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Short Communication

Persistence of viral RNA in the brain of offspring to mice infected with influenza A/WSN/33 virus during pregnancy

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Epidemiological studies have indicated an association between influenza A virus infections during fetal life and neuropsychiatric diseases. To study the potential for influenza A virus infections to cause nervous system dysfunctions, we describe a mouse model using intranasal instillation of the mouse neuroadapted influenza A/WSN/33 strain in pregnant mice. Viral RNA and nucleoprotein were detected in fetal brains and the viral RNA persisted for at least 90 days of postnatal life. We have, thus, obtained evidence for transplacental passage of influenza virus in mice and the persistence of viral components in the brains of these animals into young adulthood. *Journal of NeuroVirology* (2002) **8,** 353–357.

Keywords: orthomyxoviridae; central nervous system; persistent infection; placenta; *in utero*

Introduction

The hypothesis that the certain neuropsychiatric disorders may reflect disturbances in the maturation of the nervous system has been proposed in a number of studies, reviewed in Raedler *et al* (1998). Although controversy still exists, there is a common belief that certain neuronal populations in these patients have failed to develop normally due to disturbances in their migration and synaptogenesis. Epidemiological studies suggest that some of these disorders may be related to infectious diseases of the mother during pregnancy or during early infancy (Rantakallio *et al*, 1997). For example, influenza A virus infections during pregnancy have in many, but not all, studies been associated with the appearance of neuropsychiatric diseases in the offspring (Munk-Jorgensen and Ewald, 2001). Thus, it may be hypothesized that an infectious agent, in one way or the other, adversely affects the developing CNS leading to behavioral changes in the adult individual. It is therefore of interest to determine if a common pathogen, such as influenza A virus, can actually be transmitted from the pregnant mother to the fetus with subsequent targeting to the fetal brain. In general, influenza A virus infections are limited to the respiratory epithelium, but viremia has been observed in both humans and experimental animals (Ritova *et al*, 1979; Reinacher *et al*, 1983; Rushton *et al*, 1983; Mori *et al*, 1995). The purpose of the present study was to investigate if the mouse neuroadapted A/WSN/33 strain of influenza A virus can spread through the placenta to the brain of a fetus still allowing for viable offspring. The neurotropic influenza A/WSN/33 strain is suitable for these purposes because its spread from the respiratory tract is probably facilitated by its use of the abundant protease plasminogen in addition to the Clara cell restricted trypsin for hemagglutinin cleavage (Li *et al*, 1993; Goto and Kawaoka, 1998). Furthermore, we previously reported that the A/WSN/33 strain causes a persistent infection at levels of the upper brainstem in immunodefective mice after injection into the olfactory bulb (Aronsson *et al*, 2001). We here report that viral RNA can be detected in the brains of fetuses whose mothers were infected intranasally with this virus and that the offspring of

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Table 1 Primer sequences

Gene	*GenBank Acc no*	*Primer sequences*	*Size (bp)*
GAPDH*	NM00804	Fw 5′-ctgaggaccaggttgtctcc-3′ Rev 5′-tgtgagggagatgctcagtg-3′	267
Matrix	L25818	Fw 5′-gggaagaacaccgatcttga-3′ Rev 5′-gcaagtgcaccagcagaata-3′	274
Nucleoprotein	M30746	Fw 5′-gaatggacggagaacaagga-3′ Rev 5′-acggcagatccatacacaca-3′	224

*Glyceraldehyde-3-phosphate dehydrogenase.

such mice can harbor viral RNA at least 3 months after birth.

Passage of influenza A virus to the fetal brain In order to examine if influenza A virus can spread to the fetuses during maternal infection, C57BL/6 mice (the breeding facility at the Department of Microbiology and Tumor Biology Center, Karolinska Institutet) were inoculated intranasally with 30 μl containing 750 or 7500 plaque-forming units (PFU) of influenza A/WSN/33 virus (kindly provided by Dr S Nakajima, The Institute of Public Health, Tokyo, Japan) suspended in phosphate-buffered saline (PBS), pH 7.4, on day 14 of pregnancy (E14). Control mice were inoculated similarly with PBS. All litters were kept separately in a venticage system. These studies were conducted under institutional guidelines and ethical committee approval (Nos N192/98 and N106/01).

On day E17, the pregnant mice were sacrificed, the chorioamniotic sacs containing the fetuses and placentas sampled, put on ice, and transferred to a separate laboratory. Brains, lungs, and corresponding placentas were subsequently dissected out, using new sets of tools for every fetus in order to avoid cross-contamination. For verification of maternal infection, lungs from the mothers were dissected out in a separate room. Total RNA was extracted from whole organs using the RNeasy Kit (Qiagen, GmbH, Hilden, Germany). Following DNase I (Life Technologies, Paisley, UK) treatment, 1 μg of total RNA was reverse transcribed by random priming using Superscript II in a 20-μl reaction according to the manufacturer's instructions (Life Technologies). One μl of the cDNA template was amplified in a 25-μl reaction, containing: 1 × Advantaq Plus DNA Polymerase, 1 × Advantaq Plus PCR Buffer (Clontech Laboratories Inc., Palo Alto,USA), 1 μM of gene-specific forward and reverse primers, respectively (see Table 1), 200 μM each of dNTPs (Life Technologies). A GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA, USA) with the following cycling conditions was used: heat activation for 2 min at 94°C followed by 35–45 cycles of denaturation at 94°C for 30 s and annealing/extension at 66–69°C for 1 min, followed by a final extension at 72°C for 7 min. PCR products were electrophoresed in 2% agarose in 40 mM Tris, 20 mM acetic acid, and 1 mM EDTA (TAE) buffer. Double-stranded DNA was stained in 1 × SYBR Gold Stain (Molecular Probes, Eugene, OR, USA) in TAE buffer. Double-stranded DNA was subsequently visualized and documented on a Gel Doc 2000 system (Bio-Rad, Hercules, CA, USA).

Data from two litters ($n = 17$), using a virus titer of 750 PFU for maternal inoculation, shows that influenza A virus RNA encoding matrix (M) and/or nucleoprotein (NP) could be detected in a proportion of the fetal brains and lungs. Viral RNA was also detected in some of the placentas (Figure 1A). Using the higher dose of 7500 PFU for maternal inoculation of one animal gave similar results (data not shown). Organs from fetuses whose mothers received PBS were always negative for viral RNA. Maternal infection was verified in lung tissue by RT-PCR amplification of the RNA encoding NP. Message for the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was detected in all samples. In a fourth litter, the presence of viral protein in the fetal brains was investigated by Western blotting of homogenized whole brains with subsequent detection by a mouse monoclonal IgG1 antibody to influenza A virus nucleoprotein (ViroStat, Portland, Maine, USA), as previously described (Aronsson *et al*, 2001). As seen in Figure 1B, viral nucleoprotein was detected in 7 out of 11 brains tested.

Although a number of RNA viruses were reported to pass the placenta both in humans, and domestic and experimental animals [for review see Mims, 1968], only scattered reports exist of transplacental passage of influenza A virus. The A/PR/8 strain was reported to cross the placenta in mice (Siem *et al*, 1960; Takeyama, 1966) and the recombinant A/PR/8-A/England/939/69 strain in ferrets (Sweet *et al*, 1977; Rushton *et al*, 1983). However, in a large study of A/WSN/33 infection in mice, no evidence for transplacental passage was obtained as examined by virus cultivation (Williams and Mackenzie, 1977). Similarly, in humans, scattered case reports suggest that influenza A virus can spread via the placenta. For instance, Yawn *et al* (1971) and McGregor *et al* (1984) reported transplacental passage in humans of influenza A2/Hong Kong/8/68 and A/Bangkok, respectively. On the other hand, in a study of 138 newborns to mothers with proven influenza A virus infection during pregnancy, no serological evidence for transplacental passage was obtained as measured by cord blood IgM anti-influenza antibodies (Irving *et al*, 2000). In the present study, infectious virus could

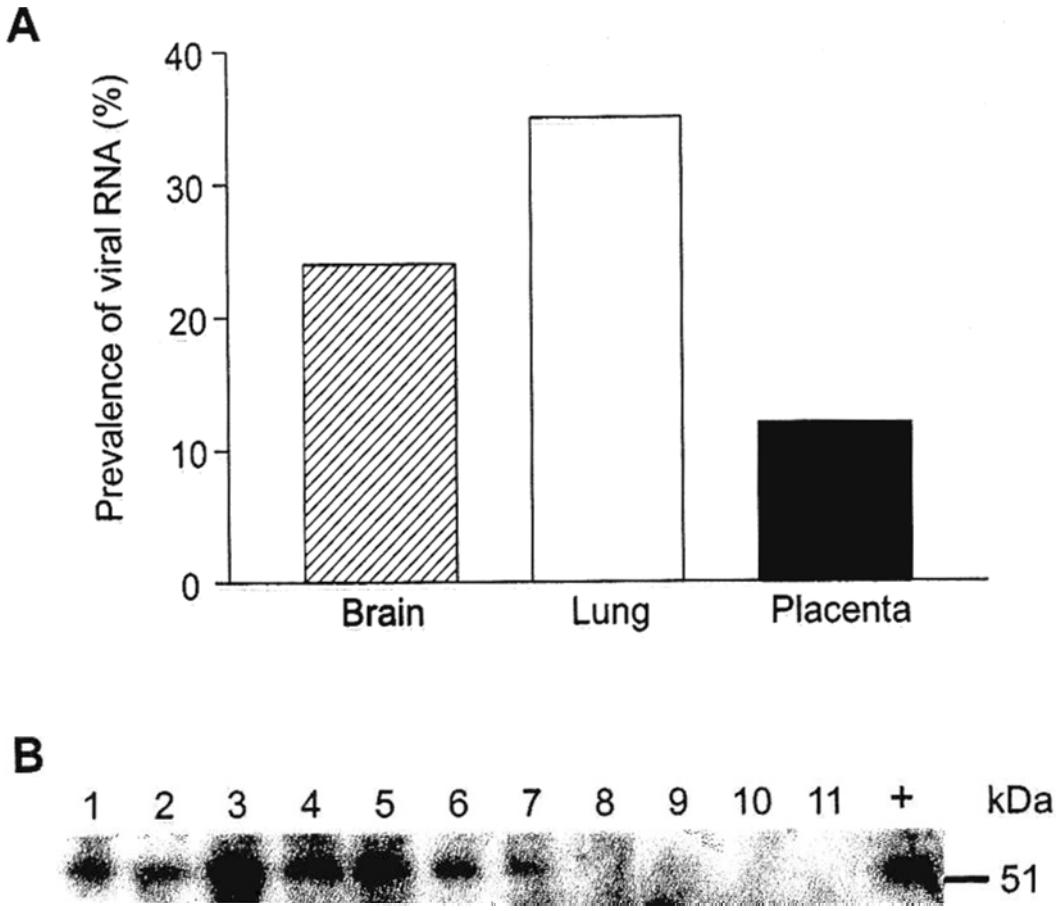

Figure 1 Transplacental passage of influenza A/WSN/33 virus. (**A**) Prevalence of viral RNA in fetal brain, lung and in corresponding placenta ($n = 17$). (**B**) Detection of the 56-kDa influenza A virus nucleoprotein in individual fetal brains (1–11) and a positive control (+). The tissues were sampled 3 days after maternal infection with 750 PFU of influenza A/WSN/33 virus.

not be grown from the fetuses by using a plaque assay on MDCK cells in the absence of trypsin (Tobita *et al*, 1975). Despite this, viral RNA and nucleoprotein could be detected in a portion of the fetal brains and lungs by RT-PCR and illustrates the need for using methods more sensitive than culture or serology in this kind of studies. The present study clearly shows that influenza A virus can spread to a fetus in which it can be targeted to the brain. We then studied the postnatal survival of these mice.

Survival of offspring to infected mice Mice born to mothers instilled intranasally with the higher dose of virus, 7500 PFU, showed signs of disease and died within 8 days of birth (Figure 2). This is in accordance with the study by Takeyama (1966) where a high rate of neonatal death within 10 days of birth was seen using the PR8 strain. Offspring to mice infected with the lower dose of virus, 750 PFU, survived the observation period of 3 months. They had normal growth rate and showed no obvious signs of disease (Figure 2). These mice were then sacrificed, and the brains were dissected out and subjected to further analyses.

Persistence of viral RNA in offspring to virus-infected mice To examine if viral RNA could persist in the brain of offspring to infected animals, mice were sampled at 10, 20, 35, 60, and 90 days of age. From these animals the brains were dissected and analyzed for the presence of viral RNA,

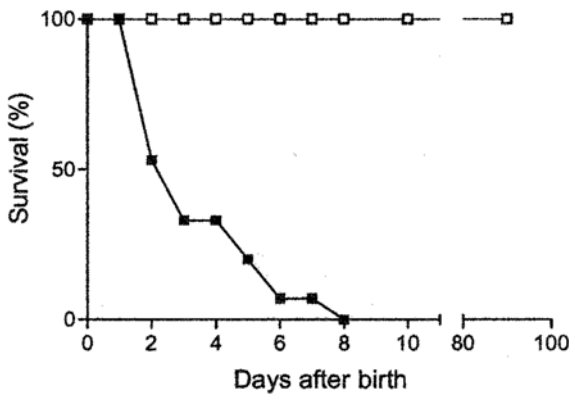

Figure 2 Survival of mice. Offspring to mothers exposed to 750 (□, $n = 17$) or 7500 (■, $n = 15$) PFU of influenza A/WSN/33 virus.

Table 2 Persistence of influenza A/WSN/33 virus RNA in brains of animals exposed in utero

Age (days)	*Virus RNA positive brains**
10	2/4
20	1/4
35	1/4
60	1/4
90	2/8

*Number of animals where viral RNA encoding M or NP was detected out of total number of animals tested at each time point of postnatal life.

as described before. As seen in Table 2, viral RNA encoding M and/or NP could be detected in animals at all points of time. Such sequences were never detected in animals born to sham-inoculated mice. Following the completion of the study, the identity of all PCR products was verified by TA cloning (Invitrogen, Groningen, The Netherlands) and sequencing on an Applied Biosystems Division 377 automated DNA sequencer (core facilities at Karolinska Institutet). The resulting sequences were compared to previously reported sequences using BLASTN algorithms.

In a previous study, RNA corresponding to all viral segments, was found to persist for up to 17 months in the brains of immunodefective TAP1 knockout mice after intracerebral inoculation (Aronsson *et al*, 2001). No sign of persistence beyond 35 days was seen in wild-type animals (Mori *et al*, 1999), which agrees with the notion that influenza A virus does not establish persistent infection in the immunocompetent host (Doherty *et al*, 1996; Hawke *et al*, 1998). Hence, in the prenatally infected animals in the present study, virus may either be tolerated or escape recognition by residing in an immunoprivileged site, such as neurons (Kristensson and Norrby, 1986; Joly *et al*, 1991; Stevenson *et al*, 1997). For a number of virus infections, the age of the host determines whether a persistent infection will be established or not. The most notable example is lymphocytic choriomeningitis (LCM) virus infections, in which virus persists in all organs for the entire life span of mice infected neonatally, while virus is rapidly cleared after infection of adult mice (Lehmann-Grube, 1988), although Ciurea *et al* (1999) recently reported the persistence of lymphocytic choriomeningitis virus, at very low levels, even in immunocompetent mice infected in adulthood. Reports on effects of congenital influenza A virus infections have previously been limited to neural tube defects in chick embryos (Hamburger and Habel, 1947; Robertson *et al*, 1967; Johnson *et al*, 1971) and fetal death with severe malformations in mice (Adams *et al*, 1956) after infection during early gestation. Our surviving mice showed no such gross changes of their brain development. The surprising finding of virus persistence in the CNS of *in utero* infected offspring suggests that virus has the potential to affect differentiated neuronal functions. This may become overt later in life due to reactivation of the virus (Levine and Griffin, 1992), or by an altered vulnerability of the nervous tissue to environmental factors. For this purpose, we will use the strategy of gene expression profiling in order to identify potential effects of the maternal virus exposure. We suggest that the present model lends itself for studies of whether a maternal infection may cause abnormalities in maturation of the central nervous system and affect behavior of the offspring.

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