INNATE IMMUNITY TO *RHODOCOCCUS EQUI*: THE RESPONSE OF ADULT AND JUVENILE EQUINE NEUTROPHILS

A Dissertation

by

JESSICA RACHEL NERREN

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

August 2007

Major Subject: Veterinary Microbiology

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Approved by:

Co-Chairs of Committee,	Noah Cohen
	Susan Payne
Committee Members,	James Derr
	David McMurray
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ABSTRACT

Innate Immunity to *Rhodococcus equi:* The Response of Adult and Juvenile Equine Neutrophils. (August 2007)

Jessica Rachel Nerren, B.S., Texas A&M University

Co-Chairs of Advisory Committee: Dr. Noah Cohen
Dr. Susan Payne

Blood was obtained from 5 adult horses and 16 juvenile horses (foals) at the time of birth and subsequently at 2-, 4-, and 8-weeks of age. Neutrophils from adult horses were purified and incubated for 2 h and 4 h with media, avirulent *R. equi*, virulent *R. equi*, or recombinant-human granulocyte-macrophage colony stimulating factor (rhGM-CSF). Neutrophils from foals were purified and incubated for 2 h and 4 h with media or virulent *R. equi*. Total RNA was extracted from both adult and foal neutrophils immediately after purification to measure baseline expression levels (0 h), and immediately after each of the prescribed incubation times. For each sample, 1 μg of total RNA was reverse-transcribed and analyzed for differential gene expression using real-time PCR. After 2 h and 4 h incubation with virulent or avirulent *R. equi*, neutrophils from adult horses expressed significantly ($P< 0.05$) greater TNFα, IL-12p40, IL-6, IL-8, and IL-23p19 mRNA relative to expression by unstimulated neutrophils, but not IFNγ or IL-12p35 mRNA. Furthermore, virulent *R. equi* induced significantly greater IL-23p19 mRNA expression than avirulent *R. equi*. Stimulation with rhGM-CSF of adult equine neutrophils failed to induce significant changes in cytokine expression. In foal

neutrophils, stimulation with virulent *R. equi* induced significantly greater expression of IFNγ, TNFα, IL-6, IL-8, IL-12p40, and IL-12p35 mRNA relative to expression by unstimulated neutrophils. Furthermore, there were significant effects of age on expression of IL-6, IL-8 and IL-12p40 mRNA. Neutrophil mRNA expression of IL-6 and IL-8 in newborn foals was significantly greater than expression at 2-, 4-, and 8-weeks of age. There was no significant difference between unstimulated and *R. equi*-stimulated neutrophils from newborn and 2-week-old foals in expression of IL-12p40; however, expression of IL-12p40 by *R. equi*-stimulated neutrophils from 4- and 8-week-old foals was significantly greater than expression by unstimulated neutrophils. These results demonstrate that *R. equi*-stimulated neutrophils are a source of many pro-inflammatory cytokines, and that the magnitude of this expression with respect to IL-6, IL-8, and IL-12p40 mRNA expression was influenced by age. Collectively, the data presented indicate a non-phagocytic role for neutrophils that may influence the type of adaptive immune response to *R. equi*.

DEDICATION

To my biggest fan, Mimi.

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INTRODUCTION

BACKGROUND

Pneumonia is a serious health problem in foals, and pneumonia caused by *Rhodococcus equi* is the most common and severe form of pneumonia in foals less than 6 months of age (Cohen and Martens, 2007). *Rhodococcus equi* is also considered an emerging opportunistic pathogen of immunosuppressed people, particularly AIDS patients (Meijer and Prescott, 2004). *Rhodococcus equi* was originally isolated from a Swedish foal in 1923 and classified as *Corynebacterium equi* (Magnusson, 1923). *Rhodococcus equi* is described as a nocardioform actinomycete, shares many characteristics with members of the genera *Mycobacterium*, *Corynebacterium*, and *Nocardia* (McNeal and Brown, 1994), and is a Gram-positive, aerobic, catalase-positive bacterium that is characterized by a rod-to-coccus life cycle (Prescott, 1991).

The impact of *R. equi* pneumonia on the equine industry is large. In the United States, respiratory disease ranked as the third most common cause of disease in foals less than 6 months of age (Anonymous, 2006). Although subclinical and acute forms of *R. equi* pneumonia have been recognized in foals, the disease most commonly occurs as a chronic bronchopneumonia with an insidious onset that makes early detection difficult. In many cases, by the time a diagnosis has been made the characteristic pyogranulomatous lesions in the lungs are well-established and substantial tissue damage has occurred.

This dissertation follows the style of Veterinary Immunology and Immunopathology.

Thus, treatment for infection with *R. equi* is generally prolonged, expensive, associated with adverse effects, and not always successful (Stratton-Phelps et al., 2000; Cohen and Martens, 2007). As a consequence, *R. equi* pneumonia has a high case fatality rate (Chaffin et al., 2003a), and foals that do survive the disease are less likely than age-matched cohorts to race (Ainsworth et al., 1998). Finally, horse-breeding farms with endemic *R. equi* may suffer a loss in clientele. For these reasons, it would be helpful to have some effective methods for preventing the disease.

To date, the only method shown to prevent the disease, or reduce its severity, is the administration of hyperimmune plasma to foals (Martens et al., 1989a; Higuchi et al., 1999; Caston et al., 2006). This procedure is controversial, as it carries some risk to the foal, and is expensive and labor-intensive (Cohen and Martens, 2007). Moreover, several field studies have shown that the procedure is not always effective, as no significant differences in the occurrence of disease were observed between foals that received hyperimmune plasma and those that did not (Hurley and Begg, 1995; Giguere et al., 2002). Numerous studies have investigated potential vaccine candidates, but have been largely unsuccessful. Because clinical, epidemiologic, and experimental observations suggest that foals with spontaneous disease are likely infected very early in life (Horowitz et al., 2001) and that foals less than 2 weeks of age are more susceptible to experimental infection (Martens et al., 1989b), traditional vaccines that rely on the development of an adaptive immune response are unlikely to be successful. In order to develop better methods for preventing and controlling *R. equi* pneumonia, more needs to be known about the pathogenesis and virulence of the organism.

PATHOGENESIS AND VIRULENCE

The basis of *R. equi* pathogenicity is its ability not only to survive, but to replicate in macrophages. The exact mechanisms by which *R. equi* does this have been the topic of debate in several published studies. Early studies conducted in foal alveolar macrophages demonstrated that *R. equi* replicated inside the phagosome by preventing phagolysosomal fusion (Hietala and Ardans, 1987a; Zink et al., 1987). Those findings were recently confirmed by a study conducted in murine macrophages, which demonstrated that intracellular vacuoles (i.e., phagosomes) containing *R. equi* were arrested sometime between the early and late stages of phagosomal maturation to a phagolysosome (Fernandez-Mora et al., 2005). In contrast, another recent study demonstrated that phagolysosomal fusion did in fact occur inside *R. equi*-infected murine macrophages, and that the bacterium was able to replicate inside of the phagolysosome by preventing its acidification (Toyooka et al., 2005). Interestingly, Hietala and Ardans found that opsonization of the bacterium with serum containing *R. equi*-specific antibody was associated with increased phagolysosomal fusion and bacterial killing (Hietala and Ardans, 1987b).

Antibody receptor-mediated phagocytosis is associated with an increase in the oxidative burst, an effective antimicrobial mechanism employed by host phagocytic cells, whereas complement receptor-mediated phagocytosis is not (Aderem and Underhill, 1999). In the absence of antibody, binding and phagocytosis of *R. equi* by macrophages is dependent on the complement receptor type 3 known as Mac-I (Hondalus et al., 1993), and it is thought that this mode of entry into the cell may play a

role in establishing intracellular infection by allowing *R. equi* to avoid the oxidative burst (Hondalus et al., 1993; Meijer and Prescott, 2004). It is important to note that the aforementioned mechanisms of pathogenesis are characteristics of virulent strains of *R. equi*, as avirulent strains of *R. equi* are unable to survive and replicate inside macrophages (Hondalus and Mosser, 1994; Giguere et al., 1999a).

In foals, virulence of *R. equi* is strongly associated with the presence of an 80- to 90-kb plasmid (Takai et al., 1993), and plasmid-cured strains of *R. equi* lose their ability to survive and proliferate in macrophages (Hondalus and Mosser, 1994; Giguere et al., 1999a). The cytotoxic effects of *R. equi* appear to occur through necrosis rather than apoptosis, and these effects are significantly greater in strains of *R. equi* that contain the virulence plasmid (Luhrmann et al., 2004). Recently, sequencing of the plasmid revealed that it contains a 27.5 kb region that bears the hallmarks of a pathogenicity island (Takai et al., 2003). The G+C content of the pathogenicity island is significantly less than the rest of the plasmid, and it is flanked by transposon resolvase genes. Furthermore, this region contains a cluster of virulence-associated genes designated *vapA, C, D, E, F, G,* and *H* (Takai et al., 2003). With the exception of *vapF*, all of these genes appear to encode functional, extracellular proteins; however, the only 1 known to be required for virulence is *vapA*, which encodes and mediates the expression of the virulence-associated protein A (VapA), a 15- to 17-kDa virulence-associated protein (Takai et al., 1991, 1992, 1993). VapA is located on the surface of the cell wall (Takai et al., 1991; Jordan et al., 2003) and its expression is up regulated in response to temperatures ≥ 34°C (Takai et al., 1992, 1996a), pH=5 (Takai et al., 1996a; Benoit et al., 2001), treatment

with hydrogen peroxide (Benoit et al., 2002) and low concentrations of iron (Jordan et al., 2003; Ren and Prescott, 2003), all of which are conditions similar to the environment inside macrophages.

Aside from the *vap* genes, most of the 18 remaining open reading frames (ORFs) on the pathogenicity island share no sequence homology with genes of known function. Consequently the function and role that these genes play in virulence of *R. equi* remains unknown (Meijer and Prescott, 2004). There is substantial evidence, however, supporting a role for VapA in virulence of *R. equi*. Deletion of the *vapA* gene attenuates virulence of this bacterium in mice (Jain et al., 2003). Serum from virtually all foals infected with *R. equi* pneumonia contains high levels of antibodies against VapA (Takai et al., 1991), and the protein is uniformly expressed by isolates of *R. equi* recovered from pneumonic foals. Interestingly, VapA is not uniformly expressed by *R. equi* isolates obtained from other sources, such as humans and other animal species (Hondalus, 1997; Takai, 1997). In 1996, Takai et al. (1996b) reported the identification of *vapB*, an isogene of *vapA* with about 85% sequence homology (Byrne et al., 2001). Expression of the VapB protein was originally observed in *R. equi* isolates obtained from humans (Takai et al., 1995), and was later observed in *R. equi* isolates obtained from the lymph node of a pig (Takai et al., 1996b). Based on murine challenge studies, *R. equi* isolates with the *vapB* genotype are classified as being intermediate in virulence (Takai et al., 1995, 2000). The clinical relevance of *vapB* isolates in foals remains unknown, as *R. equi* isolates expressing the *vapB* genotype have never been isolated from infected foals, and foals experimentally infected with *vapB* strains do not develop disease (Takai et al.,

2000). Several studies, however, suggest that *vapB* may play a role in human infections, as 5 out of 7 isolates in 1 report (Makrai et al., 2002) and 4 out of 6 isolates in another (Takai et al., 2002) tested positive for *vapB*.

Aside from the *vapA* gene, the only other known virulence factor of *R. equi* is a chromosomal gene, *aceA*, which encodes the functional enzyme isocitrate lyase (Kelly et al., 2002). Isocitrate lyase is the first enzyme of the glyoxylate shunt, which is important in fatty-acid metabolism of many bacterial pathogens following infection. Wall et al. (2005) demonstrated that an isocitrate lyase-deficient *R. equi* mutant was unable to replicate in murine macrophages, but that introduction of the intact *aceA* gene completely reversed the attenuated phenotype. Furthermore, virulence of this mutant was completely attenuated in experimentally infected foals and mice (Wall et al., 2005).

EPIDEMIOLOGY

In addition to a better understanding of virulence factors and mechanisms of *R. equi* pathogenesis, further knowledge about the epidemiology of *R. equi* will lead to improved strategies for prevention and control of the disease. In studying infectious disease, it is important to consider the triad of host, agent, and environment, and their interactions. Disease caused by *R. equi* is endemic at some farms, and occurs sporadically at other farms. On some farms the disease is absent (Prescott, 1991). DNA fingerprinting methods have been used to compare isolates of *R. equi* from different environmental and clinical sources in order to determine if a particular strain could be attributed to the differences in the occurrence of disease at farms, but epidemiologic studies have demonstrated significant heterogeneity among isolates. In 1999, Takai et al.

used restriction-fragment length polymorphism (RFLP) analysis to compare the banding patterns of virulence plasmids from 2 reference strains to the banding patterns of virulence plasmids obtained from lung lesions of infected foals, feces of healthy foals, and soil from horse breeding farms. Five countries were represented by the repertoire of isolates. The results of that study showed that *R. equi* could be divided into 5 closely related types based on the size of the virulence plasmid, and that there were differences in the geographic distribution of these isolates (Takai et al., 1999). In a similar study, 462 *R. equi* isolates from the soil at 5 endemic farms in Texas and 100 isolates from infected foals at those farms were analyzed using RFLP (Takai et al., 2001). Of the 462 soil isolates, 87 were virulent. Furthermore, 4 variations of the original 85-kb plasmid were found among these isolates. Similarly, the same 4 plasmid variants were found among the 96 foal isolates that were virulent. Of the 4 variants observed in that study, 2 had not previously been described (Takai et al., 2001). Shortly thereafter, another plasmid variant was identified in Korea (Takai et al., 2003). The findings of those 2 studies indicated that RFLP analysis of virulence plasmids might be a useful tool to assess the molecular epidemiologic distribution of *R. equi,* but because RFLP is not highly discrepate it would likely not be valuable for identifying similarities between *R. equi* isolates from a given region (Cohen and Martens, 2007). In another molecular epidemiologic study by Cohen et al. (2003), the banding patterns of chromosomal DNA were analyzed by pulsed-field gel electrophoresis (PFGE). Analysis was performed on 218 virulent *R. equi* isolates obtained from infected foals, and 72 avirulent isolates obtained from feces, soil, and respiratory tract samples. These isolates were collected

from 4 different countries over a 15-year period. The findings of that study demonstrated that isolates obtained from a farm were rarely the same strain, and that overall there was less than 80% similarity among isolates of *R. equi*. In Australia, it was reported that 44 different strains were identified by PFGE analysis of 209 virulent isolates of *R. equi* from infected foals (Morton et al., 2001). Recently a combination of RFLP and PFGE analysis of 83 virulent *R. equi* isolates obtained from foals and soil on an endemic farm in Germany revealed 3 major and 3 minor banding patterns at the same farm (Venner et al., 2007). The significant heterogeneity among *R. equi* isolates tested in these studies suggests that the occurrence of disease cannot be attributed to a particular strain of *R. equi*. Collectively, these studies indicate that with respect to agent factors, the only known requirement for disease is the presence of the virulence plasmid.

Environmental factors and their contributions to the occurrence of disease have been studied extensively. *R. equi* is a soil saprophyte with widespread distribution (Prescott, 1991). The development of a selective NANAT medium in 1979 enabled investigators to isolate *R. equi* from highly contaminated specimens, such as soil and feces (Woolcock et al., 1979). Since that time a number of studies have shown that horse feces and soil are important sources of *R. equi*. Two separate studies demonstrated that *R. equi* was present in the feces of foals during the first week of life, and that the greatest concentrations of *R. equi* were found in these foals during the first 2 months of life (Takai et al., 1986a and 1896b). Furthermore, in an epidemiologic study of foals on 3 farms, *R. equi* was isolated from 100% of fecal samples taken from 4-week old foals (Takai et al., 1987). Infected foals appear to be a major source of *R. equi*, as they shed

between 10^6- 10^8 colony-forming units (CFUs) g^{-1} of feces (Takai, 1997). A recent study conducted on an endemic breeding farm showed that dams of affected foals did not shed significantly greater numbers of virulent *R. equi* in their feces than dams of unaffected foals; however, 100% of the dams shed virulent *R. equi* in their feces at least once during the sampling period, indicating that dams may be an important source of virulent *R. equi* to their foals (Grimm et al., 2007). In 1984, Barton and Hughes demonstrated that the numbers of *R. equi* in horse feces increased 10,000-fold between 1 and 2 weeks after being freshly deposited, implicating horse feces as a rich growth medium for the organism. Thus, it is likely that horse feces are a major source of *R. equi* to the soil environment. Numerous studies have investigated the prevalence of *R. equi* in the soil at horse-breeding farms. One study showed that *R. equi* was recovered from the soil of 18 out of the 19 breeding farms sampled (Barton and Hughes, 1984). Quantitative culture of *R. equi* in soil samples collected in monthly intervals from March through December on a horse-breeding farm in Japan showed that the mean number of CFUs g^{-1} of soil decreased proportionally with the depth of the soil sample, and that surface concentrations of *R. equi* were 100 times greater than concentrations at a depth of 30 cm (Takai et al., 1986a). That study also showed that the mean concentration of *R. equi* in the soil increased significantly from 0 to 100 CFUs g^{-1} in early spring (February-March), to 10, 000 CFUs g^{-1} in late spring (April-May). Interestingly, this time of year coincides with the peak of foaling season. It has been suggested that frequency of disease is associated with the presence and concentration of virulent *R. equi* in the soil (Takai, 1997). A recently published study demonstrated that there was no significant association

between the presence and concentration of virulent *R. equi* in the soil at affected farms and the occurrence of disease; however, there was a significant association between the prevalence and concentration of airborne virulent *R. equi* and the occurrence of disease (Muscatello et al., 2006).

In addition to the aforementioned studies, studies designed to identify farm characteristics and management practices associated with the incidence of disease caused by *R. equi* have been conducted recently. The first of a 2-part prospective study conducted by researchers in Texas examined farm-based characteristics associated with the occurrence of *R. equi* pneumonia (Chaffin et al., 2003a). That study of horse breeding farms from 33 counties in Texas reported that, for the year the study was conducted, the median percentage of foals on affected farms that developed disease was 7%, and ranged from 1% to 100%; the median case fatality rate that year was 25%, and ranged from 0% to 100%. That study also showed that larger farms (i.e., > 90 acres) with a greater number and density of foals (i.e., farms with > 17 foals and > 0.25 foals acre^{-1}), and farms with greater numbers of transient dam-foal pairs were significantly associated with increased risk of *R. equi* pneumonia. The second part of the study examined management and preventative health practices associated with the occurrence of *R. equi* pneumonia (Chaffin et al., 2003b). No significant difference was observed between affected and unaffected farms as to whether mares delivered foals on the premises. Similarly, there were no significant differences between affected and unaffected farms as to where on a farm foals were born (i.e., in stalls, paddocks, or pastures) or the type of bedding used in foaling stalls. Interestingly, that study did find a significant association

between affected and unaffected farms in preventative health care: affected farms were more likely to test newborn foals for failure of passive transfer and to administer plasma IV to foals with inadequate serum IgG concentrations. Affected farms also were more likely to administer *R. equi* hyperimmune plasma to foals, to deworm dams every 12 weeks or less, to rotate anthelmintics in order to prevent parasite resistance, and to vaccinate dams and foals against *Streptococcus equi*. The aforementioned preventive health care practices are generally deemed to be desirable for preventing infectious diseases. Although their association was not deemed to be causal (i.e., use of these practices was not considered likely to cause *R. equi* pneumonia), these findings do indicate that such practices are inadequate at preventing the occurrence of *R. equi* foal pneumonia. In addition to the findings from Texas horse-breeding farms, similar findings were reported for a survey of 138 horse-breeding farms across the United States; in the farms surveyed nationwide (including Texas) the incidence of disease was 13% and the case fatality was 8.0% (Cohen et al., 2005). Collectively, the overall conclusion of these prospective studies was that lack of preventative health care at farms is not associated with an increased risk of foals developing *R. equi*.

Based on the observations made from epidemiologic-based studies of genotype, environmental distribution, farm-based characteristics, and preventative health-care practices, several conclusions about the epidemiology of *R. equi* pneumonia can be drawn: exposure to the organism is widespread; the only commonality with respect to virulence among isolates of *R. equi* obtained from clinical and environmental sources is the presence of the virulence plasmid; the occurrence of the disease at a given farm is

not attributable to a particular strain of *R. equi*; and, the occurrence of disease does not appear to be attributable to poor management practices or lack of preventative health-care. Taken together, these data suggest that host factors are critical in the outcome of infection with *R. equi*.

HOST IMMUNITY

With respect to host factors, the ontogeny of the immune response of foals is of key interest. Disease caused by *R. equi* is absent in horses > 6 months of age, and in humans and other species infection is generally associated with compromised immunity (Takai, 1997). These observations suggest a deficiency or delay in maturation of foal immunity that predisposes them to infectious disease. Indeed, there is precedence for this in other species, as immunity in neonates is known to be naïve and diminished (Morein et al., 2002). Despite immunological evidence of widespread exposure to *R. equi* (Hietala and Ardans, 1985), the incidence of disease at endemic farms is relatively low (Chaffin et al., 2003a; Cohen et al., 2005). This suggests that foals are not uniformly susceptible to infection with *R. equi*, and it is possible that susceptibility to infection is determined by differences in immune function among foals. Numerous studies, therefore, have focused on the role of immunity to *R. equi*. Although there is some evidence that humoral immunity may play a role in the outcome of infection with *R. equi*, inconsistent observations among studies have made it difficult to determine a definitive role. The findings of Hietala and Ardans (1987a) that phagocytosis and killing of *R. equi in vitro* by equine macrophages was enhanced when the bacterium was opsonized with immune serum support the hypothesis that *R. equi*-specific antibody may

block the early stages of infection by altering the mode of entry into macrophages and reducing the inhibition of phagosome-lysosome fusion (Hines et al., 1997). Parenteral administration of hyperimmune plasma to foals prior to disease can prevent or reduce the severity of experimentally-induced (Martens et al., 1989a; Caston et al., 2006) and spontaneous (Mueller and Madigan, 1992) foal pneumonia caused by *R. equi*, but the mechanisms responsible for this protection are unclear. Passive transfer of antibodies from vaccinated mares to foals through ingestion of colostrum failed to provide protection from *R. equi* (Martens et al., 1991; Mueller and Madigan, 1992), which suggests that components other than antibodies may be responsible for the protection provided by transfusion of hyperimmune plasma. In contrast, a recent study reported that administration of purified antibodies against VapA and VapC to foals 1 day prior to experimental infection with *R. equi* resulted in protection levels comparable to those observed in foals given hyperimmune plasma, whereas foals given saline developed lethal disease (Hooper-McGrevy et al., 2003a). Another study aimed at elucidating the role of humoral immunity against *R. equi* pneumonia demonstrated that foals with *R. equi* pneumonia had greater IgGb and IgGT isotype responses than healthy foals and adult horses, which had greater IgGa isotype responses (Hooper-McGrevy et al., 2003b). Based on those data, the authors concluded that foals with greater IgGb and IgGT responses were skewed toward a T helper-type 2 (Th2) response, whereas foals with greater IgGa responses were skewed toward a T helper-type 1 (Th1) response. In contrast, the same group recently reported that oral immunization of foals with virulent *R. equi* at 2, 7, and 21 days of age induced an IgGT isotype response (compared to non-

immunized foals) and provided complete protection against experimental infection at 21 days of age (Hooper-McGrevy et al., 2005).

Although experimental and epidemiologic evidence supports a role for humoral immunity in the outcome of infection with *R. equi*, immunity to intracellular infections is typically cell-mediated, and an overwhelming amount of experimental and observational evidence suggests that cell-mediated immunity plays the dominant role in protection against *R. equi*. Much of the role for cell-mediated immunity to *R. equi* has been elucidated through experiments performed in mice. In the earliest of such experiments, it was demonstrated that both $CD4^+$ and $CD8^+$ T cells were critical for resistance to *R. equi* by administration of anti-CD4 and/or anti-CD8 antibodies to mice prior to and following experimental infection (Nordmann et al., 1992). Mice receiving the antibodies had greater concentrations of *R. equi* in their tissues than control mice. That study also demonstrated that adoptive transfer of resistance by administration of immune serum from vaccinated mice to naïve mice failed, whereas adoptive transfer of resistance by spleen cells from immunized mice to naïve mice was successful. In another study, the role of T cell subsets in resistance to *R. equi* was further elucidated using transgenic mice deficient in either $CD8^+$ T cells or $CD4^+$ T cells (Kanaly et al., 1993). The authors reported that CD8-deficient mice were unable to clear pulmonary infection 3 days after experimental infection with virulent *R. equi*, but were able to overcome infection after 21 days. In contrast, CD4-deficient mice were unable to clear infection with virulent *R. equi* at any point following experimental infection. Data from that study indicated that both $CD4^+$ and $CD8^+$ T cells played an important role in controlling the acute stages of

R. equi infection, but that $CD4^+$ T cells played the dominant role in controlling the chronic stages of *R. equi* infection.

Additional studies conducted in mice pointed to a Th1 response in protection of mice against experimental infection. Nordmann et al. (1993) demonstrated that mice treated with anti-interferon gamma (IFNγ) and/or anti-tumor necrosis factor alpha (TNFα) antibodies had significantly higher tissue concentrations of *R. equi* than control mice (Nordmann et al., 1993). Kanaly et al. (1995) reported that mice treated with anti-interleukin (IL)-4 antibodies had increased levels of IFNγ mRNA that corresponded to pulmonary clearance of *R. equi*, whereas mice treated with anti-IFNγ antibodies had increased levels of IL-4 mRNA that corresponded with an inability to clear pulmonary infection with *R. equi*. The findings of these studies were strong indicators that cell-mediated immunity to *R. equi* infection in mice occurred primarily through Th1 responses, and that Th2 responses could have detrimental effects on the outcome of infection. In 1996, Kanaly et al. tested this hypothesis through adoptive transfer of spleen cells, Th1 cells, or Th2 cells from *R. equi*-immunized mice to nude mice 24 h after experimental infection (Kanaly et al., 1996). Pulmonary concentrations of *R. equi* in mice receiving Th2 cells were similar to pulmonary concentrations of *R. equi* in control mice (mice that received no cells), which confirmed the previous findings that suggested a Th2-type response was inadequate for resolution of *R. equi* pneumonia in mice. In contrast, no *R. equi* were isolated from the lungs of mice that received spleen cells or Th1 cells, which demonstrated that a Th1-type response was sufficient for pulmonary clearance of virulent *R. equi*. Although pathogenesis of infection with

virulent *R. equi* in mice does not mimic that in foals, the observations made in mice served as precedence for future studies conducted in horses.

In order to gain a better understanding of the protective immune response to *R. equi*, most studies have been conducted in adult horses because they are generally refractory to disease cause by *R. equi* (Prescott, 1991). In 1999, a study performed *in vitro* described cytokine mRNA expression of adult equine peripheral blood-mononuclear cells (PBMCs) using reverse transcription-competitive PCR (Giguere and Prescott, 1999). The authors studied expression of equine IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p35, IL-12p40, IFNγ, and β-actin, and reported that stimulation of adult PBMCs with concanavalin A (ConA) for 4 h and 24 h induced expression of IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p35, IFNγ, and TNFα, but not IL-12p40. With the exception of IFNγ mRNA, which was higher after 24 h of ConA stimulation, expression of all of the cytokines was greater after 4 h of stimulation than after 24 h of stimulation. Subsequent studies performed *in vivo* and *ex vivo* demonstrated that experimental infection of adult horses with virulent *R. equi* resulted in significant increases in the number of $CD4^+$ and $CD8^+$ T cells recovered in bronchoalveolar lavage fluid. Moreover, experimental infection of adult horses resulted in significant lymphoproliferative responses that were compartmentalized to the infected lung and typical of a secondary immune response (Hines et al., 2001).

Additional studies in adult horses have demonstrated that $CD4^+$ T cells respond to experimental infection by increasing IFNγ, but not IL-4, production (Lopez et al., 2002), that pulmonary clearance of virulent *R. equi* is associated with increased numbers

of IFNγ producing $CD4^+$ T cells (Hines et al., 2003), that these responses could be induced by *R. equi*-secreted antigens (Kohler et al., 2003), and that *R. equi*-specific $CD8^+$ cytotoxic T cells (CTLs) recognize and kill infected macrophages (Patton et al., 2004). Interestingly, the latter study demonstrated that the effector functions of these CTLs did not follow classic major histocompatibility-complex (MHC) class I restrictions, which suggests that alternative methods of antigen processing and presentation may contribute to the resistance of adult horses to infection with *R. equi* through expansion of antigen recognition by T cells (Patton et al., 2004). When similar experiments were performed in 3 week old foals, the CTL activity was deficient. By 6 weeks of age, some of the foals had *R. equi*-specific CTL activity, and by 8 weeks of age all of the foals had developed *R. equi*-specific CTL activity (Patton et al., 2005). An earlier study demonstrated that the ratio of $CD4^+/CD8^+$ T cells in blood samples from 2-week-old foals that later developed *R. equi* pneumonia was significantly lower prior to disease than in foals that did not develop the disease (Chaffin et al., 2004). Both of these studies demonstrated important age-related deficiencies in the immune response of foals to *R. equi*, which suggests that the age-related susceptibility of foals to infection with *R. equi* pneumonia is not associated with declining maternal antibodies (a once-held widespread belief), but is instead associated with immaturity of immune function early in life. Moreover, recent reports have demonstrated that foals produce relatively less IFNγ at birth, both by PBMCs (Boyd et al., 2003; Breathnach et al., 2006) and by pulmonary cells (Breathnach et al., 2006), which suggests that relatively diminished IFNγ production may contribute to the increased susceptibility of newborn foals to

infection with *R. equi*.

Although research efforts over the past 10 years have given rise to a wealth of knowledge regarding the immune response to *R. equi*, it is still unclear why some foals at endemic farms develop disease while others in the same environment do not. An increasing amount of evidence indicates that differences among foals in immune development and function may contribute to the increased susceptibility of some foals to *R. equi* pneumonia; however, most research efforts have focused on the role of adaptive immune responses in resistance to infection with *R. equi*. Unfortunately, it is becoming increasingly apparent that most foals with spontaneous *R. equi* pneumonia become infected at a much younger age (i.e., within the first few days of life) before they have time to develop an effective adaptive immune response (Horowitz et al., 2001). During primary infection with many intracellular bacteria the host must rely on innate immune defenses to prevent organisms from growing to overwhelming numbers before specific adaptive immunity can be generated and expressed (Pedrosa et al., 2000; Bennouna et al., 2003). Thus, innate immunity is likely important for controlling early *R. equi* infections. Indeed, evidence indicates that immaturity of innate immune responses of mononuclear blood cells and individual variability in those responses may contribute to increased susceptibility to infection with *R. equi* (Boyd et al., 2003; Darrah et al., 2004; Breathnach et al., 2006).

While the focus of most studies investigating innate immunity to *R. equi* has been on the role of the macrophage, neutrophils are also a critical component of innate immunity. Neutrophils are professional phagocytic cells that play a vital role in host

immunity to extracellular infections (Appelberg, 2006). The role of neutrophils in immunity to infection with intracellular pathogens has previously been disregarded for several reasons. One reason is that some intracellular pathogens, such as *R. equi*, are able to survive and replicate within macrophages and are thus protected from the bactericidal activity of neutrophils. Their short life-span is another limiting factor of the neutrophils ability to control slow-growing intracellular pathogens, such as *M. tuberculosis* and *R. equi* that are implicated in chronic infections (Pedrosa et al., 2000). Nevertheless, several lines of evidence indicate that neutrophils are critical for protection against *R. equi*. Some foals < 1 week of age have markedly reduced bactericidal capacity for *R. equi* than other foals (Martens et al., 1988). A recent study found that blood concentrations of neutrophils at 2 weeks and 4 weeks of age were significantly lower prior to disease among foals that developed *R. equi* pneumonia compared to foals at the same farms that did not develop disease (Chaffin et al., 2004). Phagocytic function of foal neutrophils is less than that of adult horses (Demmers et al., 2001; McTaggart et al., 2001). Evidence exists that this reduced phagocytic function of foal neutrophils is reversed by addition of adult sera (indicating reduced opsonic capacity of foal sera); however, foal neutrophils have also been demonstrated to have reduced phagocytic capacity relative to that of adult neutrophils despite being treated with adult sera (Grondahl et al., 1999). Furthermore, neutrophils are known play a protective role in mice experimentally infected with *R. equi* (Martens et al., 2005): tissue concentrations of *R. equi* were significantly higher in mice in which neutrophils were deleted using a monoclonal antibody than in genetically similar mice that did not undergo neutrophil depletion.

The mechanism by which neutrophils protect against intracellular infections such as *R. equi* remains unclear. Although neutrophils from horses are capable of killing *R. equi* following phagocytosis (Yager et al., 1986; Martens et al., 1988), non-phagocytic mechanisms also may play a role; it is becoming increasingly apparent that neutrophils play an important non-phagocytic role in controlling other intracellular infections, particularly early in the course of disease (Pedrosa et al., 2000). The non-phagocytic role (specifically the expression of inflammatory cytokines) of neutrophils in response to infection with *R. equi*, however, has not been reported. In other species, neutrophils produce a variety of cytokines and chemokines which modulate the immune response by recruiting and activating other effector cells of the immune system (Bennouna et al., 2003; Denkers et al., 2003; Appelberg, 2006). Consequently, neutrophils provide a crucial link between innate and adaptive cellular immune responses. Thus, the purpose of this study was to characterize changes in cytokine mRNA expression by juvenile and adult equine neutrophils in response to *in vitro* stimulation with *R. equi*.

CHAPTER II

CYTOKINE EXPRESSION BY NEUTROPHILS OF ADULT HORSES STIMULATED WITH VIRULENT AND AVIRULENT *Rhodococcus equi in vitro*

OVERVIEW

Rhodococcus equi is an intracellular pathogen of macrophages that causes rhodococcal pneumonia in foals and immunocompromised people. Evidence exists that neutrophils play a vital role in resistance to infection with *R. equi*; however, the means by which neutrophils exert their effects have not been clearly defined. In addition to directly killing bacteria, neutrophils may also exert a protective effect by linking innate and adaptive immune responses. In the present study we evaluated the cytokine expression profiles of adult equine neutrophils in response to stimulation with isogenic strains of virulent and avirulent *R. equi in vitro*. After 2 h and 4 h incubation with virulent or avirulent *R. equi*, adult equine neutrophils expressed significantly ($P< 0.05$) greater TNFα, IL-12p40, IL-6, IL-8 and IL-23p19 mRNA, but not IFNγ or IL-12p35 mRNA. Furthermore, virulent *R. equi* induced significantly greater IL-23p19 mRNA than avirulent *R. equi*. Stimulation with rhGM-CSF of adult equine neutrophils failed to induce significant changes in cytokine expression. These results demonstrate that *R. equi*-stimulated neutrophils are a source of many pro-inflammatory cytokines. Furthermore, these results suggest that IL-23 may be preferentially expressed over IL-12 in response to exposure with *R. equi*, and that this response may be more strongly induced by virulent *R. equi* than avirulent *R. equi*. Collectively, the data presented herein

suggest a non-phagocytic role for neutrophils that may influence the type of adaptive immune response to *R. equi*

INTRODUCTION

Rhodococcus equi is a Gram-positive facultative intracellular pathogen of macrophages that causes severe and often fatal pneumonia in young foals and immunocompromised people (Cohen and Martens, 2007). Infection in both horses and humans is characterized by severe pyogranulomatous pneumonia, similar to pneumonia caused by the closely related bacterium *Mycobacterium tuberculosis*. The impact of this disease is large because the associated morbidity and mortality rates are high, and treatment is generally prolonged, expensive, and associated with adverse effects (Stratton-Phelps et al., 2000; Cohen and Martens, 2007). Furthermore, affected foals that survive are less likely than age-matched cohorts to race (Ainsworth et al., 1998). Currently, there are no vaccines available to prevent disease caused by *R. equi.* The only prophylactic strategy shown to effectively control *R. equi* pneumonia is intravenous transfusion of *R. equi* hyperimmune plasma to neonatal foals (Martens et al., 1989a), a procedure that is expensive, labor-intensive, and not universally effective (Hurley and Begg, 1995; Cohen and Martens, 2007).

Many aspects of the epidemiology of *R. equi* remain unknown, despite the opportunities for prevention and control that would arise from such knowledge. In particular, it is unclear why some foals at endemic farms develop disease while other foals in the same environment do not. One possible explanation for this phenomenon is that differences in immune function among foals determine susceptibility to infection.

Recently, it has been demonstrated that the ratio of $CD4^+/CD8^+$ T cells in blood samples from foals at 2 weeks of age was significantly lower prior to disease in foals that subsequently developed *R. equi* pneumonia than in foals that did not develop the disease (Chaffin et al., 2004). In adult horses, the production of IFNγ by $CD4^+$ and $CD8^+$ T cells is required for pulmonary clearance of virulent *R. equi* (Hines et al., 2003). Furthermore, experiments conducted in mice demonstrated that both $CD4^+$ and $CD8^+$ T cells are important for protection against *R. equi*, with $CD4^+$ cells being the primary cell type involved in protection (Nordmann et al., 1992; Kanaly et al., 1993, 1996; Ross et al., 1996).

Although it is generally accepted that adaptive immune responses are critical for resistance to infection with *R. equi* (Kanaly et al., 1993, 1995, 1996; Kasuga-Aoki et al., 1999; Hines et al., 2003; Kohler et al., 2003), there is also evidence indicating that innate immunity plays an important role. Innate immune responses of mononuclear blood cells play a role in the pathogenesis of *R. equi* (Darrah et al., 2004); however, several lines of evidence indicate that neutrophils are also critical for protection. Neutrophils are known to provide protection against a number of other intracellular pathogens (Dalrymple et al., 1995; Pedrosa et al., 2000; Godaly and Young, 2005). Neutrophils from some foals < 1 week of age have markedly reduced bactericidal capacity for *R. equi* than other foals (Martens et al., 1988). Phagocytic function of foal neutrophils is less than that of adult horses (Demmers et al., 2001; McTaggart et al., 2001). Although evidence exists that this reduced phagocytic function is reversed by addition of adult sera (indicating reduced opsonic capacity of foal sera), foal neutrophils have also been demonstrated to have

reduced phagocytic capacity relative to that of adult neutrophils, even when both groups are treated with adult sera (Gröndahl et al., 1999; Demmers et al., 2001). Blood concentrations of neutrophils at 2 weeks and 4 weeks of age were significantly lower prior to disease among foals that developed *R. equi* pneumonia compared to foals at the same farms that did not develop disease (Chaffin et al., 2004). Additionally, it was recently reported that following experimental infection with *R. equi,* neutropenic mice had significantly greater tissue concentrations of *R. equi* than mice with normal numbers of neutrophils, demonstrating a protective role for neutrophils against *R. equi* infections in mice (Martens et al., 2005).

The mechanism by which neutrophils protect against intracellular infections such as *R. equi* remains unclear. Although neutrophils from most horses are capable of killing *R. equi* following phagocytosis (Yager et al., 1986, 1987; Hietala and Ardans, 1987b; Martens et al., 1988), non-phagocytic mechanisms may also play a role. In other species, neutrophils produce a variety of cytokines and chemokines that modulate the immune response by recruiting and activating other effector cells of the immune system (Appelberg, 2006). Consequently, neutrophils provide a crucial link between innate and adaptive cellular immune responses. To the authors' knowledge, the mRNA expression of pro-inflammatory cytokines by equine neutrophils in response to infection with *R. equi* has not been reported. Thus, the purpose of this study was to compare the mRNA expression of selected pro-inflammatory cytokines by adult equine neutrophils stimulated *in vitro* by isogenic strains of virulent and avirulent *R. equi*. Cytokine mRNAs examined included IL-6, IL-8, IL-12p35, IL-12p40, IL-23p19, IFNγ, and TNFα.

Although infection with *R. equi* is generally limited to foals, it is extremely important to understand the response of the resistant phenotype (i.e., adult horses) in order to identify differences that might exist in foals, which are the susceptible phenotype (Kohler et al., 2003). We hypothesized that cytokine mRNA expression by adult equine neutrophils would be significantly increased by exposure to live *R. equi* (virulent and avirulent isolates) and recombinant human (rh)GM-CSF, and that expression would not differ significantly among the different stimuli.

MATERIALS AND METHODS

Reagents

RPMI-1640, Hanks balanced salt solution (HBSS), HEPES, phosphate-buffered saline (PBS), cell-culture-grade distilled water, RNase/DNase free distilled water, and $NaHCO_3$ were obtained from Gibco-Invitrogen (Invitrogen Corporation, Grand Island, NY). Ficoll-Paque plus was obtained from Amersham Biosciences (Amersham Biosciences, Pittsburgh, PA). Bovine serum albumin (BSA), and sodium chloride (NaCl) were purchased from Sigma (Sigma Aldrich, St. Louis, MO). Primary working buffer consisted of the following: 1× HBSS, 10mM HEPES, and 8.9 mM $NaHCO_3$. Lysis buffers A and B consisted of the following: 0.2 and 1.6% NaCl, respectively, 20 mM HEPES, and 1% BSA. The pH of all buffers and reagents was adjusted to 7.4 with NaOH.

Bacteria

Virulent (ATCC 33701 P^+) and avirulent (ATCC 33701 P^-) *R. equi* were grown in *R. equi* minimal media overnight at 37°C with rocking. The bacteria were then

centrifuged at 2,000 × g for 10 min. The bacterial pellets were washed 1× with RPMI 1640, and then resuspended at an approximate concentration of 1×10^{8} CFU ml^{-1} RPMI 1640. The bacteria were aliquoted and frozen at −80°C. Prior to use, the bacteria were thawed and 10-fold serial dilutions were performed in PBS. The *R. equi* were quantitatively cultured on trypticase soy agar plates supplemented with 5% sheep red blood cells. Prior to infecting equine neutrophils, the *R. equi* were opsonized at 37°C for 1 h with 5% fresh-frozen serum obtained from an adult donor horse known to have antibodies to *R. equi*. Serum from this horse was used in order to ensure opsonization of *R. equi*.

Neutrophil purification

Whole blood was obtained from 5 healthy adult horses using acid-citrate-dextrose (ACD) as an anticoagulant. After allowing the erythrocytes to sediment at room temperature for 1 h, the leukocyte rich plasma was layered onto Ficoll-Paque and centrifuged at 500 × g for 20 min. The neutrophil pellet was washed 2 times with primary working buffer, and the residual erythrocytes were lysed 2 times with a hypotonic lysis protocol as follows: each neutrophil pellet was resuspended in 7.5 ml of lysis buffer A. After a brief incubation of 60 sec at room temperature, 7.5 ml of lysis buffer B was added to the mixture. The tubes were then centrifuged at 300 × g for 10 min. After discarding the supernatant containing lysed erythrocytes, the pellet was resuspended in RPMI-1640. The neutrophils were >98% viable as indicated by exclusion of trypan blue. Random samples of purified neutrophils were submitted to the Texas A&M Veterinary Teaching Hospital's clinical pathology laboratory for differential cell

counts to ensure that the neutrophil purification method effectively removed contaminating lymphocytes and monocytes. Contaminating cell types composed < 2% of the cell preparations and included monocytes, lymphocytes, and an occasional eosinophil.

Neutrophil stimulation

Neutrophils (~2×10^7 ml^{-1}) were incubated for 2 h and 4 h at 37°C with the following: 100 ng rhGM-CSF (positive control), virulent *R. equi* (MOI 10:1), or avirulent *R. equi* (MOI 10:1). For each time-point, equal concentrations of neutrophils were incubated with media only (unstimulated neutrophils) as a negative control. An equal volume of RPMI-1640 was added to each of the unstimulated neutrophil controls so that the volume in every sample was the same. In order to determine the baseline level of cytokine mRNA expression as a point of reference, a 0 h unstimulated sample (herein referred to as baseline) was obtained for every experiment. Following stimulation, all neutrophils were pelleted by centrifugation at 4°C for 5 min at 2,000 × g, and the supernatant was discarded.

RNA and cDNA preparation

RNA was extracted immediately from each neutrophil pellet using an RNeasy mini kit (Qiagen, Valencia, CA). The concentration and purity of the RNA was assessed by spectrophotometry. Aliquots of RNA were prepared in DNase/RNase-free dH_2O to a final concentration of 0.05 $\mu g\ \mu l^{-1}$ and frozen at −80°C until subsequent cDNA synthesis. Prior to initiating cDNA synthesis, each aliquot of RNA was treated with amplification grade DNase I according to the manufacturer's protocol (Invitrogen Corporation, Grand Island, NY). cDNA was subsequently synthesized with SuperScript III First-Strand synthesis System for RT-PCR (Invitrogen Corporation, Grand Island, NY) using the manufacturer's protocol. In order to avoid synthesis of *R. equi*-derived cDNA, the cDNA synthesis was primed with oligo $(dT)_{20}$ primers provided with the cDNA synthesis kit. Upon completion of cDNA synthesis, all cDNA was stored at −80°C until use.

Primer selection and real-time PCR conditions

The primer/probe sequences and their respective GenBank accession numbers are listed in Table 1. In order to prevent amplification of genomic DNA, primers were designed in consecutive exons with the probes crossing the exon junction. Equine β2M, IL-12p35, IL-12p40, IL-6, IL-8 and IL-23p19 specific primer/probe premixes were designed using the Assays-by-Design software program (Applied Biosystems, Foster City, CA), and sequences specific for equine IFNγ and TNFα were obtained from a previous publication (Garton et al., 2002). Each 25-µl real-time PCR reaction contained the following: 2.5 µl plasmid or template cDNA; 1.25 µl 20× primer/probe premix (Applied Biosystems, Foster City, CA); 12.5µl 1× TaqMan Universal Master Mix (Applied Biosystems, Foster City, CA); and molecular-grade water (Invitrogen Corporation, Grand Island, NY). Amplification and data analysis were carried out on a GeneAmp 7500 Sequence Detection System (Applied Biosystems, Foster City, CA). The thermal profile consisted of an initial hold at 50ºC for 2 min, followed by a single denaturation at 95°C for 10 min, and then 40 cycles of 95°C for 15 sec, 60°C for 60 sec. The resulting Ct values were normalized to the endogenous control, β2M, and the relative quantification values were determined using the ddCt method with baseline expression as the calibrator.

Table 1.
Oligonucleotide primer and probe sequences for amplification of various equine cytokines and the endogenous control

Gene (GenBank accession #)	Primer/probe	Sequence 5'-3'
β2M (X69083)	Forward	CGGGCTACTCTCCCTGACT
	Reverse	GGGTGACGTGAGTAAACCTGAAC
	Probe	CCGTCCCGCGTGTTC
IFNγ (D28520)*	Forward	AAGTGAACTCATCAAAGTGATGAATGA
	Reverse	CGAAATGGATTCTGACTCCTCTTC
	Probe	TCGCCCAAAGCTAACCTGAGGAAGC
TNFα (M64087)*	Forward	GCTCCAGACGGTGCTTGTG
	Reverse	GCCGATCACCCCAAAGTG
	Probe	TGTCGCAGGAGCCACCACGCT
IL-6 (U64794)	Forward	GAAAAAGACGGATGCTTCCAATCTG
	Reverse	TCCGAAAGACCAGTGGTGATTTT
	Probe	CAGGTCTCCTGATTGAAC
IL-8 (AY184956)	Forward	GCCACACTGCGAAAACTCA
	Reverse	GCACAATAATCTGCACCCACTTTG
	Probe	ACGAGCTTTACAATGATTTC
IL-12p35 (Y11130)	Forward	CCCGGAAAGGCCTCTTCT
	Reverse	ACCTGGTACATCTTCAAGTCCTCAT
	Probe	TAAGGCACAGCGTCATCA
IL-12p40 (Y11129)	Forward	TCACAAGAAGGAAGATGGAATTTGGT
	Reverse	CCGGAATAATTCTTTGCCTCACATTT
	Probe	TTTAAAAGACCAGAAAGAATCC
IL-23p19 (NM_001082522)	Forward	GCTGTGATCCTGAAGGACTCA
	Reverse	CCCTGGTGGATCCTTTGCA
	Probe	CAGGGCTGACTGTTGTC

* Garton et al., 2002

Plasmid selection and design

Clones containing the cDNA-derived sequences of β2M, IL-6, IL-8, and IL-23p19 were generously provided by the Pratt Laboratory (University of Georgia), and the clone containing the cDNA-derived sequence of TNFα was constructed in another laboratory by a co-author (S.P.). Cloning primers specific for IFNγ, IL-12p35, and IL-12p40 were designed from mRNA sequences published in GenBank (http://www.ncbi.nlm.nih.gov) using the Primer3 online software program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). The gene targets were amplified from equine cDNA (obtained from *R. equi*-stimulated neutrophils) and subcloned into the TOPO-TA (Invitrogen Corporation, Grand Island, NY) vector according to the manufacturer's protocol. Plasmid DNA was purified from clones corresponding to each of the 8 genes using the Fast Plasmid Mini kit (Eppendorf, Westbury, NY), and the inserts were confirmed through sequencing. Plasmid DNA concentrations were determined spectrophotometrically and DNA stocks were stored at −80ºC.

Assay validation

Assay validation was performed to determine the inter- and intra-assay variation, and to demonstrate the reproducibility of the methods used for sample preparation. Some cytokines (TNFα, IL-6, and IL-8) were validated in another laboratory by a co-author (S.P.) using methods identical to those described here. To validate the RNA extraction and cDNA synthesis method, 3 RNA extractions were performed on a single unstimulated neutrophil preparation. For each RNA extraction, 2 separate cDNA

syntheses were performed such that there were a total of 6 cDNA samples. Real-time PCR was performed in triplicate on all 6 cDNA preparations using the endogenous control, β2M. Results from the real-time PCR were evaluated in terms of the coefficient of variation (CV), which was calculated by dividing the mean Ct value by the respective standard deviation.

To confirm the product size and specificity of each primer/probe set, all PCR products from a single real-time PCR assay were run on an agarose gel. The same PCR products were purified using the Stratprep PCR purification kit (Stratagene, La Jolla, CA) and bidirectional sequencing was performed using the real-time PCR primers. The derived sequences were compared with those used to design primers/probes and with a BLAST search of GenBank sequences (http://www.ncbi.nlm.nih.gov) to confirm target specificity.

Inter-assay variability was determined for each gene of interest by running the triplicate plasmid DNA dilutions (30 reactions per gene) in 2 separate real-time PCR assays. Intra-assay variability was determined for each gene of interest by running 10 replicates of every other dilution (50 reactions per gene) in a single real-time PCR assay.

Statistical analysis

Data were transformed logarithmically for analysis because they were not distributed normally. Data were analyzed using linear mixed-effects models (Pinheiro and Bates, 2000); to account for repeated measures on individuals, horse was modeled as a random effect, and time, stimuli, and their respective interaction terms were modeled as fixed effects. Model fit was assessed by graphical methods to determine whether there was evidence of poor fit or violation of model assumptions. Post-hoc testing of between-group differences was made using the method of Scheffé. A significance level of $P < 0.05$ was used. Statistical analyses were performed using S-PLUS (version 7.0; Insightful, Inc.).

RESULTS

Assay validation

To validate the method of sample preparation, 6 cDNA samples (2 from each of 3 RNA samples) were analyzed concurrently by real-time PCR. The mean CVs for RNA and cDNA preparation across all sets were 2.8% (range, 1.4% to 4.9%) and 2.3% (range, 1.4% to 3.7%), respectively. The CVs determined for the intra- and inter-assay variation of each gene are listed in Table 2.

Gel electrophoresis of the PCR products from each primer/probe set indicated the amplification of single products that were within the expected size range (data not shown). Bidirectional sequencing and a BLAST database search of each of the sequences generated confirmed that all primer/probe sets amplified the intended gene.

Table 2.
Intra-and inter-assay variabilities

Gene	Inter-assay CV	Intra-assay CV
β2M	2.24% (range, 0.81% to 3.87%)	0.76% (range, 0.22% to 1.94%)
IL-12p35	2.49% (range, 0.11% to 11.4%)	0.81% (range, 0.62% to 0.98%)
IL-12p40	2.45% (range, 0.55% to 7.94%)	0.5% (range, 0.32% to 0.87%)
IFNγ	3.16% (range, 1.31% to 4.11%)	0.53% (range, 0.23% to 0.97%)
IL-23p19	2.37% (range, 1.27% to 3.8%)	1.77% (range, 0.61% to 3.64%)

Cytokine expression of adult equine neutrophils

There were no significant changes in expression of IFNγ or IL-12p35 mRNA by neutrophils following any stimulus relative to unstimulated neutrophils. Although expression of IFNγ mRNA tended to be increased after 4 h for all neutrophils, this difference was not significant when accounting for effects of stimulus and time (i.e., the confidence intervals for fold-change in expression always included 1, which was the baseline value).

There were significant effects of stimulus and time on expression of TNFα mRNA by neutrophils (Fig. 1). Relative to unstimulated neutrophils, expression of TNFα mRNA by rhGM-CSF-stimulated neutrophils was not significantly different at either time. Relative to unstimulated and rhGM-CSF-stimulated neutrophils, expression of TNFα mRNA by virulent and avirulent *R. equi*-stimulated neutrophils was significantly ($P< 0.05$) greater at 2 h and 4 h; however, there was no significant difference at either time in expression of TNFα mRNA between neutrophils stimulated with virulent or avirulent *R. equi*. Expression of TNFα mRNA by all neutrophils increased significantly ($P< 0.05$) at both times relative to baseline, with the exception of neutrophils stimulated with rhGM-CSF for 4 h, which were not significantly different than baseline.

There were significant effects of stimulus and time on expression of IL-12p40 mRNA by neutrophils (Fig. 2). Relative to unstimulated neutrophils, expression of IL-12p40 mRNA by avirulent *R. equi*-stimulated neutrophils was significantly ($P< 0.05$) greater at 2 h and 4 h; however, there was no significant difference in expression of IL-12p40 mRNA at either time-point by virulent *R. equi*-stimulated neutrophils relative to

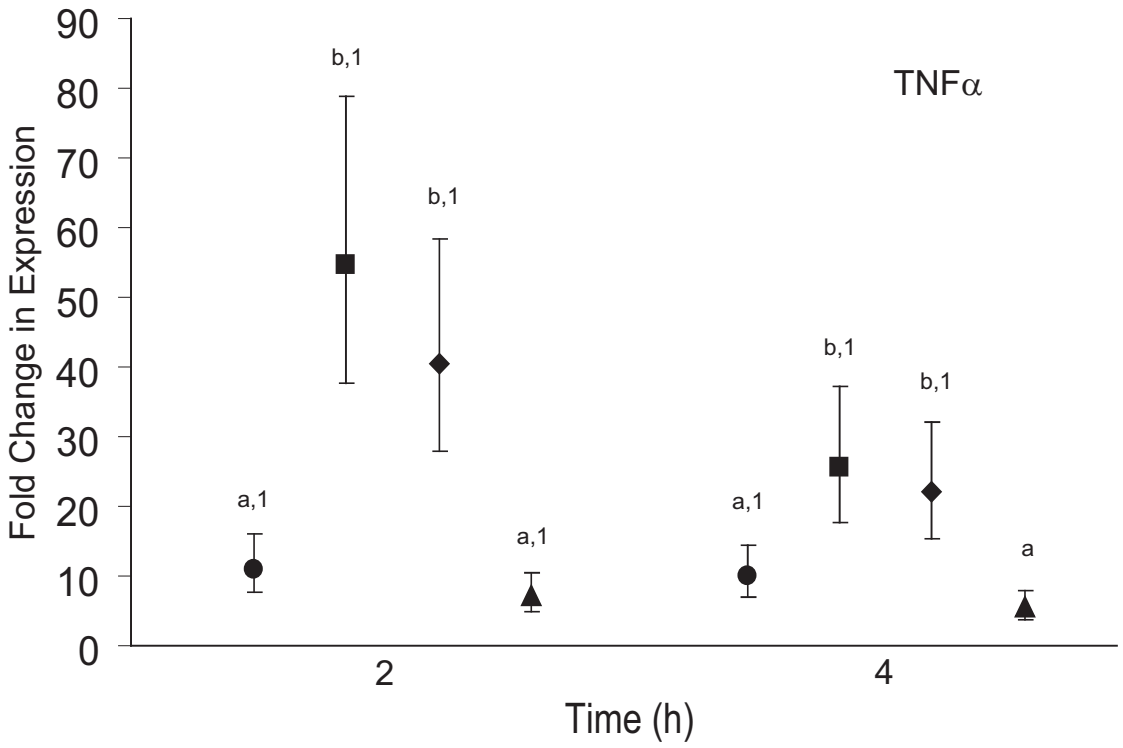

Fig. 1. TNFα mRNA expression in unstimulated (●) neutrophils and neutrophils stimulated with avirulent (■) *R. equi*, virulent (♦) *R. equi*, and rhGM-CSF (▲).Values represent the mean fold changes in expression (relative to baseline) of 5 adult horses and thin vertical lines extending to thin horizontal lines represent the time-specific 95% confidence intervals for each stimulus. Stimuli with different letters differed significantly ($P< 0.05$) from 1 another at a given time. Stimuli with a number 1 differed significantly ($P< 0.05$) from baseline.

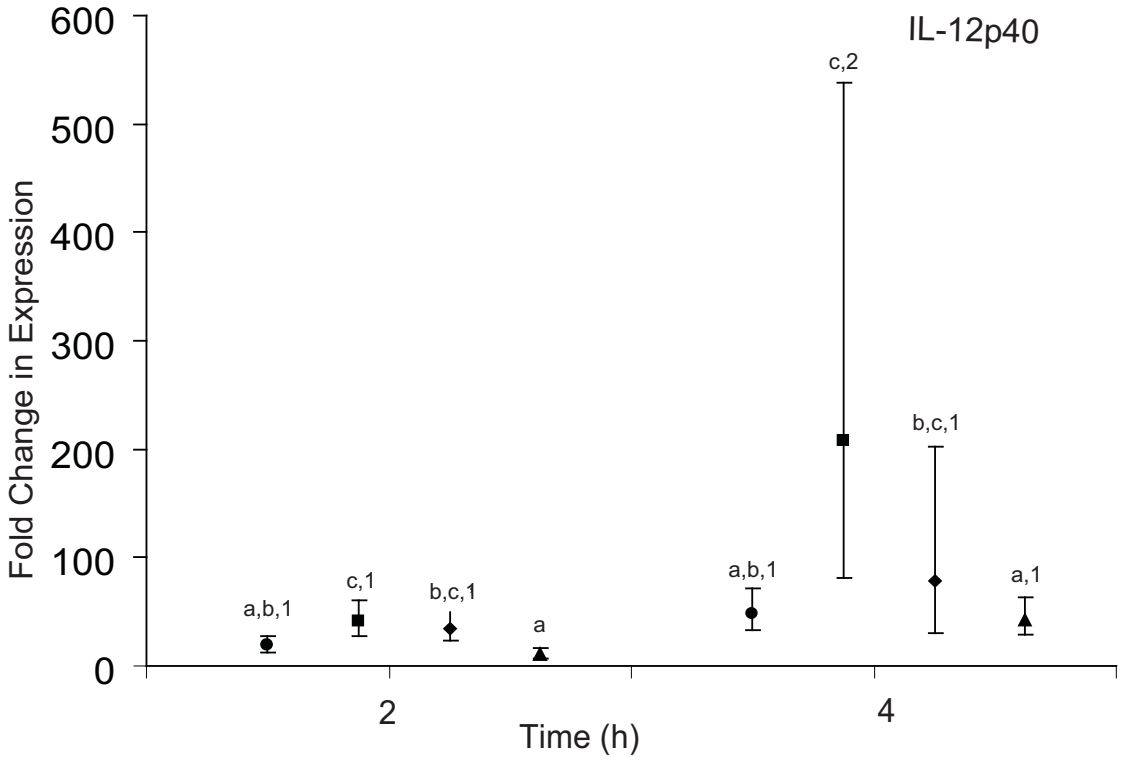

Fig. 2. IL-12p40 mRNA expression in unstimulated (●) neutrophils and neutrophils stimulated with avirulent (■) *R. equi*, virulent (♦) *R. equi*, and rhGM-CSF (▲).Values represent the mean fold changes in expression (relative to baseline) of 5 adult horses and thin vertical lines extending to thin horizontal lines represent the time-specific 95% confidence intervals for each stimulus. Stimuli with different letters differed significantly ($P< 0.05$) from 1 another at a given time. Stimuli with a number 1 differed significantly ($P< 0.05$) from baseline. Stimuli with a number 2 were significantly different between times 2 h and 4 h.

unstimulated neutrophils. Relative to rhGM-CSF-stimulated neutrophils, expression of IL-12p40 mRNA by virulent and avirulent *R. equi*-stimulated neutrophils was significantly ($P< 0.05$) greater at 2 h and 4 h. There was no significant difference at either time in expression of IL-12p40 mRNA between neutrophils stimulated with virulent or avirulent *R. equi*. Expression also did not differ significantly at either time-point between unstimulated and rhGM-CSF-stimulated neutrophils. With the exception of neutrophils stimulated with rhGM-CSF for 2 h, expression of IL-12p40 mRNA by all neutrophils increased significantly ($P< 0.05$) at both times relative to baseline. There was significantly ($P< 0.05$) higher expression of IL-12p40 mRNA by neutrophils stimulated with avirulent *R. equi* for 4 h, than those stimulated with avirulent *R. equi* for 2 h.

There were significant effects of stimulus and time on expression of IL-6 mRNA by neutrophils (Fig. 3). Relative to unstimulated and rhGM-CSF-stimulated neutrophils, expression of IL-6 mRNA by virulent and avirulent *R. equi*-stimulated neutrophils was significantly ($P< 0.05$) greater at 2 h and 4 h; however, there was no significant difference at either time in expression of IL-6 mRNA between neutrophils stimulated with virulent or avirulent *R. equi*. Relative to baseline, there was no significant difference at either time in expression of IL-6 mRNA by unstimulated or rhGM-CSF-stimulated neutrophils. Relative to baseline, expression of IL-6 mRNA by neutrophils stimulated with virulent and avirulent *R. equi* was significantly ($P< 0.05$) greater at 2 h and 4 h.

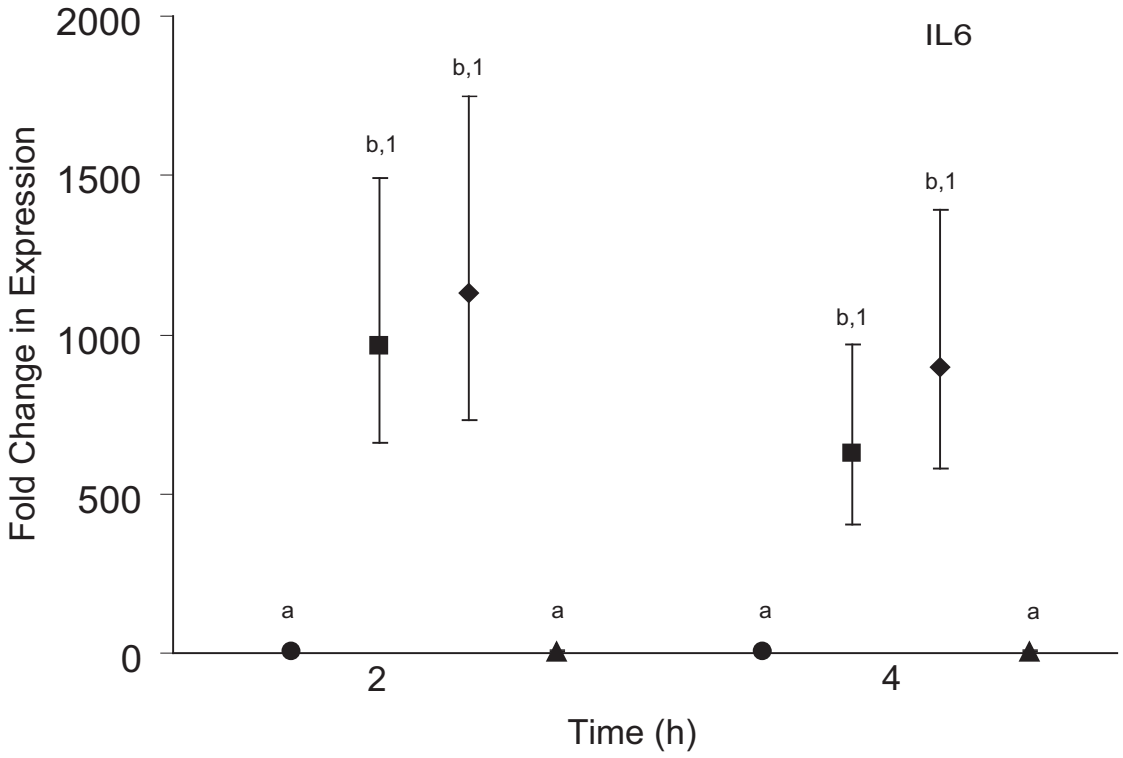

Fig. 3. IL-6 mRNA expression in unstimulated (●) neutrophils and neutrophils stimulated with avirulent (■) *R. equi*, virulent (♦) *R. equi*, and rhGM-CSF (▲).Values represent the mean fold changes in expression (relative to baseline) of 5 adult horses and thin vertical lines extending to thin horizontal lines represent the time-specific 95% confidence intervals for each stimulus. Stimuli with different letters differed significantly ($P< 0.05$) from 1 another at a given time. Stimuli with a number 1 differed significantly ($P< 0.05$) from baseline.

There were significant effects of stimulus and time on expression of IL-8 mRNA by neutrophils (Fig. 4). Relative to unstimulated and rhGM-CSF-stimulated neutrophils, expression of IL-8 mRNA by virulent and avirulent *R. equi*-stimulated neutrophils was significantly ($P< 0.05$) greater at 2 h and 4 h; however, there was no significant difference at either time in expression of IL-8 mRNA between neutrophils stimulated with virulent or avirulent *R. equi*. Relative to baseline, expression of IL-8 mRNA was increased significantly by neutrophils stimulated with rhGM-CSF for 2 h and by neutrophils stimulated with virulent and avirulent *R. equi* for 2 h and 4 h.

There were significant effects of stimulus and time on expression of IL-23p19 mRNA (Fig. 5). Relative to unstimulated and rhGM-CSF-stimulated neutrophils, expression of IL-23p19 mRNA by neutrophils stimulated with virulent and avirulent *R. equi* was significantly ($P< 0.05$) greater at 2 h and 4 h. At 2 h and 4 h, there was significantly ($P< 0.05$) greater expression of IL-23p19 mRNA by neutrophils stimulated with virulent *R. equi* than those stimulated with avirulent *R. equi* There was no significant difference in expression between unstimulated and rhGM-CSF stimulated neutrophils at any time-point. Expression of IL-23p19 mRNA by all neutrophils (irrespective of stimulus) increased significantly ($P< 0.05$) at both times relative to baseline.

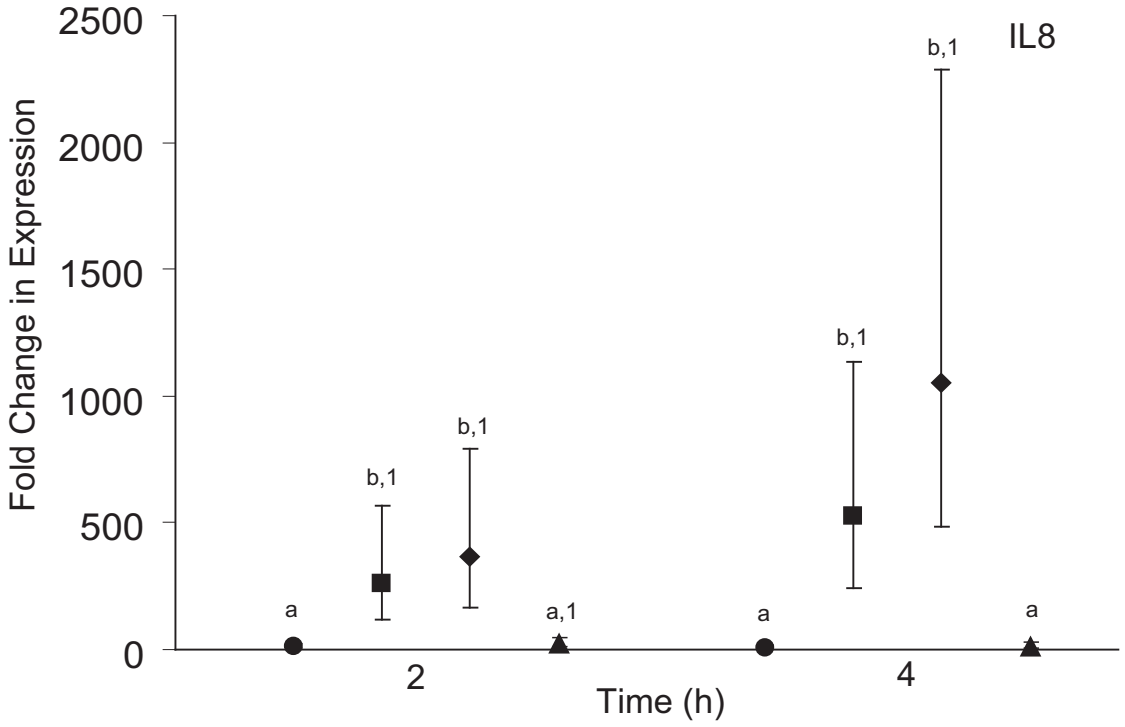

Fig. 4. IL-8 mRNA expression in unstimulated (●) neutrophils and neutrophils stimulated with avirulent (■) *R. equi*, virulent (♦) *R. equi*, and rhGM-CSF (▲). Values represent the mean fold changes in expression (relative to baseline) of 5 adult horses and thin vertical lines extending to thin horizontal lines represent the time-specific 95% confidence intervals for each stimulus. Stimuli with different letters differed significantly ($P< 0.05$) from 1 another at a given time. Stimuli with a number 1 differed significantly ($P< 0.05$) from baseline.

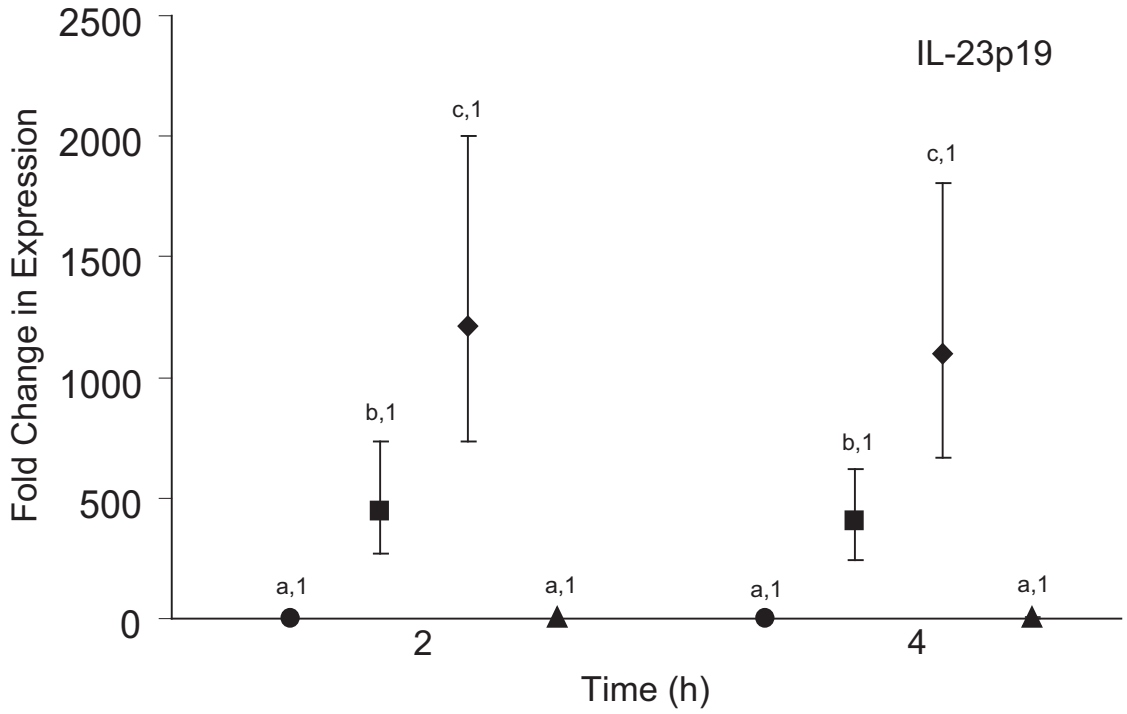

Fig. 5. IL-23p19 mRNA expression in unstimulated (●) neutrophils and neutrophils stimulated with avirulent (■) *R. equi*, virulent (♦) *R. equi*, and rhGM-CSF (▲).Values represent the mean fold changes in expression (relative to baseline) of 5 adult horses and thin vertical lines extending to thin horizontal lines represent the time-specific 95% confidence intervals for each stimulus. Stimuli with different letters differed significantly ($P< 0.05$) from 1 another at a given time. Stimuli with a number 1 differed significantly ($P< 0.05$) from baseline.

DISCUSSION

During primary infection with many intracellular bacteria, the host relies on innate immune responses to contain the replication of organisms until specific adaptive immunity develops (Pedrosa et al., 2000; Bennouna et al., 2003). Thus, innate immunity is likely important for controlling early *R. equi* infections. Evidence indicates that immaturity of innate immune responses and individual variability in those responses may contribute to increased susceptibility to infection by *R. equi* (Boyd et al., 2003; Chaffin et al., 2004; Darrah et al., 2004; Breathnach et al., 2006). It is increasingly apparent that neutrophils play an important non-phagocytic role in controlling intracellular infections, particularly early in the course of disease (Pedrosa et al., 2000). The *in vitro* bactericidal capacity and the protective role of neutrophils against *R. equi* in mice experimentally infected with *R. equi* have been demonstrated previously (Martens et al., 1988; Martens et al., 2005). The non-phagocytic role of expression of inflammatory cytokines by neutrophils in response to infection with *R. equi*, however, has not been reported.

We examined the mRNA expression of 7 pro-inflammatory cytokines that are known to play important roles in immunity to infectious disease. Adult equine neutrophils were stimulated for 2 h and 4 h with *R. equi* (virulent and avirulent) and rhGM-CSF. These incubation times were chosen based on the short life-span of the neutrophil (Pedrosa et al., 2000; Appelberg, 2006), reports that neutrophils contain stores of preformed cytokines (namely IFNγ, IL-6, and IL-12) that are rapidly released and subsequently replenished (Bliss et al., 2000; Denkers et al., 2003; Ethuin et al., 2004),

and studies done in heterophils (the avian equivalent of a neutrophil) demonstrating that expression of IL-8 and IL-18 mRNA increases significantly after 2 h stimulation and plateaus after 4 h stimulation (Kogut et al., 2003; Swaggerty et al., 2004). Maximum mRNA expression of many inflammatory cytokines within a 4 h period has also been demonstrated to occur in *R. equi*-stimulated macrophages (Giguere and Prescott, 1998). Furthermore, preliminary time-course experiments conducted in our laboratory demonstrated that, after incubation (with and without *R. equi*) for 0.5 h, 1 h, 2 h, 4 h, and 24 h, the differential expression of cytokine mRNA was most consistent after 2 h and 4 h, whereas differential expression was not consistent after 0.5 h, 1 h, and 24 h. As discussed below, the times used to evaluate expression likely may not have been optimal for all of the cytokines studied. A wide range of MOIs have been reported in the *R. equi* literature (Martens et al., 1988; Hondalus and Mosser, 1994; Giguere and Prescott, 1998). An MOI of 10:1 was chosen in order to maximize the stimulating effects of *R. equi* on neutrophils while minimizing any potential adverse effects that might arise from adding too many bacteria. A single MOI was studied because preliminary studies indicate this MOI effectively stimulated cytokine mRNA expression by equine neutrophils and financial limitations precluded evaluating additional MOIs.

We selected rhGM-CSF as a positive control on the basis of a previous report that demonstrated the activity of rhGM-CSF on equine cells (Hammond et al., 1999), and lack of a source of equine (eq)GM-CSF. The addition of rhGM-CSF, however, failed to stimulate cytokine mRNA expression in adult equine neutrophils. One possible reason for this is that rhGM-CSF displays some degree of species-specificity: it is active

in stimulating cells of dogs but not those of mice (Lee et al., 1985; Mayer et al., 1990). Recently (after our studies were initiated), it was reported that eqGM-CSF is structurally and biologically different from other mammalian homologues at the N and C termini (Mauel et al., 2006), a finding that could explain the observation in this study that rhGM-CSF failed to stimulate equine neutrophils.

The precise biological role of IL-6 is unclear. Some studies suggest that IL-6 is a Th2 cytokine (Rincon et al., 1997), while others suggest it is a Th1 cytokine (Leal et al., 1999; Saunders et al., 2000). Evidence for the latter, however, is stronger. Mice deficient in IL-6 appear to be highly susceptible to infection with the intracellular pathogens *Listeria monocytogenes* (Dalrymple et al., 1995) and *M. tuberculosis* (Ladel et al., 1997). In this study, we demonstrated that equine neutrophils exposed to virulent and avirulent *R. equi* displayed a significant increase in expression of IL-6 mRNA, and that increase was sustained throughout the period of observation. If indeed IL-6 promotes a Th1-type response, then expression of this cytokine in response to rhodococcal infection would likely be beneficial to the host, particularly if it were to promote IFNγ production early in the course of disease (Boyd et al., 2003; Breathnach et al., 2006). Additional studies will need to be conducted in order to clarify the nature of the role of IL-6 in *R. equi* infection, but the data we report here strongly support such a role.

IL-8 is a strong chemoattractant for neutrophils, monocytes, and T cells (Baggiolini et al., 1995; Zhang et al., 1995), and has been shown to enhance killing of *M. tuberculosis* by neutrophils (Godaly and Young, 2005). In this study, adult equine neutrophils demonstrated a marked increase in expression of IL-8 mRNA upon

stimulation with *R. equi.* We hypothesized that neutrophils play a vital role in early host defense against *R. equi*. It is possible that they do this through the production of IL-8, thereby recruiting additional neutrophils to the site of infection (and thus killing more *R. equi*). Furthermore, the ability of IL-8 to attract T cells may be important for facilitating antigen presentation at the immunologic synapse and initiating a cell-mediated immune response (Baggiolini et al., 1995).

IFNγ is critical for induction of a Th1-type cell-mediated immune response (Langrish et al., 2004). There is unequivocal evidence of the protective nature of IFNγ in bacterial infections such as *M. tuberculosis* (Flynn et al., 1993). Likewise, several studies have shown that IFNγ is important in protection against and pulmonary clearance of *R. equi* in experimentally infected mice (Kanaly et al., 1993, 1995) and adult horses (Hines et al., 2003). Recent reports have demonstrated that foals produce relatively less IFNγ at birth, both by PBMCs (Boyd et al., 2003; Breathnach et al., 2006) and by pulmonary cells (Breathnach et al., 2006), which suggests that relatively diminished IFNγ production may contribute to the increased susceptibility of newborn foals to infection with *R. equi*. In contrast, another recent study demonstrated that *R. equi*-stimulated bronchial lymph node (BLN) cells from foals experimentally infected with *R. equi* at 7 to 10 days of age expressed significantly greater IFNγ mRNA than *R. equi*-stimulated BLN cells from experimentally infected adults (Jacks et al., 2007). That study, however, measured IFNγ expression 15 days post-infection (at 3 to 4 weeks of age), which may account for some of the discrepancy between the report by Jacks et al. and the previously mentioned studies that demonstrated relatively lower IFNγ expression

in foals earlier in life (Boyd et al., 2003; Breathnach et al., 2006). In the present study there was an effect of time on expression of IFNγ mRNA; however, this effect was most likely attributable to stimulation of the neutrophils by processing. The expression of IFNγ mRNA by adult equine neutrophils was not significantly increased by stimulation with virulent or avirulent *R. equi*. One possible explanation for failure to induce a significant response is that maximal expression of IFNγ mRNA may have occurred after the time-points used in our study, viz., 2 h and 4 h. We selected these time-points based on preliminary data (unpublished) indicating that cytokine expression was most consistently measured within a 4 h time period, and a previous report that demonstrated a 450% increase in IFNγ mRNA expression by human neutrophils stimulated for 1 h with LPS+IL-12+IL-15 (Ethuin et al., 2004). In another report, however, neutrophils infected with *Nocardia asteroides* produced significant amounts of IFNγ as late as 3 days post-infection (Ellis and Beaman, 2002). Moreover, a previous publication demonstrated that expression of IFNγ mRNA by equine PBMCs was 24-fold higher after 24 h of ConA stimulation than after 4 h of ConA stimulation (Giguere and Prescott, 1999). Although assumptions based on the kinetics of IFNγ mRNA expression by different cell types should be made with caution, it is possible that expression of IFNγ mRNA by equine neutrophils did not occur at the time-points we measured. It is also possible that equine neutrophils contain preformed stores of bioactive IFNγ, similar to those observed in human neutrophils (Ethuin et al., 2004), but do not synthesize IFNγ *de novo*. Alternatively, equine neutrophils may not be a major source of this cytokine, a finding consistent with previous observations (Joubert et al., 2001).

In the present study, expression of TNFα mRNA by equine neutrophils was significantly increased in response to stimulation with virulent and avirulent *R. equi* following both 2 h and 4 h of incubation. The importance of TNFα in the course of disease arising from infection with *R. equi* has been demonstrated in mice. Depletion of TNFα in these mice resulted in a lethal course of infection despite the mice being given a sub-lethal dose of virulent *R. equi* (Kasuga-Aoki et al., 1999). TNFα plays an important role in protection against infection caused by other intracellular pathogens, such as *M. tuberculosis* (Flynn et al., 1995; Bekker et al., 2001). Recently, TNFα production by neutrophils infected with *M. tuberculosis* was demonstrated to coincide with the activation of alveolar macrophages and subsequent production of hydrogen peroxide (Sawant and McMurray, 2007).

Interleukin 12 is a heterodimer composed of the IL-12p35 and the IL-12p40 subunits (Langrish et al., 2004). Animal studies have shown that IL-12 is critical for the control of many intracellular infections, such as those caused by *Mycobacteria* spp. and *Salmonella* spp. (Fieschi et al., 2003). In this study, there was very little expression of IL-12p35 mRNA detected by real-time PCR. The slight mRNA expression that was present may have been due to contaminating lymphocytes and macrophages, which constituted less than 2% of the neutrophil preparations. As with IFNγ, it is possible that expression of IL-12p35 mRNA did not occur at the time-points evaluated, or that equine neutrophils contained preformed stores of bioactive IL-12, a finding that has been demonstrated in neutrophils from humans and mice (Bliss et al., 2000; Denkers et al., 2003; Ethuin et al., 2004), but do not contribute to *de novo* synthesis of this cytokine.

Despite the lack of differential expression of IL-12p35 mRNA, there was differential expression of IL-12p40 mRNA. This finding is consistent with a recent publication that demonstrated the differential expression of IL-12p40 mRNA, but not IL-12p35 mRNA, by adult equine macrophages and dendritic cells in response to stimulation with cytosine-phosphate-guanosine oligodeoxynucleotides (CpG-ODN) (Flaminio et al., 2007). The differential expression of IL-12p40 mRNA might represent a suppression of a Th1-type response, because excess production of homodimeric IL-12p40 has been documented, *in vitro* and *in vivo*, to inhibit production and function of IL-12 (Kato et al., 1996; Heinzel et al., 1997); however, given the resistance of adult horses to *R. equi* pneumonia, suppression of a Th1-type response is an unlikely consequence of *R. equi*-induced IL-12p40 mRNA expression.

Although the distinct roles of the IL-12p35 and IL-12p40 subunits are not clear, the IL-12p40 subunit is a known component of another important heterodimer (composed of the IL-12p40 and IL-23p19 subunits), IL-23 (Harrington et al., 2006). Thus, we investigated the possibility that neutrophil stimulation with *R. equi* induced the production of IL-23, rather than IL-12, by measuring the differential expression of IL-23p19 mRNA. Indeed, we found that expression of IL-23p19 mRNA was significantly increased upon exposure to *R. equi*. Interestingly, the virulent strain of *R. equi* induced significantly greater expression of IL-23p19 mRNA than did the avirulent strain. As mentioned previously, except for the presence of the virulence plasmid the strains of *R. equi* used in this study were isogenic. Thus, it is possible that the observed differences in IL-23p19 mRNA expression of neutrophils stimulated with avirulent *R. equi* versus

those stimulated with virulent *R. equi* reflect a role for the virulence plasmid in modulating the immune response, and there is some precedence for this. In a study that compared the cytokine mRNA expression profiles of $CD4^+$ T cells and pulmonary cells from foals experimentally infected with avirulent *R. equi* to those infected with virulent *R. equi*, it was demonstrated that virulent *R. equi* induced significantly greater expression of IL-12p40 mRNA in lung tissue, and a nearly significant increase in expression of IFNγ mRNA, than did avirulent *R. equi*; however, in $CD4^+$ T cells, virulent *R. equi* induced significantly less expression of IFNγ mRNA than did avirulent *R. equi* (Giguere et al., 1999b). Although that study and the present study suggest that the presence of the virulence plasmid may alter the immune response to *R. equi*, further work is needed to draw conclusions about such a role. Comparisons between the study by Giguere et al. (1999b) and the present study should be made with caution because there were important differences between studies, including the types of cells and ages of horses that were examined.

Because adult horses are generally refractory to disease caused by *R. equi*, it will be important with respect to pathogenesis of the disease to determine whether the observed difference in expression of IL-23p19 mRNA by neutrophils stimulated with virulent and avirulent *R. equi* also occurs in foals. This could have important immunological implications, because the production of IL-23 drives the expansion and survival of an alternative T-cell subset characterized by the production of the pro-inflammatory cytokine IL-17, which acts in part to mobilize neutrophils from the bone marrow and induce the acute phase response via IL-6 and TNFα production (Langrish et

al., 2004; Harrington et al., 2006). Thus, an entirely different T helper cell subset (Th17) may play a complementary role in the outcome of infection with *R. equi*. There is increasing evidence that the IL-23/IL-17 immune pathway plays an important protective role in the early immune response against pulmonary pathogens, such as *Klebsiella pneumoniae* and *M. tuberculosis* (McKenzie et al., 2006; Umemura et al., 2007). Moreover, a recent report demonstrated that Th17 cells persisted in the lungs of *M. tuberculosis* vaccinated mice and, following experimental infection, stimulated the recall response of IFNγ producing $CD4^+$ cells (Khader et al., 2007). The effect of this response was abrogated in the absence of IL-23, demonstrating a requirement for IL-23 in maintaining a persistent population of Th17 cells. The finding that adult equine neutrophils responded rapidly to *R. equi* exposure by markedly increasing IL-12p40 and IL-23p19 mRNA expression suggests that neutrophils may provide an early source of IL-23. This in turn may contribute to the resistance of adult horses to *R. equi* by recruiting the IFNγ producing $CD4^+$ T cells that are required for clearance of *R. equi* from the lungs (Hines et al., 2003). The finding by Giguere et al. that virulent *R. equi* induced significantly less IFNγ mRNA expression by $CD4^+$ T cells might reflect the ability of the virulence plasmid to modulate the immune response in foals by suppressing a Th17-induced recall response of IFNγ producing $CD4^+$ T cells, thereby allowing the organism to proliferate and cause disease.

In the present study, we demonstrated the induction of cytokine mRNA expression by adult equine neutrophils in response to *in vitro* stimulation with *R. equi*. The mechanisms by which *R. equi* stimulated that expression, however, were not

addressed in this study, and it is unknown whether phagocytosis and bactericidal capacity correlated to the observed increases in cytokine expression. Although it is possible that surface interactions between *R. equi* and equine neutrophils contributed to the observed changes in cytokine expression, it is unlikely that these interactions alone resulted in cytokine expression. A number of studies have demonstrated that equine neutrophils rapidly phagocytize and kill *R. equi in vitro* (Yager et al., 1986, 1987; Hietala and Ardans, 1987b; Martens et al., 1988). Another study showed no differences between the extent of phagocytosis of avirulent and virulent *R. equi* by macrophages (Toyooka et al., 2005). Thus, it is likely that a combination of surface interactions, phagocytosis, and bacterial killing contributed to the increased cytokine mRNA expression observed in this study. Nevertheless, the ability of live *R. equi* to stimulate the mRNA expression of pro-inflammatory cytokines by equine neutrophils strongly suggests that neutrophil-derived cytokines contribute importantly to the cytokine milieu that drives the development of an adaptive immune response. The response of neutrophils to dead *R. equi*, however, may differ and it would be helpful to know what these differences might be, as they may provide insight into host-agent interactions and the ontogeny of the immune response. As mRNA expression does not necessarily reflect release of bioactive protein, additional studies will be necessary to correlate mRNA expression with cytokine secretion by equine neutrophils (and to determine whether stores of preformed cytokines exist), to evaluate the quality and quantity of cytokine expression/secretion profiles from foal neutrophils, and to assess temporal differences in these profiles between foals that are susceptible to disease caused by *R. equi* and foals

that are not.

In summary, we have demonstrated that mRNA expression of certain pro-inflammatory cytokines by adult equine neutrophils is induced in response to exposure to virulent and avirulent *R. equi*. The findings of this study should have a significant impact on future research efforts concerning *R. equi*, because they are the first to describe cytokine mRNA expression by *R. equi*-infected equine neutrophils, and importantly the potential role of the IL-23/IL-17 pathway in immunity to infection with *R. equi*. This study will serve as precedent for future studies involving the interaction of the developing immune system of foals and *R. equi*.

CHAPTER III

AGE-RELATED CHANGES IN CYTOKINE EXPRESSION BY NEUTROPHILS OF FOALS STIMULATED WITH VIRULENT *Rhodococcus equi in vitro*

OVERVIEW

Although evidence exists that neutrophils play a vital role in resistance to infection with *R. equi*, the means by which neutrophils exert their effects have not been clearly defined. In the present study we evaluated differences in cytokine expression by unstimulated and *R. equi*-stimulated neutrophils obtained from newborn foals and subsequently from these same foals at 2-, 4-, and 8-weeks of age. Stimulation with virulent *R. equi* induced significantly ($P< 0.05$) greater expression of IFNγ, TNFα, IL-6, IL-8, IL-12p40, and IL-12p35 mRNA relative to expression by unstimulated neutrophils, and there were significant effects of age on expression of IL-6, IL-8, and IL-12p40 mRNA. Neutrophil expression of IL-6 and IL-8 mRNA in newborn foals was significantly greater than expression at 2-, 4-, and 8-weeks of age. Expression of IL-12p40 mRNA by *R. equi*-stimulated neutrophils from newborn and 2-week-old foals did not differ from that of unstimulated neutrophils; however, expression of IL-12p40 mRNA by neutrophils from 4- and 8-week-old foals was significantly greater upon stimulation with *R. equi* than without stimulation.

These results demonstrate that foal neutrophils increase mRNA expression of many pro-inflammatory cytokines, including IFNγ, in response to *in vitro* stimulation with *R. equi*, and that the magnitude of this expression with respect to IL-6, IL-8, and IL-12p40 mRNA is influenced by age. The clinical importance of the age-related difference in *R. equi*-induced expression of IL-12p40 mRNA to susceptibility of foals to *R. equi* pneumonia remains to be determined.

INTRODUCTION

Rhodococcus equi is a Gram-positive, facultative-intracellular pathogen that causes a severe form of pneumonia in foals, and has been recognized as an emerging human pathogen (Cohen and Martens, 2007). Infection in both horses and humans is characterized by severe pyogranulomatous pneumonia, similar to pneumonia caused by the closely related bacterium, *Mycobacterium tuberculosis*.

The morbidity and mortality associated with *R. equi* infection are high, and treatment is generally prolonged, expensive, and associated with adverse effects (Cohen and Martens, 2007). Furthermore, affected foals that survive are less likely than age-matched cohorts to race (Ainsworth et al., 1998). Thus, the impact of this disease on the equine industry is large. Currently, there are no vaccines available to prevent disease caused by *R. equi,* and the only method for preventing *R. equi* pneumonia is the administration of *R. equi* hyperimmune plasma (Martens et al., 1989a), which is expensive, labor-intensive, and not universally effective (Hurley and Begg, 1995; Cohen and Martens, 2007).

A better understanding of the epidemiology of *R. equi* will lead to improved strategies for prevention and control of the disease. Of particular importance is the need to understand why some foals at endemic farms develop disease while other foals in the same environment do not. It is possible that susceptibility to infection is determined by differences in immune function among foals, and there is some evidence to support this hypothesis. A previous study demonstrated that the ratio of $CD4^+/CD8^+$ T cells in blood samples from 2-week-old foals was significantly lower prior to disease in foals that later developed *R. equi* pneumonia than in foals that did not develop the disease (Chaffin et al., 2004). Both $CD4^+$ and $CD8^+$ T lymphocytes (T cells) are important in mice for protecting against *R. equi* infection, and $CD4^+$ T cells appear to play a dominant and essential role (Nordmann et al., 1992; Kanaly et al., 1993, 1996; Ross et al., 1996). In mature horses, clearance of virulent *R. equi* is dependent on IFNγ production by $CD4^+$ and $CD8^+$ T cells (Hines et al., 2003). *Rhodococcus equi*-specific CTL responses also

play a crucial role in clearance of *R. equi* by adult horses (Hines et al., 2001; Patton et al., 2004).

The aforementioned are all examples of adaptive immune responses that are critically involved in resistance to infection with *R. equi*; however, there is also evidence indicating that innate immunity plays an important role in protection against *R. equi*. Innate immune responses by macrophages and dendritic cells of mice have recently been documented to be rapidly activated in response to *R. equi* infection, leading the investigators to postulate that efficient and effective stimulation of innate immune responses are essential for the lack of virulence of *R. equi* infection in immunocompetent adult animals (Darrah et al., 2004). Several lines of evidence indicate that neutrophils may also be critical to protecting against infection by *R. equi*. Neutrophils from some foals <1 week of age were shown to have a markedly reduced bactericidal capacity for *R. equi* compared to age-matched cohorts, and at 1 month of age the capacities of all foals were similar (Martens et al., 1988). Prior to disease, blood concentrations of neutrophils at 2-weeks and 4-weeks of age were significantly lower among foals that developed *R. equi* pneumonia than foals at the same farms that did not develop disease (Chaffin et al., 2004). Furthermore, neutrophils play a protective role in mice infected with *R. equi*: mice with neutropenia had significantly greater tissue concentrations of *R. equi* than control mice (Martens et al., 2005). In addition, it has been demonstrated that the phagocytic function of foal neutrophils is less than that of adult horses (Demmers et al., 2001; McTaggart et al., 2001). Although evidence indicates that this reduced phagocytic function is a consequence of reduced opsonic capacity of foal serum (as the

addition of adult sera reversed the reduced phagocytic function), foal neutrophils incubated with adult horse sera have also been demonstrated to have reduced phagocytic capacity relative to that of adult neutrophils (Grondahl et al., 1999; Demmers et al., 2001).

Neutrophils are known to provide protection against a number of other intracellular pathogens (Dalrymple et al., 1995; Pedrosa et al., 2000; Godaly and Young, 2005), but the mechanism by which they protect against such intracellular infections remains unclear. In addition to direct effects of phagocytosis, non-phagocytic mechanisms may also play a role. In other species, neutrophils produce a variety of cytokines and chemokines that modulate the immune response by recruiting and activating other effector cells of the immune system (Denkers et al., 2003; Appelberg, 2006). Thus, neutrophils are an important link between innate and adaptive cellular immune responses.

We recently reported that adult equine neutrophils express significantly greater levels of TNFα, IL-6, IL-8, IL-12p40, and IL-23p19 mRNA in response to stimulation with virulent and avirulent *R. equi* (J.R. Harrington, S. Payne, N. D. Halbert, R. J. Martens, and N. D. Cohen, submitted for publication). The purpose of the study reported here was to compare the age-related mRNA expression of selected pro-inflammatory cytokines by foal neutrophils stimulated *in vitro* with virulent *R. equi*. Cytokine mRNAs examined included interleukin (IL)-6, IL-8, IL-12p35, IL-12p40, interferon gamma (IFNγ), and tumor necrosis factor alpha (TNFα). We hypothesized that cytokine mRNA expression by foal neutrophils would be significantly increased by exposure to virulent

R. equi, and that mRNA expression of these cytokines would increase with age.

MATERIALS AND METHODS

Reagents

RPMI-1640, HBSS, HEPES, PBS, cell-culture-grade distilled water, RNase/DNase free distilled water, and $NaHCO_3$ were obtained from Gibco-Invitrogen (Invitrogen Corporation, Grand Island, NY). Ficoll-Paque plus was obtained from Amersham Biosciences (Amersham Biosciences, Pittsburgh, PA). BSA and NaCl were purchased from Sigma (Sigma Aldrich, St. Louis, MO). Primary working buffer consisted of the following: 1× HBSS, 10mM HEPES, and 8.9 mM $NaHCO_3$. Lysis buffers A and B consisted of the following: 0.2 and 1.6% NaCl, respectively, 20 mM HEPES, and 1% BSA. The pH of all buffers and reagents was adjusted to 7.4 with NaOH.

Bacteria

Virulent (ATCC 33701 P^+) *R. equi* were grown in *R. equi* minimal media overnight at 37°C with rocking. The bacteria were then centrifuged at 2,000 × g for 10 min. The bacterial pellets were washed 1× with RPMI-1640, and then resuspended at an approximate concentration of 1×10^8 CFU ml^{-1} RPMI-1640. The bacteria were aliquoted and frozen at −80°C. Prior to use, the bacteria were thawed and 10-fold serial dilutions were performed in PBS. The *R. equi* were quantitatively cultured on trypticase soy agar plates supplemented with 5% sheep red blood cells. Prior to infecting equine neutrophils, the *R. equi* were opsonized at 37°C for 1 h with 5% fresh-frozen serum obtained from an adult donor horse known to have antibodies to *R. equi*. Serum from this horse was used

in order to eliminate potential interindividual variability in opsonic capacity of serum among foals, to eliminate the possibility that foals have less opsonic capacity than adults, and to ensure opsonization of *R. equi*.

Animals

Approximately 40 ml of whole blood was obtained from each of 16 healthy Quarter Horse foals owned by Texas A&M University. Blood samples were taken within 24 h of birth (herein referred to as newborn), and subsequently at 2-, 4-, and 8-weeks of age. Blood samples from foals were processed as described below immediately after collection. Adequate transfer of passive immunity was confirmed in foals by measuring plasma IgG concentrations using the SNAP® Test (Idexx, Westbrook, MN) by 24 h of age. All foals were deemed to be healthy by physical examination performed by an equine veterinarian from Texas A&M University within 48 h of age.

Neutrophil purification

Blood samples were centrifuged at 500 × g for 10 min. The plasma and buffy coat were layered onto Ficoll-Paque and centrifuged at 500 × g for 20 min. The neutrophil pellet was washed 2 times with primary working buffer, and the residual erythrocytes were lysed 2 times with a hypotonic lysis protocol as follows: each neutrophil pellet was resuspended in 7.5 ml of lysis buffer A. After a brief incubation of 60 sec at room temperature, 7.5 ml of lysis buffer B was added to the mixture. The tubes were then centrifuged at 300 × g for 10 min. After discarding the supernatant containing lysed erythrocytes, the pellet was resuspended in RPMI-1640. The neutrophils were >98% viable as indicated by exclusion of trypan blue. Random samples of purified

neutrophils were submitted to the Texas A&M Veterinary Teaching Hospital's clinical pathology laboratory for differential cell counts to ensure that the neutrophil purification method effectively removed contaminating lymphocytes and monocytes. Contaminating cell types composed <2% of the cell preparations and included monocytes, lymphocytes, and an occasional eosinophil.

Neutrophil stimulation

Neutrophils (~1×10^7 ml^{-1}) were incubated for 2 h and 4 h at 37°C with media (unstimulated control) or with virulent *R. equi* (MOI 10:1). In order to determine the baseline level of cytokine expression as a point of reference, a 0 h unstimulated sample was obtained for every experiment. Following stimulation, all neutrophils were pelleted by centrifugation at 4°C for 5 min at 2,000 × g, and the supernatant was discarded.

RNA and cDNA preparation

RNA was extracted immediately from each neutrophil pellet using the RNeasy mini kit (Qiagen, Valencia, CA). The concentration and purity of the RNA was assessed by spectrophotometry. Aliquots of RNA were prepared in DNase/RNase-free dH_2O to a final concentration of 0.05 $\mu g\ \mu l^{-1}$ and frozen at −80°C until subsequent cDNA synthesis. Prior to initiating cDNA synthesis, each aliquot of RNA was treated with amplification grade DNase I according to the manufacturer's protocol (Invitrogen Corporation, Grand Island, NY). cDNA was subsequently synthesized with SuperScript III First-Strand synthesis System for RT-PCR (Invitrogen Corporation, Grand Island, NY) using the manufacturer's protocol. In order to avoid synthesis of *R. equi*-derived cDNA, the cDNA synthesis was primed with oligo $(dT)_{20}$ primers provided with the

cDNA synthesis kit. Upon completion of cDNA synthesis, all cDNA was stored at −80°C until use.

Real-time PCR conditions

Gene-specific primers and probes have been previously described for equine β2M, IL-6, IL-8, IL-12p35, IL-12p40, TNFα (J.R. Harrington, S. Payne, N. D. Halbert, R. J. Martens, and N. D. Cohen, submitted for publication), and IFNγ (Garton et al., 2002). Each 25-µl real-time PCR reaction contained the following: 2 µl cDNA; 900 nM of each primer (Integrated DNA Technologies, Coralville, IA); 250 nM TaqMan probe; 12.5 µl 1× TaqMan Universal Master Mix (Applied Biosystems, Foster City, CA); and molecular-grade water (Invitrogen Corporation, Grand Island, NY). Amplification and data analysis were carried out on a GeneAmp 7500 Sequence Detection System (Applied Biosystems, Foster City, CA). The thermal profile consisted of an initial hold at 50ºC for 2 min, followed by a single denaturation at 95°C for 10 min, and then 40 cycles of 95°C for 15 sec, 60°C for 60 sec.

Statistical analysis

Data were transformed logarithmically for analysis to approximate a normal distribution. Data were analyzed using linear mixed-effects models (Pinheiro and Bates, 2000); to account for repeated measures on individuals, horse was modeled as a random effect and time, stimulus, age, and their respective interaction terms were modeled as fixed effects. Model fit was assessed by graphical methods to determine whether there was evidence of poor fit or violation of model assumptions. Post-hoc testing of between-group differences was made using the method of Scheffé. A significance level of P<

0.05 was used. Additionally, the effect of age on the Ct (the cycle at which amplification crossed the threshold) and delta Ct (ΔCt, the Ct value of the target gene – the Ct value of the endogenous control gene) values of all cytokines for unstimulated neutrophils at 0 h, 2 h, and 4 h was examined using mixed-effects modeling to assess whether there were significant differences among ages in basal cytokine expression that could have confounded observed differences of the relative cytokine expression data. Statistical analyses were performed using S-PLUS (version 7.0; Insightful, Inc).

RESULTS

Values of CT or ΔCT did not differ significantly among ages for any of the cytokines. These findings indicated that basal expression was similar among ages.

Multivariable analyses examined the effects on cytokine expression of neutrophils of ***time*** (i.e., comparison of cytokine mRNA expression among incubation times, viz., baseline, 2 h, and 4 h), ***stimulus*** (i.e., stimulated by virulent *R. equi* or unstimulated), and ***age*** of the foal. Reporting of results for each cytokine is organized to discuss each of these effects sequentially.

Expression of IFNγ mRNA by unstimulated neutrophils incubated for 2 h and 4 h did not differ significantly from each other or from baseline expression levels at any age. Foal neutrophils stimulated with *R. equi* for 2 h and 4 h demonstrated significantly greater ($P < 0.05$) IFNγ mRNA expression relative to baseline at all ages, but expression of this cytokine was not significantly different when comparing values at 2 h and 4 h at any age (Fig. 6). At each age, expression of IFNγ mRNA was significantly ($P < 0.05$) greater for *R. equi*-stimulated neutrophils than for unstimulated neutrophils; expression

was approximately 2-fold greater in the stimulated neutrophils relative to the unstimulated neutrophils. For either stimulus, there was no significant effect of age on expression of IFNγ mRNA (Fig. 6).

Expression of TNFα mRNA by unstimulated neutrophils incubated for 2 h and 4 h did not differ significantly from each other or from baseline expression levels at any age. A significant ($P < 0.05$) increase in expression of TNFα mRNA relative to baseline was observed for neutrophils stimulated with *R. equi* at all ages, but there was no significant difference at any age between expression values at 2 h and 4 h. Expression of TNFα mRNA was significantly ($P < 0.05$) greater in stimulated than unstimulated neutrophils (Fig. 7) at each age; the magnitude of effect was approximately 7-fold. For either stimulus, there was no significant effect of age on expression of TNFα mRNA by foal neutrophils.

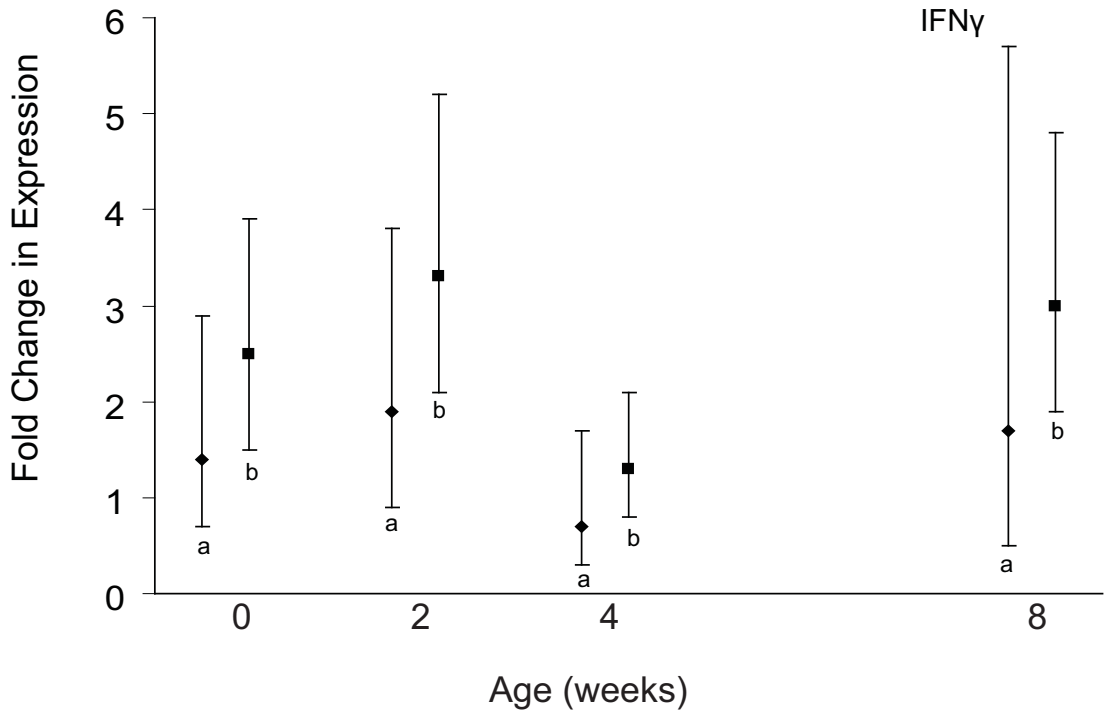

Fig. 6. Quantitative expression of IFNγ mRNA by foal neutrophils incubated without *R. equi* stimulation (♦) or with *R. equi* stimulation (■). As there were no significant differences in cytokine expression among neutrophils incubated for 2 h and 4 h, data points represent pooled values collected from 2 h and 4 h. All values were normalized to the endogenous control, β2M, and fold differences were calculated using baseline control values (unstimulated cells at time 0). For a given stimulus, values with a different letter represent significant differences within an age. Values with a different number represent significant differences between ages. Values in parentheses above each data point represent the fold-change in expression of that data point relative to a baseline value of 1. Error bars represent the 95% confidence intervals. A significance level of $P<0.05$ was used.

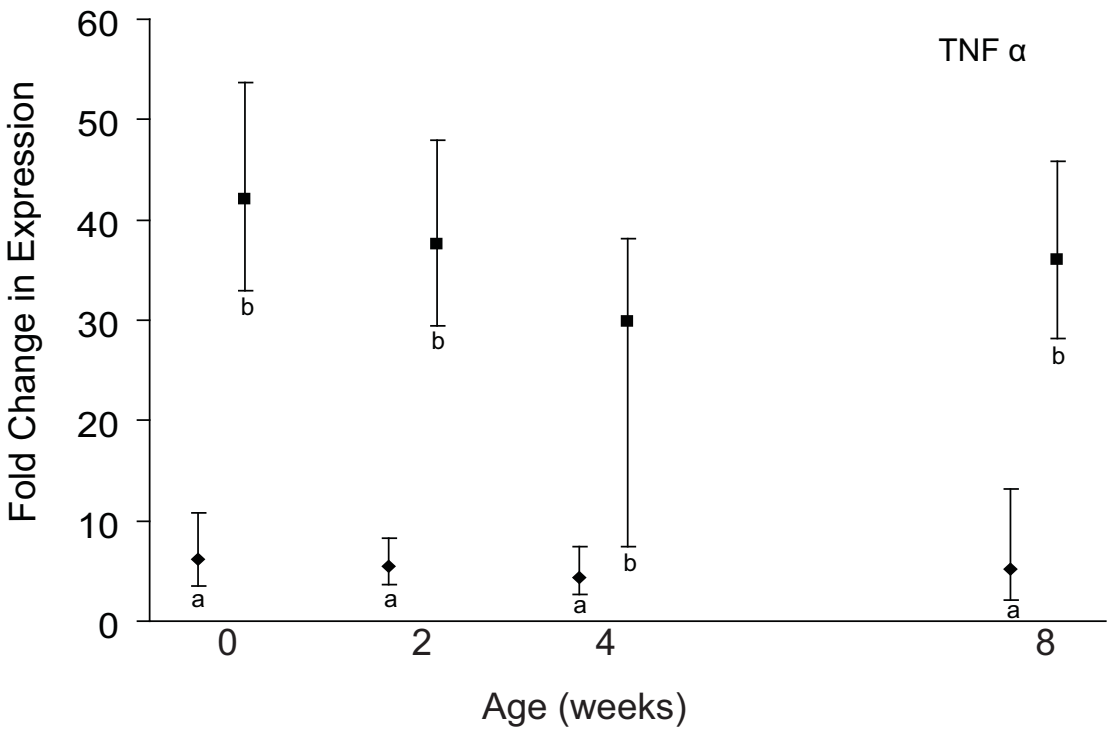

Fig. 7. Quantitative expression of TNFα mRNA by foal neutrophils incubated without *R. equi* stimulation (♦) or with *R. equi* stimulation (■). As there were no significant differences in cytokine expression among neutrophils incubated for 2 h and 4 h, data points represent pooled values collected from 2 h and 4 h. All values were normalized to the endogenous control, β2M, and fold differences were calculated using baseline control values (unstimulated cells at time 0). For a given stimulus, values with a different letter represent significant differences within an age. Values with a different number represent significant differences between ages. Values in parentheses above each data point represent the fold-change in expression of that data point relative to a baseline value of 1. Error bars represent the 95% confidence intervals. A significance level of $P<0.05$ was used.

Although expression of IL-6 or IL-8 mRNA by unstimulated neutrophils incubated for 2 h did not differ significantly from those incubated for 4 h, there was a significant ($P< 0.05$) increase in expression by unstimulated neutrophils at these times relative to baseline at all ages. Similarly, expression of IL-6 and IL-8 mRNA by stimulated neutrophils did not differ significantly at any age between 2 h and 4 h, but values at both times were significantly greater than baseline. At each age, expression of IL-6 mRNA (Fig. 8) and IL-8 mRNA (Fig. 9) was significantly ($P < 0.05$) greater by stimulated than unstimulated neutrophils. The magnitude of the effect of stimulation by *R. equi* varied by age but ranged from 20- to nearly 200-fold. There was a significant ($P< 0.05$) effect of age on expression of IL-6 mRNA (Fig. 8) and IL-8 mRNA (Fig. 9). Regardless of stimulus, mRNA expression of both cytokines in 2-, 4-, and 8-week-old foals was significantly ($P< 0.05$) lower than expression in newborn foals, but the 3 older ages did not differ significantly from 1 another.

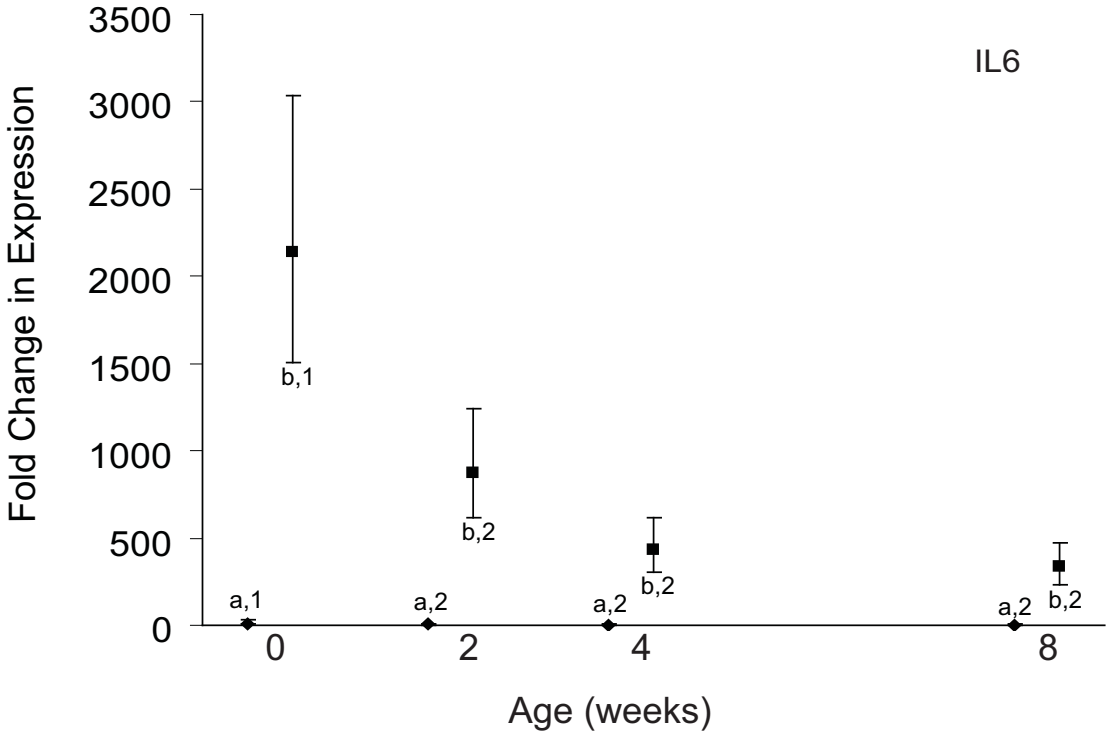

Fig. 8. Quantitative expression of IL-6 mRNA by foal neutrophils incubated without *R. equi* stimulation (♦) or with *R. equi* stimulation (■). As there were no significant differences in cytokine expression among neutrophils incubated for 2 h and 4 h, data points represent pooled values collected from 2 h and 4 h. All values were normalized to the endogenous control, β2M, and fold differences were calculated using baseline control values (unstimulated cells at time 0). For a given stimulus, values with a different letter represent significant differences within an age. Values with a different number represent significant differences between ages. Values in parentheses above each data point represent the fold-change in expression of that data point relative to a baseline value of 1. Error bars represent the 95% confidence intervals. A significance level of $P<0.05$ was used.

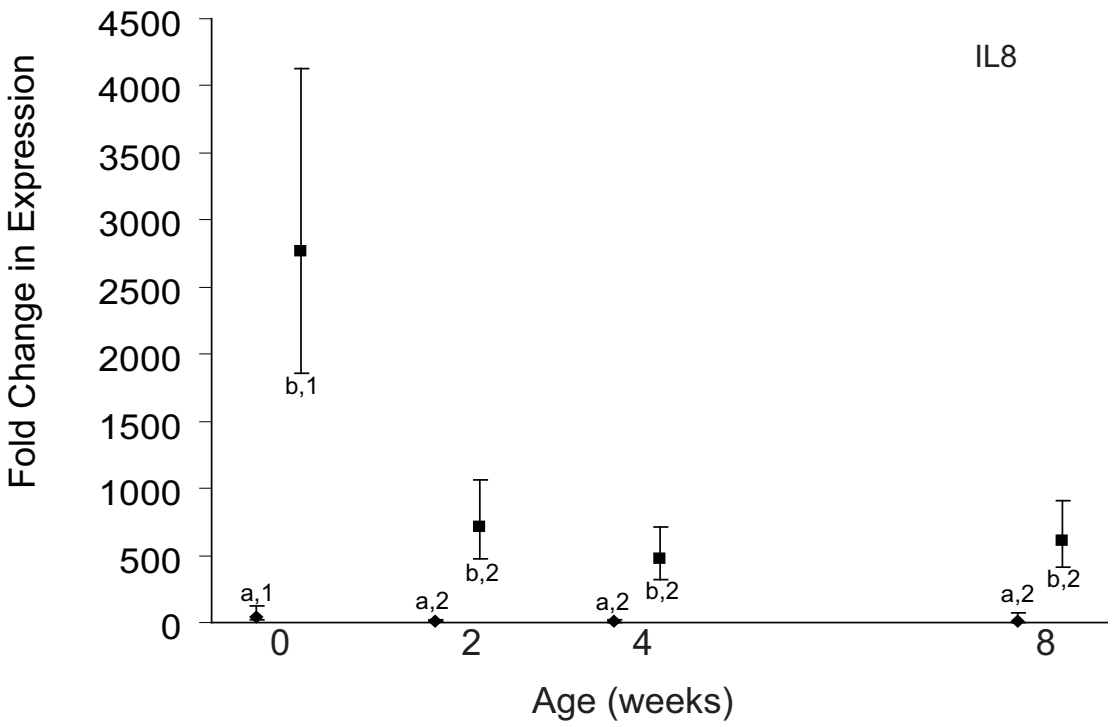

Fig. 9. Quantitative expression of IL-8 mRNA by foal neutrophils incubated without *R. equi* stimulation (♦) or with *R. equi* stimulation (■). As there were no significant differences in cytokine expression among neutrophils incubated for 2 h and 4 h, data points represent pooled values collected from 2 h and 4 h. All values were normalized to the endogenous control, β2M, and fold differences were calculated using baseline control values (unstimulated cells at time 0). For a given stimulus, values with a different letter represent significant differences within an age. Values with a different number represent significant differences between ages. Values in parentheses above each data point represent the fold-change in expression of that data point relative to a baseline value of 1. Error bars represent the 95% confidence intervals. A significance level of $P<0.05$ was used.

When compared to baseline expression levels of IL-12p35 mRNA, expression by unstimulated neutrophils was significantly ($P < 0.05$) lower than baseline at 2 h but significantly greater than baseline at 4 h (except at 4-weeks of age), and the difference in expression was greater at 4 h than at 2 h by unstimulated cells at all ages. Expression of IL-12p35 mRNA was significantly greater than baseline at 2 h and 4 h for all ages (except after 2 h of stimulation at 4-weeks), and was significantly greater at all ages between cells stimulated for 2 h and 4 h (Fig. 10). For a given age and time (i.e., 2 h or 4 h), *R. equi* induced an approximately 2-fold greater expression of IL-12p35 mRNA that was statistically significant ($P< 0.05$). Accounting for time and stimulus, there was no significant effect of age on expression of IL-12p35 mRNA (Fig. 10).

At each age, expression of IL-12p40 mRNA was significantly greater after 4 h than 2 h for both unstimulated and stimulated neutrophils (Fig. 11). The effect of stimulus varied with age (Fig. 11). In newborn and 2-week-old foals, there was no significant effect of stimulation with *R. equi* after either 2 h or 4 h; however, in 4-and 8-week-old foals, stimulation with *R. equi* induced greater than a 2-fold increase in expression of IL-12p40 mRNA that was significant ($P < 0.05$). There were significant effects of age on expression of IL-12p40 mRNA that varied by stimulus. For unstimulated neutrophils, after both 2 h and 4 h of incubation, there was a significant ($P < 0.05$) decrease in expression of IL-12p40 mRNA in 4-and 8-week-old foals relative to expression in newborn and 2-week-old foals (Fig. 11). For stimulated neutrophils after both 2 h and 4 h of incubation, however, there were no significant differences among ages in expression of IL-12p40 mRNA.

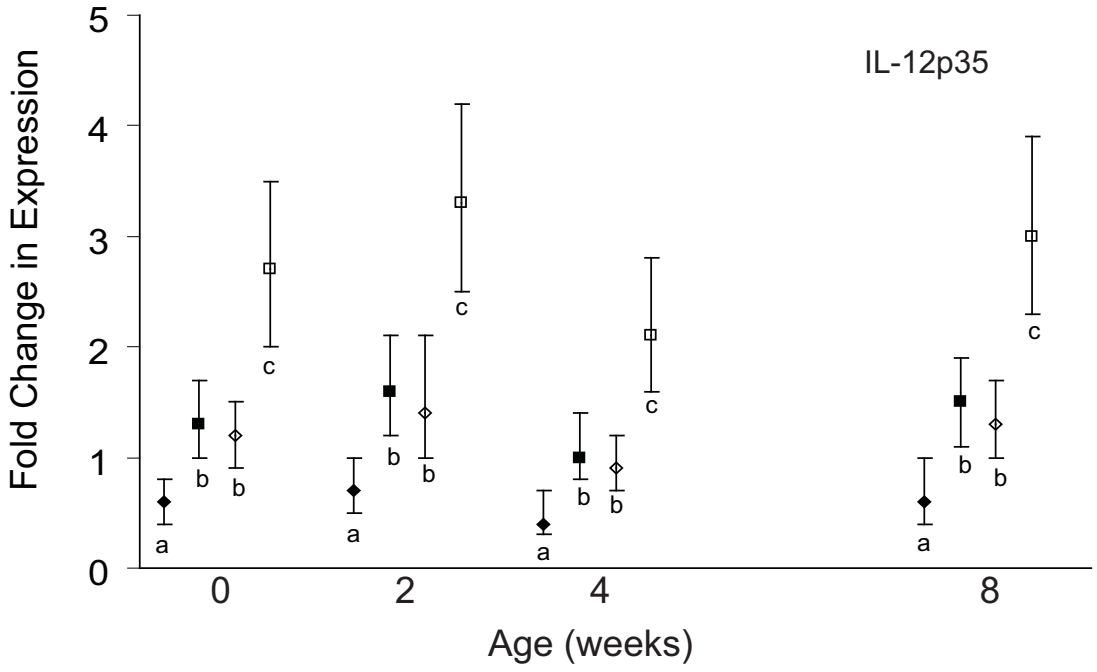

Fig. 10. Quantitative expression of IL-12p35 mRNA by foal neutrophils incubated without *R. equi* stimulation for 2 h (♦) and 4 h (◊), or with *R. equi* stimulation for 2 h (■) and 4 h (□). All values were normalized to the endogenous control, β2M, and fold differences were calculated using baseline control values (unstimulated cells at time 0). For a given stimulus, values with a different letter represent significant differences within an age. Values with a different number represent significant differences between ages. Values in parentheses above each data point represent the fold-change in expression of that data point relative to a baseline value of 1. Error bars represent the 95% confidence intervals. A significance level of $P<0.05$ was used.

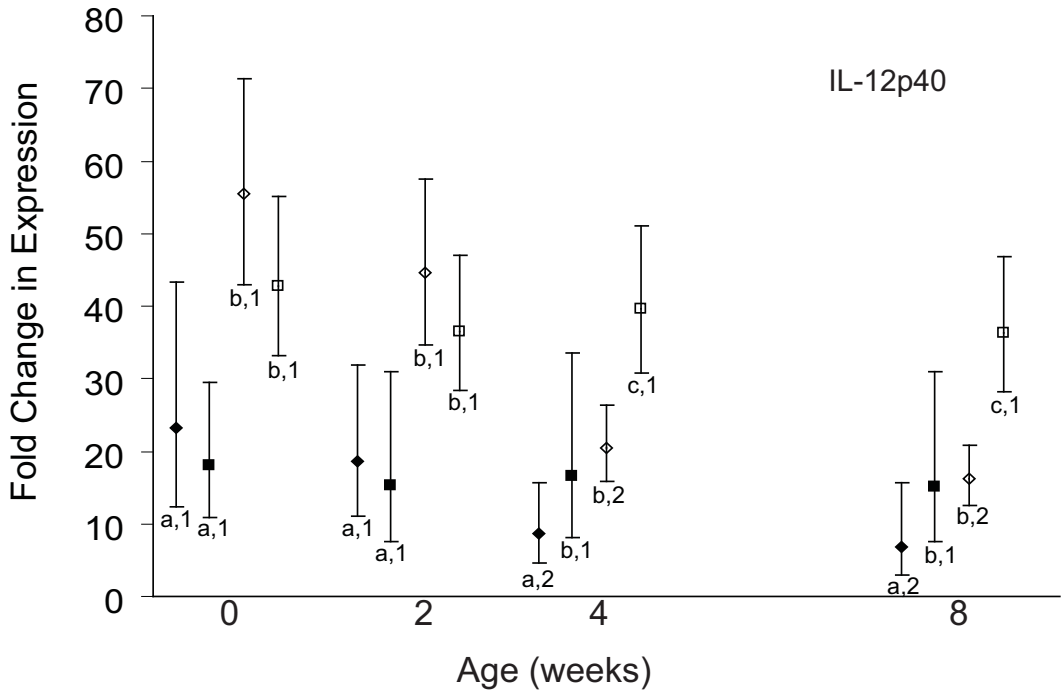

Fig. 11. Quantitative expression of IL-12p40 mRNA by foal neutrophils incubated without *R. equi* stimulation for 2 h (♦) and 4 h (◊), or with *R. equi* stimulation for 2 h (■) and 4 h (□). All values were normalized to the endogenous control, β2M, and fold differences were calculated using baseline control values (unstimulated cells at time 0). For a given cytokine, values with a different letter represent significant differences within an age. Values with a different number represent significant differences between ages. Values in parentheses above each data point represent the fold-change in expression of that data point relative to a baseline value of 1. Error bars represent the 95% confidence intervals. A significance level of $P<0.05$ was used.

DISCUSSION

The aim of the present study was to examine the mRNA expression of specific cytokines by foal neutrophils in response to virulent *R. equi*. Neutrophils were collected from foals within 24 h of birth, and subsequently at 2-, 4-, and 8-weeks of age, incubated with either media or virulent *R. equi*, and then analyzed for differential expression of IFNγ, TNFα, IL-6, IL-8, IL-12p40, and IL-12p35 mRNA. Expression of all cytokine mRNA was significantly increased, relative to baseline values, in response to stimulation with *R. equi*. These results were consistent with the response of adult equine neutrophils to virulent *R. equi (*J.R. Harrington, S. Payne, N. D. Halbert, R. J. Martens, and N. D. Cohen, submitted for publication). There also were significant age-related changes among foals in expression of IL-6, IL-8, and IL-12p40 mRNA. Collectively, these findings suggest that changes in expression of these cytokines might play a role in the outcome of infection with *R. equi*.

There are several lines of evidence indicating that both IFNγ and TNFα play an important role in the immune response to infection with *R. equi*. Experimental infections in adult horses (Hines et al., 2003) and mice (Nordmann et al., 1993; Kanaly et al., 1995; Kasuga-Aoki et al., 1999) demonstrated that IFNγ was required for clearance of *R. equi*. TNFα expression and production was induced in mice (Nordmann et al., 1993) and foals (Giguere and Prescott, 1999) experimentally infected with *R. equi*. Recently it was demonstrated that foals produce relatively less IFNγ at birth, both by PBMCs (Boyd et al., 2003; Breathnach et al., 2006) and by pulmonary cells (Breathnach et al., 2006), which suggests that relatively diminished IFNγ production may contribute to the

increased susceptibility of newborn foals to infection with *R. equi*. In contrast, a recent study demonstrated that *R. equi*-stimulated bronchial lymph node (BLN) cells from foals experimentally infected with *R. equi* at 7 to 10 days of age expressed significantly greater IFNγ mRNA than *R. equi*-stimulated BLN cells from experimentally infected adults (Jacks et al., 2007). That study, however, measured IFNγ expression 15 days post-infection (at 3 to 4 weeks of age), which may account for some of the discrepancy between the report by Jacks et al. and studies that demonstrated relatively lower IFNγ expression in foals at birth (Boyd et al., 2003; Breathnach et al., 2006). None of the studies examining IFNγ expression by foals accounted for the possibility that neutrophils provide an early source of IFNγ. The production of IFNγ (Yeamen et al., 1998; Ellis and Beaman, 2002) and TNFα (Sawant and McMurray, 2007) by neutrophils is well documented in a number of mammalian species. In adult equine neutrophils, expression of TNFα mRNA has been demonstrated, whereas expression of IFNγ mRNA was minimal (Joubert et al., 2001; J.R. Harrington, S. Payne, N. D. Halbert, R. J. Martens, and N. D. Cohen, submitted for publication). In the present study, however, we found that foal neutrophils expressed baseline levels of IFNγ and TNFα mRNA at all ages sampled, and that stimulation with *R. equi* significantly increased this expression. No age-related changes in expression by either unstimulated or stimulated neutrophils were observed for either of these cytokines. The observed increases in expression of IFNγ mRNA resulting from stimulation with *R. equi* were modest; however, because neutrophils are the most numerous cells in the blood, it is plausible that they could be a substantial source of IFNγ. This consideration, coupled with the fact that there were no

age-related differences in expression of IFNγ and TNFα mRNA, indicates that any age-related deficiency in expression of these cytokines does not appear to be attributable to neutrophil responses. It is likely, however, that other cell types (e.g., T cells), which are known to be major producers of IFNγ, play a principal role in expression of IFNγ in foals and that age-related changes in expression of this cytokine by these cells are critical for developing immunity to *R. equi*.

Expression of IL-6 and IL-8 mRNA was significantly increased following stimulation with *R. equi* at all ages sampled, and mRNA expression was significantly greater in newborn foals than in 2-, 4-, and 8-week-old foals. The role of IL-6 in infection is controversial (Rincon et al., 1997), but the majority of the evidence suggests that it is required for protection against a number of bacterial infections (Dalrymple et al., 1995; Ladel et al., 1997; Leal et al., 1999). IL-8 is a potent chemo-attractant for neutrophils, monocytes, and T cells (Baggiolini et al., 1995; Zhang et al., 1995), and has been shown to enhance killing of *M. tuberculosis* by neutrophils (Godaly and Young, 2005). It is interesting to note that the overall magnitude of expression of IL-6 and IL-8 mRNA (relative to baseline expression values) by *R. equi*-stimulated neutrophils from foals at 2-, 4-, and 8-weeks of age was similar to mRNA expression levels by *R. equi*-stimulated neutrophils from adult horses (J.R. Harrington, S. Payne, N. D. Halbert, R. J. Martens, and N. D. Cohen, submitted for publication). Thus, it is possible that in newborn foals an enhanced response with respect to IL-6 and IL-8 mRNA expression is needed for early protection against infection with *R. equi*, but that as the immune system matures and shifts toward a Th1 profile, expression of these cytokines does not need to

be as large. The biological significance of this age-dependent difference in mRNA expression awaits validation by replication of this finding and further investigation, including studies of the association of age and susceptibility to infection with expression of these cytokines.

Unlike the other cytokines studied, there was no stimulatory effect of incubation with *R. equi* on expression of IL-12p40 mRNA, relative to unstimulated neutrophils, in newborn and 2-week-old foals. In contrast, expression of IL-12p40 mRNA in 4- and 8-week-old foals was significantly higher in *R. equi*-stimulated neutrophils than in unstimulated neutrophils. These results demonstrate that stimulation of expression of IL-12p40 mRNA by neutrophils changed with age: at 4 and 8 weeks of age, foal neutrophils responded to stimulation with *R. equi* by significantly increasing expression of IL-12p40 mRNA relative to unstimulated neutrophils, whereas neutrophils from the same foals when they were newborn and 2-weeks-old did not. IL-12p40 is known to be a critical component for host defense against mycobacterial and other infections (Holscher et al., 2001; Cooper et al., 2002), and it is likely to be equally important against *R. equi* infections. Therefore, these findings could have important implications, because they may reflect an innate inability of foal neutrophils to respond appropriately to exposure with *R. equi* by producing IL-12p40. However, because there were no significant differences between ages in expression of IL-12p40 mRNA by *R. equi*-stimulated neutrophils (i.e., the levels in expression of IL-12p40 mRNA by *R. equi*-stimulated neutrophils from newborn foals did not differ significantly from *R. equi*-stimulated neutrophils from these foals at 2-, 4-, or 8-weeks of age, etc) it is also possible that these

observations occurred by chance. Additional independent studies need to be conducted in order to substantiate these findings and to exclude the possibility that the observed age-related changes occurred by chance alone.

Foal neutrophils stimulated with *R. equi* expressed significantly more IL-12p35 mRNA than unstimulated neutrophils, but the magnitude of this expression was quite small. Because expression levels were examined only up to 4 h of stimulation, it is possible that maximal expression of IL-12p35 mRNA occurred later and that the relative expression of IL-12p35 mRNA consequently was underestimated. Alternatively, this finding may indicate that minimal expression of IL-12p35 mRNA is required to make functional IL-12. In light of the fact that other cell types, such as dendritic cells, are major sources of IL-12, it is possible that limited production of IL-12 by neutrophils is of little consequence to host defense against *R. equi*. In a recent publication, however, it was demonstrated that adult horse dendritic cells do not differentially express IL-12p35 mRNA (Flaminio et al., 2007). Thus, an alternative explanation of these findings is that they reflect production of IL-23 rather than IL-12. IL-23 is a heterodimer composed of the IL-12p40 and IL-23p19 subunits that has been shown to compensate for the absence of IL-12 in mice experimentally infected with *M. tuberculosis* (Khader et al., 2005). We previously demonstrated that *R. equi* significantly induced differential expression by neutrophils of adult horses of IL-12p40 and IL-12p39 mRNA, but not IL-12p35 mRNA (J.R. Harrington, S. Payne, N. D. Halbert, R. J. Martens, and N. D. Cohen, submitted for publication). Unfortunately, we lacked sufficient cDNA from the foal neutrophils in this study to examine expression of IL-23p19 mRNA, but we are currently pursuing this line

of investigation. Differential expression of IL-23p19 mRNA in response to *R. equi*, and age-related changes in such expression, could have important immunological implications. Production of IL-23 drives the development of a Th17 response (Harrington et al., 2006), and this response has recently been shown to play a protective role in early immune responses against pulmonary pathogens, such as *Klebsiella pneumoniae* and *M. tuberculosis* (McKenzie et al., 2006; Umemura et al., 2007).

A limitation of this study was that we did not examine the response of foal neutrophils to avirulent *R. equi.* Avirulent isolates of *R. equi* generally do not cause disease in foals, and data from our previous study demonstrated that adult neutrophils responded similarly (in terms of IFNγ, TNFα, IL-6, IL-8, IL-12p40, and IL-12p35 mRNA expression) to virulent and avirulent *R. equi* (J.R. Harrington, S. Payne, N. D. Halbert, R. J. Martens, and N. D. Cohen, submitted for publication); however, the inclusion of avirulent *R. equi* would have broadened the scope and importance of the data presented in this manuscript. Another limitation of this study was that we did not measure the production of bioactive cytokines, which may not have been reflected by changes in mRNA expression levels. As neutrophils from humans and mice contain preformed stores of IFNγ and IL-12 (Bliss et al., 2000; Denkers et al., 2003; Ethuin et al., 2004), it will be important to determine whether the same observations are made in neutrophils from horses. If so, such findings could have important implications for the role of equine neutrophils in the immune response to *R. equi*.

In summary, we have described the differential mRNA expression of 6 pro-inflammatory cytokines by unstimulated and *R. equi*-stimulated neutrophils obtained

from foals within 24 h of birth, and subsequently at 2-, 4-, and 8-weeks of age. We demonstrated that mRNA expression of all cytokines was significantly increased, relative to baseline values, in response to stimulation with *R. equi*. These findings indicate that neutrophils may be an important source of these cytokines and that these cytokines may play a role in host responses to infection with *R. equi*. Furthermore, we demonstrated that changes in expression of IL-6, IL-8, and IL-12p40 mRNA were age-related suggesting that changes in mRNA expression of these cytokines might play a role in the outcome of infection with *R. equi*.

CHAPTER IV

ADDITIONAL EXPERIMENTS

In addition to the work described in Chapters II and III, 2 additional experiments were conducted. The principal objective of this chapter is to summarize that work.

BACTERICIDAL CAPACITY OF FOAL NEUTROPHILS

Blood samples obtained from foals to address the temporal changes in cytokine expression were also used to assess temporal changes in bactericidal capacity of foal neutrophils. Neutrophils were infected with live, virulent *R. equi* (opsonized with the same serum and under conditions identical to those described in chapters II and III) at an MOI of 10 bacteria: 1 neutrophil and incubated for 1.5 h at 37° C with rocking. As a control, an equal concentration of virulent *R. equi* was incubated without neutrophils. An equal volume of RPMI-1640 was added to each of the controls lacking neutrophils, such that the volume in every sample was identical. After incubation, suspensions containing bacteria without neutrophils and bacteria with neutrophils were transferred to 1.5-ml micro centrifuge tubes and centrifuged at 2,000 × g for 10 min. The supernatant was discarded, and 0.5 ml of 0.5% Tween was added to the pellet of cells. The tubes were vortexed at maximum speed for 20 sec to lyse the neutrophils. The effectiveness of this procedure to lyse equine neutrophils was previously determined in our laboratory (unpublished data) by microscopic analysis of lysates. Ten-fold serial dilutions in PBS and quantitative culture on blood agar were performed to enumerate viable bacteria. The agar plates were incubated for 48 h at 37ºC, and the number of CFUs was determined. The percent kill was determined by multiplying the following ratio by 100:

$$\frac{(\#\text{ CFUs without neutrophils}) - (\#\text{ CFUs with neutrophils})}{\#\text{ CFUs without neutrophils}}$$

The bactericidal data accumulated from foals are summarized in Table 3. Bactericidal data were analyzed using a linear-mixed effects model. There was no significant effect of age on bactericidal capacity: the bactericidal capacity of foal neutrophils did not change significantly with age during the periods of observation.

The bactericidal assay performed in this study had a number of limitations that may have contributed to the absence of a significant effect of age. First, intracellular replicating *R. equi* tend to grow in clusters within macrophages, making it difficult to quantify the number of CFUs on a plate (Hondalus and Mosser, 1994). Although it is possible that such clustering may also occur in neutrophils, it is unlikely because neutrophils are bactericidal against *R. equi* (Yager et al., 1986; Martens et al., 1988).

Table 3.
Bactericidal capacity of foal neutrophils against *Rhodococcus equi*

Age (weeks)	Mean % *R. equi* killed (range)	Std. Dev.
0	25% (0% to 56%)	18%
2	30% (5% to 62%)	17%
4	40% (10% to 78%)	19%
8	23% (0% to 62%)	32%

One potential solution to the potential problem of clustering of bacteria is sonication of the cell lysate prior to quantitative culture. This procedure, however, carries with it the risk of killing viable bacteria. Second, there may have been variation among aliquots in concentrations of *R. equi*. As described in chapters II and III, a large volume of *R. equi* was grown and separated into 1-ml aliquots for use in the study. When preparing the aliquots of bacteria for storage at -80°C, efforts were made to ensure that each aliquot contained an equal concentration of *R. equi* (i.e., the bacteria were thoroughly vortexed in between each and every aliquot, pipetting was performed slowly and carefully, etc.). Despite these precautions, it is still possible that the *R. equi* were not evenly distributed throughout the culture such that aliquots may have varied in their concentration of *R. equi*. In most cases, 1 aliquot of bacteria was sufficient to conduct the bactericidal assay for a given neutrophil preparation. Occasionally, 1 aliquot would not suffice (for example, an occasional aliquot would have a volume < 1 ml) and another aliquot would need to be used. In these situations, it was assumed that each aliquot would contain approximately the same concentration of viable bacteria. It is possible, however, that the observed variability in bactericidal capacity of foal neutrophils resulted from variability among aliquots of bacteria. In the future, this problem could be avoided by freezing larger aliquots of *R. equi* (for example, 3 ml per aliquot instead of 1 ml per aliquot) or by combining multiple aliquots prior to infecting the neutrophils.

It is possible that the observed lack of age-related changes in bactericidal capacity was real, but this interpretation must be made cautiously for a number of reasons. First, neutrophils from a number of foals had negative bactericidal capacity for

R. equi. The occurrence of these negative results appeared to be completely random and of no apparent consequence to the health of the foal. Thus, the quality of the data is questionable. Second, age-related differences in bactericidal capacity of foal neutrophils against *R. equi* have been demonstrated: the bactericidal capacity of neutrophils from some foals <1 week of age was significantly lower than age-matched cohorts, and at 1 month of age the capacities of all foals were similar (Martens et al., 1988). Thus, although there were no age-related changes in bactericidal capacity of the foals in this study, such differences may exist among other foals. Finally, the mean bactericidal capacities in this study were significantly lower than in other studies that demonstrated the bactericidal capacity of equine neutrophils against *R. equi* (Yager et al., 1986; Martens et al., 1988), which suggests that the conditions in this study were not optimal for the accurate measurement of bactericidal capacity. It should be noted, however, that the previous studies used different approaches for quantifying bactericidal capacity; thus, direct comparisons among studies is difficult if not impossible. Prior to the onset of foaling season, numerous attempts were made, but failed, to replicate the findings of Yager et al. (1986). Attempts to replicate that study were made because the methods included quantification of extracellular *R. equi* (i.e., those that were not phagocytized after 15 min incubation with equine neutrophils), and in theory should have allowed for more accurate quantification of bactericidal capacity. Due to the use of radioactive isotopes, the methods used by Martens et al. (1988) were not attempted.

VIABILITY OF ADULT EQUINE NEUTROPHILS FOLLOWING INCUBATION WITH AVIRULENT *R. equi*, VIRULENT *R. equi*, AND rhGM-CSF

In order to determine whether there were significant effects of incubation time or treatment on neutrophil viability, a separate set of experiments were conducted. Blood was collected from 5 adult horses and the neutrophils were processed using the methods described in chapter II. Because of 2 ongoing studies involving the use of the foals at the Texas A&M University Horse Center, it was not possible to obtain neutrophils from foals for this experiment. The concentrations and % viabilities of neutrophils were assessed immediately following purification, and approximately 1×10^7 viable neutrophils were incubated (under the same conditions described in chapter II) with media (unstimulated), avirulent *R. equi* (MOI 10:1), virulent *R. equi* (MOI 10:1), or rhGM-CSF (100 ng) for 2 h and 4 h. An equal volume of RPMI-1640 was added to each of the unstimulated neutrophil preparations so that the volume in every sample was identical. After each of the incubation times, the number of viable neutrophils and the % viability was determined for each neutrophil preparation. The effects of incubation time and treatment on numbers of viable neutrophils were analyzed using a 1-way analysis of variance (ANOVA). A significance level of $P<0.05$ was used. Statistical analyses were performed using Stata (version 8.0; StataCorp, LP). The data are summarized in Table 4.

Table 4.
Concentration and viability of adult equine neutrophils following incubation with media (unstimulated), avirulent *R. equi*, virulent *R. equi*, or rhGM-CSF for 2 h and 4 h.

Preparation	Mean number viable neutrophils	Std.Dev.	% Viability
Initial	1×10^7	N/A*	96.0%
2 h unstimulated	6.28×10^6	1.91×10^6	93.6%
2 h avirulent *R. equi*	5.71×10^6	1.17×10^6	92.1%
2 h virulent *R. equi*	5.44×10^6	0.994×10^6	91.9%
2 h rhGM-CSF	6.26×10^6	1.49×10^6	93.6%
4 h unstimulated	5.74×10^6	1.65×10^6	92.6%
4 h avirulent *R. equi*	4.28×10^6	1.48×10^6	90.6%
4 h virulent *R. equi*	4.30×10^6	1.43×10^6	89.75%
4 h rhGM-CSF	5.64×10^6	1.57×10^6	92.0%

* N/A = Not applicable

After 2 h and 4 h incubation, the mean number of viable neutrophils for all treatments was approximately 5.90×10^6 and 4.99×10^6, respectively. As the initial number for all treatments was approximately 1×10^7 viable neutrophils, these numbers represented a 50% reduction in the total number of viable neutrophils. Although not significant, there was a tendency for the total number of viable neutrophils and the % viable neutrophils incubated with *R. equi* (both virulent and avirulent) to be lower than the numbers incubated with media or with rhGM-CSF. The mean % of viable neutrophils among all treatments at a given time were 93.1%, 91.4%, 91.0%, and 93.0% for unstimulated, avirulent, virulent, and rhGM-CSF, respectively. There was also a tendency (although again not significant) toward a decrease in the % of viable cells during incubation: the means of the % of viable cells of all treatments after 2 h and 4 h incubation were 92.8% and 91.2%, respectively. Furthermore, pipetting and counting errors were also likely to have contributed to the reduction in the total number of viable cells. Nonetheless, the data generated from this experiment indicated that, after subjecting adult horse neutrophils to the experimental conditions outlined in chapter II, there were no significant differences in total numbers of viable neutrophils or % of viable neutrophils that account for the observed changes in cytokine expression.

CHAPTER IV

CONCLUSIONS AND DISCUSSION

Although the onset of clinical signs of *R. equi* pneumonia is most common in foals 2 to 3 months of age, experimental and epidemiologic studies suggest that most foals are infected during the first week of life (Horowitz et al., 2001). Thus, infection with *R. equi* is most commonly established before an adaptive immune response can develop, which suggests that the innate immune response is likely important for controlling early infections. Neutrophils are a major component of the innate immune response and are considered to be important in linking innate and adaptive immune functions (Denkers et al., 2003). Neutrophil activity may delay the onset of clinical disease or death, and provide the host with the opportunity to develop an adaptive immune response. A number of studies have demonstrated the ability of equine neutrophils to phagocytize and kill *R. equi*; however, only 1 study demonstrated the temporality of this capability in newborn foals. Data from that study indicated that neutrophils from 15% (2 out of 13 foals) of the foals studied had significantly lower bactericidal capacity against *R. equi* during the first week of life, but developed normal bactericidal capacity by 1 month of age (Martens et al., 1988). Those data indicate that inter-individual variability in neutrophil function early in life may contribute to increased susceptibility to disease. Thus, a better understanding of the role neutrophils play in response to infection with *R. equi* may provide insight into alternative methods for prevention and control of this disease.

Interleukin-6 is a cytokine with a variety of biological properties, including the

activation of natural killer cells, induction of the acute phase response, and stimulation of T cell proliferation and differentiation to cytolytic T cells (Melani et al., 1993). IL-6 has also been shown to influence the oxidative burst and degranulation of human neutrophils (Borish et al., 1989), which are among the many important microbicidal activities of neutrophils. The precise role of IL-6 in infection is controversial, and may vary under different host-pathogen interactions. Some reports indicate it is a Th2 cytokine because it induced expression of IL-4, which promotes the differentiation of naïve $CD4^+$ T cells into effector Th2 cells (Rincon et al., 1997; Diehl and Rincon, 2002; Diehl et al., 2002). The majority of the literature, however, indicates that IL-6 is a Th1 cytokine. For example, IL-6 induced production of IFNγ and growth of T cells protective against tuberculosis (Leal et al., 1999; Saunders et al., 2000). Mice deficient in IL-6 have been reported to be highly susceptible to infection with the intracellular pathogens *Listeria monocytogenes* (Dalrymple et al., 1995) and *M. tuberculosis* (Ladel et al., 1997).

In this study, adult and juvenile equine neutrophils exposed to *R. equi* displayed significant increases in expression of IL-6 mRNA, which suggests that it plays an important role in rhodococcal infections. Interestingly, there was a significant effect of age on expression of IL-6 mRNA by foal neutrophils; mRNA expression was significantly greater in newborn foals than in 2-, 4-, and 8-week-old foals. If indeed IL-6 does promote a Th2 response rather than a Th1 response, its production in response to infection with *R. equi* would likely be detrimental, as there is substantial evidence that clearance of *R. equi* infection requires a Th1 response (Kanaly et al., 1995, 1996; Kohler

et al., 2003). If, however, IL-6 promotes a Th1 response upon infection with *R. equi*, then it is likely to be beneficial to foals, particularly if it promotes IFNγ production early in the course of disease.

Expression of IL-8 mRNA by neutrophils from adult horses and foals followed the same pattern of expression as IL-6; *R. equi* induced significantly greater mRNA expression by adult and foal neutrophils, and this expression was significantly greater in newborn foals than in 2-, 4-, and 8-week-old foals. Interleukin-8 (neutrophil chemotactic factor) is a strong chemoattractant for neutrophils, monocytes, and T cells (Baggiolini et al., 1995; Zhang et al., 1995). IL-8 is produced by neutrophils, in addition to other leukocyte populations, and has been shown to enhance killing of *M. tuberculosis* by neutrophils (Godaly and Young, 2005). IL-8 has been shown to activate neutrophils by inducing degranulation, calcium mobilization, shape change, respiratory burst, and changes in phagocytic capacity (Baggiolini et al, 1995). Thus, IL-8 is thought to enhance the clearance of microorganisms by increasing the bactericidal capacity of immune cells, and likely plays a vital role in the acute immune response to infection with intracellular bacteria, such as *M. tuberculosis* and *R. equi*. In this study, it was hypothesized that neutrophils play a vital role in the early host defense against *R. equi*. It is possible that they do this through the production of IL-8, thereby recruiting additional neutrophils to the site of infection (and thus killing more *R. equi*). Furthermore, the ability of IL-8 to attract T cells may be important for facilitating antigen presentation and initiating a cell-mediated immune response.

It was interesting to note that the overall magnitude in expression of IL-6 and IL-

8 mRNA by neutrophils from 2-, 4-, and 8-week-old foals was similar to that of adults. In light of the well-known Th2 bias of virtually all neonates (Morein et al., 2002), it is plausible that an enhanced response with respect to IL-6 and IL-8 is needed by newborn foals for early protection against infection with *R. equi*, but that as the immune system matures and shifts toward a Th1 profile, expression of these cytokines does not need to be as large. The biological significance of this age-dependent difference, however, remains unknown and awaits validation by replication of these findings and further investigation, including studies of the association of expression of these cytokines with both age and susceptibility to infection with *R. equi*.

IFNγ is critically involved in driving a Th1-type cell-mediated immune response. It enhances Th1 polarization by facilitating production of IL-12 and synergizing with TCR signals to induce the expression of T-bet (Langrish et al., 2004). There is unequivocal evidence of the protective nature of IFNγ in bacterial infections such as *M. tuberculosis* (Flynn et al., 1993). Likewise, several studies have shown that protection against *R. equi* in mice is mediated by IFNγ, and that IFNγ is required for clearance of *R. equi* from the lungs of these mice (Nordmann et al., 1993; Kanaly et al., 1995; Kasuga-Aoki et al., 1999), and also from the lungs of experimentally infected adult horses (Hines et al., 2003). The expression and production of IFNγ in foals is controversial. Some reports indicate that foals produce relatively less IFNγ at birth, both by PBMCs (Boyd et al., 2003; Breathnach et al., 2006) and by pulmonary cells (Breathnach et al., 2006), while others suggest that levels of IFNγ expression in foals (by bronchial lymph node cells) are comparable to adult horses (Jacks et al., 2007). Despite the fact that the

production of IFNγ by neutrophils of other mammalian species is well documented (Yeamen et al., 1998; Ellis and Beaman, 2002), none of the studies examining IFNγ expression by foals accounted for the possibility that neutrophils provide an early source of this cytokine. Interestingly, the expression of IFNγ mRNA by neutrophils from adult horses was not significantly increased by stimulation with virulent or avirulent *R. equi*. Although this observation may reflect delayed kinetics of IFNγ mRNA expression, there were significant changes in mRNA expression by foal neutrophils stimulated with *R. equi* for the same duration of time. Despite differential expression of IFNγ mRNA by foal neutrophils in response to *R. equi*, no age-related changes in mRNA expression of this cytokine were observed. Nonetheless, the sheer number of neutrophils in the blood and at sites of infection suggests that they may provide a substantial source of IFNγ.

Tumor necrosis factor α (TNFα) is an inflammatory cytokine that stimulates phagocytosis by macrophages, attracts neutrophils, and stimulates the acute phase response (Dubravek et al., 1990). The importance of TNFα in the course of disease arising from infection with *R. equi* has been demonstrated in mice (Nordmann et al., 1993; Kasuga-Aoki et al., 1999). Depletion of TNFα in mice resulted in a lethal course of infection despite the mice being given a sub-lethal dose of virulent *R. equi* (Kasuga-Aoki et al., 1999). Another study demonstrated that expression of TNFα mRNA was significantly higher in the lung tissue of foals experimentally infected with virulent *R. equi* than in foals infected with avirulent *R. equi* (Giguere et al., 1999b). In the present study, expression of TNFα mRNA by neutrophils from adult horses and foals was significantly increased in response to stimulation with virulent and avirulent *R. equi*;

however, there were no age-related changes in expression of TNFα mRNA by foal neutrophils. Moreover, the magnitude in expression of TNFα mRNA by both adult and foal neutrophils was similar, which suggests that any age-related deficiency in mRNA expression of this cytokine is not attributable to neutrophil responses.

Perhaps the most interesting finding of this study was the relatively low level of IL-12p35 mRNA expression, in the face of increased IL-12p40 mRNA expression, by both adult and foal neutrophils. In order to form biologically active IL-12, both the IL-12p35 and the IL-12p40 subunits must be produced within the same cell (Gubler et al., 1991). Although many reports state that IL-12p35 mRNA is constitutively expressed (Denkers et al., 2003), many others have demonstrated that mRNA expression of the IL-12p35 subunit is inducible upon TLR3 or TLR4 ligation (Liu et al., 2003; Goriely et al., 2006). Thus, constitutive expression of IL-12p35 mRNA is an unlikely explanation for the observations in the present study. In adult neutrophils, expression of IL-12p35 mRNA in response to stimulation with *R. equi* was not significantly increased. In foal neutrophils, however, expression of IL-12p35 mRNA was significantly increased in response to stimulation with *R. equi*. Given that adult horses are generally refractory to disease caused by *R. equi*, this finding seems counterintuitive. One possible explanation for this discrepancy could be that adult neutrophils expressed higher baseline levels of the IL-12p35 subunit than foal neutrophils, such that the difference in mRNA expression of IL-12p35 by adult neutrophils upon stimulation with *R. equi* was insignificant. It is also possible that maximal expression of IL-12p35 mRNA occurred after the time-points used in this study; however, this seems unlikely given that foal neutrophils expressed

significantly greater IL-12p35 mRNA after 4 h incubation with *R. equi*. Furthermore, significant increases in IL-12p35 mRNA expression by monocyte-derived dendritic cells have been observed after 3 h incubation with LPS and PolyI:C (Goriely et al., 2006), and maximum release of IL-12p70 from human neutrophils occurred after 2 h stimulation with soluble tachyzoite antigen (Bliss et al., 1999). Because monocytes/macrophages and dendritic cells are known to be major producers of IL-12, it is also possible that equine neutrophils are a minor source of IL-12. However, a recent study demonstrated that adult horse dendritic cells stimulated with CpG-ODN significantly increased expression of IL-12p40 mRNA, but not IL-12p35 mRNA (Flaminio et al., 2007). Thus, an alternative explanation for the observations in this study is that the increased expression of IL-12p40 mRNA reflected IL-23 production rather than IL-12 production.

IL-23 is another important heterodimer that is composed of the IL-12 p40 and IL-23p19 subunits (Harrington et al., 2006). Because it is unlikely that IL-12p40 mRNA was differentially expressed for no reason, the hypothesis that neutrophils from adult horses differentially expressed IL-23p19 mRNA was tested. Indeed, there was a significant increase in expression of IL-23p19 mRNA by *R. equi*-stimulated neutrophils from adult horses. This finding could have important implications because the production of IL-23 drives the development of an alternative T cell subset characterized by the production of the pro-inflammatory cytokine IL-17, which acts in part to mobilize neutrophils from the bone marrow and induce the acute phase response via IL-6 and TNFα production (Langrish et al., 2004; Harrington et al., 2006). Allowed unregulated, the IL-23/IL-17 axis has been linked to autoimmune disease and other

immunopathological conditions (Harrington et al., 2006; McKenzie et al., 2006); however, its conservation in the immune pathway suggests that it provides some evolutionary benefit to the host. Consequently, there are increasing reports in the literature that demonstrate a protective role for IL-23 against microbial pathogens (Khader et al., 2005; Umemura et al., 2007). Moreover, in newborn humans, the IL-23 heterodimer is produced preferentially over IL-12, and is thought to compensate for an impaired Th1 response in neonates (Eijnden et al., 2006). Unfortunately, due to the lack of sufficient quantities of cDNA, analysis of IL-23p19 mRNA expression by neutrophils from foals was beyond the scope of this study. The results derived from analysis of IL-23p19 mRNA expression by neutrophils from adult horses provide compelling evidence for pursuing this line of investigation in the future.

It is important to note that the observed changes in cytokine mRNA expression (or lack thereof) by equine neutrophils may not accurately or completely reflect their contribution to the cytokine milieu. For example, translation of mRNA into protein may not occur, and if it does, post-translational modification may result in a change or loss of protein function. Another important possibility is the ability of equine neutrophils to store preformed cytokines that could be rapidly released upon degranulation. This phenomenon, which has been observed in both human and mouse neutrophils (Bliss et al., 2000; Denkers et al., 2003; Ethuin et al., 2004) and will likely be increasingly reported in other species, reflects the innate ability of neutrophils to respond rapidly to microbial invasion. In the reports cited above, the release of preformed cytokines was followed by *de novo* synthesis, as measured by mRNA expression and protein

production. Despite the absence of IFNγ and IL-12p35 mRNA expression by adult equine neutrophils in this study (and the relatively low mRNA expression levels by foal neutrophils) it is conceivable that equine neutrophils contain preformed stores of IFNγ and IL-12, but contribute little to *de novo* synthesis of these cytokines. For these reasons, it will be very important to develop the requisite immunological reagents for measuring equine cytokine secretion in future studies.

The biggest limitation of this research project was the lack of an animal model, which is a problem most scientists face. Without an acceptable animal model, scientists must either perform their work *in vitro*, or perform it in the natural host. This becomes especially problematic when dealing with human or large animal diseases because of ethical, practical, and economic issues. The cost of maintaining large animals, such as a herd of mares and foals, is significant. One particular problem with studying foals is that in the United States, most foals are born between February and May, which leaves very little time to optimize protocols that are specific for foals, and it is nearly impossible to predict when and what problems may occur. For example, at the onset of this study (which essentially began when the first foal was born), we had no way of knowing that the residual red blood cells from some foals would not only fail to lyse, but would form clumps upon addition of the red blood cell lysis buffer. This phenomenon was not observed in adult horses and made it difficult to obtain enough neutrophils for the experiments. Consequently, a number of foals had to be dropped from the study. Were there a good animal model for *R. equi* pneumonia, such as the mouse or the guinea pig, there would have been ample time to conduct experiments, encounter the problems that

inevitably arise, fix the problems and revise the experiments accordingly, and complete the studies. Moreover, the immunological reagents available to study the immune system of the mouse are much greater in number than those for foals.

Despite the lack of an animal model, the data generated in this research project are important because they provide precedence for future studies, particularly in the area of cytokine research. The real-time PCR assay developed for this study is extremely robust and should be easily duplicated by others; the primers and probes were extensively validated for repeatability, specificity, and efficiency, and the endogenous control was carefully selected based on its consistent expression levels under experimental conditions. The data generated in this research project are the first to describe cytokine mRNA expression levels of equine neutrophils in response to exposure to *R. equi*. The results of this study demonstrate that equine neutrophils are an important source of pro-inflammatory cytokine mRNA, that neutrophils respond rapidly upon exposure to *R. equi* by increasing expression of cytokine mRNA, and that in foals some of these changes are age-related. Moreover, this is the first study that has demonstrated the expression of IL-23p19 mRNA in horses. This finding, coupled with the finding that IL-23p19 mRNA expression was significantly increased by exposure to *R. equi* (and that mRNA expression by neutrophils stimulated with virulent *R. equi* was significantly greater than by neutrophils stimulated with avirulent *R. equi*), should lead to a new area of research with respect to the immunopathogenesis of *R. equi* pneumonia.

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