Immunity to *Rhodococcus equi*

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Abstract

Rhodococcal pneumonia is an important, life threatening disease of foals and immunosuppressed humans. Increased knowledge of the mechanisms of protective immunity are required in order to develop an effective immunoprophylaxis strategy for horses and immunotherapeutic regiments for people. Both humoral and cellular components of the immune system may be involved in immune clearance of *R. equi*. The susceptibility of foals less than 4–6 months of age is postulated to reflect waning maternal antibody, and passive transfer of hyperimmune plasma can provide protection on endemic farms. However, effective clearance is likely to require appropriate cellular responses, including the secretion of cytokines. In murine models, both CD4 + and CD8 + T lymphocytes can reduce bacterial counts in the lung. CD4 + cells appear to be both required and sufficient, and IFN-gamma is a primary mediator. Clearance appears to be a type 1 immune response while type 2 responses may lead to a failure to clear and lesion development. It remains to be determined how the cellular immunity experiments reported in mice relate to horses and humans. Likewise, the role of specific *R. equi* antigens in protective immunity has not been determined. © 1997 Elsevier Science B.V.

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1. Introduction

Over the past 10 years, significant progress has been made towards understanding the mechanisms of protective immunity to *R. equi*. However, critical questions remain. For example, what is the basis for the unique susceptibility of foals between 2 and 6 months of age? Virtually all horses are exposed to *R. equi* early in life, and most develop

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primary immune responses which subsequently protect them for life. Why are some foals unable to clear the infection and subsequently develop disease? Moreover, why have traditional approaches to vaccination failed to induce protective immune responses? It is likely that effective prevention of rhodococcal pneumonia will ultimately require both a more complete understanding of protective immunity to R. equi and novel methods to target those responses — especially in foals. The purpose of this paper is to review the published literature on immunity to R. equi since 1987 and to highlight the important gaps in our knowledge.

2. Pathogenesis

Factors that determine whether an animal exposed to R. equi clears the infection or develops disease include: (a) the infective dose, (b) the virulence of the infective strain and (c) development of an appropriate immune response. The key event in the pathogenesis of rhodococcal infection is considered to be the ability of virulent strains of R. equi to survive the ordinarily lethal consequences of phagocytosis by pulmonary macrophages (Takai et al., 1985; Zink et al., 1987). In vitro studies have shown that R. equi fixes complement via the alternative pathway, binds macrophages via the complement receptor type 3 (Mac-1) and is phagocytosed (Hondalus et al., 1993). Following an initial lag period, bacteria replicate intracellularly, apparently circumventing the microbicidal capabilities of the macrophage by inhibiting phagosome–lysosomal fusion (Takai et al., 1985; Hietala and Ardans, 1987; Zink et al., 1987; Hondalus and Mosser, 1994). Cellular immune responses are postulated to be critical to clearance of R. equi, due to the bacterium's status as a facultative intracellular pathogen. However, there is evidence that both antibody and T lymphocytes play a role in immunity and that secretion of appropriate cytokines is required.

3. The role of antibody

Antibody to R. equi may contribute to immunity in horses by blocking the initial stages of cellular infection, altering the route by which bacteria enter the macrophage, and/or decreasing the bacterium's ability to inhibit phagosome–lysosomal fusion (Speert, 1992). The unique susceptibility of foals less than about 6 months of age has long been thought to reflect waning of maternal antibody. Theoretically, foals with sufficient maternal antibody subsequently develop immunologic 'maturity' and mount protective primary immune responses. In support of these hypotheses, immune serum has been shown to promote the phagocytosis and killing of R. equi by equine macrophages in vitro (Hietala and Ardans, 1987). Likewise, the passive transfer of hyperimmune plasma can prevent or significantly reduce the severity of pneumonia in foals subsequently challenged experimentally or by natural exposure (Martens et al., 1989; Madigan et al., 1991). However, plasma must be present prior to challenge. Parenteral administration of hyperimmune plasma to foals seven days after challenge with R. equi does not alter the course of disease (Chaffin et al., 1991).

As a result of these experiments, passive transfer of plasma is used commonly to decrease rhodococcal disease on farms where the prevalence is high. However, the actual mechanism by which transfer of hyperimmune plasma provides protection and its relation to maternal antibody are currently unknown. Ingestion of colostrum from hyperimmunized mares does not protect foals (Martens et al., 1991). Likewise, traditional methods of immunization which are designed to elicit antibodies in pregnant mares do not provide protection to their foals. There are a number of possible explanations for these findings. The isotypes of immunoglobulin which mediate the critical effects of plasma may not be efficiently transferred in colostrum. The immunogens and/or immunization protocols may also not induce sufficient antibody or sufficient antibody with the appropriate antigen specificity. Alternatively, part of the protective effects of immune plasma may reflect factors in plasma other than antibody.

Clearly there are significant gaps in our knowledge regarding the role of antibody (including the role of immunoglobulin isotype), the importance of antibody specificity, and the mechanisms by which both antibody and immune plasma might act to prevent rhodococcal infection. Passive transfer of anti-*R. equi* immune serum does not protect mice (Nordmann et al., 1992). Therefore, if waning antibody is the key to whether foals clear infection or develop disease, the mechanisms of immune clearance would appear to differ between mice and horses. Alternatively, antibody may play a role only in the initial interactions with *R. equi*. Since bacteria are no longer accessible to antibody binding once they are within cells, ultimate control of infection may be dependent on cell mediated events. For example, *Salmonella typhimurium* is a facultative intracellular bacterium which replicates in macrophages and in mice induces a disease similar to typhoid fever. The passive transfer of antibody from immune animals enhances the inactivation of bacteria during the first few hours after challenge but does not prevent the subsequent growth of surviving bacteria. Adoptive transfer and depletion experiments have demonstrated that clearance of bacteria is mediated mainly by T lymphocytes (Nauciel, 1990). It may turn out that the most effective immunologic control strategy for rhodococcal infection in horses will involve immunization of both mares and foals and that it will be critical to target appropriate T lymphocyte responses.

4. Cell mediated immunity and cytokines

Most of what is currently known about cellular immunity to *R. equi* comes from mouse models. In general, immunocompetent strains of mice challenged with virulent *R. equi* by the natural, respiratory route are not susceptible to infection (Yager et al., 1991a,b; Kanaly et al., 1993). Instead, mice develop transient inflammatory responses, progressively clear bacteria from the lung, and resolve pulmonary lesions within 21 days — presumably through induction of a protective primary immune response (Bowles et al., 1989; Yager et al., 1991a,b). Intravenous challenge, on the other hand, can result in significant mortality which is dependent on dose and strain virulence (Takai et al., 1991). However, the relevance of intravenous challenge to natural infection is not clear.

The susceptibility of immunodeficient strains of mice to rhodococcal pneumonia underscores the importance of cellular responses in immune clearance of *R. equi* from

the lung. Both severe combined immunodeficient mice lacking T and B lymphocytes (scid/scid) and athymic nude mice lacking T lymphocytes (nu/nu) are unable to eliminate R. equi and develop pulmonary lesions (Bowles et al., 1987; Yager et al., 1991a,b). In contrast, complement (C5)-deficient A/J mice and C57Bl/6J.bg/bg (beige) mice resolve pulmonary infection, suggesting that deficiencies in complement components, phagocyte function, and NK (natural killer) cell function do not impair pulmonary clearance of R. equi (Yager et al., 1991a,b). Further evidence for the role of cellular immunity comes from AIDS patients and adoptive transfer experiments. Opportunistic R. equi infections are increasingly important in HIV-positive humans with low CD4 T cell counts (Harvey and Sunstrum, 1991; Lasky et al., 1991; Vestbo et al., 1991). Transfer of immune splenocytes from previously infected, immunocompetent BALB/c mice significantly protects both SCID/beige mice challenged by the intranasal route (Balson et al., 1992) and sublethally irradiated BALB/c mice challenged by intravenous injection (Nordmann et al., 1992).

The two principle mechanisms by which T lymphocytes mediate clearance of intracellular bacteria are direct cytotoxicity of the infected cell (classically by MHC class I restricted CD8 + T cells) and secretion of cytokines. Several approaches have been used to identify the cells involved in immune clearance of R. equi. Nordmann et al. (1992) depleted BALB/c mice of CD4 + and/or CD8 + T lymphocytes using monoclonal antibodies, then challenged by intravenous inoculation and enumerated viable bacteria in the spleen and liver at 11 days post-infection. In all experimental groups, in vivo depletion significantly increased bacterial counts compared to controls. This study indicated that both CD4 + and CD8 + T cells participated in the clearance of R. equi, and the authors suggested that CD8 + cell played the major role. However, small numbers of residual positive cells were present in all groups following depletion and could have played a significant role in immune clearance. Kanaly et al. (1993) examined the role of CD4 + and CD8 + T lymphocytes in clearance of pulmonary infection using transgenic 'knockout' mice. In this study, MHC class I-deficient mice (β_2m $-/-$) which lack class I restricted CD8 + cells cleared virulent R. equi from the lungs within 21 days. In contrast, MHC class II-deficient mice lacking class II restricted CD4 + cells reduced the number of bacteria in the lung but were unable to resolve the infection. In studies by Ross et al. (1996), adoptive transfer of positively selected CD4 + T cells from previously infected BALB/c mice effected the clearance of pulmonary bacteria in congenic C.b-17 SCID/beige mice. The experiments on T cell subsets demonstrate a central role for CD4 + lymphocytes in immune clearance of R. equi in mice, although CD8 + cells may have significant contributory or independent effects.

In mice, CD4 + T lymphocytes can be subdivided into Th1 and Th2 subsets based on distinct, non-overlapping patterns of cytokine production (Mosmann and Coffman, 1989; Mosmann and Sad, 1996). Th1 cells are defined by the ability to produce interferon gamma (IFN-□) and interleukin-2 (IL-2), while Th2 cells produce IL-4, IL-5 and IL-10. In mice and humans, many infectious agents (and allergens) produce preferential Th1 (type 1) or Th2 (type 2) immune responses (Bretsher et al., 1992). The relative balance of the type 1 versus type 2 response determines the outcome, including the ability of infected hosts to control a number of intracellular pathogens (Gajewski and Fitch, 1988;

Heinzel et al., 1989; Scott, 1991; Yamamura et al., 1991). This effect is mediated by the respective lymphokines produced.

For pathogens that survive and replicate within mononuclear phagocytes, the effects of cytokines primarily reflect their abilities to alter the microbicidal capabilities of infected cells (Trinchieri et al., 1993). IFN-□ is the major macrophage-activating factor and is capable of upregulating at least three pathways for microbial killing: the production of reactive oxygen intermediates, the production of nitrogen intermediates and the induction of indolamine 2,3 dioxygenase to catabolize tryptophan (Mosmann and Coffman, 1989). In addition IFN-□ has been shown to stimulate phagolysosomal fusion and enhance expression of Fc receptors (Nathan et al., 1983). These cell-mediated protective mechanisms can be enhanced by other cytokines (IL-2 and TNF-α), while cytokines such as IL-10 are able to down regulate macrophage function (Trinchieri et al., 1993). There is evidence that lymphocyte-mediated activation of macrophages may be involved in immune clearance of *R. equi* by horses. Hietala et al (1987) showed that conditioned medium from *R. equi* antigen-stimulated peripheral blood lymphocytes significantly increased the ability of equine alveolar macrophages to kill *R. equi*.

In mice, immune clearance of *R. equi* appears to be a type 1-like response and IFN-□ is a primary mediator. BALB/c mice treated with neutralizing monoclonal antibody to IFN-□ failed to clear pulmonary *R. equi* infection and developed pulmonary granulomas (Kanaly et al., 1995). These mice also expressed increased levels of IL-4 mRNA in CD4 + bronchial lymph node T cells and failed to express detectable IFN-□ mRNA; findings compatible with a type 2 response. In contrast, mice treated with anti-IL-4 monoclonal antibody or control antibody cleared infection by 21 days and expressed increased levels of IFN-□. These studies suggest that a type 1 response is protective in mice and that a nonprotective type 2 response is involved in disease pathogenesis. In a similar fashion, Nordmann et al. (1993) showed that anti-TNF□ and/or anti-IFN-□ antibodies significantly inhibited the ability of euthymic Swiss mice to clear an intravenous challenge at 7 days post-inoculation. In a more definitive study, adoptive transfer of a CD4 + Th1 cell line into nude mice resulted in clearance of *R. equi* from the lung and expression of IFN-□ mRNA in bronchial lymph node cells (Kanaly et al., 1996). In contrast, nude mice transfused with a CD4 + Th2 cell line failed to clear infection and developed large granulomas in the lung. In mice at least, an antigen-specific Th1 response is sufficient to effect pulmonary clearance of *R. equi* while a Th2 response (or at least the absence of a Th1 response) appears to promote lesion development.

The data suggest that secretion of IFN-□ is essential for immune clearance of *R. equi* and that it will be necessary to induce a type 1-like response in other species in order to produce protective immunity. However, a key unanswered question is how closely the type 1/type 2 paradigm (as defined in mice and humans) applies to the horse. Does the susceptibility of foals reflect an age-associated type 2 bias? If so, how is it that most foals manage to develop protective immune responses that operate throughout their adult lives?

In addition, it is becoming increasingly clear in mice that the divergence to type 1 or type 2 response is regulated by the innate immune system at the time of initial exposure (Sharton and Scott, 1993; Ladel et al., 1996). IL-12 and IL-4 are important early

inducers of type 1 and type 2 responses, respectively (Kaufmann, 1995). If type 1-like responses are protective in horses, IL-12 is likely to be an important mediator. However, little is known of the detailed mechanisms which determine whether animals develop protective or non-protective responses — especially in outbred species. This knowledge will be necessary in order to develop novel vaccines that specifically target the protective phenotype (Golding and Scott, 1995).

5. The role of antigen

Studies from other systems suggest that the ability of CD4+ T lymphocytes to eliminate intracellular pathogens depends both on the type of cytokines secreted and the antigen-specificity of the cells. In *Leishmania major* infection, adoptive transfer of parasite-specific CD4+ Th1 lymphocyte clones can protect sublethally irradiated BALB/c mice (Scott et al., 1988). However, some parasite-specific Th1 cell clones are not protective and even exacerbate the disease. Th1 clones which transferred immunity were used to identify a parasite protein which when injected with IL-12 protects susceptible mice from *Leishmania* infection (Mougneau et al., 1995). These kinds of experiments have stimulated researchers to look for 'protective' antigens in other intracellular pathogens, including *R. equi*. To date, however, no one has published studies documenting the use of either defined antigen or killed bacteria to induce significant protection against *R. equi*. T lymphocytes which transfer immunity to susceptible mice have invariably been taken from mice that previously cleared live bacteria. The only exception has been the ability of hyperimmune plasma from bacterin-immunized horses to passively protect foals.

Most of the recent efforts to identify protective *R. equi* antigens have focussed on molecules which appear to be involved in virulence. For pathogens in which humoral immunity is important, virulence factors which mediate adhesion, toxicity or evasion from phagocytosis are frequently protective antigens (Kaufmann, 1993). Antibodies against these molecules can block essential steps in pathogenesis and provide protection against disease. However, the correlation between protective antigens and virulence functions is much less clear in the case of intracellular bacteria.

VapA is a 15–17 kDa lipoprotein which is encoded by the 85–90 kb virulence plasmid of *R. equi* (Takai et al., 1991, Takai et al., 1993; Tkachuk-Saad and Prescott, 1991). The *VapA* gene has been cloned and sequenced (Sekizaki et al., 1995; Tan et al., 1995). Evidence that this protein plays a role in virulence includes: (a) expression in virulent, but not avirulent strains, (b) upregulation at 37°C so that it is expressed in the mammalian host but not the environment, and (c) exposure on the surface of the bacterium where it has the potential to interact with host components including macrophages (Takai et al., 1992). Since VapA is expressed within macrophages, it may not be readily accessible to antibody in vivo (Madarame et al., 1996). However, uptake of *R. equi* by a murine macrophage cell line is enhanced by a monoclonal antibody to VapA (Tan et al., 1995). Likewise, passive transfer of the monoclonal antibody to CD1 mice enhanced liver clearance at 1 but not at 4 and 7 days after intravenous challenge. Active immunization of CD1 mice with native VapA elicited strong antibody responses

(titers > 1:8000) and enhanced clearance of *R. equi* at 3 and 4 days after intravenous challenge. There was no significant effect from the immunization at days 1, 5 and 7 post-infection (Tan et al., 1995).

Nothing is currently known about the cellular and cytokine responses to VapA, either in infection or following immunization. Likewise, almost nothing is known about how horses respond to VapA, except that symptomatic infection tends to produce significant amounts of VapA-specific antibody which is detected by immunoblots (Takai et al., 1991). Recent experiments by Prescott et al. (1996) have shown that pregnant mares immunized with VapA antigen (using alum as an adjuvant) develop strong antibody responses and that VAP A-specific antibody is transferred to their foals in colostrum. However, experiments reporting the protective capacity of colostral anti-VAP A antibody in foals have not yet been reported. Therefore, it remains to be determined whether virulence-associated proteins like VapA will be protective antigens for *R. equi* infection.

6. Future directions

In order to develop improved control measures for rhodococcal infection, we clearly require additional basic knowledge about protective immune responses (particularly in the foal) and the host:*R. equi* interaction. Key questions remain unanswered despite significant progress over the past 10 years. For example, what determines whether an animal develops a protective or non-protective immune response? Clearly this is an active area of investigation for a number of intracellular pathogens which have resisted conventional methods of control. Determining the role of the innate immune system, antigen presenting cells, and 'initiation cytokines' are important parts of the work which may lead to a new generation of vaccines. For *R. equi*, the molecular mechanisms by which virulent strains alter the host–parasite interaction is also likely to be critical information. Other important questions, especially in horses, include: (a) what is the protective Th phenotype in outbred species? Does it differ from mice?, (b) what is the mechanism for the unique, age-associated susceptibility of foals? How can it be overcome for immunoprophylaxis?, and (c) what is the role of antigen in protective immunity to *R. equi*? The next few years are likely to provide exciting new clues regarding the answers to these questions.

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