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Amino acid substitution D222N from fatal influenza infection affects receptor-binding properties of the influenza A(H1N1)pdm09 virus

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ABSTRACT

We have analyzed the receptor binding profile of A(H1N1)pdm09 recombinant influenza viruses containing the amino acid substitution D222N which has been associated with a fatal case of infection. This mutation was investigated in conjunction with a secondary mutation, S185N. Using human tracheobronchial epithelial cells (HTBE), we found that single mutation D222N affects the binding and replication of the virus during initial stages of infection, with limited but preferred tropism to non-ciliated cells expressing α2,6-SA. However, in conjunction with the S185N change, the (D222N, S185N) virus shows a remarkable increase in binding and replication efficiency, with tropism for both ciliated and non-ciliated cells. Glycan microarray analysis demonstrated correlation between the binding profile and the cell tropism observed in the HTBE cells. These findings suggest that viruses with D222N required compensatory mutations such as S185N to maintain viral fitness, and in combination, affect the pathogenicity of the virus and the clinical outcome.

Introduction

The 2009 influenza pandemic in Mexico was characterized by an unusual increase in the number of severe and fatal respiratory infections associated with the isolation of the novel influenza A (H1N1)pdm09 virus (Chowell et al., 2009). The influenza outbreak in southern Mexico between June and August of that year was considered the second wave of the pandemic, with the highest number of infections reported in Yucatan (Chowell et al., 2011). During this time and in the following months, the health authorities in Yucatan recorded a total of 92 cases of severe or fatal influenza infection (Ayora-Talavera et al., 2012). Molecular markers of pathogenicity, including amino acid changes mapping to the receptor binding site (RBS) of the viral hemagglutinin (HA) were found in some of the patients with severe or fatal influenza infections. Isolates of the new A(H1N1)pdm09 virus containing a D222G amino acid change in the RBS were reported in different geographic regions at frequencies varying from 5% up to 24% (Chen et al., 2010; El Moussi et al., 2013; Ledesma et al., 2011; Rykkvin et al., 2013; Vazquez-Perez et al., 2013). The pathogenic effects of viruses with the D222G mutation have been analyzed in mice, Guinea pigs, and ferrets, but the results and conclusions differ depending on the study and animal model utilized (Abed et al., 2011; Casalegno et al., 2014; Chutinimitkul et al., 2010; Vazquez-Perez et al., 2013).

The genetic polymorphism 222D/G/N in HA amino acid 222 of the A(H1N1)pdm09 virus has been identified in circulating viruses, and for virus populations within the same host a higher frequency of mutants with G, N, or both, is suggested to relate to the severity of the infection (Baldanti et al., 2011; Resende et al., 2014). Interestingly, the D222N mutation has generally been detected in combination with D or G heterogeneity at this position, and in only few reports as the major population (Baldanti et al., 2011; Casalegno et al., 2014; Drews et al., 2011; Resende et al., 2014; Wang et al., 2011). The presence of any polymorphism at residue 222 may confer changes to the virus receptor binding profile and the cell/tissue tropism, as D222G HA mutants of A(H1N1)pdm09 virus showed a preference for infection of ciliated cells from HTBE (Chutinimitkul et al., 2010), consistent with α2,3-SA specificity (Matrosovich et al., 2004; Thompson et al., 2006), and with changes in the tissue tropism as demonstrated for the highly pathogenic H5N1 virus (Shinya et al., 2006; Van Riel, et al., 2006). However, the A(H1N1)pdm09 virus with D222G also retained ability to infect non-ciliated cells expressing α2,6-SA, showing dual receptor

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specificity, as well as dual binding to α2,3-SA and α2,6-SA glycans (Chutinimitkul et al., 2010; Liu et al., 2010; Yang et al., 2010).

A(H1N1)pdm09 viruses with the HA D222N substitution have also been isolated and characterized since 2009 (Wang et al., 2011), and viruses with this mutation have been detected during outbreaks of severe and fatal influenza in Mexico, Ecuador, and the U.S. Interestingly, these cases were associated with a prominent D222N and not the polymorphic trait of past isolates. Phylogenetic analysis identified a new sub-clade containing additional amino acid changes within the HA: N31D, S162N, A186T and V272I (Houng et al., 2012). The role of these mutations on the biology of the pandemic virus is unknown.

Although the glycan binding profiles of both natural and recombinant A(H1N1)pdm09 viruses containing the mutation D222N have been reported (Bradley et al., 2011; Chen et al., 2011), the precise role of the amino acid change D222N on the severity of the infection is unclear. In this study, we address further the receptor binding phenotype of A(H1N1)pdm09 viruses with polymorphisms 222D/N, and the effect on the cell tropism using the in vitro system of human airway epithelial cells.

Results and discussion

Mutation D222N affects the early replication of A(H1N1)pdm09 virus in HTBE cultures but is compensated by secondary mutations in the antigenic site Sb

In order to identify mutations in the HA RBS that might affect binding phenotype and pathogenicity, we performed sequence analysis of HA genes from 13 viral isolates from severe and fatal cases from Yucatan, Mexico. From this, relevant mutations were identified in only two viruses, A/Yucatan/5783/2009 (Yuc/5783) and A/Yucatan/3882/2009 (Yuc/3882). The Yuc/5783 virus was isolated from the bronchial lavage of a hospital patient with a severe case of Influenza. This virus contained a single mutation, D190Y, at the HA protein. The effect of this mutation on the receptor binding properties of the virus was recently described by our group (Ayora-Talavera et al., 2014). The virus Yuc/3882, isolated by throat swab from patient that subsequently died from the infection, contained two mutations in the receptor binding region of HA, S185N and D222N. Due to the relevance of the mutation D222N and the lack of data about its specific role on the pathogenicity of the pandemic virus, we examined the receptor binding profile and replication properties of viruses containing this mutation. We also addressed the relevance of the mutation S185N, located at the antigenic site Sb (Xu et al., 2010). A different amino acid change, S185T, is reported to be conserved in all A(H1N1)pdm09 viruses in current circulation, and its role in the enhancement of receptor-binding avidity of the early A(H1N1)pdm09 virus has been addressed (de Vries et al., 2013; ECDC 2013; Elderfield et al., 2014; Klimov et al., 2012).

Therefore we generated a series of mutants based on the United Kingdom prototype A(H1N1)pdm09 virus, A/England/195/2009 (Eng/195), altered only in the HA gene (Baillie et al., 2012). Each mutant virus contained either amino acid substitution S185N (rEng/195-S185N), D222N (rEng/195-D222N) or S185N+D222N (rEng/195-S185N+D222N). Sequence analysis and comparison between the Eng/195 and Yuc/3882 viruses indicated a 99.7% homology for the HA protein with only three amino acid differences S185N, S203T and D222N.

The growth properties of the recombinant mutant viruses were compared to recombinant Eng/195 (rEng/195-wt) in differentiated pseudostratified human tracheal–bronchial epithelial cells (HTBE, MatTek – Massachusetts, USA). Mutants with S185N whether as a single mutant, or in combination with D225N (rEng/195-S185N and rEng/195-S185N+D222N), replicated to higher titers at early time points than did the wild type virus rEng/195-wt (Fig. 1A). At 12 h post-infection (h.p.i.) the two mutants with S185N already displayed recoverable virus, whereas rEng/195-wt virus did not, and at 24 h, the higher titers for the two mutants were significant ($p < 0.0001$). The slow replication of the rEng/195-wt virus is probably due to differences in adaptation to the HTBE system; our data are in agreement with recent results that showed a delayed viral growth for the rEng/195-wt used as prototype virus from the first pandemic wave compared to a recombinant virus from the third wave (Baillie et al., 2012; de Vries et al., 2013; Elderfield et al., 2014). Similar to the wt, the rEng/195-D222N showed null viral growth at 12 h.p.i. ($p < 0.0001$), although at 24 h.p.i. and at later timepoints, the virus reached a maximum viral titer similar to the other two mutants. Overall, these results suggest that the presence of mutation D222N alone has a negative effect on the viral replication during the initial rounds of replication. However, the presence of mutation S185N alone, or in combination with D222N, correlated with faster replication of these viruses in HTBE cells at early time points.

In MDCK cells the growth curve (Fig. 1B) showed that rEng/195-wt virus replicated most efficiently. Significant differences were observed between the rEng/195-wt viral titer versus rEng/195-S185N at 12 ($p < 0.01$), and rEng/195-222N and rEng/195-S188N+D222N ($p < 0.0001$) at 24 h post-infection. The rEng/195-S185N virus displayed higher titers at 24 h than the other two mutants. These data suggest that in MDCK cells, recombinant viruses did not show replication constraints, even in the presence of specific mutations. The delayed viral replication we previously observed for the rEng/195-wt it was likely to be associated with the complex HTBE culture system, a feature that allows the rEng/195-D222N virus to replicate efficiently after 24 h post-infection.

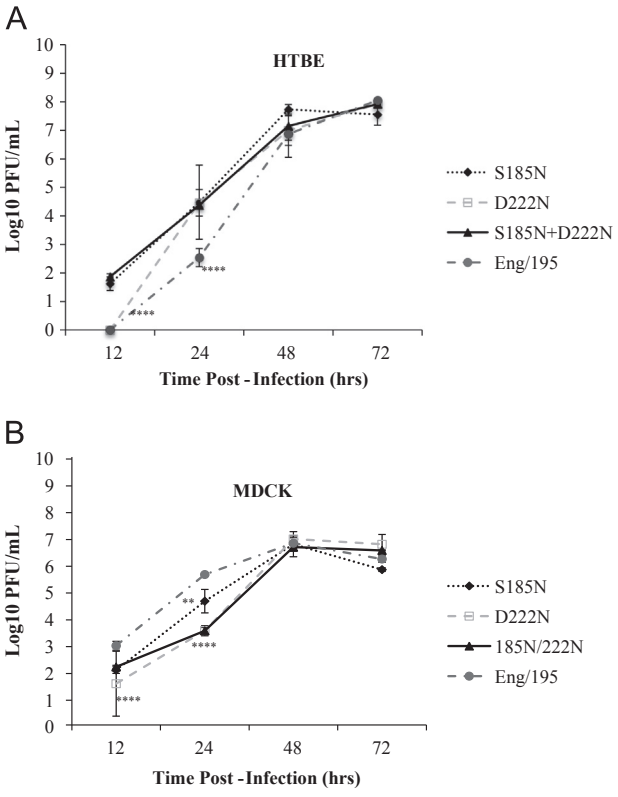

Fig. 1. Replication of viruses in cell culture. Growth kinetics of WT and mutated viruses in (A) HTBE and (B) MDCK cultures. Cells were infected at an MOI of 0.001 or 0.01. Data represent the average ± SD of three different experiments. Statistics were calculated with the ANOVA test for repeated measures using GraphPad Prism v5.0. Significant differences were considered $^{****}p < 0.0001$; $^{**}p < 0.001$.