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Inflammatory cytokine responses in a pregnant mouse model of *Chlamydophila abortus* infection

Karen Kerr [a], Nicholas Wheelhouse [a], Morag Livingstone [a], Ian E. Anderson [a], Gary Entrican [a], Declan McKeever [b], David Longbottom [a,*]

[a] Moredun Research Institute, Pentlands Science Park, Bush Loan, Penicuik, Midlothian EH26 0PZ, UK
[b] Pathology & Infectious Diseases, The Royal Veterinary College, Hawkshead Lane, North Mymms, Hatfield, Herts AL9 7TA, UK

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ABSTRACT

Chlamydophila abortus (*C. abortus*) is the aetiological agent of ovine enzootic abortion (OEA). The highly elevated expression of the pro-inflammatory cytokine tumour necrosis factor-alpha (TNFα) and low-level expression of interferon-gamma (IFNγ) that are detected in *C. abortus*-infected placentas have been implicated in the pathogenesis of OEA. Late-term abortions similar to those occurring in sheep have also been observed in mouse models of *C. abortus* infection. Since mouse studies have contributed significantly to our understanding of the immunological responses to chlamydial infections and serve as a good model for rapidly assessing candidate vaccines for OEA, we investigated local expression of TNFα and IFNγ in infected mice. At various time points over the course of infection mice were sacrificed, serum samples obtained for serum antibody and cytokine analyses, and livers and placental tissues were removed and fixed to determine *C. abortus* colonisation and cytokine expression. Immunostaining for *C. abortus* was significantly greater in placenta compared to liver ($P < 0.001$), whereas local IFNγ expression was lower and TNFα expression was absent in the placenta compared with the liver across all time points. Serum concentrations of both IFNγ and TNFα increased throughout pregnancy in infected mice. These data suggest that a protective systemic inflammatory immune response controls maternal *C. abortus* infection but not placental/fetal infection in mice. In contrast to sheep, murine placental TNFα expression does not correlate with *C. abortus* infection, suggesting that the immunopathogenesis of chlamydial abortion differs in these species.

1. Introduction

Chlamydophila abortus is the aetiological agent of ovine enzootic abortion (OEA), the single most common infectious cause of ovine abortion in the UK (Aitken and Longbottom, 2007), and also presents a potential zoonotic risk to pregnant women causing abortion and severe life threatening complications (Buxton, 1986; Longbottom and Coulter, 2003). Although abortion occurs late in pregnancy, infection with *C. abortus* is thought to occur prior to pregnancy (Entrican et al., 2001). Current evidence suggests that a persistent infection is established in non-pregnant sheep under the control of interferon-gamma (IFNγ) (Brown et al., 2001) and at some critical point during pregnancy the infection recrudesces. Infection with *C. abortus* stimulates a strong inflammatory response in both maternal and placental tissues (Navarro et al., 2004). This coordinated response consists of a mixed inflammatory infiltrate comprising neutrophils, macrophages, lymphocytes and plasma cells (Sammin et al., 2006) and the production of the pro-inflammatory cytokine tumour necrosis factor-alpha (TNFα) (Buxton et al., 2002). Although the immune response

* Corresponding author. Tel.: +44 131 445 5111; fax: +44 131 445 6235.
E-mail address: david.longbottom@moredun.ac.uk (D. Longbottom).

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of the ewe appears to be sufficient to resolve the infection in maternal tissues due to systemic production of the Th1 cytokine IFNγ, the fetal immune response is insufficient to control infection and may contribute to the pathological damage which results in abortion (Rocchi et al., 2009).

Late-term abortions similar to those occurring in sheep have also been observed in mouse models of *C. abortus* infection (Buzoni-Gatel and Rodolakis, 1983). Such models have been widely used to study the efficacy of commercial vaccines (Rodolakis et al., 1982), for comparison of strain virulence (Anderson, 1986; Rodolakis et al., 1989) and for the evaluation of experimental vaccines (Caro et al., 2003; de Sa et al., 1995; Hechard et al., 2003a,b). Although mouse studies have contributed significantly to our understanding of the immunological responses to chlamydial infection, the mechanisms underpinning murine abortion remain poorly understood. Therefore, this study was undertaken to compare the local production of two cytokines that are linked to pregnancy failure (TNFα and IFNγ) in maternal liver and placenta during *C. abortus* infection of mice with a view to defining this model for future use in vaccine development.

2. Materials and methods

2.1. Experimental infection

All mice were treated and cared for in accordance with UK Home Office regulations, as well as local animal health and welfare policies, and had access ad libitum to food and water. Adult (56- to 60-day-old) female CBA (H-2^k; inbred) mice were obtained from Harlan Olac Ltd., Oxford, UK. Sixteen pregnant mice were infected with 10^6 inclusion-forming units (IFUs) of *C. abortus* strain S26/3 elementary bodies (EBs) in 0.2 ml of 0.1 M PBS by intraperitoneal (i.p.) injection on day 11 after mating, as previously described (Anderson, 1986; Buendia et al., 1998). Sixteen pregnant uninfected control mice were injected (i.p.) with 0.2 ml 0.1 M PBS. Following inoculation, mice were weighed and examined every day, for clinical signs, such as febrile response, starry coat and dull eyes. Mice from each group were euthanized at days 3, 5, 6 and 7 post-infection (p.i.) (or mock infection). The remaining mice (3 from each group) were allowed to reach term (or abort).

Blood samples were obtained both pre- and post-infection for antibody isotype and serum cytokine analyses. Liver, spleen, placenta and reproductive tract samples were collected into 10% formal saline for chlamydial antigen detection and into 4% paraformaldehyde for detection of cytokine mRNA expression.

2.2. Detection of chlamydial antigen in tissues

The presence of chlamydial antigen in formalin-fixed tissue sections was assessed using convalescent sheep serum and an avidin–biotin–peroxidase complex (ABC; Vector Laboratories, Peterborough, UK) signal amplification protocol, as previously described (Buxton et al., 1996). The number of positive immunoreactions (means ± SEM) for chlamydial antigen detected by immunohistochemistry (IHC) were counted in 20 fields of view (20× objective magnification) and expressed as the number of positive counts per mm^2 of tissue. Tissue sections were also screened using negative control sera obtained from sheep from EAE-free accredited flocks.

2.3. C. abortus-specific antibody ELISAs

C. abortus elementary bodies (EBs) were prepared and purified as described previously (McClenaghan et al., 1984). 96-Well microtitre plates (Greiner Bio-one GmbH, Frickenhausen, Germany) were coated overnight at 4 °C with sonicated EBs in 0.1 M sodium carbonate/bicarbonate buffer, pH 9.6. Following 3 washes with 0.1% Tween 20/phosphate-buffered saline (PBST) the plates were blocked with 10% horse serum in 5% (non-fat) dried milk/PBST for 60 min at 37 °C. After a further 3 washes, appropriate horseradish peroxidase conjugated secondary antibodies (goat anti-mouse IgG1, IgG2a or IgM; Abd-Serotec, Oxfordshire, UK) at a 1:1000 dilution, were added and incubated for 60 min at 37 °C. Bound antibody was detected using SIGMA*FAST* OPD substrate (Sigma–Aldrich) and the reaction terminated with 3 M sulphuric acid. Optical densities were determined at 492 nm using a microtitre plate reader (Labsystems iEMS Reader MF, UK).

2.4. Cytokine detection by ELISA

Serum concentrations of IFNγ and TNFα were determined by commercial ELISA (Biosource International, Invitrogen, Paisley, UK), according to manufacturer's instructions. Samples were analysed in triplicate, at a dilution of 1:100.

2.5. In situ hybridization

For the synthesis of the IFNγ riboprobe template, mouse splenocytes were stimulated with 5 μg/ml Concanavalin A (Sigma, Poole, Dorset, UK) and RNA was isolated using an RNeasy® Kit (Qiagen, Crawley, West Sussex, UK). Reverse transcription was carried out using the AB-gene Reverse iT™ First Strand Synthesis Kit (AB-gene, UK). A 468 bp fragment of the published mouse IFNγ sequence (Genbank accession number XM_125899) was amplified by PCR using standard reaction and cycle conditions and an annealing temperature of 50 °C, with 1 ng cDNA template, forward primer 5′-ATGAACGCTACACACTGCATCTTGG-3′ and reverse primer 5′-TCAGCAGCGACTCCTTTTCCG-3′. The purified IFNγ PCR product was cloned into pGEM-T® (Promega, Southampton, UK). For the TNFα probe, a pORF9 vector containing the mouse TNFα cDNA sequence was obtained commercially (Invitrogen). The TNFα gene fragment (705 bp) was excised by digestion with BspHI and NheI and cloned into NcoI/SpeI-digested pGEM-T. A smaller 566 bp SacI fragment of this clone was subcloned into SacI-digested pGEM-T.

Both sense and anti-sense RNA probes were prepared by *in vitro* transcription from the T7 and SP6 promoter incorporating digoxigenin UTP (Roche Diagnostics Ltd., UK). *In situ* hybridization was performed on 7 μm sections of paraformaldehyde-fixed, paraffin wax-embedded tissues using an Omnislide thermal cycler and wash module