data presented here provides little or no evidence that this virus was able to transmit from an infected animal to an uninfected animal under conditions in which wild-type virus transmitted readily and supports evidence that this virus was less fit than the wild-type virus.

The assessment of fitness and transmission of R292K virus was important since this mutation has been shown to be selected by all three NAIs (zanamivir, oseltamivir phosphate, BCX 1812), and to have reduced sensitivity to all three drugs. It had previously been reported in an A/N2 background in a mutant virus with zanamivir (Gubareva et al., 1997) and in an A/N9 background in the presence of a zanamivir derivative (McKimm-Breschkin et al., 1998). R292K NA has also been selected by BCX 1812 and shown to have reduced sensitivity to inhibition by BCX 1812, zanamivir and oseltamivir carboxylate (Bantia, et al., 2000). When oseltamivir phosphate and zanamivir selected viruses carrying the R292K mutation were tested for virulence in mice all were poorly infective compared with the parent virus from which they were derived (McKimm-Breschkin, 2000). It was also the most common mutation that arose clinically, although it was still a very rare event. The clinical isolate carrying R292K NA selected by oseltamivir phosphate treatment was shown to have reduced infectivity in ferrets (Ives et al., 2000a,b).

The reduced fitness of R292K NA virus, which is directly related to the failure of this virus to transmit, is documented elsewhere (Ives et al., 2000a,b; Tai et al., 1998). Briefly, R292K mutant NA selected by oseltamivir carboxylate in vitro in an A/Victoria/3/75 (H3N2) background was about 30000-fold less sensitive to inhibition by oseltamivir carboxylate than the corresponding wild-type. The catalytic activity of the NA was decreased to about 2% of that of wild-type with the mutation affecting both K_m and V_{max}. R292K NA virus selected by oseltamivir carboxylate had reduced replicative ability in vitro in MDCK cells and in eggs.

In the design of these experiments we aimed to mimic the clinical situation as closely as possible. Since the recipient ferrets were co-housed with infected donors immediately following the infection procedure, it is possible that virus spread

from the donor occurred via direct contact between the ferrets, rather than by the airborne route described by Andrewes and Glover (1941). Allowing close contact between the ferrets from the time of donor infection onwards more closely mimics the situation for transmission of influenza virus in a family setting, in which direct contact between family members is likely to be a route of transmission equally important as the airborne route.

Donor animals were infected with wild-type and mutant virus challenge doses of equivalent infectious dose titer. The actual dose was determined from a previous range-finding experiment with wild-type virus only. We were able to demonstrate titers of between 10^4 and 10^5 in the nasal wash of all four ferrets who were infected with 2.3 $\log_{10}$ $TCID_{50}$/ml of wild-type virus. In contrast, we were unable to demonstrate any titer in two of the ferrets who were infected with 2.3 $\log_{10}$ $TCID_{50}$/ml of mutant virus. The two donor ferrets that were infected with mutant virus shed virus in the nasal wash at a titer one to two logs lower than donors infected with wild-type virus. Virus bearing a mutation of arginine to lysine at position 292 of the NA gene is, therefore, less infective than is the wild type virus with arginine at that position.

Transmission of influenza virus from man to man is by the respiratory route, and the number of infectious particles a recipient is exposed to by the airborne route or by direct contact is a determining factor as to whether or not that individual becomes infected. In a situation where a patient might develop virus carrying mutant R292K NA, the likelihood of this virus transmitting would depend on the number of mutant virus infectious units that were shed to expose others to possible infection. The replicative competence is, therefore, an integral feature of the R292K NA virus that must be taken into account in the assessment of transmission experiments.

It might be expected that if a considerably higher virus challenge dose (to compensate for the intrinsic reduced infectivity and/or replicative ability of mutant virus) had been used to infect donor animals then this would have resulted in greater viral shed in these animals, thereby increasing the likelihood of transmission to the recipients.

All 12 recipient ferrets exposed to donor ferrets that were infected with wild-type virus were infected by transmission. This data is consistent with previous experiments (Herlocher et al., 2001b) where all recipient ferrets have been uniformly infected with wild-type virus by transmission. However, since only two donor animals became infected with the mutant virus, we have only six recipient animals to assess for transmission rather than twelve. Three of those six ferrets were infected by mutant donor ferret five who, although infected with mutant virus, shed wild-type virus. The three recipients also shed wild-type virus. Thus we had only one donor ferret, ferret six, infected with mutant virus and shedding mutant virus, which failed to transmit the virus to all three recipient ferrets. We are confident from previous studies (Herlocher et al., 2001b) that, had these recipient ferrets which were exposed to mutant virus been exposed to wild-type virus, they would have become infected.

Of particular note from the experiment reported here is the demonstration that where mutant and wild-type virus were present as a mixed population in one donor animal, only wild-type virus transmitted to all three recipient animals. Whether the route of transmission of virus was airborne or by direct contact, there remains a clear difference in the capability of wild type and mutant virus to transmit. As well as providing an internal control for this experiment, this finding has important clinical implications. Wherever R292K NA virus might arise in a drugtreated patient, it would almost certainly do so as part of a mixed population with wild-type virus. In this situation, our data suggest that wild-type rather than mutant virus would be preferentially transmitted.

The marked reduction in viral fitness effected by the R292K mutation in the NA gene indicated that virus carrying this mutation would be unlikely to transmit in a clinical setting. This reduced fitness is an important feature of viruses with reduced sensitivity to NA inhibitors. It distinguishes the NA inhibitors as a class from amantadine and rimantadine, the use of which

resulted in drug-resistant virus readily arising which remained infectious and pathogenic (Hayden and Hay, 1992). The demonstration that clinically relevant levels of R292K NA virus does not transmit in ferrets provides further reassurance that this virus is unlikely to transmit from man to man to form a pool of influenza virus with cross-resistance to the NAIs.

Clinical isolates carrying the other two NA mutations (H274Y and E119V) that have arisen with oseltamivir phosphate treatment have also been assessed for fitness and shown to be compromised in terms of infectivity and replicative ability (Carr et al., 1999, 2000). Although these isolates have yet to be assessed for transmissibility in the ferret model, a mathematical model has been derived to predict the likelihood of any of the three mutant viruses transmitting during community use of NAIs for treatment and/or prophylaxis of influenza (Ferguson and Mallet, 2001). The model predicted that there would be no significant transmission of resistant virus due to the low incidence and low level of relative fitness of the mutant viruses. Therefore, the incidence of resistance is likely to remain at the de novo rate among treated individuals.

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