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Research paper

VapA-specific IgG and IgG subclasses responses after natural infection and experimental challenge of foals with *Rhodococcus equi*

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ABSTRACT

Rhodococcus equi is a common cause of pneumonia in young foals worldwide and has considerable economic effects on the global equine industry. Despite ongoing efforts, no vaccine is currently available to prevent rhodococaal pneumonia. This is due, in part, to an incomplete understanding of the protective immune response to this bacterium. While antibodies to VapA, a lipoprotein produced by virulent *R. equi*, are useful in differentiating antibody production in response to pathogenic *versus* non-pathogenic strains, the significance of the humoral response of foals to this lipoprotein remains poorly defined. The objectives of this study were to evaluate changes in VapA-specific IgG and IgG subclasses after exposure and infection of neonatal foals. Experimental foals included those challenged with *R. equi* at 1 ($n=18$), 2 ($n=4$) and 3 ($n=6$) weeks of age. Confirmed naturally infected ($n=7$) and not infected ($n=3$) foals were also included. All foals were bled 24 h after birth and weekly thereafter for a period of 8 weeks. Antibody changes over time were evaluated.

Following birth, VapA-specific IgGs significantly ($p<0.05$) decreased over time in all foals as a result of normal decay of passively transferred antibodies. Both VapA-specific IgGa and IgG(T) significantly increased ($p<0.05$) after experimental challenge, however, the rise in IgG(T) occurred earlier. Only a significant ($p<0.05$) increase in VapA-specific IgG(T) over time was seen after natural infection. Whether VapA-specific IgG(T) can be used to differentiate rhodococcal from other pneumonias requires further investigation under field conditions.

1. Introduction

Rhodococcus equi is an important pathogen worldwide that causes severe pneumonia and a variety of extrapulmonary lesions in young foals (Reuss et al., 2009; Zink et al., 1986). In contrast, adult horses are resistant to *R. equi* infection unless they are immunocompromised (Freestone et al., 1987). Nevertheless, little is known about the unique susceptibility of foals to this infection. Protective immunity to *R. equi* likely involves innate, humoral and cell-mediated immune responses (Cauchard et al., 2004; Darrah et al., 2004; Dawson et al., 2010). Antibodies, especially IgG, are important because of their opsonic activity (Hietala and Ardans, 1987; Hooper-McGrevy et al., 2001, 2003; Martens et al., 1989) since entry of *R. equi* into macrophages mediated by Fc receptors leads to enhanced bacterial killing (Hondalus et al., 1993; Hooper-McGrevy

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et al., 2001; Martens et al., 1987, 1989). Immunoglobulin G against *R. equi* increased over time in foals beginning at 3–5 weeks of age as a result of natural exposure (Prescott et al., 1996; Takai et al., 1986). Oral and intratracheal inoculation of foals also resulted in an increase in IgG (Takai et al., 1987). None of these studies evaluated IgG subclasses of which there are multiple in the horse with differing abilities to activate complement and stimulate a respiratory burst from peripheral blood leucocytes (Lewis et al., 2008). To date, little is known about the protective role these IgG subclasses play in *R. equi* infection. Previous studies also failed to discount antibody responses to non-virulent *R. equi*, subsequent assays measuring IgG to VapA, a surface lipoprotein of *R. equi* present in virulent strains (Jain et al., 2003; Takai et al., 1991), allowed differentiation between virulent and non-virulent bacteria. Nevertheless, there remains uncertainty regarding the significance of the antibody response to *R. equi*. Likewise, the role of IgG subclasses is also poorly defined. Thus, in adult horses experimentally challenged with *R. equi* various subclass responses have been reported including an increase in VapA-specific IgGa (Hooper-McGrevy et al., 2003), IgGb (Jacks et al., 2007) or both IgGa and IgGb (Lopez et al., 2002). In intrabronchially infected foals there was an increase in VapA-specific IgGa and IgGb (Jacks et al., 2007). By contrast, foals with naturally occurring rhodococcal pneumonia produced significant amounts of VapA-specific IgG(T) (Hooper-McGrevy et al., 2003). Also, a VapA-specific IgG(T) response was not seen after natural exposure of adult horses or healthy foals (Hooper-McGrevy et al., 2003). However, VapA-specific IgG(T) was significantly higher when a larger *R. equi* inoculum was used to challenge neonatal foals (Jacks and Giguere, 2010). Recently, VapA-specific IgG(T) was shown to be a good indicator of rhodococcal pneumonia in a challenge of neonatal foals using a low dose of *R. equi* which more accurately resembled natural infections (Sanz et al., 2013, 2015). While early *R. equi* studies indicated IgG(T) could not activate complement and therefore was an undesired response to infection (Hooper-McGrevy et al., 2003), it has since been shown to be the most potent complement activator in horses (Lewis et al., 2008). Furthermore, oral immunization with virulent *R. equi* that successfully protected foals was associated with increased Vap-specific IgG(T) (Hooper-McGrevy et al., 2005).

It is clear from the studies described above that further research is needed to clarify the role of IgG and IgG subclasses in the response of foals to *R. equi*. Also, an antibody response which differentiates *R. equi* exposure from infection is essential for the development of better preventive and diagnostic methods. Therefore, the objectives of this study were to evaluate changes in VapA-specific IgG and IgG subclasses after experimental challenge of neonatal foals, after natural exposure and after natural infection.

2. Materials and methods

2.1. VapA purification

Recombinant VapA protein was produced and purified using an *Escherichia coli* strain that contained a VapA plasmid fused to glutathione-S-transferase, as previously described (Hooper-McGrevy et al., 2001). The purity of the protein was assessed by sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).[1]

2.2. Source of reference control sera

Positive and negative control sera were included in each plate. A mare previously vaccinated with killed VapA$^+$ *R. equi* was used as the positive control. Negative control serum included fetal equine serum (FES) and serum obtained from a 4-month-old foal whose necropsy and lung tissue culture results were negative for *R. equi*. Commercially available *R. equi* specific hyperimmune plasmas were used to construct standard curves. The standard curves for VapA-specific IgG, IgGa and IgGb were constructed using one product (ReSolution)[2] while a different one (EquiplasREA)[3] was used for VapA-specific IgG(T). All reference sera were divided into 100 μl aliquots and stored at −20 °C until used.

2.3. VapA-specific enzyme-linked immunosorbent assay development

The ELISA for VapA-specific IgG was based on previously described methods (Hooper-McGrevy et al., 2003; Prescott et al., 1996) and slightly modified. The VapA, serum and conjugates were titrated to achieve the optimal concentration using checkerboard titrations (data not shown). Briefly, 96-well microplates[4] were coated with recombinant VapA (0.5 μg/well) in carbonate buffer[e] and allowed to incubate overnight at 4 °C. Afterwards, a mixture of blocking buffer (polyvinyl alcohol [Mowiol 6–98][5] 1% [w/v] in distilled water) and phosphate-buffered (PBS, to a final concentration of 1:1 [v/v]) was added. Serum was diluted (1:100) in PBS with 0.05% Tween-202 (PBST) and added to duplicate wells and incubated at 37 °C for 1 h. Either goat anti-horse IgG conjugated with horseradish peroxidase (HRP)[6] (1:10,000) or murine anti-IgGa (1:2, CVS48) anti-IgGb (1:5, CVS39) or anti-IgG(T) (1:2, CVS40) (all hybridomas provided by P. Lunn, North Carolina State University) were then added and incubated for 1 h at 37 °C. The subclass plates were washed and goat anti-mouse IgG-HRP[7] (1:2000) was added for an additional hour at 37 °C. Plates were washed between each step with PBST using an ELISA plate washer (MW 96/384)[8] The substrate (3,3′,5,5′-tetra-methylbenzidine, peroxidase substrate[9]) was then added for 5 min. The reaction was stopped using stop solution[9]. Absorbance was read at 450 nm using an ELISA plate reader[1]. Results from the test sera were converted to ELISA units (EU) utilizing a logarithmic trend line from the

[1] Bio-Rad, Philadelphia, PA, USA.
[2] MgBiologics, Ames, IO, USA.
[3] Plasvacc USA Inc, Templeton, CA, USA.
[4] Thermo Scientific, Rochester, NY, USA.
[5] Sigma Aldrich, St. Louis, MO, USA.
[6] Jackson ImmunoResearch, West Grove, PA, USA.
[7] Bethyl Laboratories, Inc, Montgomery, TX, USA.
[8] Beckman Coulter, Brea, CA, USA.
[9] KPL, Gaithersburg, MD, USA.