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Research paper

Variant-specific and diminishing immune responses towards the highly variable MSP2(P44) outer membrane protein of *Anaplasma phagocytophilum* during persistent infection in lambs

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ABSTRACT

Anaplasma phagocytophilum is the causative agent of tick-borne fever in small ruminants and has been identified as the zoonotic agent of human granulocytic anaplasmosis. The Norwegian strains of the rickettsia are naturally persistent in lambs and represent a suitable experimental system for analyzing the mechanisms of persistence. Variation of the outer membrane protein MSP2(P44) by recombination of variable pseudogene segments into an expression site is believed to play a key role in persistence of the organism. The goal of the present study was to analyze the dynamics of the immune response towards *A. phagocytophilum* and MSP2(P44) during persistent infection of lambs. Responses to the hypervariable region of MSP2(P44) were detected shortly after appearance of the respective variants in cyclic rickettsemic peaks, consistent with a process of antigenic variation. In addition, there was a diminishing antibody response to MSP2(P44) and to other *A. phagocytophilum* antigens overall with time of infection, that was not associated with clearance of the infection.

1. Introduction

Anaplasma phagocytophilum (*Ap*) is the causative agent of tick-borne fever in small ruminants and has been verified as the zoonotic agent of human granulocytic anaplasmosis (Bakken and Dumler, 2000; Dumler et al., 2007; Stuen, 2007; Woldehiwet, 2006). The disease has been described in sheep in Norway for at least 200 years, but human cases have been diagnosed within recent years (Bakken and Dumler, 2006; Bakken et al., 1996; Stuen and Bergstrom, 2001). *Ap* is an obligate intracellular rickettsia with primary tropism for neutrophil granulocytes (Granick et al., 2008; Woldehiwet et al., 2003). It has the ability to cause persistent infections in animals that can last for several years (Dumler et al., 2003; Dumler et al., 2001). The immunodominant MSP2(P44) outer membrane protein (OMP) of *Ap* is transcribed from a single expression site within the chromosome and this expression site is orthologous to that encoding the MSP2 OMP of *Anaplasma marginale* (Barbet et al., 2003). The *msp2(p44)* expression site encodes conserved N- and C termini of MSP2(P44) and a central hypervariable region (HVR) and is similar in structure among all *Ap* strains (Barbet et al., 2006). The genome of *Ap* consists of more than 100 *msp2(p44)* paralogs (Hotopp et al., 2006; Nelson et al., 2008), many of which are truncated, but most of which contain the HVR and some conserved flanking sequence. Segments of these functional pseudogenes are inserted into the *msp2(p44)* expression site by gene combinatorial conversion mechanisms (Barbet et al., 2003). Antigenic variation in the

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msp2(p44) gene is believed to have a major responsibility for the persistence of *A. marginale* and *Ap* (Brayton et al., 2001; Granquist et al., 2008). During the infection, modulation of antigenic properties would be expected to result in an altered immune response towards the constantly changing antigen. The mechanism of variation seems to be an intrinsic property of the bacterium and is controlled by RecF mediated gene conversion, most likely produced by insertion of partial or complete donor pseudogene sequences into the expression site as described for *A. marginale* (Brayton et al., 2002; Wang et al., 2004). However, in the absence of host immune responses as occurs in vitro with HL-60 cell cultures, homogenous expression profiles of the MSP2(P44) may be observed (Sarkar et al., 2008; Wang et al., 2004). A previous study revealed sequence variation of the expressed *msp2(p44)* gene in different peaks of rickettsemia during persistent infection in lambs (Granquist et al., 2008). The goal of the present study was to analyze the immune response towards *Ap* and MSP2(P44) during the persistent infection of lambs. We aimed to determine whether different MSP2(P44) variants were recognized at different times post-infection, as would be predicted by the prior genetic studies (Granquist et al., 2008). We also investigated how the rapidly changing OMPs might affect the overall dynamics of antibody responses to *Ap* during the different peaks of rickettsemia using different immunodiagnostic methods that have been proposed for detection of this organism in different animal species.

2. Materials and methods

2.1. Experimental infection of naive lambs and blood sampling

Four lambs were raised in an indoor environment, with barriers against tick entry and tick infestation. Two lambs (4203 and 4210) referred to as #1 and #2 were experimentally infected by intravenous injection of the Norwegian 16S rRNA variant 1 (GenBank no M73220) of *Ap* at an infection dose of approximately 2.0×10^6 infected neutrophils. The inoculum had been stored in 10% DMSO at −80 °C. Two lambs were kept as negative controls throughout the sampling period of three months. All four lambs were examined and found negative for *Mycoplasma ovis* (formerly *Eperythrozoon ovis*) by blood smear analysis before inoculation. The rectal temperature and clinical status was recorded daily throughout the experimental period. EDTA blood samples were collected from the jugular vein. Samples were taken on day 0 of the infection and every second day for a three-month period. One EDTA blood sample from each animal was used for blood smear analysis and differential blood cell counts (ADVIA, Bayer). The other was frozen at −75 °C for later PCR analysis. The blood smears were stained with May Grünwald Giemsa for visual confirmation and quantitative assessment of infection using oil immersion microscopy (Stuen et al., 2003). Serum samples were collected on the day of infection and then weekly during the entire experimental period. The serum was separated from the cell fraction and frozen at −20 °C for indirect fluorescent-antibody assay, ELISA and immunoblotting. The experiment was thoroughly planned and approved by the competent person at the animal experiment unit belonging to the Norwegian School of Veterinary Science, Section for Small Ruminant Research. The competent person and the facilities were approved by the National Animal Research Authority (Norway).

2.2. Real-time PCR for relative quantification and identification of positive samples, targeting the msp2(p44) gene

DNA from EDTA blood was isolated using a QIAamp Mini kit™ (QIAGEN). Detection and selection of samples positive by PCR for further cloning and restriction fragment length polymorphism pattern (RFLP) analyzes was performed by real-time PCR (Lightcycler 2.0, Roche), as described previously (Granquist et al., 2008).

2.3. Cloning and analysis of 2 kb msp2/p44 expression site using the TOPO™ plasmid vector

DNA was isolated using a QIAamp DNA Blood Mini Kit ™, as described by the manufacturer. A 2 kb sequence of the *msp2(p44)* expression site of *Ap* was amplified and cloned that included the central hypervariable region flanked by semiconserved and conserved 5′ and 3′ ends. The primers were (AB1221) 5′-ATA GAA CAA GAG CAG GGA GAA GAC-3′ and (AB1227) 5′-TCT GTC TTG GAG AGT ATT GAG TC-3′ diluted to a concentration of 4 μM as previously described (Granquist et al., 2008). The 2 kb cloned PCR products were analyzed by single digestion with EcoRI to release inserted DNA and determine the presence and size of cloned fragments and by double digestions with EcoRI and RsaI to generate diagnostic RFLP patterns from each clone. Clones from each rickettsemic peak were selected for DNA sequencing based on the RFLP patterns obtained (Granquist et al., 2008).

2.4. Immunoblots

Gel electrophoresis used a 15% SDS-PAGE separating gel with a 4% stacking gel. The antigen was RF6A endothelial cells infected with the NY-18 strain of *Ap* and solubilized in SDS gel sample buffer by heating at 100 °C for 5 min. The electrophoretic separation was achieved with a Bio-Rad PowerPac 3000 at 300 V for 43 min. The transfer was done overnight (15 h) at 30 V and increased to 70 V in the morning (2 h). The membranes were cut into 17 strips and 2.5 ml of serum (1:1000 dilution) was added to each strip. Pre-serum from dogs were applied to strip 1 as a negative control while dog post-infection serum was applied to strip 2 as a positive control. Pre-serum from lamb #1 was added to strip 3. Strips 4–17 were incubated with post-peak sera collected at intervals after the peaks of rickettsemia from lamb #1. The same incubations were repeated for lamb #2. The sera were allowed to bind to antigens for 1 h, then strips were washed three times before secondary antibodies (goat anti-dog and rabbit-anti-sheep) were applied to strips at a dilution of 1:150,000 (Kirkegaard and Perry Laboratories). The secondary antibodies were allowed to bind for 90 min, then