Contribution of H7 haemagglutinin to amantadine resistance and infectivity of influenza virus

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In the present study we determined the antiviral effect of amantadine against influenza A/Netherlands/219/03 (H7N7) virus in cell culture and in a mouse model. Amantadine at concentrations <100 μM failed to inhibit virus replication in Madin–Darby canine kidney (MDCK) cells. When orally administered to mice for 5 days, amantadine at 15 mg kg⁻¹ day⁻¹ did not protect animals against lethal challenge with H7N7 infection, and virus titres in mouse organs were not reduced. However, sequence analysis of the M2 protein revealed none of the mutations previously described as being associated with amantadine resistance. We used reverse genetics to generate viruses containing the haemagglutinin (HA) or M gene of A/Netherlands/219/03 virus to investigate the role of these genes in amantadine sensitivity. All recombinant viruses carrying the HA segment of A/Netherlands/219/03 (H7N7) virus were amantadine-resistant, regardless of the origin of their other genes. To study the role of fusion activity in the mechanism of drug resistance, we introduced the Gly23→Cys mutation in the H7 fusion peptide. This substitution resulted in a decrease of the pH of fusion and was also associated with reduced virus replication in both MDCK cells and mice, as compared to that of the wild-type virus. We suggest that H7 HA protein plays a role in amantadine resistance, although all HA amino acids that participate in drug resistance still remain to be characterized. Our finding reveals that sequence analysis of the transmembrane domain of M2 protein may not adequately identify all drug-resistant variants.

INTRODUCTION

There is concern that highly pathogenic avian influenza A viruses of the H7 haemagglutinin (HA) subtypes pose a pandemic threat to humans. Early in 2003, an H7N7 highly pathogenic avian influenza A virus caused a commercial poultry outbreak that spread from the Netherlands to Germany and Belgium (Fouchier et al., 2004; Koopmans et al., 2004). In the Netherlands, this virus was detected in 89 humans who had handled affected poultry, including three cases of their family members (Koopmans et al., 2004). In the Netherlands, this virus was detected in 89 humans who had handled affected poultry, including three cases of their family members (Koopmans et al., 2004). Influenza-like illness was observed in only a few infected persons, but there was one fatal case of pneumonia [A/Netherlands/219/03 (H7N7)] with acute respiratory distress syndrome (Fouchier et al., 2004). In February 2004, H7N3 influenza viruses were isolated from humans in Canada, although no deaths were reported (Kermode-Scott, 2004).

Antiviral drugs can play an important role in influenza control and prevention when an effective vaccine is unavailable. Two influenza-specific classes of drugs are currently available for prophylaxis and treatment: the M2 ion-channel blockers, amantadine and its congener rimantadine, and the neuraminidase inhibitors, zanamivir and oseltamivir (Monto, 2003). Koopmans et al. (2004) showed that the H7N7 virus that caused the Netherlands outbreak in 2003 is effectively inhibited in vitro by zanamivir and oseltamivir. However, there are no reports available on the efficacy of amantadine and rimantadine against this virus.

Previous investigations have shown that amantadine inhibits influenza virus replication at various stages (Hay & Zambon, 1984). At micromolar concentrations (0.1–5 μM), it selectively inhibits two different steps in the replication cycle, in a strain-specific manner (Appleyard, 1977). Virus entry is obstructed by inhibition of virus uncoating; this effect appears to require acidification of the interior of the virus particles (Hay & Zambon, 1984; Hay et al., 1985). Amantadine also acts on certain influenza strains that possess intracellularly cleavable HA, in particular the H5 and H7 subtypes, at a late stage of replication by preventing virus release. This effect apparently results from irreversible conversion of the HA to its low-pH conformation.
form within the trans-Golgi network in the absence of M2 function (Betakova et al., 2005; Grambas et al., 1992). Mutant viruses selected at low concentrations of amantadine contained amino acid substitutions in either the M2 transmembrane domain (positions 26, 27, 30, 31 or 34; Hay et al., 1985; Steinhauer et al., 1992) or the HA glycoprotein (Steinhauer et al., 1991), and thus suggested the existence of M2–HA functional interactions.

When cells are incubated with amantadine at concentrations >0.1 mM, endosomal pH increases and the acid-dependent activation of HA-mediated membrane fusion is inhibited (Gething et al., 1986; Steinhauer et al., 1996). Fusion of virus with endosomal membranes is thus blocked and infection is not initiated. Influenza virus mutants that are resistant to this effect of amantadine contain structural HA modifications that result from altered intersubunit contacts, and the pH threshold at which fusion takes place is concomitantly increased (Daniels et al., 1985; Weis et al., 1990; Wharton et al., 1986).

The influenza virus HA2 subunit (residues 1–25) mediates the fusion of virus with endosomal membranes (Skehel & Wiley, 2000; Wiley et al., 1987; White et al., 1983). A large body of data indicates that membrane fusion activity and HA conformational change co-vary with pH and temperature and occur between pH 5.0 and 6.0, depending on the influenza virus (Wharton et al., 1986; Wiley & Skehel, 1987). For example, the closely related H7 avian influenza viruses A/Chicken/Germany/34 (H7N1) (FPV Rostock) and A/Chicken/Germany/27 (H7N7) (FPV Weybridge) have a fusion pH of 5.9 and 5.3, respectively (Grambas & Hay, 1992). Notably, FPV Rostock and FPV Weybridge influenza viruses differ in their susceptibility to amantadine, despite the absence of resistance-associated M2 amino acid changes (Hay et al., 1985; Pinto et al., 1992). Kato & Eggers (1969) first showed that amantadine does not inhibit the multiplication of FPV Rostock virus when added 1.5 h after inoculation. Moreover, this virus is highly resistant to amantadine in plaque reduction and inhibition tests (Scholtissek & Faulkner, 1979), whereas FPV Weybridge is highly amantadine-sensitive (Hay et al., 1985).

In the present study we evaluated the sensitivity of A/Netherlands/219/03 (H7N7) influenza virus to amantadine in vitro and in vivo. To elucidate the role of the HA and M2 proteins and their functional relationship in the amantadine sensitivity of this H7N7 influenza virus, we used the eight-plasmid reverse genetics system to generate recombinant viruses that contained the HA or M genes of the A/Netherlands/219/03 (H7N7) virus in the background of the human A/Vietnam/1203/04 (H5N1) influenza strain. Our results provide biological evidence of the contribution of the H7 HA protein to amantadine resistance in emerging, potentially pandemic influenza virus strains.

**METHODS**

**Compound.** Amantadine (1-aminoadamantane hydrochloride) was obtained from Sigma-Aldrich. The compound was dissolved in sterile distilled water and aliquots were stored at −20°C until they were used.

**Viruses and cells.** Influenza A/Netherlands/219/03 (H7N7) virus isolated from the fatal human case (kindly provided by Drs Albert D. M. E. Osterhaus and Ron A. M. Fouchier) was plaque-purified in Madin–Darby canine kidney (MDCK) cells and propagated in the allantoic cavities of 10-day-old embryonated chicken eggs at 37°C for 48 h. The entire HA and M genes of the virus stock were sequenced to verify the absence of mutations as compared to the original strain. All experiments were performed in biosafety level 3 facilities approved by the US Department of Agriculture and the Centers for Disease Control and Prevention.

MDCK cells were grown in minimal essential medium (MEM) supplemented with 5% fetal calf serum (FCS), 5 mM l-glutamine, 0.2% sodium bicarbonate, 100 U penicillin ml⁻¹ and 100 μg streptomycin sulfate ml⁻¹ and 100 μg kanamycin sulfate ml⁻¹. Human embryonic kidney (293T) cells were maintained in OPTI-MEM-I (Gibco) medium supplemented with 10% FCS. African green monkey kidney (Vero) and BSR T7/5 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FCS. All cell lines were obtained from the ATCC and were cultivated at 37°C in a humidified atmosphere of 5% CO₂.

**Generation of recombinant viruses.** Recombinant viruses (abbreviated names are presented in Table 1) were generated by DNA transfection (Hoffmann et al., 2000). Briefly, 293T and MDCK cells

**Table 1.** Recombinant viruses generated in this study

<table>
<thead>
<tr>
<th>HA gene</th>
<th>Recombinant viruses*</th>
<th>Abbreviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/Vietnam/1203/04 (H5N1)</td>
<td>A/Vietnam/1203/04 (H5N1) with Asn₃₁→Ser in M2 protein</td>
<td>VN-1203</td>
</tr>
<tr>
<td>A/Vietnam/1203/04 (H5N1)</td>
<td>A/Vietnam/1203/04 (H5N1)</td>
<td>VN-1203-M₅₁₃₅</td>
</tr>
<tr>
<td>A/Netherlands/219/03 (H7N7)</td>
<td>A/Netherlands/219/03 (H7N7)</td>
<td>NL-219-HA-M</td>
</tr>
<tr>
<td>A/Netherlands/219/03 (H7N7)</td>
<td>A/Netherlands/219/03 (H7N7) with Gly₂₂→Cys in HA2 subunit</td>
<td>NL-219-HA₂₂₃₅C</td>
</tr>
</tbody>
</table>

*PR2, PB1, PA, NP, NA and NS genes of A/Vietnam/1203/04 (H5N1) influenza virus were used as the backbone of recombinant viruses generated in this study.
(0.2–1.0 × 10^6 cells of each cell line) were co-cultured and then transfected with 1 μg of each of the eight plasmids and 18 μl transit LT1 (PanVera) in a total volume of 1 ml OPTI-MEM-I. The DNA-lipid complexes were removed after 6 h and fresh medium was added. After a total of 72 h incubation, the supernatant was removed and 100 μl was injected into the allantoic cavity of 10-day-old embryonated chicken eggs. After 40 h, the allantoic fluid was harvested, RNA was extracted and analysed by RT-PCR and each virus segment was partially sequenced to confirm the identity of the reassortant virus. The point mutation HA2 Gly23→Cys (G23C) was inserted into the HA gene of A/Netherlands/219/03 (H7N7) and the mutation Asn31→Ser (N31S) was inserted into the M gene of A/Vietnam/1203/04 (H5N1) (Salomon et al., 2006).

Plaque assay. As described previously (Hayden et al., 1980), MDCK or Vero cells were inoculated with 10-fold dilutions of influenza virus. After 1 h, the cells were washed and overlaid with infection medium (MEM containing 0.9 % agar and 4 % BSA). After 3 days incubation at 37 °C, the cells were stained with 0.1 % crystal violet in 10 % formaldehyde solution, and plaque number and size were measured.

Antiviral activity of amantadine in cell culture. The drug sensitivity of influenza viruses was measured by plaque reduction assay (Hayden et al., 1980). Briefly, cells were inoculated with virus diluted in MEM to yield 80–100 plaques per well and were then overlaid with infection medium with or without the drug. Amantadine (0.1–100 μM) was present in the culture medium 1 h before, during and after infection. The results were recorded after 3 days incubation at 37 °C. Two independent experiments were performed on different days and the data were averaged to determine the drug concentration required to reduce the plaque size by 50 % relative to that in untreated wells. Drug toxicity was determined by visually comparing cytopathic changes in infected cells with those in uninfected cells in duplicate wells.

The amantadine sensitivity of A/Netherlands/219/03 (H7N7) virus and two recombinant viruses (NL-219-HA and NL-219-HA2G23C) was determined by measurement of a single cycle of virus growth after inoculation with virus at an m.o.i. of approximately 3 p.f.u. per cell. Amantadine (1–150 μM) was added 1 h before inoculation with virus and remained present throughout incubation. Virus yield was measured by plaque assay of the supernatant 8 h after inoculation.

Antiviral activity of amantadine in vivo. Groups of ten female 6-week-old BALB/c mice (The Jackson Laboratory) were anaesthetized with isoflurane and intranasally inoculated with 50 μl of 10-fold serial dilutions of A/Netherlands/219/03, NL-219-HA, NL-219-HA2G23C, VN-1203 or VN-1203-M_N31S virus in PBS. The 50 % mouse lethal dose (MLD50) was calculated after a 20-day observation period. Amantadine was administered by oral gavage twice daily for 5 days to groups of ten mice at a dosage of 15 mg kg^-1 day^-1. The first dose of amantadine was given 4 h after intranasal inoculation with 5 MLD50 of A/Netherlands/219/03, VN-1203 or VN-1203-M_N31S in 50 μl PBS. Control mice received sterile PBS (placebo) on the same schedule. Survival and weight change were observed; animals that showed signs of severe disease and weight loss >25 % were sacrificed. Three mice in the experimental and placebo groups were sacrificed on day 3 after inoculation and the lungs, brain, spleen and blood were removed, homogenized and suspended in 1 ml PBS. Viruses in each organ was titrated by inoculation of embryonated chicken eggs with serial dilutions of the suspensions. Titres were calculated by the method of Reed & Muench (1938) and expressed as mean log10[50 % egg-infective dose (EID50) ml^-1] ± SEM. The limit of virus detection was 0.75 log10[EID50 ml^-1]. Virus titres in each organ were compared by analysis of variance (ANOVA) (P=0.05). All studies were conducted under applicable laws and guidelines and were approved by the St Jude Children’s Research Hospital Animal Care and Use Committee.

Replication kinetics. Confluent MDCK cell monolayers were infected with viruses at an m.o.i. of approximately 3 p.f.u. per cell. After 1 h incubation, infection medium was added to the wells. Samples of supernatant were collected 2, 4, 6, 8 and 10 h post-infection and stored at -70 °C for virus titration by plaque assay.

Syncytium formation. Monolayers of 70–80 % confluent Vero cells in six-well plates were infected with A/Netherlands/219/03 (H7N7), NL-219-HA or NL-219-HA2G23C virus at an m.o.i. of approximately 5 p.f.u. per cell or were transfected with 1 μg of the plasmids expressing the viruses’ HA genes by using Lipofectamine plus (Invitrogen) according to the manufacturer’s protocol. Six hours post-infection or 15 h post-transfection, cells were washed with PBS, incubated for 2 min at the appropriate pH (4.0–6.0) at 37 °C, and then neutralized. After incubation in DMEM supplemented with 10 % FCS for 2 h at 37 °C, the cells were fixed and stained with the Hema3 staining system (Fisher Scientific).

Luciferase reporter gene assay. We used a luciferase reporter gene assay to quantitatively measure cell–cell fusion, as described previously (Russell et al., 2003). Briefly, six-well plates containing 70–80 % confluent Vero cells were transfected with 1.0 μg luciferase DNA under control of a T7 promoter (Promega) and 1.0 μg of plasmid expressing the HA of A/Netherlands/219/03 (H7N7) virus with or without the HA2 Gly23→Cys mutation. Sixteen hours post-transfection, BSR T7/5 cells (expressing T7 RNA polymerase) were overlaid on the Vero cells. After 1 h, the cells were incubated at the appropriate pH (4.0–6.0) for 2 min at 37 °C. After a 6 h incubation at 37 °C, the monolayers were washed, lysed and clarified by centrifugation. A 150 μl sample of each lysate was assayed. The luciferase activity resulting from the fusion of the two cell populations was quantified by using luciferase assay substrate (Promega) and a BD Monolight 3010 luminometer (BD). Experiments were done in triplicate.

RESULTS

Susceptibility of A/Netherlands/219/03 (H7N7) influenza virus to amantadine in vitro

To evaluate susceptibility of A/Netherlands/219/03 (H7N7) human influenza virus to amantadine in vitro, we measured the reduction of plaque size in the presence of the drug in MDCK cells (Fig. 1). Influenza VN-1203 virus carrying...
asparagine at position 31 of the M2 protein, which confers resistance to amantadine, and an amantadine-sensitive variant, VN-1203-M31S, which possessed serine at that position, served as negative and positive controls, respectively. We observed that amantadine at concentrations as high as 100 μM failed to inhibit the replication of the VN-1203 drug-resistant variant in MDCK cells. In contrast, the plaque size of VN-1203-M31S was reduced by 50% by concentrations as low as 0.1 μM. Amantadine at the highest concentration of 100 μM resulted in approximately 70% reduction in the plaque size of A/Netherlands/219/03 (H7N7) influenza virus (Fig. 1). Taken together, these results suggested that H7N7 virus is resistant to amantadine in vitro.

Susceptibility of A/Netherlands/219/03 (H7N7) influenza virus to amantadine in vivo

We administered 15 mg amantadine kg⁻¹ day⁻¹ to BALB/c mice and observed weight changes and survival of animals after inoculation with 5 MLD₅₀ of three different influenza viruses: A/Netherlands/219/03, VN-1203 and VN-1203-M31S. The latter two viruses were included for a direct comparison of the efficacy of amantadine against A/Netherlands/219/03 strain and both drug-sensitive and drug-resistant variants in mice (Table 2). The dosage of the drug was chosen in an attempt to approximate the plasma concentration produced in humans by the recommended dosage of 100 mg twice daily (Nicholson & Wiselka, 1991). Mice infected with A/Netherlands/219/03 and VN-1203 viruses and given amantadine showed the same weight loss as those in the control groups (>25%) and died no later than the groups that received placebo (between days 8 and 12 after inoculation). In contrast, amantadine treatment resulted in less weight loss (<5%), a higher survival rate and prevented death in 60% of animals infected with VN-1203-M31S influenza virus (Table 2).

To analyse whether amantadine affects the replication efficiency of A/Netherlands/219/03 (H7N7) virus in mice, we compared virus replication in the lungs, brain, spleen and blood in control and treatment groups on day 3 after inoculation. We also determined virus titres in organs and blood of mice inoculated with VN-1203 and VN-1203-M31S recombinant viruses (Table 2). Amantadine (15 mg kg⁻¹ day⁻¹) did not significantly inhibit virus replication in any of the organs of mice infected with A/Netherlands/219/03 or VN-1203 viruses. Virus titres were highest in lungs [6–8 log₁₀(EID₅₀ ml⁻¹)] and in blood [5–7 log₁₀(EID₅₀ ml⁻¹)]. All mice in these groups had virus titres of 2–3 log₁₀(EID₅₀ ml⁻¹) in brain and spleen (Table 2). In contrast, administration of amantadine significantly inhibited virus replication in the lungs and blood of animals inoculated with VN-1203-M31S virus (P<0.05) and completely eliminated virus replication in the brains and spleens on day 3 after inoculation (Table 2). Thus, our findings showed that amantadine at 15 mg kg⁻¹ day⁻¹ exerts no significant effect against challenge with A/Netherlands/219/03 (H7N7) influenza virus in vivo.

Generation and amantadine sensitivity of recombinant viruses

Mutations in the M or HA genes can alter the susceptibility of influenza viruses to amantadine (Daniels et al., 1985; Hay et al., 1985; Pinto et al., 1992; Weis et al., 1990; Wharton et al., 1986). To analyse the role of the HA and M genes of A/Netherlands/219/03 (H7N7) virus in amantadine resistance, we generated recombinant viruses containing the HA and/or M gene segments of this virus in the genetic background of A/Vietnam/1203/04 (H5N1) influenza virus, which possesses intracellularly cleavable HA like that of the H7N7 strain. The yield of all recombinant viruses in MDCK cells was similar (approx. 10²⁻³–10⁸ p.f.u. ml⁻¹).

Table 2. Antiviral activity of amantadine in mice inoculated with A/Netherlands/219/03, VN-1203 and VN-1203-M31S influenza viruses

<table>
<thead>
<tr>
<th>Virus</th>
<th>Dose (mg kg⁻¹ day⁻¹)*</th>
<th>No. of survivors/total (%)</th>
<th>Survival, mean ± SD (days)†</th>
<th>Mean virus titre ± so, log₁₀(EID₅₀ ml⁻¹)‡</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Lungs</td>
</tr>
<tr>
<td>A/Netherlands/219/03</td>
<td>0</td>
<td>0/10 (0)</td>
<td>9.2 ± 0.5</td>
<td>7.6 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>0/10 (0)</td>
<td>10.3 ± 0.4</td>
<td>7.4 ± 0.5</td>
</tr>
<tr>
<td>VN-1203</td>
<td>0</td>
<td>0/10 (0)</td>
<td>9.0 ± 0.3</td>
<td>6.8 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>0/10 (0)</td>
<td>8.6 ± 0.2</td>
<td>6.5 ± 0.3</td>
</tr>
<tr>
<td>VN-1203-M31S</td>
<td>0</td>
<td>0/10 (0)</td>
<td>9.7 ± 0.2</td>
<td>5.9 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>6/10 (60)</td>
<td>14.1 ± 0.4</td>
<td>2.5 ± 0.7§</td>
</tr>
</tbody>
</table>

*Amantadine or PBS was administered by oral gavage twice daily, starting 4 h after inoculation of 6-week-old BALB/c mice with 5 MLD₅₀ of influenza viruses.
†Estimated by the log-rank test.
‡Virus replication in organs of 6-week-old BALB/c mice 3 days after inoculation with 5 MLD₅₀ of H7N7 and H5N1 viruses in 50 μl PBS.
§P<0.05, analysis of variance (ANOVA).
... Below the limit of detection [≤0.75 log₁₀(EID₅₀ ml⁻¹)].
Table 3. Susceptibility of recombinant influenza viruses to amantadine in vitro

<table>
<thead>
<tr>
<th>Virus</th>
<th>EC_{90} (μM)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/Netherlands/219/03</td>
<td>&gt;100</td>
</tr>
<tr>
<td>NL-219-HA-M</td>
<td>100</td>
</tr>
<tr>
<td>NL-219-HA</td>
<td>100</td>
</tr>
<tr>
<td>NL-219-M</td>
<td>0.1</td>
</tr>
<tr>
<td>NL-219-HA2G23C</td>
<td>0.1</td>
</tr>
</tbody>
</table>

*Concentration of amantadine required to reduce plaque size by 50% was determined in MDCK cells in the presence of 0.01, 0.1, 1.0, 10 and 100 μM amantadine.

We measured the susceptibility of the recombinant viruses to amantadine by plaque reduction assay and calculated the dose of the compound required to reduce the plaque size by 50% (Table 3). The amantadine susceptibility of the NL-219-M recombinant virus, which carried the HA gene of VN-1203 and the M gene of wild-type (wt) A/Netherlands/219/03 (H7N7) virus (which lacked the HA gene of VN-1203 and the M gene of wild-type (wt) A/Netherlands/219/03 (H7N7) virus), was approximately 103 times lower than that of wt H7N7 virus. A/Netherlands/219/03 (H7N7) virus (which lacked the HA gene of VN-1203 and the M gene of wild-type (wt) A/Netherlands/219/03 (H7N7) virus (which lacked the HA gene of VN-1203 and the M gene of wild-type (wt) A/Netherlands/219/03 (H7N7) virus) was used to study the effects of altering the pH optimum of membrane fusion activity (Gething et al., 1986; Russell et al., 2003; Steinhauer et al., 1991, 1996). To introduce a mutation into H7 HA that would be expected to lower the pH of H7 HA fusion, we compared the sequences of the fusion peptides of two FPV H7 variants that differ in their pH of fusion with that of A/Netherlands/219/03 (H7N7) influenza virus. The fusion peptides of FPV Rostock and A/Netherlands/219/03 (H7N7) viruses were distinguished from that of the FPV Weybridge strain by having a single glycine to cysteine substitution at position 23 of HA2. We generated a mutant NL-219-HA2G23C recombinant virus containing this substitution. The HA2 Gly23→Cys mutation decreased the fusion activity of H7 viruses by syncytium formation assay in virus-infected Vero cells (Table 4). Syncytium formation was clearly observed approximately 1 h post-infection in monolayers that had been incubated at or below the pH of fusion. We observed that Vero cells infected with A/Netherlands/219/03 (H7N7) and NL-219-HA viruses formed syncytia at pH 5.4, whereas cells infected with NL-219-HA2G23C forms syncytia at pH 4.4 (Table 4). The pH of membrane fusion was also assayed by syncytium formation of Vero cells expressing A/Netherlands/219/03 (H7N7) influenza virus HA with or without the HA2 Gly23→Cys mutation. The wt HA mediated fusion at pH 5.4, whereas no syncytia formed above pH 4.4 in the monolayers of cells transfected with mutant HA (data not shown).

We also used a luciferase reporter gene assay to quantify cell–cell fusion mediated by transient expression of wt HA and HA with the Gly23→Cys substitution (Fig. 2). The results recapitulated those of the syncytium assays. Wild-type H7 HA protein promoted detectable cell–cell fusion at pH 5.4, whereas HA with the HA2 Gly23→Cys mutation promoted increased fusion at pH 4.4 (Fig. 2).

Taken together, the results of these three different assays indicated that the membrane fusion potential of the HA protein of the NL-219-HA2G23C recombinant virus is

**pH dependence of fusion activity**

To investigate further the mechanism by which the HA gene of A/Netherlands/219/03 (H7N7) virus contributes to amantadine resistance, we used site-directed mutagenesis to study the effects of altering the pH optimum of membrane fusion activity (Gething et al., 1986; Russell et al., 2003; Steinhauer et al., 1991, 1996). To introduce a mutation into H7 HA that would be expected to lower the pH of H7 HA fusion, we compared the sequences of the fusion peptides of two FPV H7 variants that differ in their pH of fusion with that of A/Netherlands/219/03 (H7N7) influenza virus. The fusion peptides of FPV Rostock and A/Netherlands/219/03 (H7N7) viruses were distinguished from that of the FPV Weybridge strain by having a single glycine to cysteine substitution at position 23 of HA2. We generated a mutant NL-219-HA2G23C recombinant virus containing this substitution. The HA2 Gly23→Cys mutation decreased the fusion activity of H7 viruses by syncytium formation assay in virus-infected Vero cells (Table 4). Syncytium formation was clearly observed approximately 1 h post-infection in monolayers that had been incubated at or below the pH of fusion. We observed that Vero cells infected with A/Netherlands/219/03 (H7N7) and NL-219-HA viruses formed syncytia at pH 5.4, whereas

Table 4. Effect of the HA2 Gly23→Cys amino acid substitution on pH of membrane fusion and infectivity

<table>
<thead>
<tr>
<th>Virus</th>
<th>pH of fusion*</th>
<th>Infectivity†</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Eggs</td>
<td>Mice</td>
</tr>
<tr>
<td>A/Netherlands/219/03</td>
<td>5.4</td>
<td>7.5 ± 0.4</td>
</tr>
<tr>
<td>NL-219-HA</td>
<td>5.4</td>
<td>7.1 ± 0.3</td>
</tr>
<tr>
<td>NL-219-HA2G23C</td>
<td>4.4</td>
<td>7.3 ± 0.2</td>
</tr>
</tbody>
</table>

*The pH associated with conformational change was determined by syncytium formation assay in Vero cells infected with the viruses at an m.o.i. of approximately 5 p.f.u. per cell.
†Virus titres were determined in embryonated chicken eggs and mice and are expressed as log_{10}(EID_{50} ml^{-1}) and log_{10}(MLD_{50} ml^{-1}), correspondingly. Values are means ± SD from two or three independent determinations.

![Fig. 2. Cell–cell fusion mediated by HA of wt and mutant (HA2 Gly23→Cys) virus as measured by luciferase reporter gene assay.](image-url)

Cell–cell fusion, measured as relative light units (RLU) of luciferase activity, is expressed as a function of pH. The data are normalized to 100% fusion (luciferase activity resulting from expression of luciferase control DNA; Promega). Each point is the mean ± SEM of three experiments.
activated at a pH 1.0 point below that required to activate the HA molecule of the wt A/Netherlands/219/03 (H7N7) virus.

**Amantadine dose response, replication kinetics and virulence of NL-219-HA and NL-219-HA2G23C viruses**

Previous data have shown that changes in the pH optimum of membrane fusion activity may influence the sensitivity of influenza viruses to amantadine in cell culture (Daniels et al., 1985; Steinhauer et al., 1991). In the present study we assayed the amantadine sensitivity of the recombinant viruses NL-219-HA and NL-219-HA2G23C in MDCK cells (Fig. 3a). We observed that amantadine at concentration of 1 μM failed to reduce virus yield of wt A/Netherlands/219/03 (H7N7) virus and NL-219-HA recombinant virus. In contrast, the replication of NL-219-HA2G23C virus was inhibited by approximately 1 log_{10} (p.f.u. ml^{-1}) in the presence of 1 μM of the drug (Fig. 3a).

To elucidate the effect of the HA2 Gly_{23}→Cys amino acid substitution on virus replication, we assayed a single replication cycle of the A/Netherlands/219/03, NL-219-HA and NL-219-HA2G23C viruses in MDCK cells (Fig. 3b). wt A/Netherlands/219/03 (H7N7) and recombinant NL-219-HA viruses showed similar replication kinetics, whereas NL-219-HA2G23C virus grew more slowly and, 10 h post-infection, had reached a virus yield of only approximately 10^{1.7} p.f.u. ml^{-1} (Fig. 3b). To assess the genetic stability of NL-219-HA2G23C virus, we sequenced the HA gene after 48 h growth in MDCK cells. A comparison to the initial variant revealed no mutations (data not shown).

In addition, to analyse whether the HA2 amino acid change Gly_{23}→Cys affects other biological characteristics of A/Netherlands/219/03 (H7N7) virus, we performed a direct comparison of the infectivity of the NL-219-HA and NL-219-HA2G23C recombinant viruses in embryonated chicken eggs and mice (Table 4). The EID_{50} and MLD_{50} values of NL-219-HA were similar to that of wt A/Netherlands/219/03 (H7N7) virus. In contrast, the virus carrying the Gly_{23}→Cys mutation in HA2 exhibited significantly lower virus yield in mice, with an MLD_{50} value 3.5 logs lower than that of the NL-219-HA strain. Taken together, our results have shown that a decrease in membrane fusion activity, as a result of the single HA2 mutation Gly_{23}→Cys, may be associated with a profound single-cycle virus replication defect and a change in amantadine sensitivity in vitro, as well as a statistically significant loss of infectivity in H7N7 influenza virus in vivo.

**DISCUSSION**

In the present study we found that amantadine, one of the specific anti-influenza drugs, was not effective in inhibiting replication of A/Netherlands/219/03 (H7N7) influenza virus in either cell culture or a mouse animal model. In vitro, amantadine at concentrations <100 μM failed to inhibit virus replication in MDCK cells. In contrast, typical amantadine-sensitive viruses could be inhibited almost completely by amantadine at a concentration as low as 0.1–1 μM (Belshe et al., 1989; Ilyushina et al., 2005). This study is the first, to our knowledge, to assess the effectiveness of amantadine therapy against highly pathogenic influenza H7N7 virus, which lacks any mutations associated with amantadine resistance in the M2 protein, in vivo. Our results demonstrated that orally administered amantadine at 15 mg kg^{-1} day^{-1} was not effective in protecting mice against infection with H7N7 influenza virus. A/Netherlands/219/03 virus was at least as amantadine-resistant as VN-1203 virus carrying asparagine at position 31 of M2 protein. We found no significant difference in virus replication in the lungs, brain, spleen or blood of the control and amantadine-treatment groups on day 3 after inoculation. In contrast, amantadine exerted

Fig. 3. Effect of HA2 Gly_{23}→Cys amino acid substitution on amantadine sensitivity and virus replication. (a) Dose effect of amantadine on the replication of A/Netherlands/219/03 (H7N7), NL-219-HA and NL-219-HA2G23C viruses. The compound was added to the cell medium 1 h before virus inoculation. Virus yield was determined by plaque assay 8 h after inoculation. (b) Replication kinetics of A/Netherlands/219/03 (H7N7), NL-219-HA and NL-219-HA2G23C viruses. Single-cycle growth curves were calculated after inoculation of MDCK cells with viruses at an m.o.i. of approximately 3 p.f.u. ml^{-1}. Supernatant was analysed by plaque assay at the times indicated.
a significant antiviral effect against VN-1203-M31S drug-sensitive virus.

Furthermore, we applied reverse genetics to generate viruses containing the HA or M gene of A/Netherlands/219/03 virus to investigate the role of these genes in amantadine sensitivity. The HA gene of H7N7 influenza virus was found to contribute to viral amantadine resistance. Further, we observed that a Gly23→Cys mutation in the H7 fusion peptide that decreases the optimum pH of fusion restored sensitivity to amantadine.

The mechanism of virus-mediated membrane fusion has been investigated by isolating virus mutants whose membrane fusion pH optima differed from those of their parents. Influenza virus variants that mediated fusion at a pH 0.1–0.7 units higher or lower than that of the parent strain were obtained by growth in the presence of high concentrations of amantadine, a compound that raises endosomal pH (Daniels et al., 1985; Steinhauer et al., 1991). Sequence analysis of influenza variants with high pH thresholds identified amino acid residues in the HA molecule that destabilize its initial quaternary structure at pH 7.0 and, as a consequence, lower the energy barrier for the conformational transition to the fusion-active state induced at a low pH (Daniels et al., 1985; Doms et al., 1986; Rott et al., 1984). Some of these residues were located along the interface between the subunits of the HA trimmer, whereas others were within or near the fusion peptide (Daniels et al., 1985; Doms et al., 1986). The mutants that caused membrane fusion at a reduced pH showed amino acid substitutions in the HA2 subunit that increased the acid stability of HA and allowed transfer of functional, native HA rather than inactivated, low-pH HA from the trans-Golgi network to the cell surface (Steinhauer et al., 1991).

We demonstrated that the fusion of A/Netherlands/219/03 (H7N7) influenza virus is induced at a pH optimum of 5.4. We found that the glycine to cysteine substitution at position 23 of the fusion peptide in the HA2 subunit decreases the pH of fusion and is one of the possible mutations that can be responsible for the altered viral fusion phenotype. The three-dimensional structure of H7 HA reveals that residue 23 is located near the base of the molecule and is buried, in contact with histidine at position 17 of HA1 and residues D112 and E114 of HA2 (Russell et al., 2004). The fact that these residues are highly conserved in the HAs of many different strains suggests that this structural interaction is important (Russell et al., 2004). Two oxygens of D112 and E114 on the long helix of HA2 and the group-specific residue H17 of HA1 form a hydrogen-bonded salt link to the same HA2 chain, which is in van der Waals contact with the terminal amino group of a second HA2 subunit (Daniels et al., 1985; Russell et al., 2004). Further, free cysteine residues in different proteins are well known to form disulfide linkages and, as a consequence, mediate and stabilize dimerization and multimerization of refolded proteins (Segal et al., 1992). Our results suggest that a cysteine plays a structural role in maintaining the neutral conformation of the fusion peptide, whereas the cysteine to glycine substitution at position 23 of HA2, which decreases the side-chain length by one atom, would be expected to disturb the tight packing of the amino-terminal peptide directly and, possibly, indirectly. By destabilizing the HA trimer, the glycine substitution in HA2 may allow it to undergo the fusion-inducing conformational change at a higher pH (pH 5.4) than it does with cysteine in this position (pH 4.4).

Our findings are in agreement with previous observations that M2 protein plays the important role in the maturation of H7 HA of modulating the pH environment encountered during transport of the glycoprotein to the plasma membrane of infected cells (Betakova et al., 2005; Grambas & Hay, 1992; Grambas et al., 1992). Mutations that alter the fusion pH of HA or the pH-modulating activity of M2 can influence changes in the corresponding properties of M2 and HA, respectively (Betakova et al., 2005; Grambas & Hay, 1992). Specifically, the lower pH-modulating activity and proton flux of the M2 ion channel, which reduces acidity in the trans-Golgi network and complements the greater acid stability of the HA protein, which has a lower fusion pH (Betakova et al., 2005; Grambas et al., 1992). Our observation that the pH of fusion of the highly pathogenic A/Netherlands/219/03 (H7N7) influenza virus is 5.4 and that the transmembrane domain of its M2 protein, like that of most human viruses, is almost identical to that of FPV Weybridge with low proton flux, strongly suggests that the activation characteristics of the M2 channel functionally complement the pH of membrane fusion by HA during virus entry.

The virus that we have characterized is a naturally occurring H7 influenza A virus that was isolated from a fatally infected human and is a potential pandemic pathogen. We demonstrated that the HA gene of this H7N7 virus is responsible for the amantadine-resistant phenotype in vitro and, presumably, in vivo. It is worth mentioning that the HA2 Gly23→Cys mutation has been introduced as an approach to investigate the mechanism of drug resistance by altering the pH of membrane fusion activity. This particular mutation was chosen based on the comparison of the fusion peptides of two FPV H7 variants that differ in their fusion pH. Although FPV Rostock and FPV Weybridge viruses differ from each other by more than 20 amino acid substitutions in the HA1 and HA2 subunits, the conserved residue at position 23 of the HA2 of A/Netherlands/219/03 (H7N7) influenza virus was chosen for mutagenesis because this mutation was expected to cause a decrease in the pH of fusion based on previous studies (Gething et al., 1986; Russell et al., 2003; Steinhauer et al., 1991, 1996). We observed that the growth of mutant NL-219-HA2G23C virus containing cysteine at position 23 of HA2 was significantly impaired in MDCK cells, presumably because of the 1.0 unit decrease in the pH optimum for membrane fusion activity. In addition, this virus showed less infectivity than the wt virus did in inoculated mice.
Finally, this study has provided further insight into the importance of the fusion activity in the drug-resistant viral phenotype; however, all of the HA amino acids that participate in amantadine resistance have yet to be characterized. Further studies should reveal both the common and the unique features of the mechanisms by which HA mediates drug resistance. Our results showed that newly emerging, potentially pandemic influenza viruses can have non-M2 protein–determined resistance to amantadine. Therefore, sequence analysis of the M2 gene alone is not necessarily adequate to detect drug resistance, biological drug susceptibility assays are needed as well. Our findings raise the concern that not only a putative pandemic strain may be resistant to amantadine, but amantadine resistance may not be detected as readily as previously thought.

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