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Identification of amino acids in the HA of H3 influenza viruses that determine infectivity levels in primary swine respiratory epithelial cells

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Abstract

In the late 1990s, triple reassortant H3N2 influenza A viruses emerged and spread widely within the swine population of the United States. We have shown previously that an isolate representative of this lineage of viruses, A/Swine/Minnesota/593/99 (Sw/MN), has higher infectivity and accelerated replication kinetics in pigs, compared to a human-lineage H3N2 virus isolated from a pig during the same time period, A/Swine/Ontario/00130/97 (Sw/ONT [Landolt, G.A., Karasin, A.I., Phillips, L., Olsen, C.W., 2003. Comparison of the pathogenesis of two genetically different H3N2 influenza A viruses in pigs. J. Clin. Microbiol. 41, 1936–1941]). Additional in vivo experiments using reverse genetics-generated reassortant viruses demonstrated that these phenotypes are dependent upon the HA and/or NA genes (Landolt, G.A., Karasin, A.I., Schutten, M.M., Olsen, C.W., 2006. Restricted infectivity of a human-lineage H3N2 influenza A virus in pigs is hemagglutinin and neuraminidase gene dependent. J. Clin. Microbiol. 44, 297–301). To further study the infectivity of influenza viruses for pigs, we developed a primary swine respiratory epithelial cell (SREC) culture model. In SRECs, Sw/MN infects a significantly higher number of cells compared to Sw/ONT. Using reverse genetics-generated Sw/MN × Sw/ONT reassortant viruses we demonstrate that the infectivity phenotypes of these viruses in SRECs are strongly dependent upon the HA gene. Using chimeras and point directed mutations within the HA genes, we have identified amino acids that, either alone or in combination with other amino acids, impact infectivity. In particular, amino acid 138 is the dominant factor in determining infectivity levels in SRECs.

Keywords: Infectivity; H3; Influenza virus; Swine; Epithelial cells

1. Introduction

Pigs may play an important role in the epidemiology and evolution of influenza viruses (reviewed in Subbarao et al., 2005; Webster et al., 1992). Pigs express 2,6-linked sialic acids, the receptor determinant preferred by human influenza viruses, as well as 2,3-linked sialic acids, the receptor determinant preferred by avian influenza viruses, on their tracheal epithelial cells (Ito et al., 1998). As such, it has been postulated that pigs may serve as intermediate hosts in which genetic reassortment among co-infecting human and avian, or human, swine and avian viruses can lead to the generation of pandemic influenza viruses (reviewed in Ito, 2000; Scholtissek and Naylor, 1988; Subbarao et al., 2005; Webby and Webster, 2001; Webster et al., 1992). In support of this hypothesis, transmission of avian as well as human influenza viruses to pigs has occurred under both natural (Karasin et al., 2000a,b, 2004 and reviewed in Brown, 2000; Webby and Webster, 2001; Webster et al., 1992) and experimental (Hinshaw et al., 1981; Kida et al., 1994) conditions. In addition to the potential to generate pandemic viruses, pigs can serve as a source for zoonotic influenza virus infections (Myers et al., 2007). Finally, beyond the risks to human health, influenza virus infection is a substantial cause of morbidity for pigs and economic loss for the swine industry (Olsen et al., 2006a).

H1N1 influenza viruses were first isolated from pigs in the U.S. in 1930 (Shope, 1931). These early swine H1N1 influenza viruses are related to the H1N1 "Spanish influenza" virus (Reid et al., 1999; Tumpey et al., 2005). Seroprevalence

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studies (Chambers et al., 1991; Hinshaw et al., 1978; Olsen et al., 2000; Webby et al., 2000) have shown that H1N1 viruses have continued to circulate in the U.S. swine population since 1930. In contrast, prior to 1998, seroprevalence studies indicated that H3N2 influenza virus infections in pigs in North America were quite rare (Chambers et al., 1991; Hinshaw et al., 1978), and when isolated from pigs, these H3N2 viruses were of human-lineage (Bikour et al., 1994, 1995; Hinshaw et al., 1978). However, since 1998, H3N2 viruses have spread widely and been maintained in the North American swine population, causing a serious epizootic of respiratory illness. These H3N2 influenza viruses are triple reassortant viruses that contain HA, NA and PB1 gene segments from human-lineage influenza viruses; PA and PB2 gene segments from avian-lineage influenza viruses; and M, NS and NP gene segments from classical swine-lineage influenza viruses (Karasin et al., 2000b; Olsen et al., 2006b; Richt et al., 2003; Webby et al., 2000; Zhou et al., 1999). Coincident with the appearance of these viruses, H3 influenza virus seroprevalence in the U.S. swine population increased from 1.4% during 1976–1977 (Hinshaw et al., 1978) and 1.1% during 1987–1989 (Chambers et al., 1991) to 8.0% during 1997–1998 (Olsen et al., 2000) and 20.5% during 1998–1999 (Webby et al., 2000).

To begin to understand the remarkable emergence, spread and maintenance of these triple reassortant H3N2 viruses in pigs, we previously performed infection studies in pigs, and now report in vitro studies utilizing swine respiratory epithelial cells (SRECs). We have primarily compared the infection characteristics of two isolates. A/Swine/Minnesota/593/99 (Sw/MN) is representative of the earliest, "cluster I" (Webby et al., 2000) triple reassortant viruses. A/Sw/Ontario/00130/97 (Sw/ONT) is a wholly human-lineage H3N2 influenza A virus that was isolated during the same time period from a baby pig (presumably following transmission from an animal caretaker), but which showed no evidence of spread on the farm of origin or beyond (Karasin et al., 2000b). In initial in vivo studies, Sw/MN caused more extensive pathological lesions, and exhibited remarkably greater infectivity as well as accelerated shedding kinetics, compared to Sw/ONT (Landolt et al., 2003). Additional in vivo studies using reverse genetics-generated Sw/MN (rgMN) and Sw/ONT (rgONT) reassortant viruses demonstrated that these phenotypes could be reversed by exchanging their HA plus NA genes (Landolt et al., 2006).

To define the role of the HA and NA genes and identify specific amino acids in these genes that determine infectivity, we moved from in vivo studies to an in vitro model system. Influenza virus infections have been studied previously in established cell lines such as Madin-Darby canine kidney (MDCK), Vero, A549, and mink lung cells (Fieldsteel et al., 1982; Govorkova et al., 1996; Huang and Turchek, 2000; Schultz-Cherry et al., 1998). These cells, while convenient and readily available, do not permit examination of factors that control virus infection of swine respiratory cells, the species and target cells of interest. Cultures of primary airway epithelial cells from humans have been used previously to study infections with rhinovirus (Newcomb et al., 2005; Schroth et al., 1999), respiratory syncytial virus (Becker et al., 1992; Kong et al., 2003), parainfluenza virus (Kogure et al., 2006; Zhang et al., 2005), and influenza A virus (Chan et al., 2005; Ibricevic et al., 2006; Kogure et al., 2006; Matrosovich et al., 2004a,b; Thompson et al., 2006; Wan and Perez, 2007). We now report the development of a method to cultivate monolayers of primary SRECs and the use of these cells to define viral genetic factors controlling influenza virus infectivity in swine respiratory cells.

Our prior in vivo results led us to focus on the contributions that the HA and NA genes have on infectivity, both in combination and individually. In the present study, using reverse genetics we examined infectivities of Sw/MN, Sw/ONT, and Sw/MN × Sw/ONT reassortant viruses in order to identify which gene(s) determine virus infectivity levels in SRECs. Our data identify the HA gene as the primary determinant of the relative infectivities of Sw/MN and Sw/ONT in SRECs; the remaining seven gene segments had minimal to no effect on infectivity. Using a series of viruses with chimeric HA genes (exchanging corresponding regions of the HA gene between Sw/MN and Sw/ONT) followed by viruses with point substitutions within the HA, we determined that serine at amino acid 138 is necessary, but not solely sufficient, for a high infectivity phenotype in SRECs.

2. Materials and methods

2.1. Cell lines

The MDCK cells were maintained in Eagle's minimal essential medium (MEM, GIBCO/BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS, Atlanta Biologicals, Lawrenceville, GA), penicillin–streptomycin (GIBCO/BRL), and amphotericin B (Fungizone®, GIBCO/BRL). Human embryonic kidney 293T cells were grown in Dulbecco's modified Eagle's medium (DMEM, GIBCO/BRL) supplemented with 10% FBS and antimicrobials. All cells were maintained at 37 °C in a 5% CO_2 atmosphere.

2.2. Isolation of primary swine respiratory epithelial cells

The SRECs were derived from airway tissues of healthy, untreated research pigs using a technique previously described for human tissues (Schroth et al., 1999; Wu et al., 1985). Briefly, distal tracheal/proximal primary bronchial airway specimens were rinsed with phosphate-buffered saline (PBS) and placed in a tissue-dissociation solution consisting of modified Mg^{++}- and Ca^{++}-free minimal essential medium supplemented with penicillin–streptomycin, pronase (1.4 mg/ml, Boehringer Mannheim, Indianapolis, IN) and DNAse (100 μg/ml, Sigma Chemical Co., St Louis, MO) for 72 h at 4 °C. After the incubation, FBS was added to a final concentration of 10% and cells were dislodged by gentle agitation, collected by centrifugation, and resuspended in Dulbecco's modified Eagle F12 medium (DMEM/F-12, GIBCO/BRL) supplemented with 5% FBS, penicillin–streptomycin, MEM non-essential amino acids (Sigma Chemical Co.), and recombinant human insulin (0.12 U/ml, Novolin®, Novo Nordisk, Princeton, NJ). To remove fibroblasts, cell suspensions were incubated at 37 °C and 5% CO_2 in uncoated tissue culture dishes for 2–6 h. Non-adherent