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Attenuation of the influenza virus sickness behavior in mice deficient in Toll-like receptor 3

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ABSTRACT

Certain sickness behaviors occur consistently in influenza-infected humans and mice. These include body temperature changes, somnolence, and anorexia. Several cytokines serve as mediators of the influenza acute phase response (APR), including these sickness behaviors, and one likely inducer of these cytokines is dsRNA produced during viral replication. TLR3 is known to be one of the host cellular components capable of recognizing dsRNA and activating cytokine synthesis. To determine the role of TLR3-detected viral dsRNA in the causation of viral symptoms, TLR3-deficient mice (TLR3 knockouts, or KOs) were infected with a marginally-lethal dose of mouse-adapted X-31 influenza virus. TLR3 KOs and their wild-type (WT) controls were monitored for baseline body temperature, locomotor activity, and sleep profiles prior to infection. Both mouse strains were then infected and monitored for changes in these sickness behaviors plus body weight changes and mortality for up to 14 days post-infection. Consistent with the observations that influenza pathology is reduced in TLR3 KOs, we showed that hypothermia after post-infection day 5 and the total loss of body weight were attenuated in the TLR3 KOs. Sleep changes characteristic of this infection model [particularly increased non-rapid-eye-movement sleep (NREMS)] were also attenuated in TLR3 KOs and returned to baseline values more rapidly. Locomotor activity suppression was similar in both strains. Therefore virus-associated dsRNA detected by TLR3 appears to play a substantial role in mediating several aspects of the influenza syndrome in mice.

1. Introduction

The viral acute phase response, or 'flu syndrome', is commonly experienced in varying degrees of severity following acute respiratory and intestinal viral infections. The most common symptoms of acute viral infections in humans are fever, somnolence, headache, and a general feeling of malaise. These symptoms are usually indistinguishable from those associated with bacterial infections, and are thought to be mediated by some of the same cytokines that are induced by bacteria, such as IL-1β and TNF-α (Leon, 2004).

All viruses produce double-stranded (ds)RNA during replication (Majde, 2000), regardless of the form of nucleic acid borne by the virions. DsRNA associated with single-stranded (ss)RNA viruses, such as influenza, is thought to be derived primarily from annealing of ssRNA replication intermediates (Majde, 2000), though other sources of intracellular dsRNA may be increased by viral infection such as small interfering RNAs (Matskevich and Moelling, 2007). Synthetic dsRNA (polyriboinosinic:polyribocytidylic acid, or pI:C) or virus-derived dsRNA stimulates sickness behaviors indistinguishable from influenza virus itself (Carter and De Clercq, 1974; Fang et al., 1999; Kimura-Takeuchi et al., 1992; Majde et al., 1991; Majde, 2000; Traynor et al., 2006). In addition, pI:C can substitute for influenza virus in the blockade of the acute phase response (APR) (Kimura-Takeuchi et al., 1992), suggesting that dsRNA alone can induce APR regulatory mediators similar to those induced by the virus [details of the mouse sickness, cytokine and endocrine responses to pI:C were recently described (Cunningham et al., 2007; Gandhi et al., 2007)]. Virus-associated dsRNA is thought to be an important mediator of viral cytokine induction and contribute to the influenza APR (Guillot et al., 2005).

In the last decade much has been learned about a pathogen recognition system termed the Toll-like receptors (TLRs). These receptors play a prominent role in the initiation of cytokine induction by microbes (Kawai and Akira, 2006). Cytokine induction by bacteria is known to be initiated by bacterial cell-wall products such as endotoxins/lipopolysaccharides that are recognized by TLR4 (Akira and Hemmi, 2003), or lipopeptides (Hashimoto et al., 2007) recognized by TLR2 (Kawai and Akira, 2005). One group of TLRs detects microbial nucleic acids (Akira and Hemmi, 2003), which have

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subtle structural differences from mammalian nucleic acids. Specifically, unmethylated microbial DNA is recognized by TLR9 (Akira and Hemmi, 2003). Intracellular viral ssRNA, on the other hand, is recognized by either TLR7 (in the mouse) or TLR8 (in the human) (Diebold et al., 2006). Extracellular viral dsRNA is detected primarily by TLR3 (Gantier and Williams, 2007), while intracellular dsRNA made during viral replication is also detected by cytoplasmic helicases such as RIG-I and MDA-5 (Eisenacher et al., 2008; Pichlmair and Reis e Sousa, 2007) as well as by vesicular TLR3 (Vercammen et al., 2008). Intracellular viral ssRNA and dsRNA also activate the inflammasome Nod-like receptor family (NLRP)3, which is important in host defense against influenza virus (Allen et al., 2009). Different dsRNA-recognition factors are expressed in different cell types (Kawai and Akira, 2006) and recognize different viruses (Kato et al., 2006).

TLR3s recognize both synthetic and virus-derived dsRNA (Vercammen et al., 2008). They are found on $CD8^+$ T lymphocytes (Salem et al., 2009), influenza-infected epithelial cells (Guillot et al., 2005), macrophages (Miettinen et al., 2001), myeloid dendritic cells (Schroder and Bowie, 2005), glial cells (Carpentier et al., 2007) and other immunocompetent cells. Binding of dsRNA to TLR3 induces apoptosis and autophagy of immune and cancer cells as well as cytokines and chemokines (Seya and Matsumoto, 2009).

The protective/pathogenic roles of TLR3 receptors have been analyzed using TLR3 knockout (KO) mice in several viral models (Vercammen et al., 2008), including influenza. However, the role of TLR3 signaling in the induction of the APR to a virus has not been explored. In this study we analyze several acute phase sickness behaviors in TLR3 KOs and their wild-type (WT) controls challenged intranasally with a marginally-lethal dose of mouse-adapted influenza A virus.

2. Materials and methods

2.1. Animals

Two mouse strains were employed: B6;129S1-Tlr3^{tm1Flv}/J (TLR3 KOs) and B6:129SF1/J (WT) strain controls. Three- to four-month-old male mice of each strain were purchased from Jackson Laboratory (Bar Harbor, Maine). All mice were quarantined in AAALAC-approved animal quarters and housed in filter-top cages throughout the study to prevent intercurrent infections or spread of X-31. For body temperature/locomotor activity/body weight studies, mice were housed individually at 22–23 °C ambient. During sleep data collection the mice were housed individually in a sound-attenuated environmental chamber maintained at a temperature of 29 ± 1 °C to which they were adapted for at least a week. All mice were maintained on a 12:12 h light–dark cycle with lights on at 09:00 PDT. Food and water were available *ad libitum*. All experiments were approved by the Washington State University Animal Care and Use Committee and conformed to National Institutes of Health guidelines.

2.2. Virus purification and titration

The influenza strain employed was mouse-adapted X-31 influenza A, a reassortant between A/PR/8/34 (H1N1) (PR8) and A/Aichi/68 (H3N2) (Lee et al., 2001). X-31 expresses H3N2 surface genes but contains the internal genome segments of PR8, a strain of influenza that is highly pathogenic for mice. About 10,000-fold more X-31 virus is required to kill mice in the same time-frame as PR8, largely due to the retention of glycosylated surface proteins on X-31 that are targeted by circulating host lectins (Reading et al., 1997). The X-31 virus grown in specific pathogen-free chick embryos was purified and tested for possible contaminants as previously described (Chen et al., 2004). Viral titrations were performed in Madin-Darby canine kidney cells as previously described (Chen et al., 2004) and expressed as median tissue culture infectious doses ($TCID_{50}$). The starting titer of the purified X-31 virus was 1×10^6 $TCID_{50}$/mL.

2.3. Virus inoculation

The purified virus was diluted in Dulbecco's PBS containing magnesium and calcium (Invitrogen, Carlsbad, CA) and mice were inoculated intranasally using a 100 µl micropipette with 200 $TCID_{50}$ X-31 in a volume of 50 µl/mouse (25 µl each nostril) under light methoxyfluorane (Metofane, Pitman-Moore, Inc., Mundelein, IL) inhalation anesthesia. All inoculations were performed within 10 min of light onset at 0900 h PDT. The dose of virus used was approximately 2 median lethal doses (LD_{50}) as determined by titration in 129 SvEv mice maintained at 23 °C.

2.4. Surgeries

All surgeries were conducted in mice anesthetized with intraperitoneal ketamine–xylazine (87 and 13 mg/kg, respectively). For temperature and locomotor activity studies, mice (8 TLR3 KO and 15 WT) were implanted intraperitoneally with chemically sterilized biotelemetry transmitters (Minimitter, Bend, OR) as previously described (Traynor et al., 2006). For sleep studies, mice ($n = 8$ for TLR3 KO and $n = 8$ for WT controls) were implanted with two electromyogram (EMG) electrodes and three EEG electrodes (Plastics One, Roanoke, VA) as described in reference (Chen et al., 2004). Electrodes for EEG recordings were placed over the frontal and parietal cortices and over the cerebellum. Electrodes for EMGs were placed in the dorsal neck muscles. Following surgery, mice were allowed to recover for 7 days prior to sleep recording. Baseline wake (W), non-rapid-eye movement sleep (NREMS) and rapid-eye movement sleep (REMS) characteristics were established by recording for 24–48 h prior to infection. Mice were allowed to recover for at least a week following surgeries before viral challenge.

2.5. Body temperature and locomotor activity analyses

Body temperature and locomotor activity measurements were performed using Mini Mitter (Bend, OR) biotelemetry as described in (Traynor et al., 2006). Temperature and locomotor activity data were recorded at 6-min intervals and data points representing those values were averaged over 6-h intervals. The mice were kept at 23 °C for these experiments because body temperature responses to influenza challenge are larger at this ambient temperature (Jhaveri et al., 2007).

2.6. Sleep scoring

EEG and EMG signals were digitized (128 Hz sampling rate) and stored on digital media. W, NREMS and REMS were scored by hand in 10-s epochs by defining NREMS as high amplitude EEG slow waves and low-tone muscle activity, REMS as highly regular theta EEG activity and loss of muscle tone with occasional twitches, and W as EEG activities similar to, but often less regular and with lower amplitude than, those in REMS and high EMG activity. Time spent in each state was tabulated into 2-h intervals and graphed. In infected mice sleep was analyzed over 24-h intervals prior to infection (baseline) or three days (Virus 3), 5 days (Virus 5) or 8 days (Virus 8) following intranasal X-31 challenge. These scoring days were selected based on body temperature changes and on previous studies in this model (Traynor et al., 2006), and represent the acute phase (Viruses 3 and 5) and the early recovery phase (Virus 8) of the infection, respectively.