Insertion of a multibasic cleavage site in the haemagglutinin of human influenza H3N2 virus does not increase pathogenicity in ferrets


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A multibasic cleavage site (MBCS) in the haemagglutinin (HA) protein of influenza A virus is a key determinant of pathogenicity in chickens, and distinguishes highly pathogenic avian influenza (HPAI) viruses from low pathogenic avian influenza viruses (LPAI). An MBCS has only been detected in viruses of the H5 and H7 subtypes. Here we investigated the phenotype of a human H3N2 virus with an MBCS in HA. Insertion of an MBCS in the H3N2 virus resulted in cleavage of HA and efficient replication in Madin–Darby canine kidney cells in the absence of exogenous trypsin in vitro, similar to HPAI H5N1 virus. However, studies in ferrets demonstrated that insertion of the MBCS into HA did not result in increased virus shedding, cellular host range, systemic replication or pathogenicity, as compared with wild-type virus. This study indicates that acquisition of an MBCS alone is insufficient to increase pathogenicity of a prototypical seasonal human H3N2 virus.
Govorkova et al., 2005; Kuiken et al., 2010; Maines et al., 2005; van Riel et al., 2010). In mice, it has been shown that removal of the MBCS from some HPAI H5 and lab-adapted H7 virus HAs resulted in reduced virulence (Gabriel et al., 2005; Hatta et al., 2001). Here, we followed an opposite – gain of function – approach, by inserting an MBCS into a human H3N2 virus and testing virus replication and pathogenesis in the ferret model. The goal of this work was to increase our basic understanding of the role of the MBCS and trypsin/furin-dependent cleavage of HA in influenza virus pathogenesis in mammals.

Influenza virus A/Netherlands/16190/68 (H3N2) was isolated from an individual in the Netherlands during the 1968 pandemic. Influenza virus A/Hong Kong/156/97 (H5N1) was isolated from the human index case during the 1997 H5N1 outbreak in Hong Kong (de Jong et al., 1997). The eight gene segments of these two viruses were amplified by RT-PCR and cloned in the BsmBI site of a modified version of plasmid pHW2000 (de Wit et al., 2004; Hoffmann et al., 2000). For the generation of H5N1 virus without an MBCS (H5N1\textsubscript{AMBCS}), the cleavage site PQIETR\textsubscript{G} in the H5 HA plasmid was changed to PQIETR\textsubscript{D} by RT-PCR with specific primers as described previously by Webbey et al. (2004). For the generation of H3N2 virus with an MBCS (H3N2\textsubscript{MBCS}), the cleavage site PEKQTR\textsubscript{G} in the H3 HA plasmid was changed to PEKQRRRKKR\textsubscript{G}, which is identical to that of the MBCS of the control virus A/Hong Kong/156/97 H5N1. Viruses H3N2\textsubscript{wt}, H5N1\textsubscript{wt}, H3N2\textsubscript{MBCS} and H5N1\textsubscript{AMBCS} were generated by reverse genetics as described previously (Munster et al., 2010). The genotypes of all plasmids and viruses were confirmed by sequencing. The risk potential of the H3N2\textsubscript{MBCS} virus was assessed prior to inoculation with exogenous trypsin.

First, the requirement of trypsin for the cleavability of the H3 HA proteins was tested. To this end, 293T cells were harvested 48 h after transfection with plasmids expressing HA of H3N2\textsubscript{wt} and H3N2\textsubscript{MBCS}, using HA of H5N1\textsubscript{wt} and H5N1\textsubscript{AMBCS} as controls. Cells were treated with either PBS (pH 7.4) or 2.5 µg trypsin ml\textsuperscript{-1} (Lonza) for 1 h at 37 °C. Cell lysates were subjected to electrophoresis in SDS-polyacrylamide gels (10% acrylamide) and immunoblotted. Western blots were incubated with rabbit serum reactive against A/Hong Kong/1/68 (H3) or A/Hong Kong/156/97 (H5) and a peroxidase-labelled swine anti-rabbit antibody as previously described (Munster et al., 2010). In the presence of trypsin, the HA of H3N2\textsubscript{wt} and H3N2\textsubscript{MBCS} were cleaved to completion (Fig. 1a). In contrast, in the absence of trypsin, the HA of H3N2\textsubscript{wt} remained uncleaved while a large fraction of the HA of H3N2\textsubscript{MBCS} was cleaved. Thus, upon insertion of an MBCS into H3 HA the cleavage of HA was no longer dependent on exogenous trypsin. H3 HA with and without an MBCS thus behaved essentially the same as H5 HA with and without an MBCS (Fig. 1a, b).

Next, virus replication of reverse genetics-derived H3N2\textsubscript{wt} and H3N2\textsubscript{MBCS} viruses in the presence and absence of 1 µg trypsin ml\textsuperscript{-1} was tested in Madin–Darby canine kidney (MDCK) cells, again using H5N1\textsubscript{wt} and H5N1\textsubscript{AMBCS} as controls. Cells were inoculated in duplo at a m.o.i. of 0.01 TCID\textsubscript{50} per cell. Supernatant samples were harvested at 6, 12, 24 and 48 h after inoculation and virus titres were determined by end-point titration in MDCK cells (Munster et al., 2010). The H3N2\textsubscript{wt} and H5N1\textsubscript{AMBCS} viruses replicated to comparable titres in the presence of trypsin. In the absence of trypsin both viruses failed to replicate to high titres. The H3N2\textsubscript{MBCS} and H5N1\textsubscript{wt} viruses replicated to comparable titres in MDCK cells in the presence and absence of trypsin (Fig. 1c, d). The trypsin-independent phenotype of the human influenza H3N2 virus with an MBCS in vitro was also observed with a second H3N2 virus, A/Netherlands/178/95 (data not shown). Thus, trypsin-independent HA cleavage by insertion of an MBCS can be achieved, not only in avian influenza viruses but also in human influenza A/H3N2 virus (Ohuchi et al., 1991).
Next, we investigated whether the trypsin-independent cleavage of H3N2_{MBCS} resulted in enhanced pathogenicity in ferrets. The ferret model is generally thought to be a good animal model for influenza in humans, because ferrets are naturally susceptible to infection and develop respiratory disease and lung pathology similarly to humans when infected with seasonal, avian or pandemic influenza viruses (Bodewes et al., 2010; Maher & DeStefano, 2004; van den Brand et al., 2010). Groups of six influenza-seronegative female ferrets (Mustella putorius furo) were inoculated intranasally with 10^6 TCID_{50} of H3N2_{wt} or H3N2_{MBCS} virus, divided over both nostrils (2 × 250 μl). Animals were observed for the development of clinical symptoms and weighed daily. The percentage mean maximum weight loss was 8.8 ± 1.4 % (SD) for animals inoculated with H3N2_{wt} virus. Animals inoculated with H3N2_{MBCS} had a maximum weight loss of 7.0 ± 1.4 % (SD) (Fig. 2a). No differences were observed for clinical parameters, such as lethargy, sneezing, ruffled fur, interest in food and runny nose, between the two groups of ferrets. Thus, H3N2_{MBCS} does not appear to cause more severe disease as compared with H3N2_{wt}.

Throat and nasal swabs were collected daily to determine virus excretion from the upper respiratory tract. Infectious

Fig. 2. Weight loss and virus replication in ferrets inoculated with H3N2_{wt} and H3N2_{MBCS} virus. (a) Weight loss of ferrets inoculated with H3N2_{wt} (○) and H3N2_{MBCS} (●) virus. Mean body weight and SD were calculated as percentages of body weight at time of inoculation for each group. After day 3, only three animals remained in each group. (b and c) Virus shedding from the nose (b) and throat (c) of ferrets inoculated with H3N2_{wt} (black bars) and H3N2_{MBCS} (grey bars). (d) Virus detection in tissues of ferrets inoculated with H3N2_{wt} (black bars) and H3N2_{MBCS} (grey bars) virus at day 3. Geometric mean titres are shown, with error bars indicating the SD. The lower limit of detection is indicated by the dotted line. The number of positive tissues from the ferrets are shown.
virus shedding from the nose peaked at day 2 for both viruses and continued until days 7 and 6 for H3N2wt and H3N2MBCS, respectively (Fig. 2b). Shedding from the throat peaked at day 1 and continued until day 6 for both viruses (Fig. 2c). We observed a consistent decline in viral shedding from the throat, compared with the nose. Nucleotide sequencing was performed to confirm that the HA cleavage site remained unchanged during the experiment.

Three animals from each group were euthanized at 3 and 7 days post-inoculation (p.i.), and the nasal turbinates, trachea, lungs, liver, spleen, kidney, colon and brain were collected to study virus distribution. Necropsies and tissue sampling were performed according to a standard protocol and infectious-virus titres were determined by end point titration in MDCK cells (Munster et al., 2010). At 7 days p.i., virus was undetectable in samples from the organs of all groups of ferrets. At 3 days p.i., virus was not detected in lungs, spleen, liver, kidney and intestine of ferrets inoculated with H3N2wt or H3N2MBCS. Both groups of inoculated ferrets revealed low virus titres in the trachea. Virus titres in the nasal turbinates were high and similar for both groups of ferrets. Virus was detected in the brain of 1 of 3 animals inoculated with H3N2wt and 3 of 3 animals inoculated with H3N2MBCS, but only at low titres (Fig. 2d). Immunohistochemistry was performed to confirm virus replication in nasal turbinate, trachea and brain at 3 days p.i. After fixation in 10% neutral-buffered formalin and embedding in paraffin, tissue sections were stained using a mAb against the nucleoprotein of influenza A virus (Munster et al., 2010). Influenza virus antigen expression was not detected by immunohistochemistry in the brain and trachea of either group of ferrets. In contrast, virus antigen was readily detected in the nasal turbinates of all three ferrets in both groups at 3 days p.i. There was no evidence for different cellular tropisms between H3N2MBCS and H3N2wt virus in the nasal turbinates of infected ferrets (Fig. 3). These results indicated that replication of H3N2MBCS, like replication of H3N2wt, is mostly restricted to the upper respiratory tract of ferrets.

The current study demonstrates that, with the insertion of an MBCS identical to that of currently circulating HPAI H5 viruses, a human H3N2 virus becomes trypsin-independent in vitro. The introduction of an MBCS into other human and LPAI HA subtypes (H3 and H6) also resulted in trypsin-independent cleavage of HA0 in vitro (Kawaoka, 1991; Munster et al., 2010; Ohuchi et al., 1991; Stech et al., 2009). Introduction of an MBCS into an LPAI H6N1 virus resulted in an increased intravenous pathogenicity index in chickens (Munster et al., 2010). However, the consequences of the introduction of the MBCS for human H3N2 virus replication and pathogenesis in animal models have not been investigated previously. Surprisingly, the trypsin independence of a human H3N2 virus did not result in enhanced pathogenicity, tissue tropism or cellular tropism in the ferret model, as compared with wild-type H3N2 virus. If anything, the H3N2MBCS virus was slightly attenuated compared with H3N2wt virus, based on observations of weight loss and virus shedding from the upper respiratory tract.

Both groups of inoculated ferrets revealed no virus replication in the lower respiratory tract. This is not unusual, as strain-to-strain variability among human H3N2 viruses with respect to infection of the lower respiratory tract of ferrets has been demonstrated (Jackson et al., 2009; Parks et al., 2007; Svitek et al., 2008; Zitzow et al., 2002). Although the H3N2MBCS virus was detected in the brain of ferrets, virus titres were very low and virus-infected cells were not detected by immunohistochemistry. Given that low virus titres were also detected for a ferret inoculated with H3N2wt virus, and that neither of the groups of infected ferrets developed neurological symptoms, we conclude that the MBCS in the context of H3 HA did not result in systemic virus replication and enhanced disease. In ferrets infected with more recent wild-type human H3N2 viruses, low virus titres were also detected in the brains (Zitzow et al., 2002).

The goal of the present work was to increase the basic understanding of the role of the MBCS and trypsin- or furin-dependent cleavage of HA in influenza virus...
pathogenesis in mammals. Our results indicate that an MBCS is not sufficient to increase the virulence of a human H3N2 virus. For HPAI H5N1 viruses, additional virulence determinants in HA and other viral proteins have been shown to affect virulence independently of the MBCS in ferrets and chickens (Bogs et al., 2010; Gohrbandt et al., 2011; Govorkova et al., 2005; Imai et al., 2010). It is possible that an MBCS in H3 HA in combination with other genetic changes, such as changes in the receptor binding site, could result in increased pathogenicity of human H3N2 virus as well, by facilitating replication in extra-respiratory tissues.

Acknowledgements

We thank R. Dias-D’Ullois, D. de Meulder, S. Chutinimitkul, P. van Run and M. de Graaf for excellent technical assistance, and E. Sorrell for helpful discussions. V. J. M. and E. d’W. are currently supported by the Division of Intramural Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health. This research was financed through VICI grant 91896613 from ZonMW and National Institute of Allergy and Infectious Diseases–NIH contract HHSN266200700010C.

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