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Detection of mouse-adapted human influenza virus in the olfactory bulbs of mice within hours after intranasal infection

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Influenza pneumonitis causes severe systemic symptoms in mice, including hypothermia and excess sleep. The association of extrapulmonary virus, particularly virus in the brain, with the onset of such disease symptoms has not been investigated. Mature C57BL/6 male mice were infected intranasally with mouse-adapted human influenza viruses (PR8 or X-31) under inhalation, systemic, or no anesthesia. Core body temperatures were monitored continuously by radiotelemetry, and tissues (lung, brain, olfactory bulb, spleen, blood) were harvested at the time of onset of hypothermia (13 to 24 h post infection [PI]) or at 4 or 7 h PI. Whole RNA from all tissues was examined by one or more of three reverse transcriptase–polymerase chain reaction (RT-PCR) procedures using H1N1 nucleoprotein (NP) primers for minus polarity RNA (genomic or vRNA) or plus polarity RNA (replication intermediates). Selected cytokines were assayed at 4, 7, and 15 h in the olfactory bulb (OB). Minus and plus RNA strands were readily detected in OBs as early as 4 h PI by nested RT-PCR. Anesthesia was not required for viral invasion of the OB. Cytokine mRNAs were also significantly elevated in the OB at 7 and 15 h PI in infected mice. Controls receiving boiled virus expressed only input vRNA and that only in lung. Immunohistochemistry demonstrated localization of H1N1 and NP antigens in olfactory nerves and the glomerular layer of the OB. Therefore a mouse-adapted human influenza virus strain, not known to be neurotropic, was detected in the mouse OB within 4 h PI where it appeared to induce replication intermediates and cytokines. *Journal of NeuroVirology* (2007) **13,** 399–409.

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Introduction

Many respiratory and intestinal viral infections are marked by abrupt onset of fever, somnolence and malaise, commonly termed the "flu," within 2 to 3 days following infection. There is little evidence in the majority of cases that such viruses replicate outside the mucosal surfaces that they target, though their systemic symptoms can be quite severe. It is assumed that acute viral symptoms are a consequence of cytokine release from infected target tissues acting upon the brain, though evidence for this assumption is minimal.

Influenza virus is among the respiratory viruses thought to be restricted to the upper respiratory tract in classical human "flu" and to the entire respiratory tract in more severe influenzal pneumonitis. However, viremia does occur in mild human influenza, though it can only be detected prior to onset of clinical symptoms using classical virus

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isolation techniques (Stanley and Jackson, 1966). Furthermore, clinical observations made during the influenza pandemics of 1918 (Kristensson, 2006) and 1957–1958 (reviewed in Schlesinger *et al*, 1998; Ward, 1996) indicate that influenza virus can invade the brain in certain lethal infections. Psychiatric sequelae to influenza are also seen (Kristensson, 2006). In the last 10 years, influenza-associated encephalitis/encephalopathy has been diagnosed relatively frequently in Japanese children (Kristensson, 2006; Sugaya, 2002), but no particular viral strain has been implicated. A review of 84 hospitalized children in Taiwan with documented influenza A reported that 31% had neurological symptoms (Wang *et al*, 2003); influenza A is more commonly seen in children with neurological involvement than is influenza B (Romero and Newland, 2003). Viral RNA is occasionally detected in cerebral spinal fluid by the reverse transcriptase–polymerase chain reaction (RT-PCR) in encephalopathy patients (Fujimoto *et al*, 1998; Steininger *et al*, 2003). Japanese case reports have led to a greater awareness of these neurological complications of influenza, with the consequence that adult and pediatric cases in Europe (Rantalaiho *et al*, 2001; Steininger *et al*, 2003) and pediatric cases in the United States (Maricich *et al*, 2004; Weitkamp *et al*, 2004) have been recognized. More recent studies have detected influenza-associated neurological complications, primarily seizures, in 4 pediatric cases per 100,000 person-years, risk factors include neurological or neuromuscular diseases (Newland *et al*, 2007). Studies in Finland suggest that from 4% to 7% of human viral encephalitides are associated with influenza infections (Rantalaiho *et al*, 2001).

Infections of mice with mouse-adapted human strains of influenza virus by the intranasal (IN) route are also assumed to be restricted to the respiratory tract, though viremia with the most commonly employed human strain, mouse-adapted A/PR/8/34 H1N1 (PR8), can be detected with sensitive methods within the first 24 h post infection (PI) (Frankova and Rychterova, 1975; Ishida *et al*, 1959; Mori *et al*, 1995). Mouse central nervous system (CNS) infections with human strains have been primarily studied using the neurovirulent WSN or NWS strains derived by serial intracerebral (IC) passages in mice (Ward, 1996). These strains are studied using the IC route in adult mice or the IN route in neonates (Schlesinger *et al*, 1998); WSN does not appear to invade the brain when inoculated by the IN route in immunocompetent adult mice (Garcia-Sastre *et al*, 1998a). Other studies of influenza infections of the mouse brain have employed avian strains or avian recombinants that are intrinsically neurotropic as well as neurovirulent in susceptible birds and mammals (Schlesinger *et al*, 1998). Neurotropism in mice is also a notable feature of human isolates of avian H5N1 viruses from Hong Kong in 1997 (Tanaka *et al*, 2003) and Southeast Asia in 2004 (Maines *et al*, 2005).

In order to clarify the role of extrapulmonary virus in the viral "flu" syndrome (or acute-phase response), we have employed one-step RT-PCR, or the more sensitive two-step nested RT-PCR (nPCR) or real-time quantitative PCR (qPCR) techniques to detect both minus-polarity (genomic vRNA) and plus-polarity (replication intermediates) PR8 nucleoprotein (NP) RNA. RNA was extracted from tissues collected from mature male C57BL/6 mice infected IN with PR8 or X-31 mouse-adapted human H1N1 influenza A strains, with or without anesthesia. Tissues were harvested at or near the onset of hypothermia, the earliest quantifiable manifestation of systemic influenzal disease in mice (Fang *et al*, 1995), or at 4 or 7 h PI when no symptoms were perceptible. PCR analyses were performed in lung, whole brain minus intact olfactory bulb (OB), isolated intact OB, spleen, and blood from infected mice or from controls inoculated with heat-inactivated virus. In addition, cytokine mRNA expression was examined by qPCR in selected 4-, 7-, or 15-h OB samples. Following detection of viral RNA in the OB by nPCR, we employed immunohistochemistry (IHC) to detect viral antigens in the OB at 15 h PI. To our knowledge this study represents the first demonstration of neurotropism of a human influenza virus in immunologically mature mice within the first 15 h PI. The results reported here may provide a biological basis for the severe systemic symptoms associated with influenza infections as well as influenza-associated neuropathologies.

Results

Body temperature response to PR8

We and others have demonstrated that a rapid fall in body temperature occurs early after lethal influenza infection of mice (Conn *et al*, 1995; Fang *et al*, 1995). In our model the time of onset of hypothermia was observed in order to ascertain the time at which the mice manifested influenza symptoms following infection with various doses of PR8. Precipitous hypothermia began at 11 to 13 h PI (three experiments, $n = 20$) in mice given 2.5×10^6 median tissue culture infectious doses ($TCID_{50}$) of PR8 (the highest dose of PR8 employed) inoculated IN under Metofane anesthesia (representative experiment Figure 1). The body temperature continued to decline steadily until it reached about 32°C on day 4 (data not shown), at which point the animals began to die (Wong *et al*, 1997). Tenfold dilutions of virus induced a qualitatively similar pattern of hypothermia beginning at 23.5 and 25.2 h, respectively (data not shown). Based on these observations, mice infected with 2.5×10^6 $TCID_{50}$ PR8 were harvested at 15 h to assure that all animals were manifesting hypothermia, or at 4 or 7 h PI (prior to the onset of hypothermia). Mice

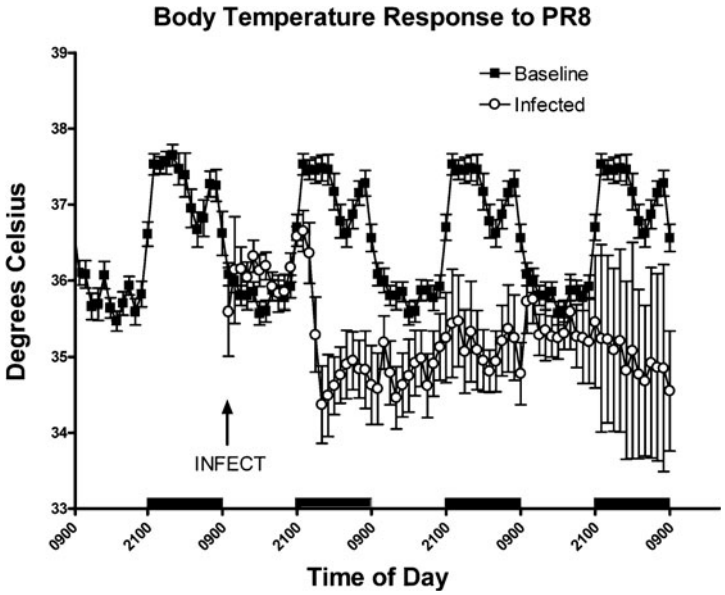

Figure 1 Body temperature curves of mice ($n = 6$) unexposed to virus (baseline) or infected IN with 2.5×10^6 $TCID_{50}$ PR8 influenza virus under Metofane anesthesia. Error bars denote SEM.

infected with 1.5×10^6 $TCID_{50}$ X-31 under Metofane anesthesia were harvested at 14 h PI after onset of hypothermia.

Viral RNA detection using heat-inactivated virus

The standard procedure for inactivating virus at 56°C for 30 min did not reliably eliminate detection of hemagglutinin (HA) plus-strand RNA in lung as determined by one-step RT-PCR (data not shown). Thus heat inactivation in subsequent experiments was conducted by suspending the virus preparation in a boiling water bath for 15 to 25 min. The more intense heat inactivation procedure eliminated the detection by nPCR method 1 or 2 of nucleoprotein (NP) plus strand in all lung samples regardless of whether lungs were harvested at 4, 7, or 15 h PI (data not shown). Neither RNA strand was detected by any PCR method in the OBs of 66 mice inoculated with boiled virus (e.g., Table 1) nor was either RNA strand detected in spleen, blood, or whole brain (data not shown) in mice challenged with the boiled virus. In contrast, the HA minus strand was detected by RT-PCR in 5/6 infected lungs at 13 h PI and the NP minus strand was detected in 2/3 infected lungs using nPCR method 1 at 12 h PI in mice challenged with 2.5×10^6 $TCID_{50}$ of PR8 under Metofane anesthesia (data not shown). There was no detection by nPCR method 1 of NP minus- or plus-strand RNA in any tissue obtained from 6/6 mice unexposed to the virus, boiled or live (data not shown).

Viral RNA detection by nPCR in whole brain (lacking an intact OB)

An early experiment using one-step RT-PCR and primers for the PR8 hemagglutinin (see Table 3) detected minus strand in 2/6 whole brain samples harvested at 13 h (data not shown). Subsequent experiments with whole brains employed the more sensitive two-step nPCR procedure (method 1) and established primers for PR8 NP (see Table 3). Whole brain was positive at hypothermia onset for both NP RNA strands in a majority of samples from mice receiving high-dose PR8 using nPCR method 1 (10/12 minus strand; 8/12 plus strand). Both NP plus and minus strands were detected sporadically using the lower PR8 doses as well (3/12 minus strand; 4/12 plus strand) in mice sampled at the time of hypothermia onset. X-31 NP minus- and plus-strand RNA were

Table 1 Frequency of NP detection by nPCR in olfactory bulbs using different anesthesia protocols

Hours post infection of tissue harvest	*Anesthesia used*	*Boiled PR8*[a]		*Live PR8*[a]	
		Minus strand	*Plus strand*	*Minus strand*	*Plus strand*
4	INH	0/6	0/6	6/6	6/6
7	INH	0/6	0/6	6/6	5/6
15	INH	0/6	0/6	6/6	2/6
4	IP	0/6	0/6	3/6	0/6
7	IP	0/4	0/4	3/4*	2/4*
15	IP	0/6	0/6	6/6	3/6
7	None	0/6	0/6	5/6*	4/6*

[a]No. positive/no. tested.

Data show the frequency of detection of minus or plus strand NP RNA using method 2 in olfactory bulbs harvested at 4, 7, or 15 h post infection using either Metofane inhalation anesthesia (INH), intraperitoneal ketamine/xylazine anesthesia (IP), or no anesthesia (None). Boiled PR8 represent control mice, whereas live PR8 represent infected mice. Ratios marked with an asterisk (*) indicate that a single mouse was negative for both minus and plus strands.

Table 2 Comparison of frequency of PR8 NP detection in various tissues using method 1 or method 2 nPCR

Hours post-infection of tissue harvest	Tissue sampled	Method 1		Method 2	
		Minus strand	Plus strand	Minus strand	Plus strand
4	Spleen	1/6	0/6	0/6	0/6
7	Spleen	0/4	0/4	0/4	0/4
15	Spleen	2/6	4/6	1/6	0/6
4	Blood	2/3*	2/3*	0/6	0/6
7	Blood	3/6	3/6	0/6	0/6
15	Blood	2/3*	2/3*	0/6	0/6
4	OB	6/6	2/6	3/6	0/6
7	OB	3/4	3/4	3/4	2/4
15	OB	6/6	2/6	6/6	3/6
4	SCtx	ND†	ND	1/6	0/6
7	SCtx	ND	ND	0/4	0/4
15	SCtx	ND	ND	0/6	0/6

Note. Data compare the frequency of detection of minus or plus strand NP RNA using either method 1 or method 2 nPCR on the same cDNA samples from various tissues (spleen, blood, olfactory bulb [OB], or somatosensory cortex [SCtx]) harvested at 4, 7, or 15 h post infeciton using intraperitoneal ketamine/xylazine anesthesia. All mice were challenged with live PR8. Ratios marked with an asterisk (*) indicate that inadequate cDNA was available from three of the mice to repeat with method 1.
†ND = not done.

also detectable by nPCR method 1 in 3/3 samples of whole brain harvested at 14 h PI.

Viral RNA detection in the intact OB

We examined the OB for NP expression in a series of mice infected with high dose PR8. OBs yielded positive nPCR method 2 signals (e.g., Tables 1 and 2) in most mice sampled at 4, 7, or 15 h PI, regardless of the anesthesia protocol employed. The NP minus strand was more consistently detected than NP plus strand using either method of nPCR (Tables 1 and 2); NP plus strand was never detected in the absence of NP minus strand in the OB. Nested PCR method 1 increased the frequency of NP detection in OB over method 2 (Table 2), especially at 4 h PI. In qPCR analyses, a few mice expressed 100-fold the NP level (both strands) seen in other mice in the same experiment, suggesting highly variable invasion of the OB by PR8 in quantitative terms (data not shown).

Effects of different anesthesia protocols on OB invasion by virus

The frequency of viral NP expression in the intact OB using nPCR method 2 for mice anesthetized or unanesthetized during infection is shown in Table 1. These nPCR data, obtained from OBs sampled at 4, 7, or 15 h PI, indicate that OB invasion by PR8 (minus strand) is not substantially enhanced by inhalation anesthesia, though plus strand expression is less consistent in the IP-anesthetized mice (Table 1). In unanesthetized mice, 5/6 OB samples obtained at 7 h PI were positive for viral RNA (Table 1), indicating that anesthesia was not required for viral invasion of the OB.

Comparison of viral RNA detection in OB and somatosensory cortex

Somatosensory cortex (SCtx), which lacks direct projections from the olfactory bulb, was examined for viral RNA by nPCR Method 2 (Table 2). Positive signal (minus strand only) was detected in only 1/6 mice at 4 h PI and no other animals at 7 or 15 h PI. In contrast, the same method applied to the OBs of the same mice detected minus strand in 12/16 mice and plus strand in 5/16 mice at the various time periods PI (Table 2).

Viral RNA detection by nPCR in lung, blood, and spleen

In mice challenged IN with live PR8, both NP strands were detected at 4 h PI by nPCR method 2 in 16/16 lungs infected under either inhalation ($n = 6$), IP ketamine/xylazine (K/X) ($n = 6$) anesthesia, or no anesthesia ($n = 4$) (data not shown but same mice as used for Tables 1 and 2). Similarly in another experiment, lungs of 12/12 mice challenged with live PR8 were positive using PCR method 1 for both RNA strands when lungs were harvested at the time of hypothermia onset (data not shown). In yet another experiment, mice infected with live X-31 were also positive for both strands in 3/3 lung samples harvested at 14 h PI.

In the mice represented in Table 1, whole blood and spleens were negative for viral NP RNA at all time periods using nPCR method 2 with the exception of one spleen sampled at 15 h PI (Table 2). However, when PCR method 1 was used, viral NP RNA was sporadically detected in both blood and spleen at the time of hypothermia onset, regardless of the dose of virus employed (data not shown). Detection frequency was similar at 4, 7, and 15 h PI in a second experiment where nPCR method 1 was used (Table 2). Mice positive for NP in blood did not correlate with the mice positive for NP in spleen. NP minus strand detection was more frequent in the OB than in blood, spleen or SCtx (Table 2); plus strand was never detected in the absence of minus strand in any tissue.

Using copy number analysis, nPCR method 1 was shown to detect as few as 18 copies of viral RNA, both minus and plus strands, whereas the lowest level of detection by nPCR method 2 was 1800 copies of plus and 18,000 copies of minus strand RNA. The relative sensitivity of the two methods is consistent with our detection frequency findings.

Cytokine mRNA detection in the OB

Expression of mRNAs for two proinflammatory cytokines (interleukin-1 beta [IL1β] and tumor necrosis factor alpha [TNFα]) and two enzymes efficiently induced by type I interferons (IFNs) 2′,5′-oligoadenylate synthase 1a [OAS] and myxovirus

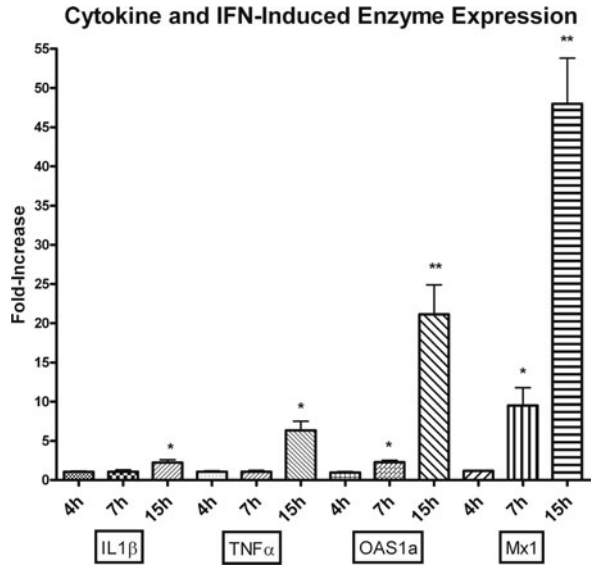

Figure 2 Olfactory bulb cytokine and IFN-induced enzyme mRNA qPCR data from three experiments (corrected for boiled virus control values) at 4 ($n = 6$), 7 ($n = 11$), and 15 ($n = 15$) h. All mice were inoculated IN with $2.5 \times 10^6 TCID_{50}$ PR8 under Metofane anesthesia. The *x* axis shows the cytokine/enzyme evaluated and the time-point at which the mouse was killed PI. Error bars denote SEM. Asterisks denote statistically significant differences ($^*P < .05$; $^{**}P < .001$).

resistance-1 GTPase [Mx1]) was evaluated. The two enzymes represent sensitive markers for type I IFN expression (Samuel, 2001). The results from three experiments are shown in Figure 2. No cytokine or enzyme mRNAs were elevated at 4 h PI. IL1β and TNFα mRNA levels also were not elevated at 7 h PI, but both cytokine mRNAs were significantly up-regulated at 15 h PI (Figure 2). Significant up-regulation of both IFN-induced enzymes occurred at both 7 and 15 h PI (Figure 2). We were unable to reliably detect the most common type I IFNs themselves in the OB at 7 or 15 h PI using IFNα consensus primers that detect all IFNα isoforms or with IFNβ primers (Traynor *et al*, 2004) (data not shown).

OB Immunohistochemistry at 15 h PI

A large number of cells intensively stained for H1N1 were seen in the olfactory nerves and OB glomerular layer (GL) but seldom in the internal layers of the OB in mice inoculated with live PR8 (Figure 3C and D). In contrast, in mice inoculated IN with boiled PR8, very few influenza-H1N1-immunoreactive cells were evident 15 h PI (Figure 3A and B). A similar distribution of stain was seen when OBs were reacted with an anti-N1 NP antibody in infected mice (Figure 3G and H) or control mice (Figure 3E and F).

Discussion

Our results confirm and extend the studies of Mori *et al* (1995) using PR8 in mice, but we have examined the brain when objective illness is first detected

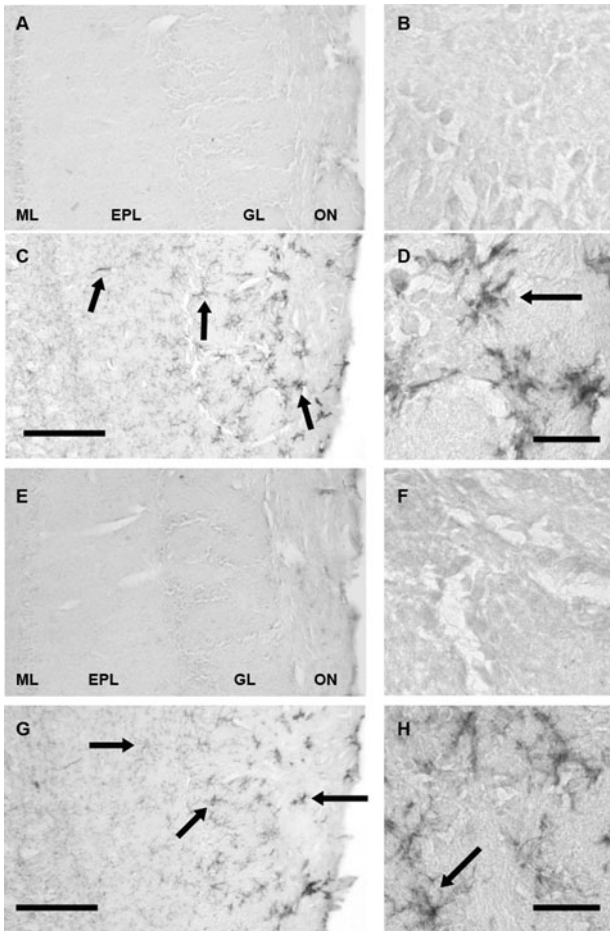

Figure 3 Photomicrographs of OB coronal sections from mice killed 15 h PI after IN challenge stained either for H1N1 influenza A (**A–D**) or for N1 NP (**E–H**). Mice receiving boiled virus are shown in **A**, **B**, **E**, and **F**. Mice receiving live PR8 are shown in **C**, **D**, **G**, and **H**. Long arrows in **C**, **D**, **G**, and **H** indicate some of the stained cells in the GL, whereas short errors point to some of the stained cells in the ON layer. The scale bar for lower magnification pictures (**A**, **C**, **E**, and **G**) is 0.2 mm; for higher magnification pictures (**B**, **D**, **F**, and **H**) the scale bar is 0.025 mm. M, mitral cell layer; EPL, external plexiform layer; GL, glomerular layer; ON, olfactory nerve.

at 13 to 15 h (Figure 1), or well before detectable illness at 4 h or 7 h PI, rather than at 5 days when the infection is advanced. We show by RT-PCR that virus is undergoing at least partial replication (expression of the NP plus strand) in brain and in the intact OB (Tables 1, 2), and that expression of the IFN-induced enzyme transcripts is up-regulated in the OB by 7 and 15 h (Figure 2). IL1β and TNFα mRNAs are both significantly up-regulated at 15 h PI in infected OBs (Figure 2) when symptoms are apparent.

The IHC analysis at 15 h PI indicates that an antibody to H1N1 (reported to bind to PR8, and presumably reactive with a viral surface protein) and an antibody to N1 NP stains cells mainly within the glomerular layer (G) of the OB as well as cells within the olfactory nerves (Figure 3). The stained cells morphologically resemble microglial cells. Preliminary data also indicate that these microglia-like cells

are IL1β-immunoreactive, and thus they may be the source of that cytokine in the OB that we demonstrated by qPCR at 15 h (Figure 2).

Our heat-inactivation studies also extend those of Bussfeld *et al* (1998), who have demonstrated that virus heated at 56°C for 30 min (a commonly employed heat inactivation regimen) is capable of cytokine induction and that boiling the virus for 15 min eliminates cytokine induction. Our nPCR data indicated that virus heated to 56°C for 30 min is still capable of at least partial replication in that plus strand was often detected in the lung. Boiling the virus eliminated the plus strand expression in the lung and gave consistently negative findings in the OB when examined by nPCR and qPCR, as well as negative cytokine mRNA induction and negative IHC findings.

It is noted that the intact OB is often not collected in studies of viral neurotropism (as was the case in our initial studies) and the rostral portion of the OB may not be included in whole-brain analyses. To acquire the intact OB along with the bulk of the mouse brain the olfactory nerves must be carefully cut along the cribriform plate, which is not always done. From our results it would appear that the OB is an important brain region for analysis of IN infections, regardless of whether the agent is defined as neurotropic. Further, examination of the OB with extremely sensitive techniques such as nPCR earlier than is traditionally done (usually no earlier than 2 to 3 days PI) can be informative as abortive viruses may not be detectable after one replication cycle even though they may induce cytokines *in situ*.

Viremia has been reported early in mouse PR8 infections (Frankova and Rychterova, 1975; Ishida *et al*, 1959; Mori *et al*, 1995), and we sporadically saw viral NP RNA in whole blood and spleen by nPCR as early as 4 h PI (Table 2). Therefore residual blood as a source of the PCR signals in the brain must be considered. Virus may enter the blood by penetrating the fenestrated endothelium of the olfactory mucosa following IN inoculation (Schlesinger *et al*, 1998). From the blood the virus may potentially traverse the blood-brain barrier via extracellular channels (Banks, 2004). However, blood sources are unlikely in view of the focal distribution of the viral antigen in a very circumscribed area of the rostral OB as visualized by IHC (Figure 3) and the very infrequent detection of viral RNA in the SCtx (Table 2), a brain region lacking direct neural projections from the OB. Instead, viral antigen localization within the olfactory nerves and at the site of the first synapse of those nerves (the GL) strongly points to invasion of the OB by PR8 via the olfactory nerve. Olfactory nerves have some unique anatomical features (Mori *et al*, 2005), discussed below, that may promote viral transport.

Olfactory nerve transport of virus has been demonstrated by two established mechanisms: the axonal transport pathway and the olfactory epithelial pathway (Dahlin *et al*, 2000). Mouse hepatitis virus (Barnett and Perlman, 1993), vesicular stomatitis virus (Huneycutt *et al*, 1994), herpes simplex (Johnson, 1964), and large protein complexes (Thorne *et al*, 1995) can enter the OB via axonal transport through olfactory receptor neurons (Mori *et al*, 2005). Axonal transport would allow influenza virus to move a distance of about 8 mm in 4 h, assuming the transport speed is similar to that of herpes virus (Maratou *et al*, 1998); the length of the olfactory receptor axons in the mouse is likely to be less than 8 mm and thus this pathway may be relevant to our observations. Axonal transport would be consistent with the dense localization of viral antigen in the GL (Fig. 3B) where the first synapse of olfactory neurons takes place.

The olfactory epithelial pathway is relevant when a virus enters into the nasal lamina propria (Dahlin *et al*, 2000). From this tissue layer the virus can enter the perineural space of nerves projecting to the subarachnoid space of the OB and other regions of the brain. Herpes virus can use both axonal transport and perineural transport simultaneously (Johnson, 1964).

A newly discovered route to the GL, which has been demonstrated to permit passive transport of ultrafine carbon particulates in the size range (100 nm) of viruses (Oberdorster *et al*, 2004), are the channels formed by a fibroblastic sheath surrounding the network of olfactory ensheathing cells that wrap olfactory nerve fascicles (Li *et al*, 2005). Transport of carbon particles to the OB via these channels was detected as early as day 1 post challenge (Oberdorster *et al*, 2004). Viruses have not yet been demonstrated to enter the brain via these channels.

The model that we have constructed based on these observations is as follows: Virus enters the upper nasal cavity and binds to the olfactory receptor dendritic cilia that probably bear the sialic acid viral receptor (Allen and Akeson, 1985); the virus is then rapidly taken up by the olfactory receptor neurons by endocytosis and is transported by axonal flow to the glomeruli, where the axon terminates in a synapse. Virions and/or viral proteins detected by our mouse H1N1 and NP antibodies as well as viral RNA are released into the synaptic cleft, perhaps by exocytosis. After release into the extracellular environment, these viral products are taken up by resident microglia or other glial cells. The glial cells are thus activated to produce cytokines such as IL1β in response to viral RNA, which in turn initiate the acute phase response by acting upon the hypothalamus. Investigations are ongoing to resolve all involved cell types, the cytokines induced in them, and the other suppositions underlying this model.

Our studies have not yet examined whether PR8 influenza virus can be detected within the trigeminal nerve, vagus nerve, or other demonstrated routes of viral nerve transport from the respiratory system (Johnson and Mims, 1968: Park *et al*, 2002). Some neurotrophic viruses are selective as to which nerves are employed for transport (for instance, CNS invasion by mouse hepatitis virus is restricted to the olfactory nerve [Barnet *et al*, 1993]) where other viruses

such as herpes viruses may employ numerous nerves to invade the CNS (Barnet *et al*, 1993).

Although conclusive studies of the route of PR8 transport to the OB will require a more extensive time series and finer resolution of virus, our observations suggest that PR8 can be considered neurotropic but not progressively neurovirulent. This conclusion is compatible with the absence of neuropathology seen by Iwasaki *et al* (2004) in PR8 infected mice and the general lack of neurological symptoms observed in mice infected IN with PR8. Cytokines such as those that we detect by qPCR are likely to be induced by the viral RNA (single-stranded and/or double-stranded [Diebold *et al*, 2004]) that we detect in the OB by both nPCR and qPCR. Neural pathways from the OB to the hypothalamus (Aronsson *et al*, 2003) could conceivably result in OB-synthesized cytokines activating the hypothalamus to induce the viral acute phase response in the absence of actual hypothalamic infection or the action of cytokines made in the respiratory tree.

Because we detect viral RNA in the OB by 4 h, the signal we detect is likely to derive from input virus. Therefore a functional Mx1 enzyme that inhibits influenza virus replication (Haller *et al*, 1980) is not present in our mice (or most inbred strains) and is unlikely to affect OB invasion, though its absence may affect cytokine induction and viral replication. Similarly, the NS1 gene that blocks IFN induction (Garcia-Sastre *et al*, 1998b), which is present in both viral strains that we have used, would not be expected to affect passive viral invasion though it also may affect cytokine induction and viral replication. Preexisting antibody may also inhibit central nervous system (CNS) invasion. The effects of these viral and host factors on viral invasion of the OB are under investigation.

Clearly the vast majority of human influenza infections or live virus vaccinations do not lead to clinical neuropathology, and if the virus routinely invades the human brain, the brain's defense mechanisms (and/or influenza's replication properties in neural tissues) nearly always eliminate progressive infection. Perhaps the most intriguing possibility raised by these findings is that the severe systemic symptoms associated with pandemic influenzal infections may reflect direct cytokine induction in the brain resulting from viral invasion via nerves, rather than, or in addition to, localized cytokine induction in the respiratory tract. Closer scrutiny of the brain following IN infections with viruses not known to be neurotropic may help us to better understand systemic viral illness.

In summary, in these studies we have demonstrated influenza viral RNA in extrapulmonary tissues of mature mice, including the intact OB, as early as 4 h PI following IN infection. Viral RNA expression in the OB was accompanied by cytokine induction as early as 7 h PI. Detection of the NP plus strand in many samples suggested that virus is undergoing at least partial replication in the OB. IHC methods indicated that viral antigen is primarily localized to the olfactory nerve and glomerular layers of the OB, probably in glial cells, at 15 h PI. Anesthesia was not required for viral invasion of the OB, and all boiled-virus controls were negative for viral RNA and cytokine mRNA expression. Our basic observations were confirmed using two viral strains, primers for two different viral genes, three distinct RT-PCR methodologies, and two distinct virus-specific monoclonal antibodies over the course of these studies. Thus we have demonstrated rapid invasion of the brain by mouse-adapted influenza virus, apparently via olfactory nerves. Determining the relevance of these findings to human influenza encephalopathy/encephalitis or influenza symptoms will require examination of appropriate clinical specimens using virus-detection techniques of comparable sensitivity.

Materials and methods

Mice

Specific pathogen-free male C57BL/6 mice (Taconic Farms, Germantown, NY, or Jackson Laboratory, Bar Harbor, ME), 10 to 14 weeks of age at the time of inoculation, were maintained in Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC)-approved animal quarters under veterinary supervision. The procedures employed were approved by the Washington State University Institutional Animal Use and Care Committee. After 2 weeks' quarantine, mice were housed in standard plastic cages with filter tops and maintained at 29°C ± 2°C (the thermoneutral zone for mice that allows full expression of stimulus-induced temperature changes [Hoffman-Goetz and Keir, 1985]) in a closed environmental chamber on a 12:12-h light/dark cycle.

Viruses and virus preparation

PR8 influenza virus was purified from specific pathogen-free (SPF) egg allantoic fluid using endotoxin-free reagents and titered as described in (Chen *et al*, 2004). The virus stock was shown to be free of detectable endotoxin or mycoplasma contamination (Chen *et al*, 2004). In one experiment the X-31 strain of influenza A (a reassortant between PR8 and A/Aichi/68 [H3N2] [Lee *et al*, 2001]) in allantoic fluid from SPF eggs was employed. X-31 expresses H3N2 surface genes but contains the internal genome segments of PR8, including the NP. X-31 requires about 10-fold more virus to kill mice in the same timeframe as PR8 (Price *et al*, 2000).

Inoculation procedures

PR8-infected mice received 2.5×10^6 $TCID_{50}$ purified PR8 (high dose, lethal in 4 to 5 days PI) in Dulbecco's phosphate-buffered saline (PBS), or two 10-fold dilutions of this virus inoculum. X-31-infected mice virus received 1.5×10^6 $TCID_{50}$ of crude virus. All control mice received virus that was heat-inactivated using

one of two incubation techniques: a 56°C water bath for 30 min or submersion in a boiling water bath for 15 to 25 min (boiled virus). Inoculations were performed within an hour of light onset.

For IN inoculations under anesthesia, either light methoxyflurane (Metofane; Schering-Plough Animal Health, Union, NJ) inhalation (INH) anesthesia or intraperitoneal (IP) ketamine/xylazine (K/X) (Chen *et al*, 2004) were used. Anesthetized mice were handheld in a semirecumbent position and inoculated with 50-μl volumes delivered slowly to the nostrils (25 μl per nostril) with a 100-μl micropipette. Unanesthetized mice were restrained in a 50-ml conical plastic tube with the tip removed to provide access to the nose and inoculated as described above. After inoculation unanesthetized mice were held in a recumbent position for 1 min. Control animals received the same volume of high dose virus that was heat inactivated by one of the two methods described above, except in the body temperature studies where infected mice were compared against untreated mouse baseline data.

Body temperature measurements
At least 1 week prior to infection, mice (six/group) were implanted intraperitoneally with 0.5 g VM-FM radio transmitters (Mini-Mitter, Sunriver, OR) capable of monitoring body temperature and locomotor activity through receivers under the individual cages. Temperature data from individually housed mice were collected at 6 min intervals and processed using the VitalView data acquisition system (Mini-Mitter); each data point in Figure 1 is an average of values from six mice. For each mouse 10 values were collected over each 1-h period.

Tissue sampling
A total of 127 boiled virus controls and 131 live PR8 inoculated mice were sampled for RT-PCR analysis. Tissues (lung, blood, spleen, intact OB, or whole brain with only the caudal root of the OB*) were harvested at the time of onset of hypothermia (drop in body temperature of 1°C or more of at least two animals in the group compared to baseline temperatures at that time of day, cf. Figure 1). In mice not monitored for temperature change, tissues were harvested at 14 or 15 h PI. Lungs and OBs from high-dose PR8-infected mice were also harvested prior to onset of hypothermia at 4 and 7 h PI. For molecular analysis mice were exsanguinated by open cardiac puncture under Metofane anesthesia and tissues were harvested into liquid nitrogen (taking care to clean the dissecting tools in dilute sodium hydroxide between each organ dissection), and stored at −80°C for subsequent RNA extraction.

For IHC analysis OBs were harvested from whole brains taken at 15 h after IN inoculation under light Metofane anesthesia with high dose live PR8 or boiled PR8. Mice under deep Metofane anesthesia were perfused transcardially with 20 ml of warm normal saline followed by 40 to 60 mL of ice-cold 4% paraformaldehyde in PBS. Control mice unexposed to either inoculum were perfused in a similar manner. Whole brains, including intact OBs, were removed, allowed to postfix for 2 h, and then immersed in 20% sucrose overnight. The brains were then frozen in crushed dry ice and stored at −80°C until sectioned.

RNA isolation for RT-PCR procedures
Total RNA was extracted from frozen tissues except blood for RT-PCR and nPCR using TRIzol reagent (Invitrogen, Carlsbad, CA; catalog number 15596-018) according to the manufacturer's recommended protocol. Total RNA was extracted from whole blood samples (approximately 0.7 ml) using the SV Total RNA Isolation System (Promega, Madison, WI; catalog number Z3100) following the manufacturer's protocol. Alternatively, RNA from blood was extracted using the RiboPure-Blood kit (Ambion, Austin, TX) according to the manufacturer's instruction.

Primers employed
Primers used for NP RT-PCR, nPCR, and qPCR studies are listed in Table 3. Primer sequences used for qPCR analysis of IL1β, TNFα, OAS and Mx1 are reported in Traynor *et al* (2004). Our NP primers were examined to determine if their products would include the human protein that shares sequence homology with influenza NP (Cooper *et al*, 1996); the PCR products of our NP primers do not include this potentially confounding region.

Detection of viral genes by RT-PCR and nPCR analysis
One-step RT-PCR and two-step nPCR procedures were adapted from the methods of Mori *et al* (1995). Two nPCR methods were employed, which differed primarily with respect to the amount of cDNA template used. Method 1 used 10-fold more cDNA template for the first step and 5-fold less for the second step than method 2.

Copy number analysis
To determine the sensitivity of our nPCR techniques, we performed copy number analysis using both method 1 and method 2. Viral RNA was isolated from a sucrose-gradient-purified PR8 preparation using Trizol (Invitrogen). Viral RNA (0.25 μg) was reverse transcribed with primers complementary to the 5′ or 3′ ends using superscript II (Invitrogen). Additional cytosines were designed into the 5' primer region to

*Our dissection methods for removing the brain or OB changed over time; early experiments where whole brain was examined were not dissected in a manner that reliably removed the rostral OB with olfactory nerve roots together with the caudal regions of the brain from cortex through the brain stem. Studies involving the OB *per se* were performed with intact OBs where the olfactory nerves were dissected away from the cribriform plate.

Table 3 Primer sequences for RT-PCR and nPCR analysis

Gene	*Product size (bp)*	*Bases spanned*	*Sequence 5′-3′*	*Refs.*
HA sense	524	1–31	ACGCATCAATGCATGAGTGTAACACGA AGTG	Lab§
HA antisense		1366–1400	GATTCTTCACATTTGAGTCATGGA AAT CCAGAGTC	Lab§
NP1 sense*	1097	301–320	GGGAAAGATCCTAAGAAAAC	Mori *et al*, 1995
NP2 antisense*		1398–1379	TGCACTTTCCATCATCCTTA	Mori *et al*, 1995
NP3 sense†	450	649–668	AATGATCGGAACTTCTGGAG	Mori *et al*, 1995
NP4 antisense†		1098–1079	CTTCGTCCCTTTGATGAAGC	Mori *et al*, 1995
NP5 sense‡	98	669–688	GGGTGAGAATGGACGAAAAA	Lab§
NP6 antisense‡		764–745	TCCATCATTGCTTTTTGTGC	Lab§
NP 5′ terminus sense (copy no.)	1570	1–20	CCAGCAAAAGCAGGGTAGATAA	Lab§
NP 3′ terminus antisense (copy no.)		1546–1565	CCCAGTAGAAACAAGGGTATTTT	Lab§
Cyclophilin A sense	430	90–113	GTCTCCTTCGAGCTGTTTGCAGAC	Lab§
Cyclophilin A antisense		497–519	GTCCACAGTCGGAGATGGTGATC	Lab§

*PR8 NP primers used for cDNA synthesis for RT-PCR and first step (external) nPCR.
†PR8 NPprimers used for second step (internal) nPCR.
‡ PR8 NP primers used for qPCR.
§Primers developed in our laboratory using the criteria described in Materials and Methods.

increase the T_m in order to make the primers useful for PCR amplification. cDNA was PCR amplified with the sense and anti-sense primers and the products cloned into pCR 4.2 TOPO vector (Invitrogen catalog number K4575-01). Plasmids were linearized with Not-I or Spe-I restriction enzymes. Plus and minus GS-5 RNA was amplified with MEGAscript T7 or MEGAscript T3 kits (Ambion) using the manufacturer's instructions. RNA was purified with MEGAclear Kit (Ambion). Approximately 100 μg of RNA was obtained per 1 μg of linearized plasmid. RNA was mixed with yeast tRNA (0.2 μg/ml in 10 mM Tris, 1 mM EDTA buffer, pH = 7.6) prior to 10-fold serial dilutions to establish a standard curve. Nested PCR method 1 was shown to detect as few as 18 copies of viral RNA, both minus and plus strands. Method 2 detected 1800 copies of plus- and 18,000 copies of minus-strand RNA.

Quantification of RNA transcripts by qPCR
Five microliters of cDNA (12.5 ng of total lung RNA) or 5 μl of cDNA (25 ng of total brain RNA) were amplified by PCR using NP and cyclophilin primers provided in Table 3 or cytokine primers provided in Traynor *et al* (2004). The total reaction volume was 25 μl containing 0.2 μM sense and antisense primers, 12.5 μl Platinum qPCR Supermix-UDG (Invitrogen, Carlsbad, CA), a 1:100,000 dilution of SYBR green (Molecular Probes, Eugene, OR), and a 1:100,000 dilution of Fluorescein Calibration Dye (Bio-Rad Laboratories, Hercules, CA). qPCR was performed and analyzed as described in Bohnet *et al* (2004). Data are expressed as fold-increase of experimental over control ± the standard error of the mean (SEM). The Student's *t* test was used for statistical analysis of the fold-increase data and $P < .05$ was considered to indicate a statistically significant difference.

Olfactory bulb immunohistochemistry
Coronal 30-μm frozen sections of the OB were cut and stained by the immunoperoxidase procedures described in Churchill *et al* (2005) using diaminobenzidine as a chromophore. Viral antigen studies were performed with either the mouse monoclonal PR8-reactive anti-influenza virus A H1N1 antibody (Chemicon, Temecula, CA; catalog number MAB8261, lot number 24050638; dilution 1:100) or the mouse monoclonal anti-influenza N1 NP antibody (Chemicon catalog number MAB8257F-5, lot number 0506002204; dilution 1:100) as the first antibody. The second antibody for viral antigen studies was biotinylated horse anti-mouse immunoglobulin G (IgG) (Vector Labs, Burlingame, CA; catalog number BA2001, lot number N0109; 1:500) with 2% normal horse serum as a blocking agent. Control sections were treated with the antibody to mouse IgG but no primary antibody. No specific immunoreactivity was observed in the control sections where the primary antibody was omitted. Rostral portions of the OB were examined at various magnifications and photographed using a Leica DMLB microscope and a Spot camera. Pictures are shown as Figure 3.

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