Vet. Res. 35 (2004) 383–396

DOI: 10.1051/vetres:2004024

Review article

Rhodococcus equi

Wim G. MEIJER[a], John F. PRESCOTT[b]*

[a] Department of Industrial Microbiology, Conway Institute of Biomolecular and Biomedical Research, University College Dublin, Dublin 4, Ireland
[b] Department of Pathobiology, University of Guelph, Guelph, Ontario N1G 2W1, Canada

(Received 26 June 2003; accepted 3 October 2003)

Abstract – *Rhodococcus equi* is an important cause of subacute or chronic abscessating bronchopneumonia of foals up to 3–5 months of age. It shares the lipid-rich cell wall envelope characteristic of the mycolata, including *Mycobacterium tuberculosis*, as well as the ability of pathogenic members of this group to survive within macrophages. The possession of a large virulence plasmid in isolates recovered from pneumonic foals is crucial for virulence. The plasmid contains an 27 kb pathogenicity island (PI) that encodes seven related virulence-associated proteins (Vaps), including the immunodominant surface-expressed protein, VapA. Only PI genes are differentially expressed when the organism is grown in macrophages in vitro. Ten of the PI genes, including six Vap genes, have signal sequences, suggesting that they are exported from the cell to interact with the macrophage. Different PI genes are regulated by temperature, pH, iron, oxidative stress and probably also by magnesium, all environmental changes encountered after environmental *R. equi* are inhaled in dust and are ingested into macrophages in the lung. The basis of pathogenicity of *R. equi* is its ability to multiply in and eventually to destroy alveolar macrophages. Infectivity is largely or exclusively limited to cells of the monocyte-macrophage lineage. Current evidence suggests that infection of foals with virulent *R. equi* results in some foals in subversion of cell-mediated immunity and development of an ineffective and sometimes lethal Th2-based immune response. Significant progress has been made recently in the development of *R. equi-E. coli* shuttle vectors, transformation and random and site specific mutagenesis procedures, all of which will be important in molecular dissection of the mechanisms by which *R. equi* subverts normal macrophage killing mechanisms and cell-mediated immunity.

***Rhodococcus equi* / virulence / cell biology / immunity / genetic tools**

Table of contents

* Corresponding author: prescott@uoguelph.ca

1. INTRODUCTION

Rhodococcus equi is an important pathogen of young foals aged up to 3–5 months old [53]. Infection causes a subacute or chronic abscessating bronchopneumonia, sometimes with ulcerative typhlocolitis, and may include mesenteric lymphadenitis, osteomyelitis, purulent arthritis, reactive arthritis, and ulcerative lymphangitis. Tuberculosis-like lesions caused by *R. equi* may also occur in the submandibular and other lymph nodes of cattle and pigs and, in young goats, granulomatous lesions in the liver are associated with wasting and death. Infection in other animal species is rare and usually the result of immunosuppression. *Rhodococcus equi* is an important cause of AIDS-associated pneumonia in HIV-infected humans.

The distribution of *R. equi* is highly variable, being endemic on some horse farms and only occuring sporadically or not at all on others. This seems to be due to differences in foal population density, farm management and environmental factors, such as temperature dust and soil pH, as well as the presence of a high proportion of virulent strains among *R. equi* in the soil. Manure is an important reservoir of infection as this pathogen rapidly grows on volatile organic acids contained within it [38]. The majority of cases of *R. equi* infection are diagnosed during dry, warm summers; not only are these conditions optimal for bacterial multiplication but also give rise to a dusty environment causing foals to inhale contaminated dust particles.

The pyogranulomatous lung lesions characteristic of *R. equi* infections reflect its ability to survive in macrophages, a characteristic also of *Mycobacterium tuberculosis*, to which it is closely related. The latter is highlighted by a partial genome sequence of *R. equi* which showed that the majority of *R. equi* genes are most similar to those of *M. tuberculosis* [55]. *R. equi* and *Mycobacterium* species belong to the Mycolata, a phylogenetically distinct group of high G+C Gram-positive bacteria that contains a number of pathogens, including species of the genera *Rhodococcus*, *Nocardia*, *Corynebacterium* and *Mycobacterium* [26]. Mycolata are characterised by a unique cell envelope that consists of mycolic acids linked to arabinogalactan wall polysaccharide and (glyco)lipids. The lipoarabinomannan (LAM) of *R. equi* is smaller than mycobacterial LAM, and does not display the extensive branching seen in the latter. But as is the case in mycobacterial LAM, it displays the terminal mannose-containing side chains [22]. The unique cell envelope of the Mycolata is completely different from those of Gram-negative and other Gram-positive bacteria. It forms a permeability barrier to hydrophilic compounds, resulting in the formation of a periplasmic space [9, 13, 63]. Obviously this has consequences for the import of small hydrophilic molecules, which is highlighted by the presence of porins which have been identified in the cell walls of *Mycobacterium*, *Corynebacterium*, *Rhodococcus* and *Nocardia* [42, 45, 46, 58]. The mycolic acid containing cell wall is likely to be of importance for survival of these bacteria under harsh enviromental conditions as occur for example within macrophages. It has been shown that *R. equi* is extremely resistant to environmental stress conditions, such as oxidative stress and low pH [6, 7].

2. VIRULENCE PLASMID

Although *R. equi* has been recognised as an important foal pathogen since the early 1920's, it was not until 1991 that the first major breakthroughs in identifying the virulence mechanisms were made. Takai et al. showed that serum of foals infected with *R. equi* invariably has high levels of antibodies directed against a highly immunogenic protein of approximately 15–17 kDa [71]. This Virulence Associated Protein (VapA) is susceptible to trypsin digestion and accessible to biotin labeling, suggesting a surface localization for the protein. In addition, VapA could be radiolabeled with

[9,10-^{3}H] palmitate, showing that the protein is lipid modified, a feature that may serve to anchor the protein to the cell wall [69, 73]. Lipoproteins, which are abundant in Gram-positive bacteria are generally involved in transport of solutes into the cytosol, adherence to surfaces, or have enzymatic activity [64]. The signal sequence of lipoproteins contains a "lipobox", which consists of the amino acid sequence (S/T/G/L/A/V)L(A/S)(G/A)C. The cysteine residue is the first amino acid of the mature protein and is lipid modified. Analysis of the genomic sequence of *Mycobacterium tuberculosis* revealed the presence of 65 putative lipoproteins containing a lipobox, suggesting that mycolic acid containing actinomycetes employ a conventional lipid modification system as found in other bacteria [64]. However, the VapA protein does not contain cysteine nor a lipobox, suggesting that *R. equi* may employ an additional mechanism to lipid modify proteins.

Following the identification of VapA as a virulence associated protein, a second major breakthrough it was shown that the *vapA* gene is located on an indigenous *R. equi* plasmid of approximately 80 to 85 kb [72, 76]. The third major breakthrough was the discovery that *R. equi* strains cured of these plasmids are unable to survive and replicate in macrophages, and are avirulent for foals and mice [23, 35]. Isolates from the submaxillary lymph nodes of infected pigs also contain a large plasmid. However, these plasmids do not encode VapA, but a related protein of 20 kDa (VapB). Interestingly, pig isolates are less virulent in mice than foal isolates harbouring the *vapA* gene, suggesting host specificity of strains harbouring different plasmids [67]. These studies clearly establish that the virulence plasmid is essential for virulence in foals and pigs, and therefore encodes one or more virulence factors. However, *R. equi* isolates from cattle and goats lack a virulence plasmid and human isolates contain either VapA or VapB encoding plasmids or lack a virulence plasmid altogether [21, 65, 70, 75]. This suggests that the pathogenesis of *R. equi* infection in these hosts is different than in foals or pigs.

Nucleotide sequencing of the virulence plasmid of two foal isolates revealed the presence of 69 ORF [68]. Based on comparisons with genes previously identified in other organisms three functional regions of the virulence plasmid can be recognised. Two of these harbour genes that are similar to those encoding proteins involved in conjugation and in plasmid replication, stability and segregation. Interestingly, ORF 34, 38, 39 and 40 of the virulence plasmid are similar to open reading frames found on an indigenous plasmid of *Micrococcus* sp 28 (Accession number AY034092), and are organised in a similar fashion suggesting a common origin. The identification of genes that resemble genes required for conjugation suggests that the virulence plasmid can be mobilised from virulent to avirulent strains. Although this has not yet been demonstrated, it is highly significant as it would provide a mechanism for the virulence plasmid to spread through a population of avirulent strains. The third region of 27.5 kb bears the hallmarks of a pathogenicity island (Fig. 1): it is characterised by a significantly lower G+C content than the rest of the plasmid, and is flanked by genes similar to transposon resolvases. In addition, it harbours the *vapA* gene strengthening the notion that the third region is indeed a pathogenicity island. Analysis of the pathogenicity island resulted in the surprising finding that it encodes six *vapA* homologues (*vapC, D, E, F, G, H*). With exception of *vapF*, which is not functional, all *vap* genes encode proteins with a clear signal sequence, indicating that these are extracellular proteins. Byrne et al. showed that VapC, D and E are indeed secreted, but in contrast to VapA are not anchored to the cell wall [10]. In addition to the six *vap* genes, four more genes encode proteins with a clear signal sequence, bringing the total of extracellular proteins encoded by the pathogenicity island to ten. As extracellular proteins can interact with the host

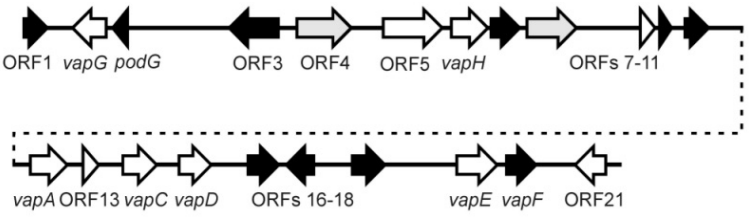

Figure1. Schematic view of the 27.5 kb pathogenicity island of the virulence plasmid of *Rhodococcus equi*. The arrows indicate the position and direction of transcription of the genes located within the pathogenicity island. White arrows indicate that the genes encode proteins with a signal sequence and may therefore be secreted. Grey arrows indicate the two transcriptional regulators encoded by the pathogenicity island. The *podG* gene was identified by Benoit et al. [5] and is not included in the numbering of the open reading frames, which is the original numbering by Takai et al. [68].

environment, these ten proteins could be important virulence factors.

Although the discovery that virulence of *R. equi* is dependent on a large plasmid, the subsequent determination and analysis of the nucleotide sequence did not provide the immediately expected insight into the virulence mechanisms of *R. equi.* The reason is that the majority of the proteins encoded within the pathogenicity island do not share any similarity with proteins from other organisms, suggesting that *R. equi* employs a novel virulence mechanism. Two of the proteins that do have homologues in other bacteria are transcriptional regulators.

ORF8 encodes a response regulator, which usually interact with a sensor kinase protein to regulate gene expression. The latter autophosphorylates a conserved histidine residue in response to an environmental signal; the phosphate is transferred to a conserved aspartate residue in the response regulator resulting in transcriptional activation. Although the virulence plasmid encodes a response regulator, it does not encode a sensor kinase, indicating that the ORF8 gene product interacts with a chromosomally encoded sensor kinase protein. The PhoP/PhoQ system is an example of a two-component system in *Salmonella typhimurium* that is induced within the phagosome of the macrophage [2]. PhoQ, an inner membrane sensor kinase, responds to changes in cation concentration and transduces this signal to the cytoplasmic response regulator, PhoP. The latter regulates invasion genes on the pathogenicity island I, SPI-1 [29]. This regulatory system has also been identified in *Yersinia pestis* and was shown to be important for survival under conditions of macrophage-induced stress [51].

ORF4 encodes a protein homologous to the LysR type transcriptional regulators (LTTR). LTTR are, after the two component response regulators, the second largest class of bacterial regulatory proteins [61]. The more than 100 proteins assigned to this family control a wide range of bacterial processes, for example bacterial carbon dioxide fixation (CbbR) and oxygen stress response in pathogens (OxyR). A number of LTTR have been identified which are associated with virulence gene regulation. For example, SpvR encoded by the 90 kb virulence plasmid of *Salmonella typhimurium*, regulates the *spv* operon in response to conditions such as late growth phase [11]. Interestingly, the *spv* operon has been shown to be induced in bacteria within macrophages [57].

Three other genes, ORF 3, 5 and 21, encode proteins with clear homologues in other bacteria. The protein encoded by

ORF21, which has a *M. tubercolosis* homologue (Rv1885c), is a secreted chorismate mutase, an enzyme of the shikimate pathway for biosynthesis of aromatic amino acids. The extracellular location of this protein is enigmatic, as this pathway is usually located inside the cytoplasm. Secreted chorismate mutase proteins have also been encountered in *S. typhimurium*, *Erwinia herbicola*, and *Pseudomonas fluorescens* [12]. The nematode plant pathogen *Meloidogyne javanica* produces an extracellular chorismate mutase that appears to interfere with the shikimate pathway of plants, which is required for plant growth and defense [44]. Interestingly, overexpression of this nematode enzyme in plants alters plant cell development, leading the authors to speculate that it is involved in allowing nematodes to establish a parasitic relationship with the host plant [20]. An alternative possibility is that the protein encoded by ORF21 is not involved in the biosynthesis of aromatic amino acids, but may catalyse a reaction similar to the chorismate mutase reaction using a substrate structurally related to chorismate. The protein encoded by ORF5 is predicted to be an integral membrane protein, displaying a strong similarity to sugar permeases (Pfam 00083). ORF3 encodes an O-methyl-transferase protein, that lacks a signal sequence and is therefore likely to be located in the cytoplasm. Although it has not yet been shown whether these genes are required for virulence, their presence in the pathogenicity island might suggest that virulent *R. equi* either produces a metabolite that is secreted by the cell or that *R. equi* takes up a modified host metabolite. The former could serve to alter the macrophage response, the latter could be involved in signaling.

3. REGULATION OF VIRULENCE PLASMID GENE EXPRESSION

Successful invasion and survival within macrophages are totally dependent on an adequate response to environmental signals. Bacteria therefore employ complex regulatory networks to perceive and integrate environmental signals which ultimately leads to a pattern of gene expression uniquely adapted to meet environmental challenges. The first gene for which it was shown that its expression is indeed controlled by environmental parameters is *vapA*. The expression of this gene was shown to respond to temperature and pH, with maximal expression occuring at high temperature (37 °C) and low pH (6.5) [66, 69]. It was subsequently shown that the *vapA* homologues, *vapC, D, E*, are also thermoregulated in a similar fashion as *vapA* [10]. Benoit et al. demonstrated that *vapA* and *vapG* are upregulated under oxidative stress conditions, by exposing *R. equi* to H_2O_2 [7]. Following uptake of *R. equi* by its host it is subjected to oxidative stress, a decrease in pH, an increase in temperature, signaling that virulence factors must be induced. Some of these factors are discussed under Section 4. Cell biology. The pattern of gene regulation of the *vap* genes is therefore consistent with their presumed role as virulence factors.

Recently Ren and Prescott [56] constructed a DNA microarray to analyse virulence plasmid gene expression. Using this array they identified the concentration of iron and magnesium as additional environmental parameters influencing the expression of genes within the pathogenicity island. Of the genes that were up-regulated by an increase of growth temperature from 30 to 37 °C, twelve genes, including the six *vap* genes were further up-regulated under low-iron conditions, and were down-regulated at decreased magnesium concentrations. A second group displayed the converse regulatory pattern in response to iron and magnesium: ORF 3, 9 and 10 were down-regulated by low-iron and up-regulated by low magnesium concentrations. A key question is whether virulence plasmid genes are transcribed following internalization by macrophages. Using the virulence plasmid DNA array, it was shown that only the genes within the pathogenicity island

were differentially transcribed inside equine macrophages compared to growth of *R. equi* under non-limiting growth conditions at 30 °C. Not surprisingly these included the *vap* genes. Two genes unique to *R. equi* and of unknown function, ORF 9 and 10, were the most abundantly expressed.

These studies show that the regulation of expression of genes within the pathogenicity island is complex, and depends on at least five environmental signals: temperature, pH, oxidative stress, magnesium and iron. It seems very likely that the two transcriptional regulators encoded by ORF4 and ORF8 play an important role in transducing these environmental signals to the transcription apparatus, although this has not yet been established. To date two transcriptional repressors, Fur and IdeR, controlling iron dependent gene expression have been identified [31]. Although these proteins are unrelated, they operate in a similar manner. Both bind to their cognate binding sites in the presence of Fe^{2+}, and repress transcription. In the absence of Fe^{2+}, the proteins release from their binding sites and repression is alleviated. An *ideR* homologue of *R. equi* was recently cloned and over-expressed in *E. coli*. It was shown that the *R. equi* IdeR protein is functional; it binds to a consensus IdeR recognition sequence and regulates gene expression in an Fe^{2+} dependent manner [8]. Interestingly the promoter region of the *vapA* gene contains an IdeR consensus binding site [56], suggesting that the iron dependent expression of this gene may be controlled by IdeR.

4. CELL BIOLOGY

The basis of pathogenicity of *R. equi* is its ability to multiply in and eventually to destroy alveolar macrophages. Infectivity is largely or exclusively limited to cells of the monocyte-macrophage lineage, whereas neutrophils from foals are fully bactericidal. How virulent *R. equi* survive and replicate in macrophages offers fertile ground for future study.

Since opsonisation of *R. equi* with specific antibody is followed by increased phagosome-lysosome fusion and by significantly enhanced *R. equi* killing by equine macrophages [32, 79], macrophage entry through non-Fc receptors may be important in determining the fate of the bacteria. The general limitation of the disease in foals to the period between 4 and 12 weeks after birth coincides with the time of major decline of maternally-derived antibodies, supporting the concept that entry of *R. equi* into macrophages by non-Fc receptors may be important in allowing it to avoid antibody-associated macrophage killing pathways. The mechanisms of bacterial ingestion vary with the phagocyte receptor involved, which in turn also affects activation of the macrophage.

Complement receptor (CR)-mediated phagocytosis of bacteria involves several macrophage receptors (CR1, CR3, CR4) which bind one or more of C3b, C3bi, and C4b deposited on the bacterial surface. Whereas in antibody (FcR)-mediated phagocytosis, pseudopodia tightly engulf opsonized bacteria before drawing them into the macrophage, in CR-mediated phagocytosis, opsonized bacteria sink into the macrophage without marked pseudopodia [1]. FcR-induced phagocytosis is strongly associated with production of reactive oxygen intermediates (oxidative burst) and pro-inflammatory mediators, whereas CR-mediated phagocytosis does not induce this effect [1]. The effect of the phagocyte receptor involved in mediating differences in internalization mechanisms is illustrated also by the mannose receptor (MR), which recognizes mannose and fucose on bacterial surfaces. Unlike FcR and CR-mediated phagocytosis, MR-mediated phagocytosis is not associated with accumulation of the cytoskeletal proteins vinculin and paxillin around the phagosomes [1].

Optimal binding of *R. equi* to macrophages in vitro requires complement and is

mediated by Mac-1, a leukocyte complement receptor type 3 (CR3) [35]. The lipoarabinomannam of *R. equi* (ReqLAM) binds to recombinant mannose-binding protein, which may activate complement C3b deposition onto *R. equi* via the lectin pathway, and thus may also promote Mac-1 mediated uptake into macrophages [22]. ReqLAM may bind other collectins, since *M. tuberculosis* lipoarabinomannam is a ligand for human pulmonary surfactant protein A, and equine surfactant proteins bind mannose [22]. In addition, entry to macrophages through ReqLAM binding to the mannose receptor may occur.

Once bacteria are internalized, the phagosome undergoes a series of fusion and fission events with vesicles from the endocytic pathway, a complex maturation process which leads to the formation of phagolysosomes. The usual progressive movement of the phagosome on microtubules into the cell during maturation allows the phagosome to interact with the endosomal system. During maturation, the phagosome membrane increasingly changes to resemble that of late endosomes and lysosomes. This follows a regulated and sequential pattern in which phagosomes change from ready fusion with both early and late endosomes, to fusion most readily with lysosomes, and finally to failure to fuse with any endocytic organelle [74]. The evidence supports phagosome-endosome fusion as occurring as brief exchanges ("kiss-and-run") of content and membranes [18, 19]. This maturation process is characterized by changes in the molecules associated with the phagosomal membrane from those of early endosomes (Rab5, annexin, NSF, SNAP [soluble NSF-attachment proteins], transferrin receptor, mannose receptor) to those of late endosomes and lysosomes (Rab5, Rab7, mannose-6-phosphate receptor, cathepsin D, LAMP1 [lysosome-associated membrane-protein1], LAMP2). One or more proteins involved in budding from endosomal vesicles and their fusion with the phagosome may be important targets for *R. equi* but this requires to be investigated.

The presence of transferrin in early endosomes likely explains the importance of iron restriction in regulating transcription of some virulence plasmid pathogenicity island genes. NRAMP1 (natural-resistance-associated macrophage protein 1) confers innate resistance to macrophages against the growth of certain intracellular pathogens [60]. A divalent cation transporter that shows preference for iron or manganese, and pumps iron into the phagosome [80], NRAMP1 is associated with efficient acidification of the phagocytic vacuole, although details of its mechanism of action are unclear [60]. Once inside the phagosome, iron stimulates formation of biotoxic reactive oxidative species via the Fenton/Haber-Weiss reactions [52]. The limited quantities of iron transported into the phagosome by NRAMP1 to catalyze the generation of antimicrobial oxidative radicals do not increase iron availability for bacteria since iron in the phagosome is removed during the process of nitric oxide (NO) formation. Interestingly, dramatic down-regulation of NRAMP1 transcription was observed in equine macrophages following infection with *R. equi* [77], but whether this is different between plasmid positive and negative strains was not explored. In *M. tuberculosis*, IdeR controls the transcription of genes involved in macrophage survival, as well as in iron acquisition and iron storage [25].

Maturation of the phagosome is also characterized by progressive acidification with delivery of the vacuolar proton pump from early and late endosomes, and removal of the Na^+/K^+-ATPase from the late endosome, so that the vacuolar pump works unopposed in the phagolysosome (pH $\leq$ 5.5) [15]. For bacteria which do not interfere with the process, phagocytosis to phagolysosome formation takes about five minutes [49]. Phagocytosed bacteria are degraded in the late phagolysosome by the same mechanisms employed by lysosomes, involving a wide range of acid-resistant hydrolases within the acid environment of the phagolysosome. Materials may also be transported for further degradation from the

phagolysosome to late endosomes or lysosomes.

Intramacrophage *R. equi* appear to be located exclusively within membrane-enclosed vacuoles and persistence correlates with the absence of phagosome-lysosome fusion [32, 79]. It has been shown that plasmid-encoded products contribute to the ability of *R. equi* to survive and replicate in macrophages. Preliminary observations by Haas et al. [30] suggest that maturation of the phagosome is more efficiently diverted in strains possessing the virulence plasmid compared to plasmid-negative isogenic strains that do not, and that cytotoxicity of *R. equi* for J774E murine macrophages is strongly up-regulated by the possession of the virulence plasmid.

Although details are sparse, *R. equi* may therefore behave like *M. tuberculosis* and related mycobacteria which interfere with phagosome maturation, residing in a vacuole which is only mildly acid (pH 6.5) and which resists lysosomal fusion [3, 16]. *M. tuberculosis* interferes with the acquisition of the fusion facilitating molecules, either by avoiding loss of Rab5 and/or early endosome SNARES (soluble N-ethylmaleimide-sensitive fusion factor attachment protein receptor), or by blocking acquisition of other facilitating molecules (such as Rab7) [16, 58]. The specific lack of the vacuolar proton pump [59] appears to be responsible for the mildly acid conditions. Inhibition of acidification blocks vesicular delivery to lysosomes [14], and the pH of the phagosome is probably important in controlling fusion events. pH in the phagosomes containing mycobacteria is maintained mildly acidic, possibly by persistence of the Na^+/K^+-ATPase or by ammonia production within the phagosome. Ammonia, which can be produced in several ways by *M. tuberculosis* inhibits phagolysosome formation [27]. Whether the prominent urease of *R. equi* has a similar role is unknown.

Besides lysosomal degradation, macrophages can inhibit or kill bacteria by producing toxic reactive oxygen and nitrogen species through the respiratory burst phagocyte oxidase and inducible nitric oxide synthase (iNOS). As noted earlier, *R. equi* may avoid the oxidative burst through its mode of entry into macrophages. Macrophage production of toxic superoxides from the relatively inert oxygen molecule involves production by a membrane-bound NADPH oxidase that is activated in the "respiratory burst" which occurs when opsonized bacteria initiate the phagocytosis process. Nitric oxide synthetases, especially iNOS, catalyse the oxidation of a guanidino nitrogen of L-arginine to NO. iNOS is usually transcribed in response to cytokines (IFN-γ, IL-1, TNF-α) released in response to microbial products or through immune cell interactions. Both IFN-γ and TNF-α are required for clearance of virulent *R. equi* from mice [43]. Besides sometimes synergizing with the oxidative burst to produce peroxynitrite, NO and its derivatives have a longer lasting bacteriostatic effect on bacteria in the phagosome. Killing of *R. equi* by murine macrophages was shown to be dependent on IFN-γ, which activated macrophages to produce reactive nitrogen and oxygen intermediates which in turn combined to peroxynitrite ($ONOO^-$), the essential bactericidal factor [17]. IFN-γ activation of macrophages prevented growth of *R. equi*, in contrast to their replication in non-IFN-γ activated macrophages, consistent with the role of IFN-γ in activating the high-output iNOS [17]. The study suggested a two-step model for efficient killing of *R. equi* in immune animals [17]. The first step, macrophage activation by IFN-γ and TNF-α results in nitric oxide production following iNOS transcription, and the second step, signalled by bacterial phagocytosis, results in the respiratory burst and the production of, and killing by, $ONOO^-$ produced by reaction between nitrogen and oxygen intermediates.

For *M. tuberculosis* and possibly for *R. equi*, once macrophages are activated by IFN-γ, these cells overcome the block that

the bacterium imposes on endosomal maturation. *R. equi* is highly resistant to hydrogen peroxide, an oxidative stress which reflects the production of reactive oxygen intermediates effect during the oxidative burst of phagocytes following bacterial phagocytosis [7]. This resistance may result from its catalase activity. Although resistance to hydrogen peroxide is not a function of the virulence plasmid, treatment with hydrogen peroxide resulted in enhanced *vapA* and *vapG* transcription [7], supporting a role for VapA and VapG in survival of *R. equi* under the oxidative stress imposed by macrophages.

5. IMMUNITY

Current evidence suggests that infection of foals with virulent *R. equi* may result in subversion of cell-mediated immunity and development of an ineffective and sometimes lethal immune response. Although details are not clear, the pathogenicity island on the virulence plasmid must play a crucial role in this immunomodulatory process.

Immunity to *R. equi* pneumonia in foals likely depends on both the antibody and cell-mediated components of the immune system but its exact basis remains to be determined. Antibodies to *R. equi* are widespread in horses. Evidence for a role of antibody in protection against *R. equi* is the protective effect of passively transferred anti-*R. equi* hyperimmune equine plasma which is used extensively on endemically affected farms to reduce morbidity and mortality. Hooper-McGrevy et al. [36] showed that purified VapA- and VapC-antibody provided protection of foals against experimental pneumonic infection equivalent to that obtained with hyperimmune plasma. Interestingly, however, foals born to vaccinated mares are not protected against infection despite passive transfer of *R. equi* antibody. This failure may relate to the isotype of antibody produced by the vaccines used experimentally but this has not been investigated in detail.

Because of the facultative intracellular nature of *R. equi*, cell-mediated immune mechanisms are thought to be of major importance in resistance. Key studies by Kanaly et al. [39–41] have confirmed this in mice. For example, immunocompetent BALB/c mice experimentally infected with virulent *R. equi* developed a Th1 cytokine response and progressively cleared the infection. Mice in which a Th2 response was induced by administration of monoclonal antibodies against IFN-γ, failed to clear the infection and developed pulmonary granulomas. The two major mechanisms by which T lymphocytes mediate clearance of intracellular pathogens are secretion of cytokines and direct cytotoxicity (usually mediated by MHC class I restricted $CD8^+$ T lymphocytes). Kanaly et al. [39] conclusively demonstrated the central role of $CD4^+$ T-cells since MHC class I transgenic mice deficient in $CD8^+$ T-lymphocytes cleared virulent *R. equi* from the lungs whereas infection persisted in MHC class II transgenic mice deficient in $CD4^+$ T-lymphocytes and led to the formation of granulomas.

It is apparent that infection of foals by virulent *R. equi* can result in an immunomodulatory effect which drives an ineffective, Th2-like, rather than an effective, Th1-like, response. For example, Giguère et al. [24] found that foals experimentally infected with a virulent *R. equi* strain showed marked reduction in IFN-γ production by $CD4^+$ lymphocytes isolated from bronchial lymph nodes compared to $CD4^+$ lymphocytes similarly isolated from foals infected with an avirulent, plasmid-cured derivative of the same strain and that concentrations of IL-10, a Th1-immune response down-regulatory cytokine, were significantly higher in the lungs of foals infected with the virulent strain. The conclusion was that virulent *R. equi* have an immunomodulating effect which drives an ineffective, Th2-like, immune response. Interestingly, Hines et al. [34] observed that clearance of virulent *R. equi* from the lung of experimentally-infected adult horses was associated

with increased numbers of IFN-γ producing $CD4^+$ and $CD8^+$ lymphocytes. This difference between immune adults and non-immune foals may reflect unique features of the foal's immune system, differences between immune and non-immune animals, or differences in the experimental procedures.

Hooper-McGrevy et al. [37] observed an IgGb- and IgGT-dominant response to Vap proteins in the serum of foals sick with *R. equi* compared to healthy, immune, foals and to adults, both of which had an IgGa-dominant response. This finding was interpreted as supporting the concept that foals that become sick with *R. equi* do so because of an ineffective, Th2-dominated, immune response and that this is the reason for the IgGb and IgGT dominant response in these foals. Because of the reciprocal relationship between these two types of immune response, or perhaps also because of an interference effect of IgGT on complement activation by IgGa, immunization with VapA in an adjuvant which drives a Th2-response would be expected to result in development of disease. In support of this concept, immunization of foals under natural settings with a VapA extract in aluminium hydroxide adjuvant resulted in development of pneumonia in immunized but not in nonimmunized foals, and a IgGb- and IgGT-dominated isotype response compared to an IgGa response in immune foals [54].

Adult immune horses showed marked lymphoproliferative responses to recombinant VapA following intrabronchial challenge with virulent *R. equi* [33], as well as a marked $CD4^+$ and $CD8^+$ T-lymphocyte response, suggesting that both subsets play a role in clearance of infection from the lung, reinforcing also earlier conclusions of the importance of VapA as an immunodominant antigen. Stimulated cells showed significant increases in antigen-specific IFN-γ but not in IL-4 expression, and local levels of IgGa and IgGb isotypes against VapA were dramatically enhanced after challenge, suggesting that these are important correlates of protective immunity. Interestingly, IgGa and IgGb fix complement whereas IgGT does not, and indeed IgGT may inhibit complement fixation by IgGa and IgGb [47].

6. DEVELOPMENT OF GENETIC TOOLS

Although considerable progress has been made in understanding important aspects of virulence of *R. equi*, there is an almost complete lack of genetic tools for this bacterium that will be required to make further progress. Recently, this problem has been addressed, and significant progress has been made in the development of *R. equi–E. coli* shuttle vectors, transformation and random and site specific mutagenesis procedures. To date three plasmids have been described that efficiently replicate in *R. equi*. As these are based on different replicons, they are compatible, allowing stable maintenance of these plasmids in the same cell. pRE7 is a shuttle vector based on the origin of replication of the *R. equi* virulence plasmid, and is therefore not maintained in virulent *R. equi* strains [78]. A shuttle vector based on an indigenous plasmid from the plant pathogen *R. fascians* containing a chlorampenicol resistance marker was shown to efficiently replicate in *R. equi* [62]. Plasmids containing an origin based on the pAL5000 plasmid of *Mycobacterium fortuitum* subsp. *fortuitum* were also shown to replicate in *R. equi* [23]. All plasmids are introduced into *R. equi* by electroporation [62, 78], however, conjugation using the *Escherichia coli* strain S17-1 has also been shown to be an efficient method to introduce plasmids (Boland and Meijer, unpublished results).

An essential technique to identify virulence factors is random mutagenesis, in which a selectable marker is randomly integrated into the genome. A mutagenesis system based on the insertion of a protein-DNA complex of transposase and a DNA fragment harbouring a kanamycin resistance gene flanked by transposase binding

sites [28] was shown to integrate efficiently and randomly into the genome of *R. equi* [48]. Transposome mutagenesis therefore allows the generation of mutant libraries which can be screened for virulence. This approach led to the isolation of an *R. equi* mutant defective in haem utilisation (Graham and Meijer, unpublished results). Recently, a random mutagenesis system for *R. equi* based on the Himar1 transposon, which belongs to the Mariner family of transposable elements, was described. This highly efficient system was used to isolate an attenuated mutant of *R. equi*, underscoring the usefulness of transposon mutagenesis [4]. Navas et al. [50] developed a highly efficient system based on homologous recombination to disrupt the *choE* gene encoding cholesterol oxidase of *R. equi*. The development of random and site directed mutagenesis procedures for *R. equi* was a major development which will facilitate a detailed analysis of *R. equi* virulence.

7. CONCLUSION

The last decade has seen a number of important breakthroughs in understanding the mechanisms used by *R. equi* to allow it to proliferate in the host. Current work is focusing on identifying the molecular basis of how *R. equi* subverts the macrophage and subverts the foal's immune response, as well as on how to develop an effective vaccine for foals based on forcing a Th1-like immune response to Vap proteins.

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