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Influenza Virus Pathophysiology and Brain Invasion in Mice with Functional and Dysfunctional Mx1 Genes

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Abstract

Mice with a dysfunctional myxovirus resistance-1 (dMx1) gene transport intranasally-instilled PR8 influenza virus to the olfactory bulb (OB) within 4 h post-infection. To determine if the presence of a functional Mx1 (fMx1) gene would influence this brain viral localization and/or disease, we infected mature C57BL/6 dMx1 and fMx1 mice under the same conditions and observed sickness behaviors, viral nucleoprotein (NP) RNA expression and innate immune mediator (IIM) mRNA expression in selected tissues at 15 and 96 h post-infection. Virus invaded the OB and lungs comparably in both sub-strains at 15 and 96 h as determined by nested PCR. In contrast, virus was present in blood and somatosensory cortex of dMx1, but not fMx1 mice at 96 h. At 15 h, sickness behaviors were comparable in both sub-strains; by 96 h dMx1, but not fMx1, were moribund. In both 15 h and 96 h lungs, viral NP was significantly elevated in the dMx1 mice compared to the fMx1 mice, as determined by quantitative PCR. OB expression of most IIM mRNAs was similar at both time periods in both sub-strains. In contrast, lung IIM mRNAs were elevated in fMx1 at 15 h, but by 96 h were consistently reduced compared to dMx1 mice. In conclusion, functional Mx1 did not alter OB invasion by virus but attenuated illness compared to dMx1 mice. Inflammation was similar in OBs and lungs of both strains at 15 h but by 96 h it was suppressed in lungs, but not in OBs, of fMx1 mice.

INTRODUCTION

Influenza virus is an important pathogen of humans and many mammalian and avian species. While human strains are not generally thought to invade the brain, growing evidence suggests that neurological complications of influenza (distinct from Reye's syndrome) are not rare in children (Newland et al., 2007; Studahl, 2003; Sugaya, 2002; Wang et al., 2010; Webster et al., 2010). Neurological complications have also been reported in adults infected with pandemic strains to which the victims had no pre-existing immunity (Fugate et al., 2010; Kristensson, 2006; Majde et al., 2007). Despite these reports, the relationship of human influenza viruses and the brain during severe infections has received little attention.

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The innate immune system is the first line of defense against all infectious agents (Germain, 2004; Kumagai et al., 2008). This system employs numerous innate immune mediators (IIMs), including eicosenoids, lectins, cytokines, chemokines, defensins, and anti-viral enzymes, to impair viral replication. Selected cytokines activate pathophysiological changes, often termed the acute phase response, 'flu' syndrome, or sickness behavior.

Sickness behaviors are manifestations of pathophysiological changes in the brain that accompany overt viral infections and include changes in body temperature and sleep, headache, anorexia and cognitive impairment (Majde and Krueger, 2005; Nicholson, 1998). Severe influenza in mice is characterized by a precipitous reduction in body temperature (Wong et al., 1997) together with a disappearance of circadian temperature rhythms (Conn et al., 1995; Majde et al., 2007). In addition, infected mice demonstrate reduced locomotor activity that is also accompanied by a loss of circadian rhythms (Conn et al., 1995). These mice also lose up to 20% of their body weight as a consequence of reduced water and food intake (Conn et al., 1995). The time of onset and severity of these responses to mouse-adapted influenza virus in a particular mouse strain is determined by the dose of virus administered (Conn et al., 1995).

Key cytokines produced in response to viral challenge are the well-characterized proinflammatory cytokines interleukin-1 beta (IL1 β) and tumor necrosis factor alpha (TNF α) as well as anti-inflammatory type I interferons (IFNs) (Amadori, 2007). Influenza virus induces these cytokines through at least two Toll-like receptors (TLRs): intracellular TLR7, which binds to single-stranded viral RNA released into the cytosol, and TLR3, which responds to both extracellular and intracellular double-stranded (ds)RNA made during viral replication (Majde et al., 2010).

The type I IFNs include several isoforms of IFN α and one isoform of IFN β that are widely recognized for their antiviral activity. These IFNs stimulate the expression of several dozen known antiviral factors (De Veer et al., 2001). These include enzymes with broad-spectrum antiviral properties, such as 2',5'-oligoadenylate synthetase (OAS) (Silverman, 2007), and an enzyme that blocks transcription of influenza and closely related myxoviruses, the myxovirus resistance-1 (Mx1) guanine triphosphatase (Haller et al., 2007).

Influenza A/PR/8/34 (PR8) is an H1N1 human strain adapted to efficiently replicate in and kill mice. Following intranasal instillation PR8 is found in the olfactory bulb (OB) of the brain within 4 h post-infection (PI) (Majde et al., 2007). Pro-inflammatory cytokine mRNAs, as well as IFN-induced enzymes OAS1a and Mx1 mRNA, are also elevated in the OB at 15 h PI when the mice become overtly ill with the high dose of virus employed (Majde et al., 2007). Virus appears to be restricted to glia of the outer layers of the OB, but the numbers of IL1 β - and TNF α -expressing neurons are increased in olfactory and autonomic pathways with projections from the olfactory system (Leyva-Grado et al., 2009). Illness onset correlates with increased numbers of IL1 β -expressing neurons in the temperature regulating nuclei of the hypothalamus (Leyva-Grado et al., 2009). However, the C57BL/6 mice used in the study, like other commercially-available strains of inbred mice, carry a dysfunctional Mx1 (dMx1) gene (Haller et al., 2007). Because Mx1 plays a vital role in the innate resistance of mice to influenza, a fully functional Mx1 protein (here termed fMx1) may influence uptake of influenza virus into the mouse OB and/or sickness behavior in response to the virus. Because humans express a MxA gene that appears to be a homolog of the functional mouse Mx1 gene (Haller et al., 2007), determining the effect of fMx1 gene product on virus uptake by the OB may shed light on the relevance of our mouse brain invasion studies to human disease.

To investigate the impact of the fMx1 gene product on the effects of PR8 influenza viral infection, we compared three categories of responses in mice bearing either dMx1 or fMx1 genes: 1) sickness behaviors (hypothermia, reduced locomotor activity, and weight loss) in response to the infection; 2) invasion of PR8 into the lung, blood, OB, and a control brain region, the somatosensory cortex, as detected by nested RT-PCR (nPCR) analysis of the PR8 nucleoprotein (NP) gene segment; and 3) expression of IIM genes in the OB and lung and of virus in lung as measured by quantitative real-time RT-PCR (qPCR).

MATERIALS AND METHODS

Animals

C57BL/6 male mice with a defective Mx1 gene (hereafter referred to as dMx1 mice) were procured from Jackson Laboratories (Bar Harbor, ME). Congenic C57BL/6 male mice with a functional Mx1 gene (fMx1 mice), derived from influenza-resistant A2G mice (Koerner et al., 2007), were obtained from a breeding colony that was kindly provided by Prof. Peter Staeheli of the University of Freiburg, Germany. Mice were infected at 2–3 months of age. Breeding mice were maintained on a 14:10 h light-dark cycle and experimental mice were on a 12:12 light-dark cycle. All mice were maintained in plastic filter-top cages at 23–24° C and were given food and water *ad libitum*. All animal procedures were approved by the Washington State University Animal Care and Use Committee and conformed to National Institutes of Health guidelines.

Infection procedures

All mice received the same dose (2.5×10^6 median tissue culture infectious doses) of purified mouse-adapted PR8 influenza virus, either live or heat-inactivated by suspension in boiling water for 25 min (Majde et al., 2007). Virus delivery occurred by intranasal instillation of 50 μ L of purified PR8, 25 μ L per nostril under 20% isoflurane/80% polyethylene glycol (isoflurane-PEG) anesthesia (Itah et al., 2004) and was performed within one hour of light onset. Mice were held in their cages for either 15 h (boiled virus: 6 dMx1 and 7 fMx1 mice; live virus: 6 dMx1 mice and 8 fMx1) or 96 h (boiled: 6 dMx1 and 6 fMx1 mice; live: 6 dMx1 and 6 fMx1) prior to euthanasia and tissue collection.

Body temperature, locomotor activity, and body weight determinations

A total of 7 dMx1 and 6 fMx1 mice were surgically implanted with intraperitoneal (IP) Mini Mitters (TA Emitters, Mini Mitter, Bend, OR) following anesthesia with an IP injection of ketamine/xylazine at a dose of 87/13 mg/kg, respectively. After surgery, mice were allowed two weeks of recovery time. Mice were housed individually in plastic cages with filter tops resting on Mini Mitter radio receivers (ER-4000 Energizer/Receiver, Mini Mitter). Body temperature and locomotor activity data were collected and processed with the VitalView computer program (Mini Mitter). Baseline temperature and activity data were collected for 48 h, and then the mice were infected as described above and monitored for 96 h for illness. One group was followed for two weeks to assess mortality. Temperature and activity data were recorded in 1- or 6-min intervals and averaged over 6 h for plotting and statistical analysis. Mice were weighed on a pan balance prior to anesthesia at the time of infection and before euthanasia to obtain body weight data.

Tissue collection and processing for PCR studies

At 15 or 96 h PI the mice were anesthetized by isoflurane-PEG inhalation and euthanized by open-heart cardiac blood collection. Tissues collected included the OB, somatosensory cortex (Sctx), lung (upper lobes) and heart blood; fresh tissues were collected in liquid nitrogen and stored at –80° C until RNA extraction. Sctx, a brain region lacking neuronal

projections from the OB, was collected as a control tissue for blood virus contamination of the brain.

RNA was extracted from 15 or 96 h PI solid organ samples (OB, Sctx and lungs) using Trizol reagent (Invitrogen, Carlsbad, CA) and processed for nPCR or qPCR as described previously (Majde et al., 2007). RNA extractions were conducted for boiled samples first followed by live virus samples in a laminar flow hood treated with UV light between samples to minimize cross-contamination. Solid tissues were homogenized with a Tissue-Tearor (Bio Spec Products Inc., Bartlesville, OK) for 2 min over ice in 1 mL of Trizol per sample (2 mL of Trizol for lung samples). The remainder of the RNA extraction procedure was continued according to the Trizol instruction manual. RNA was extracted from blood samples using the Mouse RiboPure-Blood RNA Isolation Kit (Ambion, Austin, TX) according to the manufacturer's protocol. DNA was removed from samples by treating the RNA solution with a DNA-free kit (Ambion).

All PCR procedures were initiated with 1 µg of tissue RNA. Nested PCR and qPCR procedures for viral NP minus strand (NP⁻, virion RNA) and NP plus strand (NP⁺, replication intermediate RNA) RNA were conducted as previously described (Majde et al., 2007). For qPCR of viral RNA only lung samples obtained from mice receiving live virus were examined due to low levels of NP-RNA in lung samples obtained from mice receiving boiled virus. In this case, NP⁻ and NP⁺ values are expressed as fold increases in the dMx1 mice compared to fMx1 mice using cyclophilin A as the housekeeping gene and the delta-delta Ct method to calculate fold increase. For IIM mRNA quantification, previously published qPCR primers for IFN-α consensus, IFN-β, IL1β, TNFα, Mx1, OAS and cyclophilin (Traynor et al., 2004) were employed using the qPCR procedures described in (Majde et al., 2007). Quantitative IIM PCR data were expressed as fold-increase of infected mouse IIM mRNA compared to RNA levels in boiled virus control mice using the reference gene product cyclophilin A as previously described (Taishi et al., 2008).

Statistical procedures

Mini Mitter data were organized in Microsoft Office Excel, averaged over 120 minute intervals. Repeated measures two-way ANOVAs were performed on Mini Mitter data using NCSS Statistical and Power Analysis Software using Neuman-Keuls Multiple-Comparison post-hoc test. nPCR results were expressed as frequency of NP⁻ or NP⁺ 450 base pair signals detected in gels. qPCR results were analyzed as in previous studies (Majde et al., 2007) and expressed as fold-increase of infected over boiled control levels (IIM values). GraphPad InStat 3 was used to conduct unpaired t-tests with Welch corrections comparing responses in boiled virus samples versus live virus samples or dMx1 mice versus fMx1 mice. All graphs were prepared using SigmaPlot 8.0. A value of $p < 0.05$ was considered significant for all tests performed.

RESULTS

Body temperature changes

There was a significant temperature difference between sub-strains from 36 h PI on $[F(1,17) = 6.13, p = 0.03]$ (Fig. 1A). Body temperatures of fMx1 mice remained near baseline values whereas body temperatures of dMx1 mice continued to decline for the remainder of the observation period, with a nadir of 33.6° C (Fig. 1A). After 42 h PI, the dMx1 temperatures did not exceed 34.8°C.

By 36 h PI circadian temperature rhythms disappeared in the infected dMx1 mice and were not detectable for the duration of the 96 h observation period (Fig. 1A). This loss of circadian temperature rhythms did not occur in the infected fMx1 mice (Fig. 1A).

Locomotor activity changes

Peak locomotor activity occurs in mice at dark onset, as illustrated by the pre-infection values shown in Fig. 1B. In this study, locomotor activity declined significantly at 12 h PI (dark onset) from peak baseline values at 12 h before infection ($p = 0.003$) in both sub-strains. Dark onset locomotor activity levels in both dMx1 mice and fMx1 mice remained substantially lower than baseline dark onset values for the duration of the 96 h observation period (Fig. 1B). The locomotor activity decline was somewhat attenuated in fMx1 mice from 12 h PI onward compared to dMx1 mice (significant differences becoming apparent at 36 h and 84 h PI, Fig. 1B), and circadian activity rhythms were retained in fMx1 but not dMx1 mice throughout the 96 h observation period (Fig. 1B).

Weight loss

Weight loss over 96 h following infection was determined from the body weights of mice on the day of euthanization subtracted from the weights taken immediately prior to viral inoculation. dMx1 mice lost a significant amount of body weight (mean = 7.3 ± 0.2 g, $n = 11$, $p < 0.0001$), as is characteristic of this model. In contrast, fMx1 mouse weights at 96 h PI were not significantly different from baseline weights (mean loss = 2.2 ± 0.8 g, $n = 11$, $p = 0.24$). The weight loss difference between the two sub-strains was statistically significant ($p = 0.007$).

Mortality

The dose of PR8 used in this study resulted in a decrease of body temperature below 34°C in all dMx1 mice by days 5–6 PI. A temperature value this low is associated with mortality (Wong et al., 1997) and these mice were euthanized at this time. In contrast, all fMx1 mice maintained a night-time peak of body temperature of at least 37°C and survived the infection for 2 weeks.

Detection of viral RNA in brain regions and blood using nPCR

In 15 h PI samples, the OBs from both dMx1 and fMx1 mice contained viral NP– RNA (virion or vRNA) and NP+ RNA (replication intermediate RNA) 450 bp bands as determined by nPCR (Table 1, Fig. 2). These 450 bp NP bands were not detected in OBs of mice of either sub-strain receiving boiled virus (Fig. 2). The percentage of detectable bands was similar in both sub-strains for both NP– and NP+ RNA, and the frequency of NP– bands exceeded that of NP+ bands. These results are similar to those reported previously in dMx1 mice infected for 15 h (Majde et al., 2007).

In contrast, in 15 h PI somatosensory cortex (Sctx) samples 1/6 dMx1 mouse showed a NP– band (Table 1). The rest of the 15 h PI Sctx samples from infected dMx1 and fMx1 mice were negative for viral NP RNA (Table 1). These data are comparable to those previously shown in dMx1 mice (Majde et al., 2007) and demonstrate that virus is not generally detectable in a brain region lacking projections from the OB at 15 h PI.

All blood samples from both mouse strains were negative for viral RNA at 15 h PI (Table 1), indicating that detectable viremia was not present at 15 h PI and that viral NP expression in the OB is not due to blood virus in the brain tissue.

At 96 h PI, all the OB samples from infected dMx1 mice expressed NP– and NP+ viral RNA (Fig. 2, Table 1), along with 5/6 OBs from fMx1 mice, as determined by nPCR (Table 1). [The fMx1 animal lacking an NP signal in Fig. 2 showed no evidence of infection (i.e., no illness or viral RNA in the lung) and was omitted from subsequent analyses.] No viral NP bands were found at 96 h PI in mice of either sub-strain treated with boiled virus in any of the tissues analyzed by nPCR (e.g. Fig. 2).

At 96 h PI in Sctx and blood samples, there were distinct differences between the two sub-strains of mice. In dMx1 mice, 4/6 Sctx samples contained NP⁻ RNA, whereas no viral RNA was found in the Sctx samples obtained from 6 fMx1 mice (Table 1). NP⁺ viral RNA was found in the Sctx of 3/6 dMx1 but no fMx1 mice expressed Sctx NP⁺ RNA at 96 h PI. Similarly, viral NP RNA was detected in the blood of dMx1 mice (4/6 NP⁻ and 3/6 NP⁺ RNA, Table 1) but no viral RNA was detected by nPCR in the blood of fMx1 mice at 96 h PI (Table 1).

Quantification of viral NP RNA levels in lungs at 15 and 96 h PI

At 15 h PI, NP⁻ RNA was 10.6-fold higher in the dMx1 mice than in the fMx1 mice ($p < 0.001$); the comparable value for the NP⁺ strand was 21.1-fold higher in the dMx1 mice ($p < 0.001$). At 96 h PI, the differences between the strains were greater with NP⁻ RNA being 14.9- and NP⁺ RNA being 36.8-fold higher in the dMx1 mice than in the fMx1 mice ($p < 0.001$ in both cases).

Quantification of IIM mRNA levels in OBs and lungs at 15 and 96h PI

At 15 h PIOB mRNA levels for OAS1a, Mx1 and TNF α were significantly higher than boiled virus controls of both sub-strains while IFN α -con, IFN β and IL1 β mRNA levels were not significantly different from control levels in either mouse sub-strain (Fig. 3A). [Note that while the Mx1 gene is expressed in both mouse strains, the Mx1 gene in dMx1 mice (and other common inbred strains) has a spontaneous mutation that results in synthesis of a dysfunctional protein lacking enzymatic activity (Haller et al., 2007).] Comparison of the OB IIM mRNAs between the two sub-strains indicated that Mx1 mRNA was not quite significantly increased ($p = 0.05$) and TNF α mRNA was significantly decreased ($p = 0.0003$) in fMx1 mice compared to dMx1 mice (Fig. 3A). The other IIM mRNA levels (IFN α -con, IFN β , OAS1a and IL1 β) measured at 15 h PI in the OB were not significantly different between the two sub-strains (Fig. 3A).

In the 15 h PI lungs, all tested IIM mRNAs were significantly up-regulated in infected mice of both sub-strains compared to values expressed in mice challenged with boiled virus (Fig. 3B). Comparison of IIM mRNA expression between dMx1 and fMx1 mice revealed that IL1 β and TNF α mRNAs were significantly elevated in fMx1 vs. dMx1 mice, but IFN mRNAs (IFN α -con, IFN β) were significantly suppressed in the fMx1 vs. dMx1 mice (Fig. 3B). No significant differences were seen for OAS1a or Mx1 enzyme mRNAs between the two sub-strains (Fig. 3B).

In 96 h PI OBs only OAS1a and Mx1 mRNAs levels were elevated above boiled virus control levels in both sub-strains, while TNF α mRNA was elevated only in dMx1 mice (Fig. 4A). This pattern was similar to that seen in 15 h PI OBs (Fig. 3A). IFN α -con, IFN β and IL1 β mRNA levels were not significantly elevated above boiled control levels. No significant differences were detected between dMx1 and fMx1 mice for any OB IIM mRNA measured at 96 h PI (Fig. 4A).

In the 96 h PI lungs, most IIM mRNAs (IFN β , OAS-1a, Mx1, IL1 β and TNF α) were significantly elevated above boiled control levels in both mouse sub-strains (Fig. 4B). In general the dMx1 mRNA values were higher than fMx1 values, but this trend was not statistically significant other than for IL1 β because of the large variance between individual mice (Fig. 4B).

DISCUSSION

Previous studies in comparable mouse PR8 influenza models have shown a striking protection against mortality when a functional Mx1 gene product is expressed (Grimm et al.,

2007; Haller et al., 2007). The protective effects of fMx1 in PR8 models have been based on mortality and not illness profiles, though one would expect that surviving mice would become less ill than those that die. This report shows that several facets of sickness behavior, i.e., hypothermia, reduced locomotor activity and body weight loss are attenuated in fMx1 mice compared to dMx1 mice.

It is probable that this attenuation of symptoms is related to the inhibition of influenza virus transcription by functional Mx1 gene product (Haller et al., 2007). Because the fMx1 mice do become mildly ill following PR8 challenge it is apparent that functional Mx1 does not completely block viral replication in this model. Mx1 is one of several IFN-induced genes with anti-viral action, but studies in IFN-receptor knockout dMx1 mice have shown that PR8 and a recombinant virus with the internal genes of PR8 (X31) are both minimally inhibited by IFN (Price et al., 2000; Traynor et al., 2006). The influenza NS1 allele found in PR8 and X31 viruses plays a major role in the resistance of these viral strains to IFN (Kochs et al., 2007). This resistance depends on a defective dsRNA-binding motif on the PR8 NS1 protein (Steidle et al., 2010). The PR8 NS1 variant inhibits expression of cytokines regulated by nuclear factor kappa B as well as IFN β induction and IFN-induced signal transduction factors (Geiss et al., 2002). Interestingly, one of the few influenza gene products found in PR8-infected fMx1 mouse cells is NS1 (Krug et al., 1985). A truncated NS1 gene product that fails to bind viral dsRNA obviates cytokine inhibition as well as the pathogenicity of PR8 in dMx1 mice (Donelan et al., 2004). In our model, therefore, the NS1 gene variant in the PR8 strain used in these studies probably plays a role in mild influenza syndrome detected in our fMx1 mice.

Previously it was shown that a hyper-virulent mutant of PR8 can kill fMx1 mice because it replicates to high levels prior to activation of Mx1 by IFN (Grimm et al., 2007). Perhaps our PR8 stock contains such a variant, though continued viral replication at 96 h post-infection in fMx1 mice suggests another mechanism of Mx1 escape by PR8, such as the NS1 blockade discussed above.

Our nPCR results reveal that the frequency of PR8 influenza virus detection in the OB does not differ between fMx1 and dMx1 mice, suggesting that the presence of the functional Mx1 gene does not affect virus uptake by the OB. We previously demonstrated that viral RNA is detectable in the OB within 4 h PI (Majde et al., 2007). Another report has noted the presence of PR8 RNA in the Balb/c mouse OB at 24 h after intranasal instillation but not after sub-lingual administration (Cuburu et al., 2007). This rapid appearance of viral RNA in the OB strongly suggests a passive route of infection of this brain region by input virus, perhaps via channels formed by olfactory ensheathing cells and fibroblasts within the olfactory nerves (Majde et al., 2007). Such a passive route would not be expected to be affected by a gene product that acts upon viral transcription.

The lack of viral NP in the fMx1 Sctx and blood at 96 h PI (in contrast to dMx1 mice) as determined by nPCR suggests that fMx1 mice, but not dMx1 mice, control viral replication sufficiently to block viremia.

The decreased level of NP+ viral RNA in the 15 h PI fMx1 lungs suggests that viral replication is inhibited by the functional Mx1 gene product even at this early time point (2–3 replication cycles) in the infection. The difference between the two sub-strains in viral replication is marked in 96 h PI lung, where viral replication continues in the dMx1 mouse lung but is less in the 96 h PI fMx1 lung when the mice are beginning to recover. In general, the lung NP RNA levels tend to correlate with sickness symptoms while the presence of NP RNA in the OBs does not.

With respect to IIM mRNA expression in the OBs, the same IIMs (OAS1a, Mx1 and TNF α) were elevated in the OB of both sub-strains at both 15 h PI and 96 h PI. However, sub-strain expression levels of Mx1 and TNF α at 15 h PI differed quantitatively between the two sub-strains: Mx1 was increased and TNF α decreased in the fMx1 mice compared to dMx1 mice. At 96 h PI the two IFN-induced enzyme levels were similar and TNF α remained suppressed in the fMx1 mice. At both time periods IFN α -con, IFN β , and IL1 β mRNAs were not significantly elevated above boiled virus background levels. Though TNF α antibody provides some protection against influenza illness (Hussell et al., 2001), influenza infections in mice deficient in both TNF α receptors show minimal differences in sickness behaviors from mice with both functional TNF α receptors (Kapas et al., 2008). It is unlikely that the small differences in TNF α mRNA that we have detected between sub-strains are biologically significant. The single IIM most closely associated with influenza illness in mice is IL1 β (Kozak et al., 1995) and conceivably the significant reduction of IL1 β in the fMx1 lung at 96 h PI is related to the reduced illness (and reduced NP expression) in this sub-strain.

An unexpected finding in these studies was the persistence of viral RNA, including plus strand RNA, in the OB, though this tissue lacks epithelial cell types generally targeted by influenza virus (Julkunen et al., 2001). In our previous immunohistochemistry studies we found virus associated with microglia-like cells and astrocytes in the outer layers of the OB (Leyva-Grado et al., 2009). A human H1N1 influenza virus not adapted to mice is capable of complete replication in both mouse microglia and astrocytes (Wang et al., 2008), and these cells may be the source of the viral NP $^-$ and NP $^+$ that we detect at 96 h PI. Previous efforts in our laboratory to isolate live virus from brains of PR8-infected mice were unsuccessful (Chen et al., 2004), though the technique used may not be adequately sensitive to detect live virus at the levels that we can detect by the molecular methods used. Another possible mechanism by which viral replication intermediates could persist in the OB through 96 h PI without complete replication is continued uptake of virus from the nasal cavity and infection of additional cells using the same route by which the virus initially infects the OB. Though NP RNA is substantially reduced in fMx1 lung, it is not known if the levels in the upper respiratory tract are similarly suppressed.

While the route of virus infection of the OB has not been defined, its association with hypothalamic neuroinflammation at 15 h PI when disease onset occurs suggests (Leyva-Grado et al., 2009) that OB virus could play a role in the precipitous onset of sickness behavior characteristic of pandemic influenza (Bloomfield and Harrop, Jr., 1919). Whether this neuroinflammation in response to brain virus has long-term consequences is yet to be determined (Majde, 2010). However, it is noteworthy that intranasal live virus vaccines have infrequently been associated with neurological conditions such as Bell's palsy (Izurieta et al., 2005) and the possibility of live vaccine virus invasion of the brain should be considered.

Early in the recent swine-origin H1N1 pandemic a number of cases of neurological complications associated with the infection were reported (Centers for Disease Control and Prevention (CDC), 2009; Gonzalez Duarte, A. et al., 2010; Iwata et al., 2010; Mariotti et al., 2010; Martin and Reade, 2010; Wang et al., 2010; Webster et al., 2010). Many of these cases resembled the acute encephalopathy associated with influenza that has been extensively reported in Japanese children (Mizuguchi et al., 2007). Virus has not been isolated from the brains of these encephalopathy patients and the condition may reflect metabolic changes in these patients associated with selected polymorphisms (Wang et al., 2010). A recent report has tied an HLA variant to the development of the sleep disorder narcolepsy following swine-origin H1N1 exposure, either via an adjuvanted vaccine or actual infection (Dauvilliers et al., 2010). There is a growing awareness of the association of

influenza with neurological complications (Newland et al., 2007), though the role of the virus itself in these conditions remains unclear.

In summary, this report demonstrated that the presence of a functional Mx1 gene product did not alter the invasion of the OB by PR8 influenza virus, indicating that blockade of virus transcription by Mx1 protein has no impact on this process. However, influenza disease was attenuated in the fMx1 mice, and all of the animals survived the infection that was uniformly lethal in dMx1 mice. There were no marked changes in OB or lung IIMs measured at either 15 h or 96 h PI in either sub-strain. OB virus persists throughout the observation period, but its role in viral disease remains unclear.

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Research Highlight

A functional Mx1 gene product inhibits viral replication and reduces sickness behavior but does not affect viral invasion of the brain.

Fig. 1. Changes in body temperature (A) and locomotor activity (B) over 96 h following PR8 infection. Locomotor activity data were normalized by averaging the number of locomotor activity counts across 2-h time periods for each mouse and then dividing by 1/12th of each mouse's pre-infection 24-h average. * $p < 0.05$ comparing dMx1 to fMx1 using repeated measures two-way ANOVA.

Fig. 2. OB viral NP⁻ and NP⁺ RNA detection by nPCR at 96 h in dMx1 (A) and fMx1 (B) mice. After live virus challenge (lower gels) the bands near the 500 base pair (bp) standard are viral NP⁻ and NP⁺. No viral NP bands were detected in the OB if mice were challenged with boiled virus (upper gels).

Fig. 3. Quantitative mRNA expression at 15 h PI of innate immune mediators in PR8-infected OB (A) and lung (B) comparing dMx1 (black bars) to fMx1 (gray bars) values. Asterisks designate significant differences from boiled virus control values, plus signs designate significant differences between mouse sub-strains. Boiled virus values have been normalized to 1.0 and therefore are not included in this Figure. Note that while the Mx1 mRNA is expressed in both mouse strains, the protein product of the mutated Mx1 gene in dMx1 mice has no enzymatic activity. See Results text for analysis. * $p < .05$, ** $p < 0.01$, + $p < 0.05$. ++ $p < 0.01$. (Note scale differences between OB and lung.)

Fig. 4. Quantitative mRNA expression at 96 h PI of innate immune mediators (IIMs) in PR8-infected OB (A) and lung (B) comparing dMx1 (black bars) to fMx1 (gray bars) values. Asterisks designate significant differences from boiled virus control values, plus signs designate significant differences between mouse sub-strains. Boiled virus data have been normalized to 1.0 and therefore are not included in this Figure. Note that while the Mx1 mRNA is expressed in both mouse strains, the protein product of the mutated Mx1 gene in dMx1 mice has no enzymatic activity. See Results text for analysis. * $p < .05$, ** $p < 0.01$, + $p < 0.05$, ++ $p < 0.05$. (Note scale differences between OB and lung.)

Table 1

Summary of nPCR Results for NP Expression in Olfactory Bulb, Somatosensory Cortex, Blood and Lung

Tissue	Sub-strain	H PI	NP Minus Strand Ratio	NP Plus Strand Ratio
OB	dMx1	15	6/6 (100%)	2/6 (33%)
OB	fMx1	15	7/8 (88%)	3/8 (38%)
OB	dMx1	96	6/6 (100%)	6/6 (100%)
OB	fMx1	96	5/6 (83%)	5/6 (83%)
Sctx	dMx1	15	1/6 (16%)	0/6 (0)
Sctx	fMx1	15	0/6 (0)	0/6 (0)
Sctx	dMx1	96	4/6 (66%)	3/6 (50%)
Sctx	fMx1	96	0/6 (0)	0/6 (0)
Blood	dMx1	15	0/6 (0)	0/6 (0)
Blood	fMx1	15	0/6 (0)	0/6 (0)
Blood	dMx1	96	4/6 (66%)	3/6 (50%)
Blood	fMx1	96	0/6 (0)	0/6 (0)
Lung	dMx1	15	6/6 (100%)	6/6 (100%)
Lung	fMx1	15	6/6 (100%)	6/6 (100%)
Lung	dMx1	96	6/6 (100%)	6/6 (100%)
Lung	fMx1	96	6/6 (100%)	6/6 (100%)

Note: Boiled virus (control) samples are not included in this Table as all OB, Sctx and blood samples were negative for both minus and plus strand viral RNA. In lung boiled virus samples, NP– was detected sporadically in both strains as previously reported (Majde et al 2007)..