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Characterization of non-lethal West Nile Virus (WNV) infection in horses: Subclinical pathology and innate immune response

Helle Bielefeldt-Ohmann [a, b, c, *], Angela Bosco-Lauth [d], Airn-Elizabeth Hartwig [d], M. Jasim Uddin [b], Jean Barcelon [c], Willy W. Suen [b], Wenqi Wang [b], Roy A. Hall [a, c], Richard A. Bowen [d]

[a] Australian Infectious Diseases Research Centre, University of Queensland, Australia
[b] School of Veterinary Science, University of Queensland, Australia
[c] School of Chemistry and Molecular Biosciences, University of Queensland, Australia
[d] College of Veterinary Medicine & Biomedical Sciences, Colorado State University, Fort Collins, CO, USA

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ABSTRACT

Most natural West Nile virus (WNV) infections in humans and horses are subclinical or sub-lethal and non-encephalitic. Yet, the main focus of WNV research remains on the pathogenesis of encephalitic disease, mainly conducted in mouse models. We characterized host responses during subclinical WNV infection in horses and compared outcomes with those obtained in a novel rabbit model of subclinical WNV infection (Suen et al. 2015. *Pathogens*, 4: 529). Experimental infection of 10 horses with the newly emerging WNV-strain, $WNV_{NSW2011}$, did not result in neurological disease in any animal but transcriptional upregulation of both type I and II interferon (IFN) was seen in peripheral blood leukocytes prior to or at the time of viremia. Likewise, transcript upregulation for IFNs, TNFα, IL1β, CXCL10, TLRs, and MyD88 was detected in lymphoid tissues, while IFNα, CXCL10, TLR3, ISG15 and IRF7 mRNA was upregulated in brains with histopathological evidence of mild encephalitis, but absence of detectable viral RNA or antigen. These responses were reproduced in the New Zealand White rabbits (*Oryctolagus cuniculus*) experimentally infected with $WNV_{NSW2011}$, by intradermal footpad inoculation. Kinetics of the anti-WNV antibody response was similar in horses and rabbits, which for both species may be explained by the early IFN and cytokine responses evident in circulating leukocytes and lymphoid organs. Given the similarities to the majority of equine infection outcomes, immunocompetent rabbits appear to represent a valuable small-animal model for investigating aspects of non-lethal WNV infections, notably mechanisms involved in abrogating morbidity.

1. Introduction

West Nile virus (WNV), a mosquito-borne flavivirus in the family *Flaviviridae*, is widely distributed throughout Africa, the Middle East, Asia, Southern Europe, Australia and the Americas. Since the 1990s, WNV has been the causative agent for numerous equine and human encephalitis outbreaks worldwide, highlighting it as an important re-emerging neurotropic virus. However, the current rodent (mouse and hamster) models for studying the pathogenesis of WNV infection have several shortcomings limiting their ability to accurately model equine and human infection. These include markedly different levels of virus replication in the central nervous system (CNS), as well as in peripheral tissues in rodents compared to horses [1–4]. The much exaggerated level of especially

Abbreviations: CNS, central nervous system; CXCL10, chemokine (C-X-C motif) ligand 10; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HE, hematoxylin and eosin; HIF-1α, hypoxia inducible factor 1α; IFN, Interferon; IL, interleukin; IRF, interferon regulatory factor; ISG, interferon-stimulated genes; IU, infectious units; mAb, monoclonal antibody; MyD88, myeloid differentiation 88; NF-κB, nuclear factor kappa- B; NSW, New South Wales; NZW, New Zealand white; PFU, plaque forming units; p.i., post infection; PRNT, plaque reduction neutralization test; PTX3, pentraxin-related protein 3; qRT-PCR, quantitative reverse transcriptase polymerase chain reaction; STAT, Signal transducer and activator of transcription; $TCID_{50}$, 50% tissue culture infectious dose; TLR, Toll-like receptors; TNFα, tumor necrosis factor α; TRAF, TNF receptor-associated factor; VEGF, vascular endothelial growth factor; WNV, West Nile virus.

* Corresponding author. School of Veterinary Science, University of Queensland, Gatton 4343, Australia.
E-mail address: h.bielefeldtohmann1@uq.edu.au (H. Bielefeldt-Ohmann).

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CNS infection in rodents, and the variability of responses seen in mice depending on mouse strain, age and inoculation route in addition to virus strain [2], suggests a potentially different mechanism(s) of disease in these animals compared to the natural hosts, horses and humans. The prevalence of infections resulting in clinical symptoms in horses, including ataxia, weakness, recumbency and muscle fasciculations [1], is not known, but seroepidemiological studies suggest that *asymptomatic infections* in equines occur with a frequency similar to that in humans, i.e., ~80% of infections [5–8]. However, infections with neurological symptoms appear to have a high case-fatality rate (up to 30–40%) in horses [4] compared to ~1% in humans, although the number for equines is likely highly inflated by the propensity to euthanatize horses with neurological signs for humane and/or financial reasons.

WNV is a small, enveloped, single-stranded, positive sense RNA-virus. The genome of ~11 kb encodes three structural and seven non-structural proteins. After inoculation of the vertebrate host via a mosquito bite, WNV may initially replicate locally in the skin, presumably followed by further amplification in draining lymph nodes, viremia and spread to other organs including CNS. WNV strains are phylogenetically separated into two main genetic lineages (I and II), containing virulent strains associated with neurological disease. Several WNV-like strains, clustering outside these two lineages, have also been identified and form an additional five proposed lineages. However, little is known about whether these strains have the potential to induce disease. Lineage I strains are found in North America, North Africa, Europe and Australia, while lineage II strains are endemic in southern Africa and Madagascar, and in recent years have caused epidemics in southern Europe [1,9]. The WNV transmission cycle involves birds as the natural vertebrate hosts and ornithophilic mosquitoes as maintenance vectors. Humans and horses are incidental hosts for WNV [1].

A significant expansion of WNV has occurred in the last few decades, most notably to the Americas, where the first cases were observed in New York, USA, in 1999 caused by a new strain, WNV_{NY99}, which subsequently spread epidemically throughout North- and South-America. WNV is now regarded as endemic in the region and remains a significant risk. Recurring outbreaks of WNV-encephalitis are also seen in Southern Europe and the Middle East [9,10]. In Australia, strains of WNV, known as Kunjin viruses (WNV_{KUN}), are relatively attenuated and only rare mild cases of non-fatal neurological disease have been reported in humans [11]. However, in 2011, following severe flood-events in Eastern Australia, a new strain of WNV_{KUN} emerged (hereafter named $WNV_{NSW2011}$), causing a severe epidemic of equine encephalitis in New South Wales (NSW), Victoria and South Australia [12]. Interestingly, while this strain is also virulent in mice (the common approach to virulence determination of flaviviruses), albeit not as virulent as WNVNY99 [12,13], no severe human cases were reported at the time of the outbreak amongst equines, of which more than 1000 cases of neurological disease with a fatality rate of 10–15% were recorded [12]. Our recent studies have shown that this and other potentially virulent strains of WNV have circulated in Australia for $\geq$30 years [13], and that the Australian public [14] as well as the equine population [8] remain as vulnerable as ever to emerging virulent WNV strains.

Asymptomatic infection may be due to pre-existing immunity (prior exposure or cross-reactive immunity) or inherent 'resistance' due to a rapid, robust innate and adaptive immune response. So far the research focus has been on the mechanisms of WNV neuroinvasive disease using mouse models [2,15–17], while the mechanisms underlying "resistance" remain largely unknown. Yet, it is exactly those mechanisms of the inherently resistant individuals, and most likely relating to innate immune reactions, which should be targeted in development of new vaccines, adjuvants and treatments [18–20]. The much exaggerated level of infection in mice suggests a potentially different pathogenesis of disease in this species compared to horses and humans [2,16,17], and may, at least in part, be explained by differences in immune responses between horses, humans and mice [15,21]. We therefore hypothesized that the innate immune response in horses is of greater potency and/or faster kinetics compared to mice, ensuring that the virus rarely replicates to levels conducive to viral neuro-invasion and at the same time facilitate a strong virus specific immune response and elimination of the virus, including from the CNS. We established a rabbit model of non-lethal WNV infection, which mimics the course of the majority of equine (and human) WNV-infections, including low, transient viremia, and negligible morbidity despite histopathological evidence of mild infection-induced inflammation in some animals and virus replication in peripheral tissues [22,23]. In this report we describe outcomes of experimental, sub-lethal infection in horses with the equine-pathogenic $WNV_{NSW2011}$ strain and compare to results obtained in the rabbit model as well as draw comparisons to findings in natural equine WNV-infections.

2. Materials and methods

2.1. Virus

Detailed characterization of the Australian equine-pathogenic $WNV_{NSW2011}$ strain has been previously published [12,13]. The stock virus was fourth *in vitro* passage in alternating C6/36 mosquito cells and Vero cells, with the last passage in C6/36 cultures. The virus was titrated by plaque assay (PFU) and 50% endpoint of tissue culture infective dose ($TCID_{50}$)-assay on Vero and C6/36 cells, respectively [22].

2.2. Experimental infection of horses with WNV

Ten quarterhorse stock horses, 1–2 years old (equal numbers of geldings and mares) and seronegative for WNV, were challenged by intradermal inoculation on the left shoulder with 3×10^4 PFU ($n = 5$) or 9×10^5 PFU ($n = 5$) of $WNV_{NSW2011}$. The horses were monitored clinically by twice daily observations and rectal temperature taken. The horses were bled twice daily during the first 9–12 days p.i., thereafter once daily until day 21–28 p.i., and serum as well as whole blood stored at −80 °C until virus-isolation and RNA-extraction, respectively. Three horses per dose were euthanatized 9–12 days p.i., while the remaining 4 horses were terminated at day 21 ($n = 2$) and 28 p.i. ($n = 2$), respectively. Samples from CNS (cerebral cortex, medulla oblongata, pons, three levels of spinal cord), spleen, lymph node (the injection-site draining prescapular), kidney, and heart were collected at necropsy for RNA isolation, histopathology assessment and immunohistochemistry. The studies were approved by the Animal Use and Care Committee of Colorado State University (Approval no. 12–3837 A), and carried out in accordance with the NIH guidelines for ethical use of animals in research.

2.3. Histopathology & immunohistochemistry (IHC)

Tissues collected at necropsy of experimentally $WNV_{NSW2011}$ infected horses were fixed for 48 h in neutral-buffered formaldehyde and routinely processed through to paraffin-embedding. In instances where samples could not be processed immediately, they were transferred into and stored in 70% ethanol. A section of each