The 2009 Pandemic H1N1 D222G Hemagglutinin Mutation Alters Receptor Specificity and Increases Virulence in Mice but Not in Ferrets

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Background. The D222G (H1 numbering) hemagglutinin (HA) mutation within the receptor-binding site was detected with higher frequencies in severe cases of 2009 pandemic H1N1 (pH1N1) infections. We investigated the impact of this mutation in vitro and in animal models using recombinant pH1N1 viruses.

Methods. The recombinant D222G HA mutant was generated from a wild-type (WT) clinical strain by using reverse genetics and site-directed mutagenesis. Replicative capacities were determined in MDCK and MDCK-α2,6 cells. Antigenicity was characterized by HA inhibition and microneutralization assays. HA titers were determined using human, chicken, and resialylated turkey red blood cells (RBCs). Virulence and contact-transmissibility were analyzed in mice and ferrets.

Results. The recombinant D222G virus grew to significantly higher titers and generated larger viral plaques compared with the WT in MDCK but not in MDCK-α2,6 cells. The mutant also showed a significant reduction in HA titers using α2,6-expressing RBCs. The 2 recombinants were antigenically similar. The D222G mutant virus induced higher lung viral titers and alveolar inflammation in mice whereas the 2 recombinants had similar impacts in ferrets.

Conclusions. The D222G HA mutation alters receptor binding specificity, resulting in higher lung titers in mice. This could contribute to the higher case fatality rates reported in humans.

In April 2009, a novel swine-origin influenza A/H1N1 virus emerged in Mexico and spread worldwide to become the first influenza pandemic (pH1N1) of this century. The pH1N1 virus was found to be genetically unrelated to seasonal influenza A/H1N1 viruses. Indeed, the HA, NP, and NS genes of the 2009 pH1N1 virus originated from the classical swine H1N1 lineage, the NA and M genes from the Eurasian swine H1N1 lineage, and the polymerase (PB2, PB1, and PA) genes from the North American H3N2 triple reassortant lineage [1]. Unlike seasonal A/H1N1 viruses, which bind mainly to α2,6-linked sialic acid (SA) receptors, the pH1N1 virus can bind to both α2,3- and α2,6-linked SA receptors [2].

The pH1N1 virus was shown to cause mild disease in the majority of cases but it has also been associated with fatal cases [3]. Despite its lower virulence compared with highly pathogenic H5N1 strains or the 1918 pandemic H1N1 virus, a concern was raised that pH1N1 could acquire virulence traits through mutations in HA and/or other genes [4]. Consequently, as the pH1N1 virus has already acquired efficient interhuman transmissibility, constant search for a more virulent genotype is an important public health priority.
MATERIALS AND METHODS

Rescue of Recombinant pH1N1 Viruses

Reverse transcription-polymerase chain reaction (RT-PCR) was used to amplify the 8 genes of A/Quebec/144147/09, which is an A/California/07/2009 pH1N1-like virus isolated in Quebec City (Canada) on 3 May 2009 (GenBank accession numbers FN434457–FN434464). The PB1, PB2, and PA segments were cloned into pLLBG whereas the HA, NA, NP, M1/M2, and NS1/NS2 segments were cloned into pLLBA bidirectional expression/translation vectors as described [10]. The pLLBA plasmid containing the HA gene was used for the introduction of the D222G mutation using appropriate primers and the QuiChange site-directed mutagenesis kit (Stratagene). The resulting pLLB-HA222G plasmid was sequenced to ensure the absence of undesired mutations. The 8 bidirectional plasmids were cotransfected into 293T human embryonic kidney cells using the Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer’s instructions. Supernatants were collected 72 hours after transfection and used to inoculate ST6Gall Madin-Darby canine kidney cells overexpressing the α2,6 SA receptors (MDCK-α2,6 cells kindly provided by Dr Y. Kawaoka, University of Wisconsin–Madison). The recombinant wild-type (WT) and D222G HA mutant viruses were subsequently sequenced and titrated by standard plaque assays in MDCK-α2,6 cells.

In Vitro Replication Kinetics Experiments

Replicative capacities of the recombinant viruses were evaluated by infecting unmodified MDCK or MDCK-α2,6 cells with a multiplicity of infection of 0.001 plaque-forming units (PFU)/cell. Supernatants were collected every 12 hours until 72 hours after inoculation (PI) and titrated by plaque assays. The mean viral plaque area of recombinants produced in MDCK or MDCK-α2,6 cells was determined from a minimum of 20 plaques obtained after 60 hours of incubation under agarose overlay as previously described [11].

Hemagglutination Assays

HA assays were performed in U-bottom microtiter plates using 50 μL of serial 2-fold dilutions of virus and 50 μL of a 1% suspension of unmodified human red blood cells (hRBCs), chicken red blood cells (cRBCs), and turkey red blood cells (tRBCs) as well as rRBCs that were treated with the Vibrio cholerae neuraminidase (VCNA) (Sigma-Aldrich) before resialylation with 2 μM of α2,3- or α2,6-(N)-sialytransferase (CosmoBio) and 1.5 mM cytidine monophospho-N-acetylneuraminic (CMP) sialic acid (Sigma-Aldrich) as previously described [12].

Hemagglutination Inhibition and Microneutralization Assays

Serum samples that were positive or negative for pH1N1 (A/California/07/2009 strain) were used in HA inhibition (HAI) and microneutralization (MN) assays. The latter procedures were performed essentially as described [13]. The HAI titer was expressed as the reciprocal of the last serum dilution achieving complete inhibition of agglutination. Neutralizing antibody titers were defined as the reciprocal of the highest dilution of serum that completely neutralized the infectivity of the recombinant pH1N1 virus as determined by the absence of cytopathic effects on day 4 PI.

Mouse Studies

Groups of twelve 6–8-week-old female BALB/c mice (Charles River) were infected by intranasal inoculation of 5 × 10^5 PFU of the recombinant WT or D222G mutant viruses. Animals were weighed daily for 12 days and monitored for clinical signs. Three mice per group were sacrificed on days 3 and 6 PI and lungs, kidneys, spleen, and colon were removed aseptically. Tissues were homogenized and RNA was extracted using the RNeasy Mini Kit (Qiagen). RNA was reverse-transcribed and amplified using the LightCycler 480 RNA Master Hydrolysis Probes (Roche) kit with primers and probes targeting a conserved region of the pH1N1 NA gene (primers/probes sequences are available upon request). Reaction conditions were as follows: reverse transcription at 63°C for 3 minutes, activation at 95°C for 30 seconds, and cycling of 95°C for 15 seconds and 60°C for 30 seconds for 45 cycles. The lower limit of detection for this assay is 2.6 × 10^1 genomic copies.

For determination of viral titers, lung tissues were harvested and homogenized in modified Eagle’s medium/bovine serum albumin using a bead mill homogenizer (Tissue Lyser, Qiagen). Debris was pelleted by centrifugation (2000g, 5 minutes) and supernatants were titrated in plaque assays using MDCK-α2,6 cells.

The levels of cytokine/chemokine (interleukin [IL]–4, IL-5, IL-6, IL-10, keratinocyte chemottractant [KC], and interferon [IFN]-γ) transcripts on days 3, 6, and 12 PI were determined by
RESULTS

Characteristics of Recombinant WT and D222G HA Mutant pH1N1 Viruses In Vitro

In MDCK cells, the recombinant D222G mutant pH1N1 virus grew to significantly higher titers during the first 36 hours PI compared with the recombinant WT virus (Figure 1). However, the maximum viral titer reached by the D222G mutant at 48 hours PI was the same as that obtained with the recombinant WT virus. In MDCK-α2,6 cells, viral titers obtained with the recombinant D222G mutant at 24 hours and then after 48 hours PI were significantly lower than those obtained with the recombinant WT virus (Figure 1). There was a significant increase in viral plaque size obtained with the recombinant D222G compared with the recombinant WT in MDCK cells (P < .001) whereas the 2 recombinants produced plaques of similar sizes in MDCK-α2,6 cells (P = .85) (Figure 1).

As shown in Table 1, the D222G mutation did not affect the antigenicity of the recombinant pH1N1 virus as both the recombinant WT and the D222G mutant had similar titers in HAI and MN assays using sera from pH1N1-vaccinated individuals.

The impact of the D222G mutation on the receptor-binding specificity was first assessed by HA tests using hRBCs, which are known to contain predominantly α2,6 SA receptors and cRBCs containing predominantly α2,3 SA receptors [15]. As shown in Table 1, there was a significant decrease in reciprocal HA titers for the D222G mutant compared with the WT using hRBCs (32 vs 128), whereas the 2 recombinants had the same HA titer (64) using cRBCs. Furthermore, by using TRBCs containing only α2,3 or α2,6 SA receptors on their surface, the HA activity of the D222G mutant was totally abolished whereas the WT agglutinated the α2,6-tRBCs and had no HA activity against α2,3-tRBCs (Table 1).

Impact of the D222G HA Mutation in Mice

Mice infected with the recombinant D222G HA mutant virus showed more signs of illness compared with the WT group. As shown in Figure 2, body weight losses observed in the mutant group were significantly higher compared with those in the WT group between days 1 and 11 PI. There was no significant difference in lung viral titers between the WT and the D222G mutant groups on day 3 PI (P = .1), whereas viral titers were significantly greater (by 1 log) for the recombinant D222G mutant compared with the recombinant WT on day 6 PI (P < .05) (Figure 3). Viral RNA was detected by real-time RT-PCR only in lung but not in kidney, colon, and spleen of mice infected with the WT or the D222G recombinants on days 3 and 6 PI (data not shown).

Compared to mock controls, IFN-γ, IL-6, IL-10, and KC transcript levels were significantly increased in the lungs of the 2 infected groups (Figure 4), whereas IL-4 and IL-5 were not (data not shown). Levels of IFN-γ transcripts on days 3, 6, and 12 PI
were similar between the WT and D222G mutant groups. On the other hand, the levels of IL-6 and KC transcripts were significantly higher in the mutant group compared with the WT group on day 3 PI (\( P < .05 \) for both cytokines) but not on day 6 PI. IL-10 was similarly expressed in the WT and mutant groups on days 3 and 12 PI whereas there was a significant

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**Figure 1.** In vitro replicative capacities of recombinant wild-type (WT) and D222G hemagglutinin (HA) mutant pH1N1 viruses. Viral titers were determined at the indicated time points from supernatants of Madin-Darby canine kidney (MDCK) (A) or MDCK-\( \alpha \)2,6 (B) cells infected with pH1N1 recombinants at a multiplicity of infection of 0.001 plaque-forming units (PFU) per cell. Mean viral titers ± SEM from triplicate experiments were determined by using standard plaque assays. *, \( P < .05 \); **, \( P < .01 \) between the recombinant WT and D222G viral titers. Viral plaque area was determined after a 60-hour incubation period in infected MDCK (C) or MDCK-\( \alpha \)2,6 (D) cells. A significant increase in plaque area was seen with the D222G mutant in MDCK (5.2 mm\(^2\) vs 0.5 mm\(^2\); \( P < .001 \)) but not in MDCK-\( \alpha \)2,6 (0.3 mm\(^2\) for both recombinants; \( P = .85 \)) cells after measuring areas of at least 20 plaques.
increase in the mutant group compared with the WT group on day 6 PI ($P < .01$).

The most noteworthy histopathological feature was the increased intra-alveolar inflammation in the D222G mutant group compared with the WT group on days 6 (mean scores of 2 vs 0.87, $P < .001$) and 12 (0.75 vs 0.0, $P < .051$) (Figure 5). On day 12, the pleural inflammation was also increased in the D222G mutant group (1.75 vs 1.0, $P < .05$). Meanwhile, comparable histopathological scores between the 2 groups were obtained with regard to bronchial/endobronchial, peribronchial, perivascular, and interstitial inflammation.

Impact of the D222G HA Mutation in Ferrets

Intranasal inoculation of ferrets with recombinant WT and D222G HA mutant pH1N1 viruses resulted in a slight pyrexic response that appeared on days 2 (mutant) and 5 (WT) PI (data not shown). Although the mutant group exhibited a higher temperature on day 2, the area under the curve of temperatures over the course of the 14-day experiment was similar for both groups of ferrets (534 ± 0.8 for the WT and 536.9 ± 1.1 for the D222G mutant) ($P = .19$). There was also no significant difference in body weight between the 2 groups (data not shown). As shown in Figure 6, viruses were recovered in nasal wash samples of ferrets up to 7 days PI with a peak ($10^5–10^6$ PFU/mL) on day 2 PI. There was no significant difference in nasal wash viral titers at any time points between the WT and D222G mutant groups of ferrets. Also, there was no significant difference in lung viral titers between the 2 recombinants on day 4 ($P = .38$) or 6 ($P = .40$) PI (Figure 6).

The contact-transmission study demonstrated efficient transmission of both recombinant WT and D222G mutant viruses in ferrets. As shown in Figure 6, viruses could be recovered in nasal wash samples of contact ferrets from both WT and D222G mutant groups between days 2 and 9 PI with a peak ($10^5$ PFU/mL) on day 4 PI. All contact ferrets seroconverted for A/California/07/2009 when tested 14 days after infection.

Table 1. Receptor binding and antigenic properties of recombinant wild-type (WT) and D222G mutant pH1N1 viruses.

<table>
<thead>
<tr>
<th>Recombinant pH1N1 viruses</th>
<th>HA titer&lt;sup&gt;a&lt;/sup&gt;</th>
<th>HAI titer&lt;sup&gt;b&lt;/sup&gt;</th>
<th>MN titer&lt;sup&gt;b&lt;/sup&gt;</th>
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<tr>
<td></td>
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<td>cRBC</td>
<td>tRBC</td>
</tr>
<tr>
<td>WT</td>
<td>128</td>
<td>64</td>
<td>64</td>
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<tr>
<td>D222G mutant</td>
<td>32</td>
<td>64</td>
<td>32</td>
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<sup>a</sup> Hemagglutination (HA) assays were performed using unmodified human (hRBC), chicken (cRBC) and turkey red blood cells (tRBC) or tRBC resialylated with α2,3 or α2,6 transferases after treatment with the Vibrio cholerae neuraminidase.

<sup>b</sup> Antisera used in hemagglutination inhibition (HAI) and microneutralization (MN) tests originated from humans who received or not the pH1N1 (A/California/07/2009) vaccine.

Figure 2. Mean body weight loss ± SEM in mice infected intranasally with $5 \times 10^5$ plaque-forming units of the recombinant wild-type (WT) or D222G mutant pH1N1 viruses. Percent body weight losses as compared with initial weights were determined daily up to day 12 after inoculation. *, $P < .05$; **, $P < .01$; ***, $P < .001$ between the recombinant WT and D222G groups.
DISCUSSION

During the early stages of infection, the receptor-binding site of the HA protein mediates the attachment of the virus to the host cell SA receptors [16]. Amino acid residue 222 (H1 numbering or 225 in H3 numbering), which is located in the influenza receptor-binding site, has been shown to be an important determinant of receptor-binding specificity [17]. The single D222G substitution in the HA protein of the 1918 pandemic H1N1 virus resulted in a shift in receptor-binding specificity [18]. Receptor binding is also a major determinant of influenza virus transmission efficiency [19]. Interestingly, the D222G mutation was identified in the HA of recent pH1N1 strains circulating in different regions of the world [9], representing 1% of contemporary pH1N1 viruses [20]. Some clinical reports have suggested a link between the D222G HA mutation and the severity of pH1N1 infections despite the detection of D222G variants in some mild cases [5, 8]. The D222G mutation was also identified in 2 mouse-adapted A/California/4/09 pH1N1 strains that led to significantly increased virulence in mice compared with the parental strain [20]. However, besides the D222G mutation, these 2 mouse-adapted (A/California/4/09-MA1 and -MA2) variants shared 2 other HA mutations (G155E and S183P) and 1 NP mutation (D101G) mutations in addition to other mutations in the polymerase (PA and PB2) proteins. Another highly virulent mouse-adapted pH1N1 strain (A/Hong Kong/41742/09) containing only the D222G HA mutation has also been reported [21]. More recently, the impact of D222G/E...
substitutions on receptor specificity and virulence was also investigated using recombinant A/Netherlands/602/09 pH1N1 variants [22].

In this study, we developed a reverse genetics system for the rescue of A/Quebec/144147/09 (an A/California/7/209-like isolate) and its D222G HA variant in order to investigate whether the mutation affects in vitro and in vivo properties of pH1N1. The reverse genetics approach precludes eventual confounding effects of changes elsewhere in the influenza genome such as mutations that may arise during lung-to-lung passage cycles. Our in vitro results demonstrate an altered receptor-binding specificity for the recombinant D222G mutant virus as suggested by a significant reduction in HA titers using hRBCs containing predominantly α2,6 SA receptors and an absence of HA activity with tRBCs resialylated with α2,6 SA receptors. Furthermore, we found higher replication for the mutant virus in MDCK cells that contain mainly α2,3 SA receptors. However, the use of α2,3 resialylated tRBCs revealed no HA activity for both the WT and mutant viruses and thus such a method may be insensitive to reveal an increased affinity of the D222G mutant for α2,3 SA receptors. Similar findings were reported by others [22].

Recently, carbohydrate microarray analyses demonstrated that the D222G pH1N1 variant was able to bind to a broader range of α2,3-linked SA receptors on ciliated bronchial epithelial cells and on the lung epithelium than were WT viruses [23]. The D222G mutation did not affect antigenicity as there were no significant differences in titers using HAI and MN assays. Similar results were obtained using A/Netherlands/602/09 pH1N1 variants [22]. This contrasts with the altered antigenicity observed in mouse-adapted variants (A/California/4/09-MA1 and -MA2), suggesting a predominant role for the additional 2 HA mutations (G155E and S183P) on the antigenic change described in that study [20].

The D222G HA mutation also resulted in increased virulence in our mouse model. In this animal, clinical signs of disease such as loss of body weight appeared more rapidly and were more pronounced with the recombinant D222G pH1N1 virus. The weight loss correlated with more persistent lung viral titers and higher proinflammatory cytokine (IL-6) and chemokine (KC) levels observed at day 3 PI but not at day 6 PI in the mutant-infected mice. IL-6 levels have been previously shown to correlate with fever and influenza symptoms in humans [24].
level of IL-10 was also higher in the recombinant D222G mutant group at day 6 PI. Despite its anti-inflammatory effect and its role in promoting tissue repair, certain reports have suggested a negative effect for this cytokine in influenza infections [25–27]. The presence of pulmonary edema and alveolar inflammation observed at days 6 and 12 PI in the recombinant D222G mutant group could also explain enhanced morbidity. Interestingly, the recombinant A/Netherlands/602/09 D222G variant was found to attach with a higher efficiency to alveolar macrophages and type II pneumocytes in the alveoli of the human respiratory tract [22]. In our study, intranasal inoculation of mice with $3 \times 10^5$ PFU of the recombinant D222G mutant virus did not result in significant mortality, which is in sharp contrast with experimental infections using $10^8$ PFU of A/California/4/2009-MA1 and -MA2 variants that resulted in 100% mortality [20]. Therefore, the D222G mutation is unlikely to be the sole determinant for the lethality in that study, and other mutations in the HA and polymerase genes could have also contributed to enhanced disease. Of interest, in the 2 other mouse-adapted viruses derived from another pH1N1 strain (A/Tennessee/1-560/2009-MA1 and -MA2) that also induced enhanced virulence and lethality, mutations other than HA D222G were also identified [20]. In contrast to Chutinimitkul et al, who reported ocular disease associated with their D222G recombinant pH1N1 mutant [22], we did not observe extrarespiratory disease in our mouse studies, suggesting that other genetic changes or different inoculums could influence pathogenicity.

It has been previously described that infection of ferrets with pH1N1 virus did not result in severe disease [28]. Accordingly, there were minimal clinical signs observed in our ferret experiments and no significant differences between the recombinant WT and its D222G variant in terms of weight and pyrexic responses. Viral titers recovered from nasal wash samples were also comparable between the 2 groups. Furthermore, the recombinant D222G mutant virus was transmitted by direct contact with a similar efficiency compared with the recombinant WT virus. This observation is in agreement with a recent clinical report demonstrating efficient transmission of the pH1N1 isolate containing the D222G HA mutation in household contacts [29] and it suggests the retained capability of this HA mutant to attach to $\alpha$2,6 SA receptors.

In conclusion, our findings suggest that the pH1N1 D222G mutant has altered receptor specificity resulting in improved binding to SA receptors (presumably of the $\alpha$2,3 linkage) predominantly found in the lower respiratory tract of various mammals, including humans. However, although the D222G mutation conferred a replicative advantage in some cell lines and in mice, it did not enhance the virulence (weight loss, temperature, upper and lower respiratory tract titers) in ferrets. It was recently reported that $\alpha$2,6 SA receptors were predominantly found in the respiratory tract of ferrets including in lung alveoli, which could partly explain the absence of enhanced disease conferred by the D222G mutant in that model [30]. Fortunately, this HA mutation did not change the virus antigenic properties, suggesting continuous protection by actual pH1N1 vaccines. Real-time monitoring of pH1N1 viruses for the D222G HA mutation appears relevant to predict clinical outcomes, although additional well-controlled epidemiological studies are needed to confirm our animal observations.

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**References**


