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Innate and adaptive immune responses to tick-borne flavivirus infection in sheep

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ABSTRACT

The flaviviruses tick-borne encephalitis virus (TBEV) and louping ill virus (LIV) are closely-related genetically and antigenically, have broadly similar host ranges that include rodents and other mammals (including sheep), and are both transmitted by the same tick species, *Ixodes ricinus*. Although human infection with TBEV results in a febrile illness followed in some cases by encephalitis, humans appear to be much less susceptible to infection with LIV. However, these viruses demonstrate different susceptibilities in sheep; LIV infection causes encephalitic disease, whereas TBEV infection generally does not. To investigate the role of the immune response in this mixed outcome, groups of sheep were inoculated with either virus, or with a primary inoculation with one virus and secondary inoculation with the other. Markers of both adaptive and innate immune responses were measured. In each group studied, infection resulted in seroconversion, demonstrated by the detection of virus specific neutralising antibodies. This appeared to control infection with TBEV but not LIV, which progressed to a febrile infection, with transient viraemia and elevated levels of serum interferon. This was followed by neuroinvasion, leading to up-regulation of innate immune transcripts in discrete areas of the brain, including interferon inducible genes and chemokines. Prior inoculation with TBEV did not prevent infection with LIV, but did appear to reduce disease severity and viraemia. We postulate that LIV has adapted to replicate efficiently in sheep cells, and disseminate rapidly following infection. By contrast, TBEV fails to disseminate in sheep and is controlled by the immune response.

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1. Introduction

Louping ill virus (LIV) and tick-borne encephalitis virus (TBEV) are zoonotic members of the genus *Flavivirus*, family *Flaviviridae* (Calisher 1988). Both viruses are members of the tick-borne flavivirus lineage, transmitted by the tick species *Ixodes ricinus* (*I. ricinus*) in Europe (Gritsun et al., 2003). Although TBEV is not detected in the United Kingdom (UK), it is indigenous across a vast geographical area from Western Europe to Asia, where it causes many cases of human disease. Human infection with TBEV leads to fever and encephalitis, which can be fatal (Mansfield et al., 2009). Wild mammals participate in the ecological cycle of TBEV and have been shown to seroconvert (Klaus et al., 2010). However, neurological disease in wild and domestic sheep following infection with TBEV is exceedingly rare (Bago et al., 2002).

LIV causes encephalitis in sheep, with the highest prevalence in upland areas of the UK, (Jeffries et al., 2014). LIV has also been reported from Norway and Spain (Gao et al., 1993; Balseiro et al., 2012), and closely-related viruses have been detected in Spain, Greece and Turkey (Hartley et al., 1969; Papadopoulos et al., 1971; Gonzalez et al., 1987; Mansfield et al., 2015). Seroprevalence studies have indicated the exposure of wildlife to LIV, but instances of disease are rare (Reid et al., 1978). Occasional cases of human disease caused by LIV have also been reported (Davidson et al., 1991). LIV infection in sheep leads to pyrexia and viraemia,

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resulting in widespread dissemination of virus. Infected sheep may show a partial recovery before developing severe neurological disease symptomatic of encephalitis (Jeffries et al., 2014). An extensive series of studies into LIV infection in sheep demonstrated relationships between viraemia, neutralising antibodies and the pathology of disease in the infected brain (Doherty and Reid, 1971; Reid and Doherty, 1971). Later studies confirmed the presence of perivascular cuffing and mononuclear infiltration of the brain parenchyma (Sheahan et al., 2002). TBEV causes similar lesions in the brains of infected humans, typical of viral encephalitis (Gelpy et al., 2005), but there is no evidence to suggest that this virus can cause neuroinvasion in other mammalian species.

The geographical area affected by TBEV has expanded across Europe (Mansfield et al., 2009), and the incidence of LIV cases in UK sheep has also increased (Jeffries et al., 2014), prompting an investigation into the factors underlying the different outcomes of infection in ruminants. Aside from the neutralising antibody response, little is known about the immune response to flavivirus infection in sheep. Through an *in vivo* model of LIV and TBEV infection, the immune response to infection in sheep was investigated, including the response to serial infection with both viruses to assess whether prior infection with one virus modifies the outcome of infection with a second. Infections with LIV and TBEV in a sheep model have dramatically different outcomes, with LIV progressing to overt neurological disease associated with extensive encephalitis, whereas TBEV infection appears to be controlled at an early stage with no evidence of virus dissemination. We also report evidence for innate immune responses to LIV infection within the central nervous system (CNS) of infected sheep. However, this does not appear to control virus spread or disease progression.

2. Materials and methods

2.1. Ethics statement

All animal work was undertaken in accordance with Home Office (UK) guidelines and legislation, and was covered by the Home Office Animals (Scientific Procedures) Act 1986, project license number PPL 70/6411. Ethical approval was granted by the APHA Ethical Review Committee. Humane endpoints were identified prior to undertaking these experiments and euthanasia applied when these endpoints were reached.

2.2. Virus isolates

LIV strain LI 3/1 (designated Arb 126) was originally isolated from a sheep in Oban, Scotland and TBEV strain Neudorfl (designated Arb 131) was isolated from an *I. ricinus* tick in Austria in the early 1950s. Both virus isolates were a kind gift from Professor John Stephenson (Public Health England, formerly Centre for Applied Microbiology and Research, Porton Down, UK). The TBEV isolate originated from Dr Christian Kunz, University of Vienna, Austria, and was subsequently passaged four times in an outbred strain of mice. The LIV isolate originated from Dr Hugh Reid, Moredun Institute, Scotland, and was passaged four times in sheep and six times in an outbred strain of mice. Subcutaneous inoculation of CD-1 mice with either virus resulted in neuroinvasion within 5 days (data not shown). Both LIV strain LI3/1 and TBEV strain Neudorfl have been fully sequenced and can be obtained using GenBank Accession numbers KP144331 and U27495, respectively.

2.3. Experimental infection in sheep

For direct comparison between viruses, two groups of Poll Dorset/Poll Dorset cross male lambs ($n = 5$), aged approximately 6 months, were inoculated subcutaneously with 5×10^6 PFU of either LIV or TBEV, diluted in Eagle's minimum essential medium (E-MEM).

Additionally, two groups were inoculated in order to investigate heterologous flavivirus infection, with a lower dose of 5×10^3 PFU of LIV or TBEV administered at day 0, followed by the higher dose of 5×10^5 PFU of the heterologous virus at 11 days post-infection (DPI).

A control group ($n = 3$) received a subcutaneous inoculation of E-MEM. Clinical course was monitored, and clinical signs were classified as follows:

(−): no clinical signs observed

(+): lethargic, lying down, inappetence, pyrexia, panting, spontaneous nibbling, sensitivity to noise

(++): ataxia, profuse diarrhoea, head-tremor, body-tremor, head-shaking, dis-jointed gait, circling, louping.

(+++): paralysis, convulsions

Serial blood samples were also taken every 48–72 h, including a pre-bleed 6 days prior to inoculation. Post-mortem tissue samples were taken for analysis.

2.4. Detection of virus particles in serum

Virus was detected in serum samples by plaque assay on Vero C1008 cells using standard techniques, to determine titre as PFU/ml of sera.

2.5. Detection of virus by RT-PCR

Total RNA was extracted from serum using TRIzol® LS (Invitrogen), and from tissue using TRIzol® reagent (Invitrogen). Extracted RNA was re-suspended in nuclease-free water and treated with 0.3 units/μl DNAse using the RNeasy Mini Kit (Qiagen). cDNA was prepared through reverse transcription with Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT) (Promega) and 10X random hexamers (or 0.5 μg Oligo dT_{15} primer). Amplification was undertaken using Amplitaq Gold® DNA polymerase (Applied Biosystems), with 10 pmol of sense and antisense primers TBEV-E F1 (5′-CCTATGAGTGTGTGACCATAG-3′) and TBEV-E R1 (5′- CAGCCTCGATCACTCTG-3′).

2.6. Quantification of virus in CNS tissues

Equivalent weights of tissue samples were homogenised in tissue culture medium using a Tissue Lyser II (Qiagen). Homogenates were centrifuged for 10 min/14,000 rpm/4 °C, and 30 μl of the supernatant was titrated in Vero C1008 cells, as described above, to determine virus titre as PFU/mg tissue.

2.7. Quantification of type-1 IFN bioactivity in sheep serum

Type I interferon (IFN) was detected in sheep serum using an Mx/CAT reporter assay. This assay used the transfected cell line MDBK-t2 which encodes the enzyme chloramphenicol acetyltransferase (CAT) when serum type-1 IFN binds to the cell. Subconfluent MDBK-t2 cells were seeded in 96-well plates, at 2.5×10^4 cells/well (50 μl volume). A porcine IFN-α standard curve was prepared on each plate tested, with doubling dilutions from 2500 U/ml to 39.0625 U/ml. 50 μl of each standard was added in triplicate to each plate tested, along with a media-only negative control. Serum samples were diluted 1/2.5 in serum-free Dulbecco's Modified Eagle Medium (D-MEM), and 50 μl was added to the plate in triplicate. Plates were incubated for 24 hours at 37 °C/5% CO_2. The cell monolayer was washed with pre-cooled Dulbecco's Phosphate-Buffered Saline (DPBS). A commercial CAT ELISA (Roche) was then performed with lysed cell extracts, to

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