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Antiviral Research 55 (2002) 307–317

Antiviral Research

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The H274Y mutation in the influenza A/H1N1 neuraminidase active site following oseltamivir phosphate treatment leave virus severely compromised both in vitro and in vivo

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Received 21 November 2001; accepted 1 April 2002

Abstract

Oseltamivir carboxylate is a potent and specific inhibitor of influenza A and B neuraminidase (NA). Oseltamivir phosphate, the ethyl ester prodrug of oseltamivir carboxylate, is the first orally active NA inhibitor available for the prophylaxis and treatment of influenza A and B. It offers an improvement over amantadine and rimantadine which are active only against influenza A and rapidly generate resistant virus. The emergence of virus resistant to oseltamivir carboxylate in the treatment of naturally acquired influenza infection is low (about 1%). The types of NA mutation to arise are sub-type specific and largely predicted from in vitro drug selection studies. A substitution of the conserved histidine at position 274 for tyrosine in the NA active site has been selected via site directed mutagenesis, serial passage in culture under drug pressure in H1N1 and during the treatment of experimental H1N1 infection in man. Virus carrying H274Y NA enzyme selected in vivo has reduced sensitivity to oseltamivir carboxylate. The replicative ability in cell culture was reduced up to 3 logs, as was infectivity in animal models of influenza virus infection. Additionally, pathogenicity of the mutant virus is significantly compromised in ferret, compared to the corresponding wild type virus. Virus carrying a H274Y mutation is unlikely to be of clinical consequence in man.

Keywords: Oseltamivir phosphate; Influenza; Neuraminidase; Resistance; Animal models

1. Introduction

The neuraminidase inhibitors (NAI) are a new class of anti-influenza agent. Currently there are

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0166-3542/02/$ - see front matter
PII: S0166-3542(02)00053-0

two NAI's, zanamivir (Relenza) and oseltamivir phosphate (Tamiflu, Ro64-0796, GS4104), in clinical use. The compounds are specific and potent inhibitors of influenza viral neuraminidase (NA) (Kim et al., 1997). Oseltamivir carboxylate, the active metabolite of oseltamivir phosphate, is the first orally active inhibitor of influenza NA (Kim et al., 1997; Mendel et al., 1998; Hayden et al., 1999). This affords ease of administration compared with the inhaled formulation of zanamivir, for which efficacy depends upon correct use of an inhaler delivery system. Oseltamivir carboxylate binds directly to the NA active site. This binding relies partly on hydrophobic interactions of the inhibitor with conserved amino acid residues within the enzyme active site (Kim et al., 1997). One residue within the viral NA active site shown to be important for oseltamivir carboxylate hydrophobic binding is glutamic acid 276. This lies in close proximity to histidine 274 (N2 numbering) (Lentz et al., 1987; Wang et al., 2000b). The implications of their interaction will be discussed here.

As with all antiviral drugs, selection of drug resistant virus in the clinic presents a potential problem; for example, in treatment of Human Immunodeficiency Virus infection where multiple drug resistant viruses emerge. The experience of resistance generation in influenza virus prior to the introduction of NAI was with the M2 channel blockers, amantadine and rimantadine. Drug resistant virus emerges rapidly following one or two passages in vitro in the presence of amantadine and rimantadine or within 48 h of treatment in man. These drug resistant viruses remain highly pathogenic and transmissible (Oxford et al., 1970; Monto and Arden, 1992; Hayden and Couch, 1992; Hayden, 1996; Sidwell and Huffman, 2000). In contrast, selection of drug resistant virus to NAI in vitro is difficult and many passages are required under continual increasing drug pressure (Bantia et al., 2000; Barnett et al., 1999; Blick et al., 1995; McKimm-Breschkin et al., 1996; McKimm-Breschkin, 2000; Tai et al., 1998). Decreased susceptibility of influenza virus to NAI in antiviral assays following in vitro selection can also arise due to mutational changes in the haemagglutinin (HA), but will not be discussed here.

In N1 NA, a histidine to tyrosine substitution at position 274 has been selected in vitro in two strains of influenza H1N1 using oseltamivir carboxylate. A single amino acid change H274Y occurred in the NA of A/WS/33 whereas in A/Texas/36/91 the H274Y mutation arose following an earlier I222V substitution. This existed as a mixed population with wild type. The I222V mutation alone afforded only a twofold change in sensitivity of enzyme to oseltamivir carboxylate, whereas sensitivity of the double mutant was reduced more than 1000-fold (Wang et al., 2000a). To date there are no reported data on NA mutations selected by zanamivir or BCX-1812 (RWJ-270201) in H1N1 influenza virus in vitro. However, mutation H274N, derived in N1 by point mutation, gave resistance to zanamivir but not oseltamivir carboxylate (Wang et al., 2000b).

Consistent with in vitro studies, the emergence of influenza virus resistant to amantadine and rimantadine in a clinical setting occurs with a high incidence (25–38%) (Hayden, 1996; Daly et al., 2000). In contrast, the emergence of resistant virus in adults with naturally acquired influenza infection treated with oseltamivir phosphate has a low incidence ($\sim$1%) (Covington et al., 2000). The features of NA resistance that has arisen during NAI use in man were largely predicted from in vitro selection studies.

Here we describe the full characterisation, in vitro and in vivo, of an oseltamivir resistant virus with a H274Y mutation in the N1 NA that arose during a study of efficacy of oseltamivir phosphate in experimental influenza A/Texas/36/91 (H1N1) infection in adults (Hayden et al., 1999; Gubareva et al., 2001).

2. Materials and methods

2.1. Cells and virus

Madin Darby canine kidney (MDCK) cells were obtained from the laboratory of Dr Alan Hay at the National Institute of Medical Research (London, UK). Cells were grown in Eagles minimum essential medium (MEM) supplemented with 10% fetal calf serum (FCS), 2 mM L-glu-

tamine, 1% non-essential amino acids and 100 Units penicillin–100 μg streptomycin.

Influenza A/Texas/36/91 was obtained as a chicken egg allantoic fluid grown virus from Dr Brian Murphy at the National Institute of Allergy and Infectious Diseases (Bethesda, MD). This was used to infect healthy volunteers on a clinical trial. First and last (day 6) virus positive nasal wash samples from a subject treated with 200 mg qd oseltamivir phosphate were expanded in MDCK cells before undergoing three rounds of plaque purification to ensure homogeneity. Purified virus was passaged twice further in MDCK cells, and the resulting expanded virus stocks were stored at −80 °C prior to use in these experiments.

2.2. NA sequencing

The NA gene of the first and last day positive purified virus preparations was sequenced to confirm genotype at position 274. Viral RNA was isolated from these samples using a QIAamp viral RNA kit (Qiagen), and Ready-To-Go You-Prime First Strand Beads (Pharmacia) were used with the synthetic primer 5′-CTTTGTCCTATC-CGTGGGTGG-3′ to generate cDNAs. The NA gene was amplified using the Expand PCR System (Boehringer Mannheim) and included the coding and non-coding oligonucleotides 5′-CTTTGTCC-TATCCGTGGGTGG-3′ and 5′-AAAGACACC-CAAGGTCGATTCGA-3′, respectively. Manual sequence of the PCR products were carried out using the Thermo Sequenase system (Amersham). The NA gene sequence of mutant virus samples were compared to the master sequence of NA gene from wild type virus in the original inoculum.

Nasal wash samples (day 6 post-infection) from ferrets infected with the 10^{-3} challenge dose of both wild type and mutant virus (see below) were sequenced using a method similar to that described above.

2.3. HA sequencing

Viral RNA was isolated using the Ultraspec-3 RNA system. First strand c-DNA synthesis kit was then used with the synthetic primer 5′-CAAT-GAAACCGGCAATGGCTC-3′ to generate cDNA's. The HA gene was then amplified using an Amplitaq DNA polymerase kit, before sequencing of these PCR products on an A.L.F. DNA sequencer (Amersham Pharmacia). HA sequences were compared to H1 master sequence and original H1 sequence of A/Texas/36/91 recovered from the patient in this study.

2.4. NA enzyme assay

NA activity was assayed using a modified version of a method which has been previously described (Potier et al., 1979; Tai et al., 1998). Virus supernatant from fully cytopathic cell cultures, clarified by centrifugation (800 G for 10 min), was solubilised using Nonidet P-40 and used as the source of NA enzyme. Briefly, sensitivity (IC_{50}-50% inhibitory concentration) determinations to oseltamivir carboxylate were carried out at 37 °C using methylumbelliferyl-*N*-acetylneuraminic acid (MUNANA) as substrate. Dilutions of virus and oseltamivir carboxylate (0.1 nM–1 μM) in 33 mM MES (2-[*N*-morpholino]ethanesulfonic acid) pH 6.5 containing 4 mM $CaCl_2$ were mixed and incubated at room temperature prior to addition of substrate. The reaction was stopped after 30 min at 37 °C, by the addition of 150 μl 14 mM NaOH in 83% ethanol and fluorescence was quantified on a fluorimeter. Ki values were determined as previously described (Tai et al., 1998; Mendel et al., 1998).

2.5. Virus growth in MDCK cells

Equivalent particle numbers (as determined by reactivity with human erythrocytes) of both 274H (wild type) and 274Y (mutant) virus isolates were used to infect confluent MDCK cells in a 96-well format. Briefly, cells were washed free of FCS-containing culture medium using sterile PBS, and then replaced with FCS free medium supplemented with 0.14% BSA (fraction V) and 1.25 μg/ml TPCK-treated trypsin. Serial 10-fold dilutions were made for each virus inoculum and virus was allowed to adsorb for 2 h at 34 °C/5% CO_2. Excess virus was removed from the plates

and 200 μl of fresh culture medium replaced. The plates were then incubated for up to 10 days 34 °C/5% CO_2 and aliquots of supernatant removed daily to test for virus positivity by human erythrocyte agglutination. Mean virus titres for each time point for both wild type and mutant viruses were calculated using the Spearman–Karber equation (Finney, 1952) and expressed as a $\log_{10}$ $TCID_{50}$/ml ($\log_{10}$ dilution of virus that causes 50% infection of the cell culture).

2.6. Antiviral assay

Oseltamivir carboxylate (concentration range 1 nM–300 μM), diluted in FCS-free Eagles MEM media supplemented with 0.14% BSA (fraction V) and 1.25 μg/ml TPCK-treated trypsin was applied to confluent MDCK cell monolayers in quadruplicate (96-well plate) for 1 h prior to addition of either wild type or mutant virus, at a challenge dose of three times the $TCID_{50}$. Cell cultures were incubated with virus for 2 h at 34 °C after which time the virus inoculum was removed and replaced with medium containing appropriate concentrations of oseltamivir carboxylate. Plates were then incubated for a further 7 days and supernatants tested for virus titre by their ability to agglutinate 2% human erythrocytes.

2.7. Mouse model of influenza virus infection

Female 6–8 weeks old specified pathogen free BALB/c mice, were obtained from Charles River, UK. Groups of mice ($n = 3$) were gently anaesthetised by an inhaled mixture of isoflurane and oxygen, then infected intranasally with 10 μl of either wild type or mutant virus in each nostril. A range of virus challenge doses were used, equated between wild type and mutant on particle number (highest dose 5.3 and 3.5 $\log_{10}$ $TCID_{50}$/ml, respectively). On day 3 post-infection mice were sacrificed by cervical dislocation and lungs were aseptically removed and weighed. Each lung was homogenised in 1 ml of media and the resultant homogenate was clarified by centrifugation for 5 min at 2000 rpm. Supernatants were then assayed for infectious virus by titration on MDCK cells in 96-well plates. Serial 10-fold dilutions were made for each virus inoculum. Virus was allowed to adsorb for 1–2 h at 34 °C/5% CO_2. Excess virus was removed from the plates and 200 μl of fresh medium replaced. The plates were then incubated for 6 days and virus titres were calculated on the ability of supernatant containing virus to agglutinate 2% human erythrocytes. Mean virus titres ($\log_{10}$ $TCID_{50}$/ml) for each dilution group of wild type and mutant virus were calculated using the Spearman–Karber equation (Finney, 1952).

2.8. Ferret model of influenza virus infection

Female ferrets weighing between the range of approximately 600–1100 g were obtained from Foxfield Farms UK Ltd., and randomised into groups. Groups of ferrets ($n = 4$) were anaesthetised by an inhaled mixture of Halothane and oxygen. Animals were then infected intranasally with 500 μl of virus (either wild type or mutant virus at a range of challenge doses equated on particle number, highest dose being 4.8 and 3 $\log_{10}$ $TCID_{50}$/ml, respectively), and allowed to recover from anaesthesia before being placed back in their cages. Ferrets were weighed and their body temperatures recorded daily until day 6 post-infection. Nasal washes were obtained daily under light anaesthesia. Nasal washes were assayed for infectious virus by titration on MDCK cells in 96-well plates, as described above, and for inflammatory cell counts. Mean virus titres ($\log_{10}$ $TCID_{50}$/ml) for each dilution group of wild type and mutant virus were calculated using the Spearman–Karber equation (Finney, 1952).

2.9. Statistical analysis

Area under the curve (AUC) values were calculated for all parameters including viral growth curves, temperatures, inflammatory cell counts and body weight. Mutant virus AUC was compared to wild type virus AUC using Student's *t*-test.

Table 1
Enzyme activity of wild type and mutant NA

NA	MUNANA substrate		Oseltamivir carboxylate Ki (nM)
	Km (μM)	Relative Vmax	
Wild type 274H	105	1.0	0.5
Mutant 274Y	109	0.9	200

3. Results

3.1. Effect of the H274Y mutation on NA activity

Substrate affinity (Km) and enzymatic activity (Vmax) of mutant NA derived from virus isolated during this clinical study (Gubareva et al., 2001) was equivalent to wild type (Table 1), although the inhibition constant (Ki) for oseltamivir carboxylate was markedly increased (400-fold).

3.2. Virus growth in MDCK cell monolayers

Comparison of the growth characteristics of wild type and mutant virus were carried out on confluent MDCK monolayers. Mean viral titres ($n = 4$) were determined over time for each virus (Fig. 1).

The mutant virus was found to grow in MDCK cells to a significantly lower titre ($P < 0.001$) than wild type virus, even though an equivalent inoculum dose was added (equivalent on HA units). From day 3 onwards the titre of mutant virus was reduced by at least 2 logs compared to wild type, and the AUC of mutant virus totalled just 51% (on a $\log_{10}$ scale) of the AUC for wild type virus indicating a reduction in infectivity of the mutant virus of between 90 and 99%.

3.3. Antiviral activity

The 50% effective antiviral concentration (EC_{50}) of oseltamivir carboxylate against wild type influenza A/Texas/36/91 isolated pre-treatment from the patient was 100 nM, whereas antiviral activity against mutant virus from the same patient had an EC_{50} greater than 300 μM. Thus, influenza virus carrying H274Y in the NA gene results in a greater than 3000-fold reduction in sensitivity to oseltamivir carboxylate in this cytopathic effect cell culture antiviral assay.

3.4. Influenza virus infection in the mouse

An in vivo titration of the wild type virus and mutant virus was carried out in mice to determine their relative infectivity. Lungs were removed from mice on day 3 post-infection for assessment of virus titre (day 3 approximating to when peak virus titres occur in this model). Fig. 2 shows the mean virus titre recovered from the lungs of mice

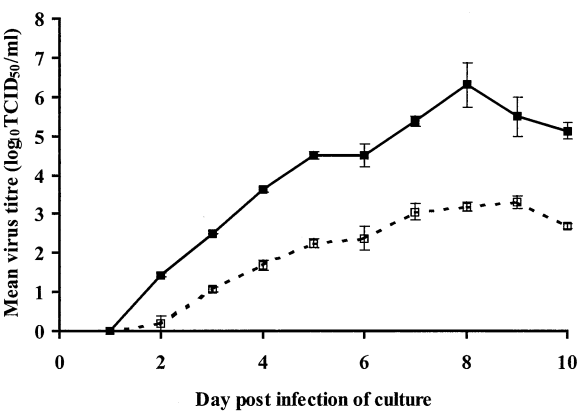

Fig. 1. Growth of influenza A/Texas/36/91 virus carrying wild type or mutant NA in MDCK cells. Presence of virus in the culture supernatant was determined daily using haemagglutination of human erythrocytes as the end point. $TCID_{50}$/ml was calculated using the Spearman–Karber equation. Each point represents the mean of four individual $TCID_{50}$ determinations. Solid line denotes wild type virus and broken line denotes mutant virus. Error bars denote standard error of the mean.

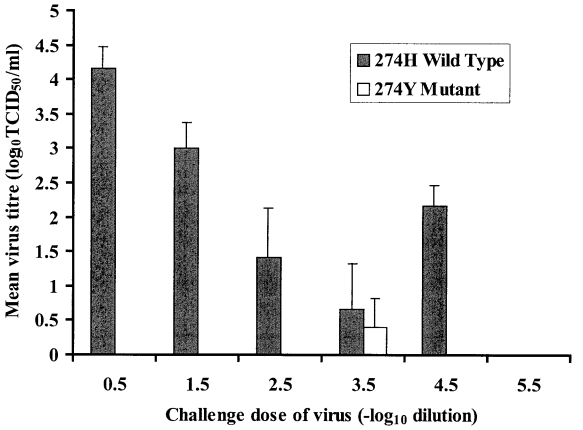

Fig. 2. Replication of influenza A/Texas/36/91 virus carrying wild type or mutant NA in mouse lung on day 3 post-infection. Mice were infected intranasally with a series of virus challenge doses equated on HA reactivity. Virus titres were determined from lung homogenates titrated on MDCK cells and represented as mean TCID$_{50}$/ml. Mean TCID$_{50}$/ml from three individual animals are shown. Error bars represent standard error of the mean.

($n = 3$) infected with a range of challenge doses of either virus.

Virus was recovered from all but the lowest challenge dose group of mice infected with the wild type virus. In contrast virus was barely recoverable from the lungs of mice infected with virus carrying the H274Y mutation. A low virus titre was recovered from only one out of 18 animals infected. Lung tissue from all other mice, including those infected with the highest virus dose, assayed negative for virus.

These data indicate that influenza virus carrying the H274Y mutation is compromised at least 1000-fold, in infecting and replicating in the mouse model of influenza infection compared to the corresponding wild type influenza virus.

3.5. Ferret model of influenza virus infection

This model was used to further compare the infectivity of wild type and mutant virus in vivo and also compare pathogenicity by measuring inflammatory cells recoverable from nasal wash, febrile responses and whole body weights.

3.6. Virus titres

The measurement of virus titres in the nasal washings from ferrets not only provides information about the relative infectivity or frequency of infection of a virus but also the replicative ability.

The profile of virus recovery from the nasal washes of ferrets infected with either wild type or mutant virus is represented in Fig. 3. Both wild type and mutant virus were recovered at equivalent titres from ferrets infected with the two highest challenge doses of virus (10^{-1} and 10^{-2} dilutions from virus stocks equated on particle number). Infection of ferrets with a mid range (10^{-3}) challenge dose resulted in a significant reduction ($P < 0.05$) in virus recovery of the mutant virus compared to the wild type virus. The profile of mutant virus recovery at this 10^{-3} challenge dose closely resembles the profile of wild type virus recovery from the 10^{-5} challenge dose, 100-fold lower. The virus titres recovered from the nasal washes of the three lowest challenge dose groups (10^{-4}, 10^{-5} and 10^{-6}), showed an even greater difference in recovery of wild type virus compared to mutant virus. Mutant virus was undetectable in all ferrets at all time points at these low challenge doses. However, wild type virus in these lower dose challenge groups could be recovered at titres similar to that seen at higher virus challenge, although a 1–2 days shift in time to reach peak titres was observed.

Viruses in day 6 nasal wash samples from ferrets infected with wild type virus (10^{-3} challenge group) were all found to have retained their wild type NA sequence and had the predicted amino acid sequence that included histidine at position 274. Viruses in day 6 nasal wash samples taken from ferrets infected with mutant virus (10^{-3} challenge group) were all found to have retained mutant genotype, i.e. tyrosine at position 274.

Gubareva et al. (2001), have shown that the challenge virus used in the clinical study from which this resistant virus was derived carried mutations at position 225 of the HA. These arose by adaptation of the virus to eggs during virus production for the study. A D225N mutation in HA was carried through to the resistant NA mutants studied here. In a separate experiment (data not

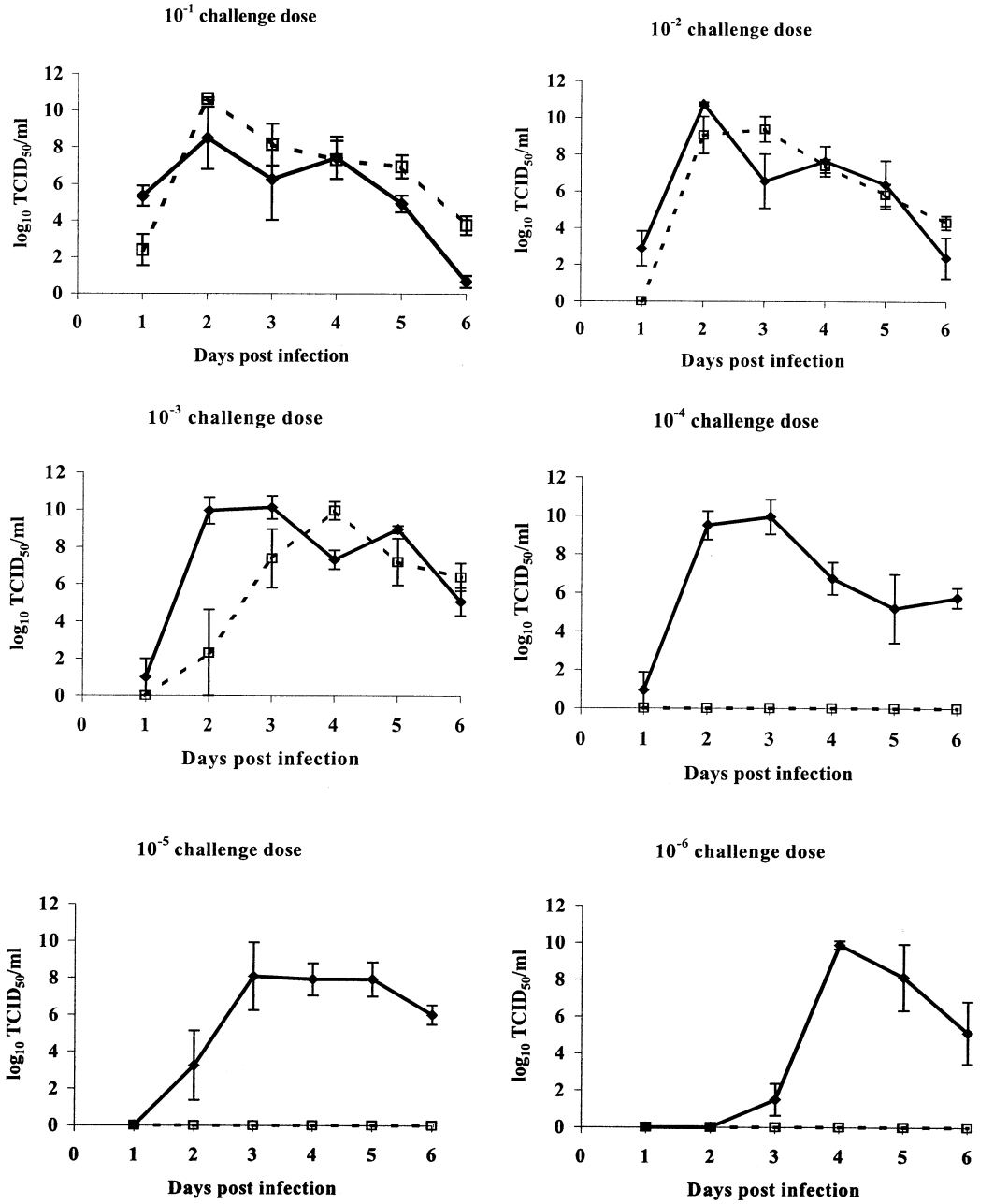

Fig. 3. Replication of influenza A/Texas/36/91 virus carrying wild type or mutant NA in ferrets. Ferrets were infected intranasally with range of infectious doses equated on HA reactivity. The relative infectivity of wild type and mutant virus were compared by titration of infectious virus, recoverable from the nasal washes of ferrets, on MDCK cells. Nasal washes were sampled daily from day 1 through to day 6 post-infection. Each point represents mean virus titer as determined by haemagglutination of human erythrocytes and expressed as $TCID_{50}$/ml of four individual ferrets per group. Solid line denotes wild type virus and broken line denotes mutant virus. Error bars represent standard error of the mean.

shown) we have shown that the HA D225N mutation was still present in virus in the nasal wash of ferrets 8 days after infection with the double mutant virus (HA D225N and NA H274Y). Thus, both wild type and NA mutant virus used in this study carry an additional and common HA mutation, and both NA and HA mutations are stable in the ferret. The differential properties seen are therefore due to NA mutations H274Y on this particular A/Texas H1N1 background.

The data shown here indicates that the H274Y mutant virus is severely compromised (by 2 logs) in infective and/or replicative ability in the ferret model of influenza virus infection, compared to the wild type counterpart.

3.7. Inflammatory cell counts, febrile response and body weight

The mean inflammatory cell counts in nasal washes throughout the duration of infection for each group are presented in Fig. 4. The reduction in inflammatory cell response in ferrets infected with mutant virus was significant ($P < 0.05$) at the four highest challenge doses compared to wild

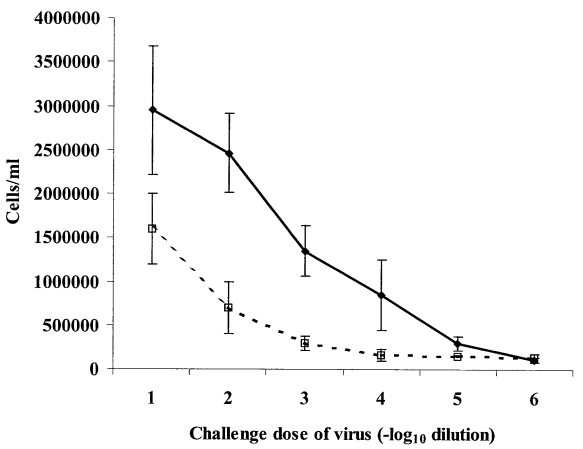

Fig. 4. Wild type and mutant influenza A/Texas/36/91 virus induction of inflammatory response in ferrets. Whole cell counts in the nasal washings collected from ferrets ($n = 4$) daily for each group were determined using trypan blue exclusion. The mean inflammatory cell counts for each group averaged over the duration of infection are shown here. Solid line denotes wild type virus and broken line denotes mutant virus. Error bars represent standard error of the mean.

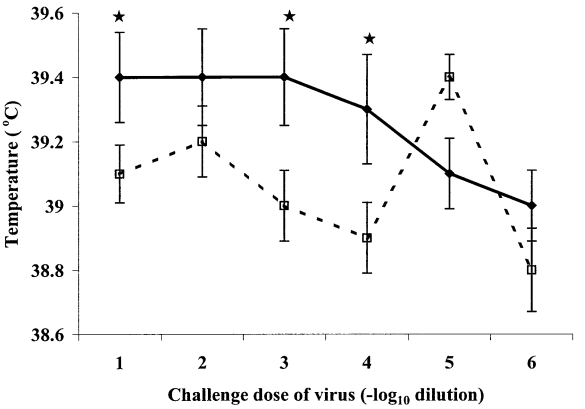

Fig. 5. Wild type and mutant influenza A/Texas/36/91 virus induction of febrile response in ferrets. The mean changes in body temperature over time for each challenge dose of wild type and mutant virus are shown. Solid line denotes wild type virus and broken line denotes mutant virus. Error bars represent standard error of the mean. $*P < 0.05$.

type infected ferrets. This includes the two highest challenge doses of virus that achieved comparable infection between mutant and wild type virus as measured by the recovery of virus from nasal wash (Fig. 3). The fact that the inflammatory response induced in mutant virus infected ferrets is reduced in comparison to the corresponding wild type despite similar viral burden indicates the reduced pathogenicity of the mutant virus.

Fig. 5 shows the febrile response throughout the duration of infection (6 days), for each challenge dose. The febrile response to mutant virus infection at high virus challenge was consistently lower than for wild type virus infection at an equivalent challenge dose. This difference was significant ($P < 0.05$) for three of the four highest challenge doses (it is not thought that the temperature spike of approximately 0.4 °C at 10^{-5} is significant). Again these data indicate that mutant virus is inherently less pathogenic than wild type virus since the febrile response is reduced even where viral titres are comparable.

Total body weight loss throughout the course of infection (6 days) for each challenge dose group is shown in Fig. 6. A trend for a reduction in weight loss during influenza infection was observed when comparing mutant to wild type virus, even though such weight changes can be highly

variable in influenza infected ferrets. This variability in weight was observed in our study, nevertheless it does indicate a trend towards a reduction in weight loss for mutant infected ferrets. The exact relevance of these changes is unknown, but might be consistent with mutant virus being less pathogenic than wild type virus since the two highest challenge dose groups showed a reduction in weight loss.

All the data taken together indicate that influenza A/N1 carrying a H274Y mutation in the NA gene is compromised in both its ability to infect and replicate in ferrets, and at high challenge doses where replication of mutant virus is comparable to wild type, the pathogenicity of the mutant virus is reduced.

4. Discussion

These preclinical studies address the possible consequence of drug resistant H1N1 influenza virus that may emerge following use of oseltamivir phosphate in the clinic. The data here describe the further characterisation, both in vitro and in vivo, of a clinically derived influenza virus A/Texas/36/91 carrying a H274Y NA mutation that arose during an experimental influenza challenge and treatment study in man (Gubareva et al., 2001).

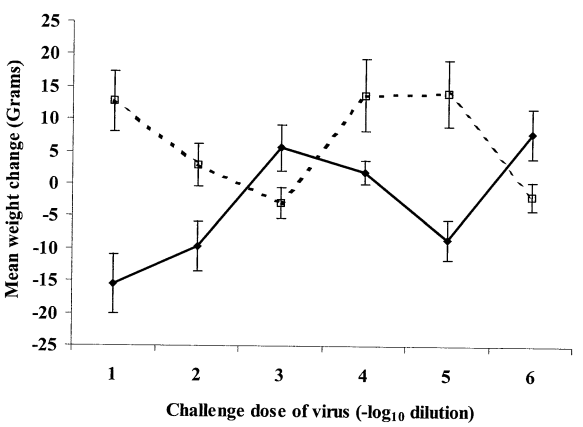

Fig. 6. Effect of wild type and mutant influenza A/Texas/36/91 virus infection on body weight in ferrets. The means changes in whole body weight over time (6 days post-infection) for each challenge dose of wild type and mutant virus are shown. Solid line denotes wild type virus and broken line denotes mutant virus. Error bars represent standard error of the mean.

Gubareva has drawn attention to the fact that this virus has an egg-adaptive HA mutation, which results in virus preferentially binding cellular $\alpha 2-3$ sialyl receptors as opposed to human $\alpha 2-6$ sialyl receptors. This mutation may have predisposed the virus to selection of the H274Y NA mutation in the presence of oseltamivir carboxylate. In the placebo group of patients the virus reverts from the egg-adapted challenge virus to replicate more readily in human respiratory epithelia. Results also strongly suggest that human adapted virus (to $\alpha 2-6$) is more susceptible to the inhibitory effects of oseltamivir, than egg-adapted virus. The results in this study exemplified the difficulties of monitoring resistance emergence by antiviral assay in a challenge study setting, where it is common practise for egg-grown and hence potentially egg-adapted virus to be used (Gubareva et al., 2001).

The H274Y mutation occurs in the NA enzyme active site, and confers decreased sensitivity of both enzyme and virus to inhibition by oseltamivir carboxylate in vitro. Although mutant enzyme activity appears comparable to that of wild type virus, the mutant virus is compromised in its ability to replicate in cell culture. Lentz has shown that an H274Y mutation engineered into A/Tokyo/3/67 NA (N2), a different NA enzyme, effected a reduced enzyme activity particularly related to a change in pH profile, substrate size (fetuin vs NANL) and enzyme source (solubilsed vs non-solubilised) (Lentz et al., 1987). All these factors taken together may account for the differences seen between this study and those of Lentz.

Histidine 274 was found to interact with glutamic acid 276 a residue that is involved in binding substrate and was therefore deemed an important binding residue. Recent studies on the mechanism by which the H274Y mutation confers resistance to NA inhibitors have been reported. Replacement of histidine 274 with amino acids having side chains of differing size showed that larger residues, such as tyrosine, prevented glutamic acid 276 from reorientation such that it was unable to form a salt bridge with arginine 224. This normally occurs on the binding of os-

eltamivir carboxylate to wild type NA. Smaller residues such as glycine or asparagine resulted in higher or unaltered sensitivity to oseltamivir carboxylate (Wang et al., 2000b).

These properties translate in vivo to infectivity or replicative ability of the mutant H274Y virus in mice being compromised. In fact virus was barely recoverable from the lungs of mice challenged with mutant virus. The ferret model of influenza virus infection closely reflects the course of human disease (Smith and Sweet, 1988). Ferrets infected with virus containing H274Y showed not only a decrease in infectivity and/or replicative ability but also a decrease in pathogenicity compared to wild type virus. The reduced pathogenicity of mutant virus was not entirely a consequence of reduced infectivity since the differences observed between symptomatic responses induced by wild type and mutant virus were apparent at higher challenge doses, where there was no difference in virus titre in nasal washes. These results can be attributed to H274Y since the mutation was stable throughout replication in vivo.

The properties of the H274Y NA mutant virus that has been presented here are closely similar in character to those of other NA mutant viruses that have been identified following treatment with both oseltamivir phosphate and zanamivir (e.g. R292K, E119V, R152K). Most importantly they have been shown to be less infective/virulent in animal models (Ives et al., 2000a,b; Gubareva et al., 1998) and hence unlikely to be transmitted in man. Additionally, evidence from a study of the R292K virus in a ferret model of influenza virus transmission shows that the R292K virus is unable to transmit a productive infection at concentrations of virus where wild type virus transmits completely (Carr et al., 2001).

In a study in children with naturally acquired H1N1 infection, a single patient on treatment with oseltamivir phosphate developed virus carrying a H274Y NA mutation (Whitley et al., 2001). This patient's course of virus infection was no different to that of the rest of the treated population. This data in conjunction with the data presented here, indicates that virus carrying H274Y NA will unlikely be of clinical significance.

The incidence of the emergence of virus resistant to oseltamivir carboxylate is low compared to amantadine and rimantadine. The virus studied here, obtained from a patient who took part in an experimental challenge study, was found to be less virulent and infectious in vivo in mice and ferrets than the corresponding pre-treatment wild type virus. In contrast rimantadine was found by Sweet et al. (1991), to retain resistant influenza virus with wild type growth characteristics and virulence. It is unlikely that the development of the NA mutation, which is restricted to the H1N1 subtype, will have clinical consequence in man.

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