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The Interaction of Neuraminidase and Hemagglutinin Mutations in Influenza Virus in Resistance to 4-Guanidino-Neu5Ac2en

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We have previously described a 4-guanidino-Neu5Ac2en (zanamivir)-resistant neuraminidase (NA) variant G70C4-G, with an active site mutation Glu 119 to Gly. This variant has been found to also harbor a hemagglutinin (HA) mutation in the receptor binding site, Ser 186 to Phe. Examination of early passages of the G70C4-G virus revealed that this HA mutation had arisen by the first passage. From a subsequent passage two transient variants were isolated which had each acquired a different second HA mutation, Ser 165 to Asn and Lys 222 to Thr. Both were highly drug resistant and drug dependent and their ability to adsorb to and penetrate cells was decreased. Comparison of drug sensitivities between the variant, with the additional HA mutation at Ser 165, and viruses with either mutation alone revealed that these two HA mutations acted synergistically to increase resistance. To determine the contribution to resistance of each of the NA and HA mutations in G70C4-G, the NA mutation was separated from the HA mutation by reassorting. The NA mutation and the HA mutation each conferred low-level resistance to zanamivir, while the two mutations interacted synergistically in the double mutant to give higher resistance *in vitro*. Infectivity was not adversely affected in the double mutant and while there was a small decrease in sensitivity to zanamivir in the mouse model, there was no detectable resistance to zanamivir in the ferret model.

INTRODUCTION

Influenza viruses have two surface glycoproteins, the hemagglutinin (HA) and neuraminidase (NA). The HA facilitates virus entry into the cell via its receptor, glycoconjugates with sialic acid as the terminal sugar (Gottschalk, 1959). Viral NA removes terminal sialic acids from glycoproteins, on both cell receptors and viral HA and NA, thereby allowing the release of progeny virus from infected cells (Seto and Rott, 1966; Palese *et al.,* 1974; Palese and Compans, 1976).

Determination of the crystal structure of influenza virus NA complexed with sialic acid (Varghese *et al.,* 1992) led to the rational design of specific inhibitors (von Itzstein *et al.,* 1993), 4-guanidino-Neu5Ac2en (zanamivir) and 4-amino-Neu5Ac2en. Zanamivir is a potent inhibitor of influenza virus replication *in vitro* (Woods *et al.,* 1993; Thomas *et al.,* 1994) and *in vivo* (Ryan *et al.,* 1994, 1995). Experimental human trials have demonstrated its safety and efficacy (Hayden *et al.,* 1996).

Resistant variants to zanamivir have been generated *in vitro,* with mutations found both in the active site of the NA and/or in the region of the receptor binding pocket of the HA (Blick *et al.,* 1995; Staschke *et al.,* 1995; McKimm-Breschkin *et al.,* 1996a, 1998; Gubareva *et al.,* 1996, 1997). These HA mutations were proposed to affect its affinity or specificity, resulting in a weakly binding HA and allowing for release of progeny virions from infected cells with less dependence on the NA (McKimm-Breschkin *et al.,* 1996a).

We have previously described an NA variant, G70C4-G, with the active site mutation Glu 119 to Gly (Blick *et al.,* 1995). This variant showed decreased sensitivity to zanamivir in enzyme assays, but not to 4-amino-Neu5Ac2en. Surprisingly this variant demonstrated cross-resistance to both zanamivir and 4-amino-Neu5Ac2en in cell culture. It was subsequently found also to have an HA mutation. To determine the contribution to resistance of each mutation, variants with either the HA or NA mutation were obtained. The interaction of the two mutations in combination was assessed by comparing the drug sensitivity of the G70C4-G variant to the sensitivity of the single HA or NA variants. Evolution of this double mutant was further studied by selection and characterization of progeny at each passage. The mouse and ferret models were used to determine the *in vivo* infectivity of G70C4-G and to measure its sensitivity to zanamivir.

RESULTS

The G70C4-G virus showed resistance to zanamivir in enzyme inhibition assays, but not to 4-amino-Neu5Ac2en.

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However, G70C4-G was partially cross-resistant to 4-amino-Neu5Ac2en in a plaque reduction assay, with resistance to zanamivir being greater than that to 4-amino-Neu5Ac2en (Blick *et al.,* 1995). We have previously shown variants with HA mutations to be equally cross-resistant to zanamivir and 4-amino-Neu5Ac2en in cell culture (McKimm-Breschkin *et al.,* 1996a). These observations caused us to reexamine the HA sequence, and a single mutation, Ser 186 to Phe, was in fact detected.

Examination of passage history

Early passages from the generation of the G70C4-G virus were also examined to elucidate the evolutionary dynamics involved in the generation of the double mutant. Each passage was amplified and the drug sensitivity was determined in a plaque reduction assay, with mixed populations separated by plaque purification. A variant with a relatively small change in drug sensitivity, P1-1, arose at passage 1. Passage 3 consisted of a major population of P1-1 and two highly resistant minor populations, each $<$10% of the overall population, P3-1 and P3-2. These two variants were not present in passage 4. Passage 5 was a mixed population of P1-1 and G70C4-G. Only G70C4-G was present by passage 6.

Sequencing of the HA genes

The HA of each variant was sequenced. All the variants, including G70C4-G, previously reported not to have an HA mutation (Blick *et al.,* 1995), had a Ser 186 to Phe mutation (H3 numbering). P3-1 and P3-2 each had an additional mutation; P3-1 had a Ser 165 to Asn mutation and P3-2 had a Lys 222 to Thr mutation.

Generation of virus with only the NA mutation

The G70C4-G mutant was reassorted with its NWS/G70C parent virus to generate a virus with the NA mutation, but with a wild-type HA and the same background genes. Slow elution from chicken erythrocytes was used to select virus with a wild-type HA. Resistant virus was distinguished from wild-type NWS/G70C on the basis of resistance to zanamivir in a plaque inhibition assay. Selection of the mutant, G70C4-G/NA, with the Glu 119 to Gly NA mutation, but a wild-type HA, was confirmed by NA inhibition assay and sequencing of the HA and NA. This genotype is identical to that described by Staschke *et al.* (1995). The plaque size of G70C4-G/NA was smaller than the NWS/G70C plaque size.

Sensitivity of virus in plaque assay

Sensitivity to zanamivir and 4-amino-Neu5Ac2en was determined by a plaque inhibition assay (results for zanamivir shown in Fig. 1). While the G70C4-G double mutant was 100-fold less sensitive to zanamivir (EC_{50} = 0.3 μg/ml, based on the concentration of drug required to give a 50% in plaque size) than the wild type (EC_{50} = 0.003 μg/ml), P1-1 (HA mutant) and G70C4-G/NA (NA mutant) were each only 10-fold less sensitive to zanamivir (EC_{50} = 0.03 μg/ml). The two mutations therefore acted in a synergistic way to decrease drug sensitivity. G70C4-G and P1-1 were 10-fold less sensitive to 4-amino-Neu5Ac2en (EC_{50} = 3 μg/ml) than wild-type NWS/G70C (EC_{50} = 0.3 μg/ml), but G70C4-G/NA was as sensitive as the wild-type.

Both P3-1 and P3-2 were $\geq$1000-fold less sensitive to zanamivir than NWS/G70C and were equally cross-resistant to 4-amino-Neu5Ac2en. Both exhibited drug dependence, having fewer plaques in the absence of inhibitor. In addition, P3-1 also had small, diffuse plaques in the absence of inhibitor.

Effect of exogenous NA

The NA mutation Glu 119 to Gly does not affect specific activity (Blick *et al.,* 1995), but does reduce NA stability (McKimm-Breschkin *et al.,* 1996b). To determine whether the small plaque size of G70C4-G/NA was caused by a deficiency of NA activity *in vitro,* exogenous NA was added to the agar overlay in a plaque assay (Fig. 2). With exogenous NA the plaque size of the G70C4-G/NA virus increased to that of wild-type virus.

Kinetics of replication

To ascertain if there were other effects of the *in vitro* NA deficiency, the kinetics of replication of G70C4-G/NA was followed over 48 h and compared to that of wild-type virus. Results showed that the replication of G70C4-G/NA was not adversely affected.

Stability of the resistance phenotype

P3-2, P1-1, and G70C4-G/NA did not revert after passaging in the absence of drug, as determined by drug sensitivity in a plaque reduction assay and NA inhibition assay for G70C4-G/NA. However, after 10 limiting dilution passages in the absence of drug, P3-1 was now only 10-fold less sensitive to zanamivir than wild-type virus. Sequencing revealed reversion to wild-type sequence at residue 165 with maintenance of the Ser 186 to Phe mutation.

Kinetics of adsorption

We have previously shown that virus may overcome drug inhibition by HA mutations which decrease affinity, allowing progeny virions to elute from cells with a reduced requirement for NA activity (McKimm-Breschkin *et al.,* 1996a; Sahasrabudhe *et al.,* 1996). To determine if these HA variants had a reduced efficiency of adsorption compared to the wild-type virus, the kinetics of adsorption and penetration were studied. The efficiencies of adsorption of G70C4-G and P1-1 were no different to that

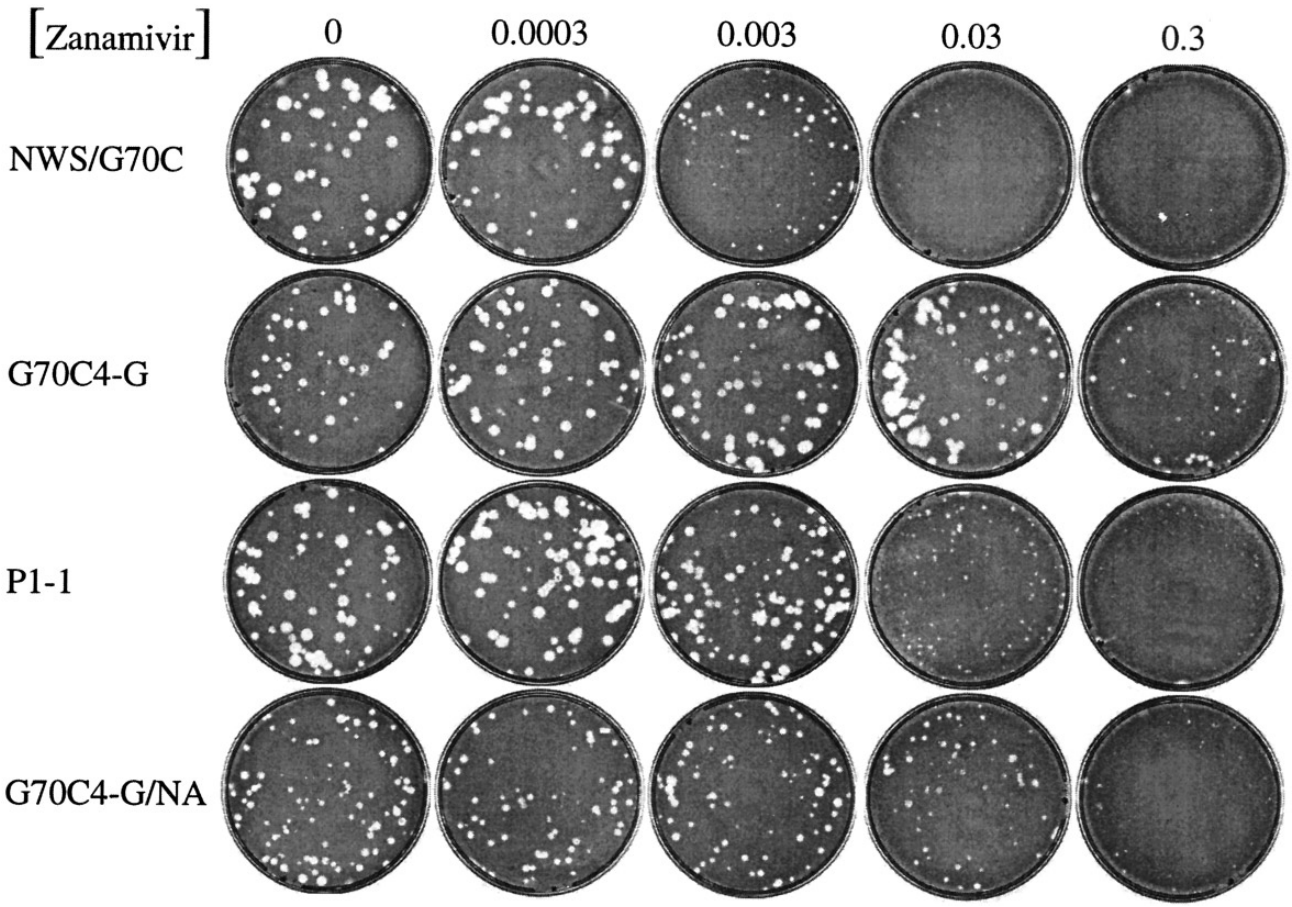

FIG. 1 Plaque inhibition assay of NWS/G70C, the double mutant G70C4-G, and the single HA (P1-1) or NA (G70C4-G/NA) variants. Concentrations of zanamivir are in μg/ml.

of the wild-type. The adsorption efficiencies of P3-1 and P3-2 were reduced to 10–15% of that of the wild-type virus.

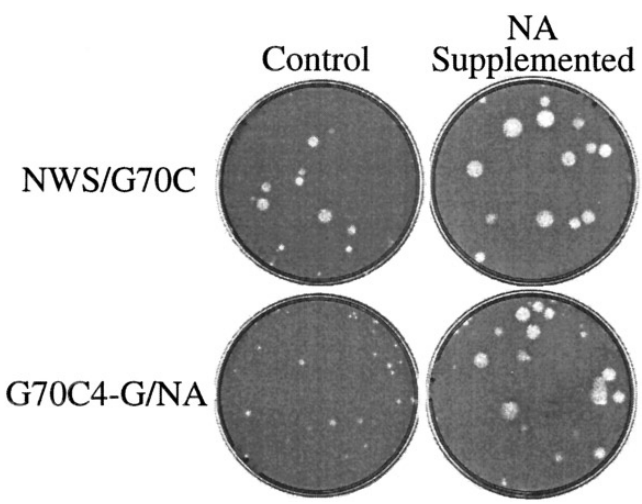

FIG. 2. Effect of exogenous NA on plaque size on NA only mutant and wild-type viruses. One of each pair of plates was supplemented with 10 mU/ml of NA from *Clostridium perfringens.*

Thermal stability of HA

We have previously shown mutations in the HA can result in thermolability (McKimm-Breschkin *et al.,* 1996a). For each virus the thermal stability of the mutant HA was assessed by incubating virus at elevated temperatures for 1 h. Binding to fetuin was compared to a control incubated at 4°C. None of the mutations lead to a marked decrease in the thermal stability of the HA.

Infectivity and drug sensitivity in the mouse model

To determine whether the mutations had an effect *in vivo* on virus replication or drug sensitivity, infectivity and sensitivity to zanamivir were determined in the mouse model (Fig. 3). In the absence of drug, NWS/G70C and G70C4-G grew to comparable titers in animals infected with the same inoculum, indicating that the *in vivo* infectivity of G70C4-G was not affected. In drug treated mice a reduction in mean virus lung titers was seen at both 1 mg/kg/dose ($P = 0.03$) and 10 mg/kg/dose ($P = 0.02$) zanamivir for NWS/G70C. Mean virus lung titers for G70C4-G were only reduced at the highest drug concentration ($P = 0.004$). G70C4-G was less sensitive to inhib-

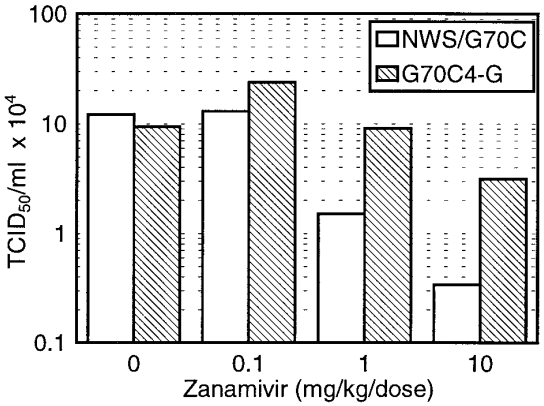

FIG. 3. Infectivity and zanamivir sensitivity in the mouse model. Mice were infected i.n. with 10^5 PFU of either the NWS/G70C virus or G70C4-G virus. Animals were dosed with PBS or zanamivir at −20 and −4 h prior to infection and at 4, 20, 28, and 44 h postinfection. Four to five mice were used per group. Lungs were harvested at 48 h postinfection. Lung virus titers were determined by $TCID_{50}$. Data were collated from three separate experiments.

itor than NWS/G70C at both 1 mg/kg/dose ($P = 0.04$) and 10 mg/kg/dose ($P = 0.02$). Overall G70C4-G was 10-fold less sensitive to zanamivir than NWS/G70C.

Drug sensitivity in the ferret model

As influenza virus infection in ferrets more closely resembles the clinical situation in humans, the drug sensitivity of the G70C4-G mutant was determined in the ferret model (Fig. 4). All three doses of zanamivir reduced nasal virus titers over the course of infection, though to differing degrees. There was no detectable difference between the responses of ferrets infected with either NWS/G70C or G70C4-G to any drug dose. All sham-treated control animals, infected with either NWS/G70C or G70C4-G, had a pyrexic response within the period 48–96 h postinfection. In contrast, the temperature responses of zanamivir-treated ferrets were completely suppressed at doses of 5, 0.5, or 0.05 mg/kg in all ferrets infected with either NWS/G70C or G70C4-G.

DISCUSSION

We have previously described a drug-resistant variant, G70C4-G, with the active site mutation Glu 119 to Gly, generated by passaging virus in the presence of zanamivir (Blick *et al.,* 1995). While it was only resistant to zanamivir in an enzyme inhibition assay, it also showed some resistance to 4-amino-Neu5Ac2en in a plaque inhibition assay. This variant was subsequently found to also harbor an HA mutation in the receptor binding site, Ser 186 to Phe. To determine the contribution of each mutation to resistance we separated the two mutations by reassorting. These viruses, with a single mutation, were used to measure the cooperative effects of mutations in both the NA and the HA.

While the NA mutation Glu 119 to Gly and the HA mutation Ser 186 to Phe each conferred low level resistance to zanamivir, they interacted synergistically to give higher *in vitro* resistance in the G70C4-G variant with both mutations. Synergistic resistance to antiviral compounds has previously been described for herpes simplex virus (HSV) (Darby *et al.,* 1984) and human immunodeficiency virus (HIV) (Lacey and Larder, 1994). In acyclovir-resistant HSV, mutations in the thymidine kinase and the DNA polymerase behave synergistically to give high-level resistance. Two HIV reverse transcriptase mutations have been found to interact synergistically in regard to resistance to zidovudine. More common are synergistic resistance mechanisms to antibiotics in gram-negative bacteria (reviewed in Stratton and Tausk, 1987; Hancock, 1997) and yeast (Howell *et al.,* 1974; Rowlands and Turner, 1977; Subik *et al.,* 1977).

The NA only mutant showed no resistance to 4-amino-Neu5Ac2en in a plaque inhibition assay, which corre-

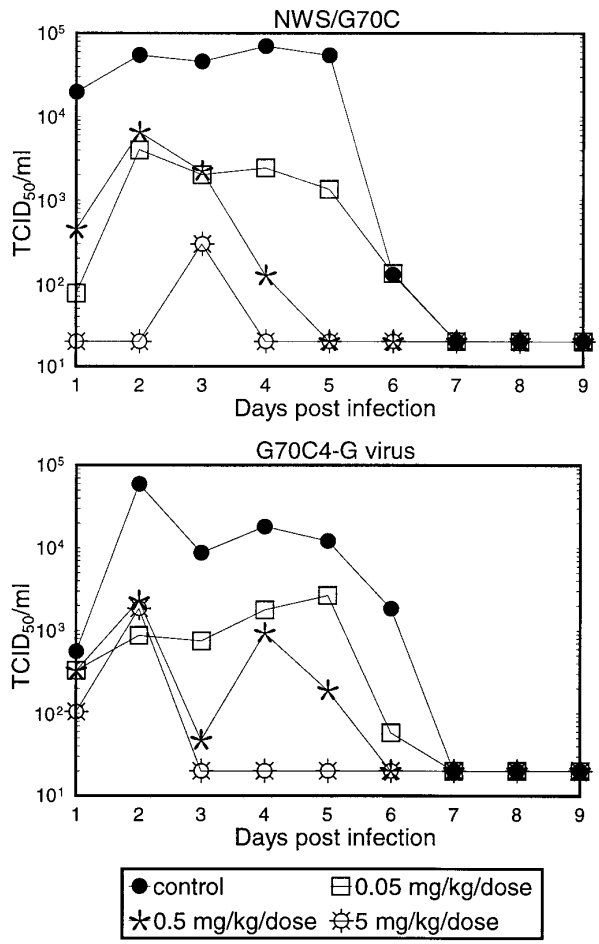

FIG. 4. Zanamivir sensitivity in the ferret model. Ferrets were challenged i.n. with 2.5×10^5 PFU of either the NWS/G70C virus or G70C4-G virus. Animals were dosed with distilled water or zanamivir twice daily from the day before infection to day 5 after infection. Viral titers were determined by $TCID_{50}$ from nasal wash samples taken up to 9 days after infection.

TABLE 1

Sequence Changes in the HA and NA Proteins[a] and *in Vitro* Zanamivir Sensitivity[b]

Virus	NA amino acid at position 119	HA amino acid at position: 165	186	222	EC_{50} (zanamivir μg/ml)
NWS/G70C parent	E	S	S	K	0.003
P1-1	E	S	F	K	0.03
P3-1	E	N	F	K	≥3
P3-2	E	S	F	T	≥3
G70C4-G	G	S	F	K	0.3
G70C4-G/NA	G	S	S	K	0.03

[a] Amino acid numbering is based on the H3 HA and the N2 NA. Sequence changes are shown in boldface.
[b] EC_{50} based on the concentration of zanamivir required to give a 50% reduction in plaque size.

lated with complete sensitivity to the compound in an NA inhibition assay (Blick *et al.,* 1995). The 10-fold resistance of G70C4-G to 4-amino-Neu5Ac2en seen previously (Blick *et al.,* 1995) correlates with the resistance of the HA only mutant P1-1 to the inhibitor and thus derives solely from the HA mutation.

We examined plaque progeny from early passages of G70C4-G to determine when these individual mutations arose. The HA mutation of this double mutant had arisen by the first passage. However from a subsequent passage two other new HA variants, P3-1 and P3-2, were isolated, which had each acquired a different additional mutation in the HA, Ser 165 to Asn and Lys 222 to Thr, respectively. These additional mutations increased resistance significantly, but interestingly both caused drug dependence. Neither of these variants were isolated from the subsequent passage, whereas the single Ser 186 to Phe mutation was maintained and the NA mutation was subsequently acquired at passage five.

The decreased drug sensitivity and drug dependence of P3-1 and P3-2 were similar to those of two resistant variants with HA mutations reported by McKimm-Breschkin *et al.* (1996a). We have previously shown that the HA and NA mutations in G70C4-G were stable when the virus was passaged in the absence of drug (Blick *et al.,* 1995). Here we have found that this is also true for viruses with either one of these two mutations.

The two HA mutations in P3-2 were also stable when passaged in the absence of drug. In the most drug-dependent variant, P3-1, the weak Ser 186 to Phe was stable, but there was enough selective pressure to force the reversion of residue 165 to wild-type sequence, removing the potential glycosylation site in the mutant, correlating with loss of drug-dependence and a decrease in drug-resistance.

An NWS/G70C HA variant derived from passaging in another NA inhibitor, a 6-carboxamide derivative of zanamivir, has been generated, having a single Ser 165 to Asn mutation. This mutant had 30-fold resistance to NA inhibitors and was not drug dependent (McKimm-Breschkin *et al.,* 1998). As the Ser 186 to Phe mutant P1-1 was only 10-fold resistant to zanamivir, while the double HA mutant P3-1 was ≥1000-fold resistant, the two HA mutations must act synergistically to increase resistance.

Previously three resistant variants with mutations in their HA (McKimm-Breschkin *et al.,* 1996a) have been shown to have reduced efficiencies of adsorption (Sahasrabudhe *et al.,* 1996). Based on the small change in resistance due to the Ser 186 to Phe mutation, this mutation is unlikely to have a large impact on the affinity of the HA for its receptor. The efficiencies of adsorption of G70C4-G and P1-1 were no different from that of the wild-type. The adsorption efficiencies of the more resistant P3-1 and P3-2 were significantly reduced compared to the wild-type virus, correlating with a significant decrease in the affinity of the HA for its receptor. For these drug-dependent variants, as the adsorption and penetration steps were not as efficient as for the wild-type virus, the virus NA may cleave receptors on cell surfaces before virus can infect. Presence of drug prevents cleavage of cell surface receptors, allowing virus to adsorb to and penetrate cells.

Some HA mutations have previously been shown to lead to thermal instability (McKimm-Breschkin *et al.,* 1996a). These current mutations affected the affinity but not the stability of the HA trimer.

Here we report mutations at three residues in the HA (Table 1). In terms of the structural implications of these HA mutations, Ser 186 is not conserved across HA subtypes, but it is in close proximity to the receptor binding pocket. A change at this residue to Ile has been implicated in egg adaptation (Meyer *et al.,* 1993) and mutation at residue 186 has been postulated as an important switch in the specificity of the receptor binding site from an avian receptor to a human receptor (Matrosovich *et al.,* 1997). Therefore it is feasible that the Ser 186 to Phe mutation has a small impact on receptor binding, correlating with its biological properties. In addition to Ser 186 to Phe, the P3-1 mutant had a Lys 222 to Thr mutation. Residue Lys 222 is flanked by a pair of known conserved

residues (220–221) and a stretch of residues 224–229 described as the left edge of the receptor binding pocket (Nobusawa *et al.,* 1991). In H3, residue Trp 222 has been shown to be directly involved in the binding of 2,6-sialyllactose (Eisen *et al.,* 1997). The biological properties of this mutant indicate a significant additional effect of this mutation which would correlate with its close association with other residues involved in receptor binding. The P3-2 virus with the additional Ser 165 to Asn mutation introduces a potential glycosylation site on the globular head of the HA. Residue 163 is frequently a glycosylation site in human H1, as is residue 165 in H3. Carbohydrate from this glycoslyation site could extend across the receptor binding site of adjacent monomers of the HA homotrimer. Increased glycosylation of the globular head of the HA has been shown to decrease binding affinity (Matrosovich *et al.,* 1997), which would correlate with our findings.

The NA mutation Glu 119 to Gly caused a small plaquing phenotype, which was rescued by NA supplementation, indicating that it was due to an NA deficiency. This did not affect the kinetics of virus replication, which is in agreement with Gubareva *et al.* (1997), who also found that mutations at Glu 119 did not affect the ability of virus to replicate *in vitro,* but differs from the results of Staschke *et al.* (1995), who found that an NA-only mutant, with the same NA mutation, displayed delayed kinetics. Since the NA-specific activity of the Glu 119 to Gly mutant is no different from that of wild-type (Blick *et al.,* 1995), it is most likely that the instability of the mutant NA (McKimm-Breschkin *et al.,* 1996b) is the cause of the small plaquing phenotype of this mutant. The normal plaque size of the G70C4-G variant is probably due to rescue of the NA deficiency by the HA mutation, which reduces the dependence on the NA.

The infectivity of the double mutant, G70C4-G, was not affected in mice or ferrets, in contrast to a Glu 119 to Ala mutant which previously demonstrated slightly lower infectivity in mice (Gubareva *et al.,* 1997). There was only a 10-fold decrease in sensitivity to zanamivir seen in the mouse model with G70C4-G, while there was a 100-fold resistance seen in cell culture. Furthermore, in the ferret model, which more closely resembles the natural upper respiratory tract infection of humans by influenza, there was no detectable reduction in sensitivity to zanamivir and pyrexia was still prevented. The different sites of infection in the two models may have contributed to the different results. Only one of these two mutations may lead to resistance *in vivo.* Another group has reported an HA mutation that caused high *in vitro* resistance, but no change in zanamivir sensitivity in the mouse model (Penn *et al.,* 1996). It is possible that this particular HA mutation may not contribute to resistance *in vivo.*

We have therefore demonstrated for the first time that either combinations of NA and HA mutations or two HA mutations can act synergistically to increase resistance to NA inhibitors. The role of HA and NA mutations *in vivo* is still unclear.

MATERIALS AND METHODS

Virus

The NWS/G70C virus (H1N9) was originally obtained from Dr. Robert Webster (St. Jude Children's Medical Research Center, Memphis, TN). It contains the NA gene of the A/tern/Australia/G70C/75 (N9) virus and the remainder of the genes are derived from the NWS virus.

Cells and media

MDCK cells were grown in Dulbecco's modified Eagle's medium/Ham's F12 (DMEM), supplemented with 10% fetal bovine serum, penicillin/streptomycin (Trace Biosciences, Australia), glutamine (Sigma Chemical Co., St. Louis, MO), and fungizone (Squibb, Australia). For virus culture cells were maintained in maintenance medium (MM), consisting of minimal Eagle's medium (Life Technologies, U.S.A.) and Liebovitz L15 (Trace Biosciences, Australia), 1:1, without serum, supplemented with glutamine, penicillin/streptomycin, and fungizone.

Inhibitors

Zanamivir and 4-amino-Neu5Ac2en were synthesized by Glaxo Wellcome Research and Development Ltd. (Stevenage, UK).

Examination of passage history

To determine when mutations contributing to resistance arose, the original passages 1 to 6 of the G70C4-G virus were amplified and were tested for drug sensitivity in a plaque reduction assay. For phenotypically mixed populations individual mutants were isolated by plaque purification.

Generation of virus with only the NA mutation

The G70C4-G mutant was reassorted with the NWS/G70C wild-type by coinfection at high multiplicity of infection (m.o.i.) into MDCK cells. Reassorted viruses were grown overnight in 1 mg/ml of zanamivir in MM. Progeny were plaqued in DMEM only or in DMEM containing $\log_{10}$ dilutions of zanamivir ranging from 0.0003 to 3.0 μg/ml. Plaques grown in DMEM only and each drug concentration were picked and amplified. Amplified plaques were agglutinated with chicken erythrocytes at 4°C for 1 h and were then incubated at 37°C for 3 h to allow elution of the virus. Virus with the wild-type HA elute much more slowly than the mutant virus (McKimm-Breschkin *et al.,* 1996a). On this basis, amplified slow eluting progeny with a putative wild-type HA were selected. To select progeny which retained the NA mutation, relative drug sensitivity was assayed in a plaque

inhibition assay. Conservation of the NA mutation was confirmed by an NA enzyme inhibition assay followed by sequencing of the NA gene. Loss of the HA mutation was confirmed by sequencing.

Sensitivity of virus in plaque assay

Approximately 100 PFU of virus was inoculated onto MDCK cells in 55-mm petri dishes. Virus was adsorbed for 1 h before cells were overlaid with agarose containing $\log_{10}$ dilutions of zanamivir ranging from 0.0003 to 3 μg/ml or 4-amino-Neu5Ac2en ranging from 0.01 to 100 μg/ml. Cells were fixed after 4 days with 1% Formalin in saline, agarose was removed, and plaques were stained with 0.05% neutral red.

Sequencing of the NA and HA genes

Influenza virus RNA was extracted directly from infected tissue culture supernatants and was digested with proteinase K in 0.5% SDS for 10 min. RNA was extracted twice using phenol chloroform and was precipitated with ethanol. Full-length NA and HA cDNA were prepared from the virion RNA using AMV reverse transcriptase (Progema, U.S.A.). For each gene three segments were amplified by PCR for direct PCR sequencing. Sequences were determined using the PRISM Ready Reaction Dyedeoxy Terminator Cycle Sequencing Kit (Applied Biosystems, U.S.A.). Sequence analysis was done using the Macintosh-based DNA analysis software GeneJockey II (Biosoft Corp., UK).

NA enzyme inhibition assays

NA enzyme activity was determined using a modification of the method of Potier *et al.* (1979). Briefly, 25 μl of virus and 25 μl of dilutions of zanamivir were preincubated for 30 min at room temperature before the addition of 50 μl of 0.2 mM methylumbelliferone *N*-acetylneuraminic acid (kindly provided by Keith Watson, Biota Holdings Chemistry Laboratory) in a buffer containing 10 mM $CaCl_2$ and 100 mM sodium acetate. After 1 h reactions were stopped with 200 mM Na_2CO_3. Fluorescence was quantitated in a Perkin–Elmer fluorimeter (Model LS50B), using an excitation wavelength of 365 nm with a slit width of 2.5, and an emission wavelength of 450 nm with a slit width of 20. Virus for enzyme assays was grown in the absence of drug. NA activity was determined over a range of virus dilutions to determine virus titers for which the enzyme activity was linear.

Effect of exogenous NA

To determine whether NA activity was a limiting factor in the small plaque size of the variant with only the NA mutation (G70C4-G/NA), exogenous NA from *Clostridium perfringens* (Sigma Chemical Co.) was added at 10 mU/ml to the agar overlay in a plaque assay with G70C4-G/NA and NWS/G70C. Plaque sizes with and without exogenous NA were compared.

Kinetics of replication

NWS/G70C and G70C4-G/NA were inoculated at an m.o.i. of 0.1 onto MDCK cells in 24-well cluster dishes. After 1 h at 37°C the inoculum was removed and was replaced with 1 ml of MM. Quadruplicate samples were collected at 8-h intervals and these were titrated by $TCID_{50}$. Cells were fixed and stained after 4 days and were scored for CPE.

Stability of the resistance phenotype

Resistant viruses were serially passaged by limit dilution, in the absence of drug, for 10 passages. At each passage serial $\log_{10}$ dilutions of virus were inoculated onto MDCK cells in a 24-well cluster dish. Three rows were grown in the absence of drug, and one row was grown in the presence of drug. Virus growth was observed visually and quantitated by a slot blot immunoassay. Relative drug sensitivity could then be assessed. Virus showing growth from the lowest dilution in the absence of drug was used for each subsequent passage. After 10 passages viruses were tested for resistance in plaque assays and, for G70C4-G/NA, an enzyme inhibition assay. Reversion was confirmed by sequencing.

Kinetics of adsorption

Adsorption efficiency was determined by comparing the number of cells infected after different adsorption periods. MDCK cells were grown on quarter microscope slides. Confluent cells were washed with cold PBS and then were infected with an equivalent m.o.i. of NWS/G70C parent virus and each of the variants, G70C4-G, P1-1, P3-1, and P3-2, in a volume of 100 μl. To synchronize infection adsorption was carried out at 0°C for 20, 40, and 60 min, after which the inoculum was removed, 100 μl of MM was added, and cells were incubated at 37°C for 6 h. The control in each case was adsorption at 0°C for 1 h, followed by 37°C for 1 h, after which the inoculum was removed, MM was added and cells were incubated a further 5 h at 37°C. After incubation at 37°C slides were washed with PBS and were fixed in chilled acetone. Cells were incubated with an anti-NP monoclonal antibody (ATCC HB65), for 1 h at room temperature, washed in PBS, stained with FITC conjugate (Silenus, Australia) for 1 h, and were again rinsed in PBS, followed by a water rinse. Slides were air dried and were observed under an Olympus BH2 fluorescence microscope.

Thermal stability of HA

Virus stocks were diluted in MM to give equivalent amounts of HA as determined by titrations on fetuin

(Sigma Chemical Co.)-coated ELISA plates, with detection by E12, a monoclonal antibody to the HA (kindly provided by Margot Anders, Microbiology Department, Melbourne University). Diluted virus was incubated in triplicate at 0°, 45°, 50°, 55°, and 60°C for 1 h. Remaining native HA was measured by ELISA and was expressed as a percentage of the 0°C control.

Infectivity and drug sensitivity in the mouse model

Six-week-old female C57BL6 × C57BL10 mice (W.E.H.I., Melbourne) were infected i.n. with 10^5 PFU of either the NWS/G70C virus or G70C4-G in 50 μl of PBS. Animals were treated i.n. with PBS or were treated with zanamivir at 0.1, 1, or 10 mg/kg/dose in 50 μl PBS. Animals were dosed with drug at −20 and −4 h prior to infection and postinfection at 4, 20, 28, and 44 h. Four to five mice were used per group. Animals were sacrificed at 48 h postinfection and lungs were harvested. Lungs were washed with PBS and were homogenized in 1.5 ml PBS. Homogenates were sonicated and were clarified at 15K for 2 min. Lung virus titers were determined by $TCID_{50}$. Cells were fixed and stained after 4 days and were scored for CPE. Data were collated from three separate experiments. Relative infectivity was determined by comparing the growth of each virus in PBS-treated animals. For statistical analysis, *t* tests were performed using GraphPad Prism (GraphPad, U.S.A.).

Drug sensitivity in the ferret model

Groups of four animals were dosed with either distilled water or zanamivir at 5, 0.5, or 0.05 mg/kg/dose, twice daily (8 h apart) from the day before infection to day 5 after infection. On the day of infection the first treatment dose was given 2 h before infection. To help with intranasal dosing ferrets were sedated with gaseous isofluorane (Abbott Lab., UK). Ferrets were challenged by intranasal instillation of 0.25 ml of diluted virus containing 10^6 PFU/ml of either NWS/G70C or G70C4-G. Temperature responses were measured by telemetry. Battery-operated transmitters (Dataquest, Data Sciences, U.S.A.) were implanted into the peritoneal cavity 7 days before infection. Telemetric data were collected by dedicated receivers (RLA 2000, Data Sciences). Temperature profiles were measured prior to and during the influenza virus infectious period. Infection was monitored by taking nasal wash samples on days 1–9 after infection, and virus titers were determined as tissue culture infectivity (in MDCK cells) by limiting dilution.

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