Interaction of *Rhodococcus equi* with equine host defense mechanisms

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GENERAL INTRODUCTION

Characteristics of Rhodococcus equi

Classification

The earliest description of the bacterium now classified as Rhodococcus equi (R. equi) appeared in 1923. Magnusson, a Norwegian, isolated an organism from the lungs of foals with pneumonia and named this organism Corynebacterium equi (C. equi).[114] Later in this same year, the German workers Miessner and Wetzel isolated the same organism from foals and named it Corynebacterium pyogenes (equi).[127,128] Close resemblance of the organism to members of the genus Mycobacterium led Jensen to propose the name Mycobacterium equi in 1934.[83] Isolation of the organism from pigs prompted suggestions from Holth and Amundsen[77] and Plum[149] to name the organism Corynebacterium Magnusson-Holth. In 1945 the organism was isolated from chronic pneumonia in a calf by Holtman.[79] He subsequently proposed the name Corynebacterium purulentus as an appropriate indicator of the nature of the infection regardless of the host species. Gordon proposed the name Mycobacterium rhodochrous as a suitable classification for a bacterium which was intermediate between Nocardia and Mycobacterium.[67] His suggestion was based on the similarity of cell wall structure of these organisms.[34] Taxonomic studies completed by Harrington[73] and Stuart and Pease[186] supported

this reclassification. None of these alternative names was widely recognized,[13,88,208] even though there were generally accepted dissimilarities between C. equi and the other members of the genus Corynebacterium.[36,88,208]

Strong evidence for reclassification of C. equi was presented by several researchers in the 1970s.[14,65,66] Their taxonomic studies supported the transfer of C. equi into the newly established genus Rhodococcus. This classification was based on similarities in structure, biochemical reactivity, and habitat of C. equi and Rhodococcus coprophilus.[173,174] In 1980, R. equi was established as a new species and added to the approved list of bacterial names.[178] The DNA reassociation study performed by Mordarski et al. subsequently added to the evidence supporting the change from C. equi to R. equi.[134]

Staining reactions

In all reports of the Gram staining reaction of R. equi, the organism was found to be Gram-positive.[4] The presence of cell wall and/or capsular components composed of peptidoglycan has generally been accepted as the basis of the positive Gram-stain reaction.

Acid-fast staining of R. equi was first described by Jensen in 1934.[83] He reported on his finding of acid-fast bacteria in the cervical lymph nodes of pigs with non-tuberculous lesions. Other workers confirmed the acid-fast nature of R. equi isolated from pigs and other sources.[8,74,85,99,111,190] Two reports indicated that early

cultures of R. equi were not acid-fast.[5,187] Differences in these observations were probably caused by variations in staining technique, culture conditions, and the age and source of the bacterial culture. The cell wall structures of R. equi and acid-fast bacteria in the genera Mycobacterium and Nocardia were found to be similar.[62,63]

The presence of metachromatic granules in R. equi was first reported by Magnusson.[114] He used Neisser's stain to demonstrate numerous faintly staining granules scattered throughout the R. equi bacilli. Two reports which were published subsequently confirmed the observation.[90,124] Other workers were unable to detect granules within R. equi.[111,161,162,166] The cultural environment of the bacterium was probably a factor in the detection of metachromatic granules.

Morphology

Early literature on R. equi noted the pleomorphic nature of the bacterium.[115] The size and shape of the organism varied with temperature and culture conditions. Isolates from purulent lung lesions or solid culture media tend to be oval or coccoid in shape.[115] Broth cultures produce the longer bacillary forms of R. equi.[169] The size of the organism varies from 0.5 um to 1.0 um in diameter and from 1.0 um to 2.0 um in length. Smears of the organism frequently reveal clumping of the cells and formation of L or V shapes resembling Chinese characters.[4]

Structure

The presence of a capsule on R. equi has been documented by the use of modern techniques. In the years before such methods were available, India ink wet preparations were commonly used to outline bacterial capsules. Many workers did not detect capsules on R. equi with this technique.[32,43,90,161,162] Bruner and Edwards used India ink to demonstrate the presence of R. equi capsules in 1941.[19] This observation was supported by the studies of Wilson,[195] Knight,[95] and Smith.[181] Alcian blue staining was successfully used to stain the capsule of R. equi by Carter and Hylton in 1974.[25] Further evidence for encapsulation was obtained by Woolcock and Mutimer in 1978.[200] Their use of ruthenium red staining and electron microscopy demonstrated a laminated capsule surrounding the cell wall of R. equi. The staining reactions of this capsule with alcian blue and ruthenium red indicated its composition was primarily polysaccharide.[25,107]

The cell wall of R. equi is composed of carbohydrates, amino acids, and lipids. Like other Gram-positive bacteria, R. equi has a predominantly peptidoglycan cell wall composed of sugars and amino acids.[102] Various types of lipids are found within this peptidoglycan core. Although these major constituents are found in many bacteria, the composition and arrangement of cell wall components in R. equi is unique.

Structural carbohydrates and amino acids of the cell wall of R. equi were studied by several workers.[29,34,35,92,184,205] The reports agreed closely on the specific sugars and amino acids which composed

the peptidoglycan layer. Arabinose and galactose were found in large amounts in R. equi. Muramic acid and glucosamine were less abundant, while ribose, rhamnose, and mannose were absent. The major amino acid components in the cell wall were alanine, glucosamine, glutamic acid, and D,L diaminopimelic acid. Traces of aspartic acid, glycine, and muramic acid were present in some of the strains examined. The combination of sugars and amino acids found in the R. equi cell wall most closely resembled that of the chemotype IV cell walls of Corynebacteria, Nocardia, and Mycobacteria.[102]

Mycolic acids were found in the lipid component of the cell walls of R. equi and other bacteria having chemotype IV cell walls. Although the chromatographic mobilities of mycolic acids from R. equi and most corynebacteria were similar, the carbon atom backbone of R. equi mycolates was nearly twice as long as that of other coryneforms.[30,31,64] Thin-layer chromatography demonstrated similarities between the R. equi mycolic acids and mycolic acids from other bacteria in the the genus Rhodococcus.[62,92]

Phospholipids and glycolipids were found to be the main polar lipids of R. equi.[15,63,92] The phospholipid in highest concentration was cardiolipin. Fatty acid analysis with gas liquid chromatography revealed 33.1% saturation in the side chains.

Isoprenoid menaquinones and carotenoid lipids were the third and fourth classes of lipid isolated from R. equi.[31] The menaquinones functioned in electron transport and oxidative phosphorylation.[129, 164,204] Carotenoid lipids were associated with the formation of

pigments.[154]

The only intracellular component studied in R. equi was DNA. Early workers reported a guanine-cytosine (GC) content of 58.5 moles percent.[11,75] More recent values have ranged from 65.9 moles percent to 70.8 moles percent GC.[134,206] Results of DNA reassociation of R. equi with other members of the genus supported the classification of the bacterium as a distinct species in the genus Rhodococcus.[134]

R. equi is a non-motile organism having no flagella. One report notes the presence of scant pili on the surface of the bacterium, but the significance of these structures is unknown.[209]

Colonial morphology

On conventional agar plates R. equi is most commonly described as forming irregularly round colonies with a smooth, entire edge and having a glistening, mucoid appearance.[5,17,90,93,117,178] Colonies less than 48 hours old range from 1 to 3 mm in diameter and have a white or gray cast. Older cultures usually develop a characteristic salmon pink pigment. Variations from this typical appearance are described in the older literature. Rough, dry colonies of R. equi on agar are noted and the pigment colorations reported include yellow, pink, red, and brown.[5,33] A less-mucoid, rough form of R. equi colony can be seen following repeated passage on laboratory media.[83,99,198] The change in colonial morphology from smooth to rough is attributed to decreased synthesis of capsular material.

The growth of R. equi on potato resulted in a wide variety of pigment colorations and colony textures.[17,32,37,43,61,81,90,99,114,116,162,168] The range of pigment colors included tan, yellow, pink, red, orange, and brown. Growth on potato medium varied from light to abundant, and colony textures ranged from dry and granular to smooth and mucoid.

Growth of R. equi on egg media resulted in colonies resembling those of tubercle bacilli.[19,42,91] The dry, raised, rough-textured colonies exhibited pigments ranging from pink to dark red.

Characterization of the growth of R. equi in liquid media was attempted by many workers and resulted in a wide variety of descriptions. Several groups reported that cultures in liquid media formed a heavy pellicle and/or sediment.[17,29,32,33,37,83,99,114,198] Other reports noted that R. equi growth in broth caused turbidity of the culture medium with little or no sediment formation.[43,90,111,124]

Differences in the descriptions of the gross appearance of R. equi are the manifestations of many variables in culture media and conditions, bacterial sources, and subjective assessments of growth. The diversity of past descriptions is being replaced by increasingly uniform characterizations of R. equi.

Growth requirements

R. equi has been reported to be capable of utilizing a wide variety of carbon sources. The sources of carbon which were utilized by R. equi included acetic acid, pyruvic acid, L- and D- lactic acid,

malic acid, succinic acid, fumaric acid, propionic acid, butyric acid, and glycolic acid.[65,99,207]

The nitrogen requirements of R. equi have not been thoroughly studied. Limited trials have indicated that R. equi could be cultured with either ammonium sulfate or potassium nitrate as the sole source of nitrogen.[154,173]

A requirement for oxygen has led to the classification of R. equi as an obligate aerobe.[65,92] Growth occurred in atmospheres containing up to 40% CO_2.[32]

Optimum temperature for the growth of R. equi has been disputed for many years. The bacterium grew well at temperatures ranging from 10°C to 40°C.[62] Reported optimum temperature ranges have included 28° to 30°C,[83,99] 25° to 37°C,[17] and 37°C.[111]

Activity in biochemical tests

R. equi has been characterized as generally non-reactive in standard biochemical tests. The following reactions have been accepted for the majority of R. equi isolates: negative for liquefaction of gelatin; negative for liquefaction of coagulated serum; negative for hemolysis of erythrocytes from a wide range of hosts; positive for catalase production; negative for production of ammonia, indole, and oxidase; negative for oxidation or fermentation of sugars and alcohols.[4,36,114,163]

Tests for which the characteristic reaction of R. equi has been under dispute include oxidase production, urea hydrolysis, nitrate

reduction, hydrogen sulfide production, hippurate hydrolysis, and litmus milk reactions. 9,10,29,38,39,63,81,101,120,136,137,141, 160,175,187,193

Diffusible substances

Toxin production by R. equi has not been demonstrated. However, the presence of a diffusible substance which enhanced the hemolytic activity of several other bacterial species has been noted.[26,54,140] Hemolysis of erythrocytes was observed when cultures of R. equi were streaked against Staphylococcus aureus, Corynebacterium hemolyticum, Corynebacterium pyogenes, Corynebacterium pseudotuberculosis, and Listeria monocytogenes. The substance produced by R. equi was capable of diffusing through a membrane filter, but its chemical nature has not been characterized.

Distribution

R. equi has been isolated on every continent except Antarctica. Literature from Africa has included reports from Kenya,[142,176] the Sudan,[37] South Africa,[70,125] and Nigeria.[1,2] Documentation of R. equi infection in Asia has come from India,[131,140,141,161,162182] Russia,[165] and Japan.[72] Australians have published numerous accounts of R. equi outbreaks in their country.[3,20,23,28,39,53,111,112, 166,175,195,198,203] The bacterium appears to be widely distributed throughout Europe, with reports of R. equi isolations in the United Kingdom,[27,32,33,147,167,168] Ireland,[50,71,104] the Scandinavian

countries,[7,8,46,52,69,76,77,78,84,89,85,114,116,145,150.151,188] Germany,[59,74,93,103,108,109,126,127,128,130,197] Italy,[10] France,[12,190,191] and Yugoslavia.[60,183] Several South American countries have reported R. equi infections.[22,101,132,133,153] The majority of the literature on R. equi isolates originating from North America came from the United States and Canada.[9,16,18,21,24,41,51,56,61,79,81,82,91,95,96,110,120,159,170,177,193,194]

Habitat

Many investigators have suggested that R. equi is a saprophytic inhabitant of the soil. The majority of other Rhodococcus species are classified as primary inhabitants of soil.[63] R. equi has not been established in this classification.[5,118] Cultures of R.equi have been recovered from bovine and equine feces,[6,144,145,201] from soils recently contaminated with cattle or horse feces,[3,145,195,199] and also from soils with no history of exposure to animal feces.[83] The isolations of R. equi from feces and soil provided an explanation for the continuous presence of R. equi in equine environments, but did not elucidate the route of exposure which resulted in disease.

Resistance

Isolation of R. equi from contaminated sources was simplified by the use of selective media.[6,5,199,201] The media, which contained nalidixic acid, novobiocin, cyclohexamide, and potassium tellurite, were tolerated by R. equi. Limited studies reported that the

bacterium was relatively resistant to treatment with acids or bases.[32,90,115,144] R. equi was susceptible to disinfection with phenol or mercurochrome but not to the effects of hypochlorous acid.[79,161] Antibiotic resistance was commonly seen with clinical isolates of R. equi in response to in vitro treatment with tetracyclines, penicillin, and ampicillin.[9,38,45,61,104,160,175] In studies by Cotchin[32] and Magnusson[115], the organism retained its virulence after 15 years of yearly subculture on simple media. Soil from a lawn seeded with R. equi in broth culture yielded isolates of the bacterium for the ensuing 12 months.[195] The temperature at which R. equi was killed by one hour of heating has been reported as 58°C to 60°C.[32,79,161] One of the few mechanisms of resistance not exhibited by R. equi was the formation of spores.

Clinical Syndromes Associated with Rhodococcus equi

Infection in horses

The most commonly reported clinical syndrome associated with R. equi is suppurative bronchopneumonia in young foals.[4] This infection is generally noted in foals between the ages of 2 and 4 months, although cases occur in slightly older and younger foals as well. The disease is sporadic in nature, except on farms where R. equi infection is endemic. Clinical disease may affect up to 15% of the foals on such farms.[3,47,135,177] Inhalation[21,114,121,180] and ingestion[5,20] are considered possible routes of exposure. Most cases of R. equi

pneumonia are first detected when animals begin coughing and show increases in respiratory rate and body temperature.[180] Diagnostic measures taken at the first sign of infection generally reveal severe bronchopneumonia caused by R. equi. Symptoms associated with continuation of the disease process include mucopurulent nasal discharge, dyspnea, and weight loss.[16,20,21,43,104,118,170] As respiratory distress increases, foals become dull and listless. Death is caused by asphyxiation.

Diagnosis of R. equi pneumonia in foals is usually based on the presence of clinical signs and characteristic post-mortem lesions.[72,135,161,180] Recently the technique of transtracheal aspiration has been adapted for use in the recovery of R. equi from infected foals.[96,119] Isolation and identification of R. equi from the respiratory tract of foals is the only currently available means of making a definitive diagnosis of the disease. Radiographic evidence of pneumonia and lung abscessation in foals does not identify the etiologic agent. Tests which measure humoral or cell-mediated immunity may support the diagnosis, but cannot differentiate between exposed and infected animals.[158] The presence of characteristic post-mortem lesions can be ascribed to R. equi infection when the bacterium is cultured from the tissues.

The insidious onset of signs of R. equi pneumonia delays diagnosis and greatly complicates treatment of the disease.[47,180] Systemic antibiotic therapy has little effect on the bacteria within encapsulated lung abscesses.[45] Lesions such as these are most

effectively treated with surgical drainage, but this technique is not currently practiced in foals.[194] Treatment is usually limited to administration of systemic antibiotics, nebulization of antibiotics into the respiratory tract, and supportive care.[57,180] The choice of antibiotic, if based on the in vitro culture susceptibility patterns, will vary with the particular isolate of R. equi.[3,4,38,45,53,82,97,101,104,156,160] Neomycin, erythromycin, and gentamicin are effective against most R. equi cultures, but these drugs have had limited application in foals because of cost and toxicity.[57] Difficulties with the treatment of R. equi pneumonia are evident in the 70% mortality rate generally ascribed to the disease.[3,22,47,114,161,177]

The pathogenesis of R. equi pneumonia in foals involves destruction of lung parenchyma due to cellular infiltration, alveolar collapse, and abscess formation.[5,47,114,157] Tissue involvement generally begins in the cranial and ventral lobes and spreads to involve large portions of the lung. Cellular infiltration of infected lung parenchyma results in thickening of interlobular septa and obstruction of bronchioles with fibrinomucopurulent exudate. Macrophages in the lungs and lymph nodes fuse into multinucleate giant cells. The coalescence of the cellular response and its walling off by fibrous material result in formation of an abscess. Multiple large abscesses, caseous debris, and areas of complete necrosis replace large portions of lung tissue.[86] Resolution of these abscesses has been observed radiographically in foals which recover from R. equi

pneumonia, but the recovery process is not well-documented.[57]

Post-mortem findings in foals with R. equi pneumonia include suppurative bronchopneumonia and bronchial lymphadenopathy.[5,47,86] Cases of long duration are characterized by the presence of abscesses within the lung and bronchial lymph nodes. These abscesses contain purulent or caseous exudate. Histologic examination of the exudate reveals the presence of large numbers of polymorphonuclear leukocytes and mononuclear cells. Many phagocytic cells within this exudate and in the diseased lung tissue contain ingested R. equi bacilli.[86] The cellular reaction within the lung is granulomatous in nature.[37,121]

A second syndrome in foals caused by R. equi is enteritis.[3,5,20,27,80,177] Symptoms of the enteric infection with R. equi are diarrhea and dehydration. Lesions of the intestinal mucosa and enteric lymph nodes are frequently described in conjunction with the pulmonary disease process.[21,43,80] Some authors believe the enteric infection occurs secondary to pulmonary infection, while others describe the infectious process as a primary lesion of R. equi infection.[5,20] Enteritis involving R. equi is characterized by infiltration of phagocytic cells into the lamina propria, necrosis of the submucosa and villous tips, and ulceration of the mucosa.[87] Lesions may be present in the small intestine, colon, or cecum. Lymphoid tissues of the Peyer's patches seem especially prone to lesion development.[5,27,47,87] Lymph nodes which drain the affected areas of intestine contain numerous giant cells, large numbers of R. equi-laden macrophages and much necrotic debris.

The incidence of disease caused by R. equi in adult horses is extremely low. Disease syndromes in adults include pulmonary and enteric infections similar to those described in foals,[23,37,58,176] and genital infections.[18,41,130] Cases of R. equi infection in adults are sporadic and generally not associated with epidemics of R. equi foal pneumonia.

Infection in pigs

During the first half of this century, there was a high incidence of R. equi infection in pigs. Cultures of R. equi were obtained from encapsulated caseous lesions in the cervical lymph nodes of young pigs in the 1930s and 1940s.[8,51,77,84,85,110,115,117,148,149,150,151,190,191] The R. equi lesions in pigs were nearly identical to lesions caused by tuberculosis, another disease which was prevalent in swine during these years. Attempts to reproduce the characteristic lesions by experimental inoculation of R. equi into pigs met with little success,[32,188] although R. equi could be recovered from cervical and submaxillary lymph nodes in some inoculated pigs.[8,90,115] Ingestion was believed to be the natural route of exposure.[4] Declining prevalence of R. equi infection in pigs mirrored the decrease in swine tuberculosis in the mid- to late 1900s. Changes in swine husbandry practices probably contributed to the reduction in number of R. equi infections in pigs.

Infection in man

Eight cases of R. equi pneumonia in people have been reported.[9,24,56,61,120,175,194] Although none of the patients had similar histories of potential exposure to the organism, all were immunosuppressed by neoplasia or chemotherapy when the infection developed. Lung lesions resembled those described in foals. Treatment included drainage of abscesses, resection of affected lung tissue, and antibiotic therapy. The R. equi infection was eventually cleared from most of these patients.

Infection in other species

Among domestic species R. equi infection has been reported in cattle,[33,47,79,89,105,112,113,131,141,142,162,203] sheep[1,39,166,201] goats,[141,193] and a cat.[81] Many of these cases involved the respiratory tract or lymphatic system, resulting in abscessation. Naturally occurring R. equi infections in non-domestic animals have been described in a koala, an alligator, and a crocodile.[53,82,160] Some attempts at infecting laboratory animals with R. equi were unsuccessful,[44,79,190] but other trials produced infection in mice,[37,109,127,128,188] guinea pigs,[20,114,188] and rabbits.[79]

Immunologic Properties of *Rhodococcus equi*

Interaction with natural immune mechanisms

R. equi is not an invasive organism.[5,47,87,113] The protection afforded by an intact epithelial barrier and associated defense mechanisms appears to be adequate in the face of considerable exposure to *R. equi*.[202] Suppression of natural immune mechanisms involved in the defense of the respiratory or gastrointestinal tract would facilitate the establishment of infection by this opportunistic bacterium. There appears to be a definite link between the weakening of natural protective mechanisms by immunosuppression and the incidence of *R. equi* pneumonia in people.[9,24,56,61,120,175,194] Lung defense mechanisms which are compromised by immunosuppression include ciliary activity, mucus production, and bactericidal capabilities of phagocytic cells.[143] It is likely that *R. equi* contains components which exacerbate the immunocompromised condition of the host defense systems.

Evidence of the effect of *R. equi* on the status of natural immunity is beginning to accumulate. A water-soluble fraction obtained from *R. equi* has been shown to block the binding of IgE to mast cells and the subsequent antigen-induced degranulation of these cells.[49,55] Immunosuppression of the allergic reaction is not the only known immunomodulatory activity of *R. equi*. Research on the growth of chemically-induced tumors in hamsters has shown a reduction in carcinogenesis following administration of *R. equi* extracts to the

animals.[122,123] The nonspecific nature of these reactions indicates probable modulation of natural immunity by R. equi.

Interaction with the cell-mediated immune system

The interaction of R. equi and the immune system is believed to result in establishment of cell-mediated immunity.[5,25,47,121,157,158] A portion of the evidence supporting this theory is based on proposed similarities of R. equi and the mycobacteria which are involved in tuberculosis.[32,83,91,112,149,202,203] R. equi infection and tuberculosis are both characterized as chronic infections by opportunistic, intracellular, acid-fast bacteria which cause a mononuclear cell infiltration and granulomatous reaction in the lung.

The cell-mediated immune response of horses to R. equi has been studied in vivo and in vitro. Wilson related R. equi-induced delayed-type hypersensitivity skin reactions in mares to their exposure to R. equi-infected foals.[195] Prescott has attempted with marginal success to demonstrate cell-mediated immunity using in vitro lymphocyte blastogenesis assays to test R. equi sensitization of naturally and experimentally infected foals.[157,158] These studies provided preliminary evidence that the cell-mediated response could be important in resistance to R. equi infection.

Interaction with the humoral immune system

The study of the humoral response of horses to R. equi has been inconclusive. Many sources reported that agglutinating antibody to R.

equi was absent or difficult to produce in horses, but that rabbits were suitable for the production of agglutinating R. equi antisera.[12,18,20,42,90,115,124,155,198] Low titers of anti-R. equi antibody were detected in serum from naturally infected or experimentally inoculated horses in gel diffusion[139] or passive hemagglutination tests.[25,158] Reports on serotyping schemes which utilized whole organisms and saline or hot-acid extracts of the bacterium contain a wide variety of conclusions about the cross-reactivity of R. equi capsular strains.[12,18,19,25,90,155,198] Discrepancies in the results of serologic testing obstruct the interpretation of studies on the antibody response to R. equi.

Research Objectives

The objective of this research was elucidation of the response of the equine immune system to R. equi. Three assays were developed to study the interaction of equine defenses with the bacterium. Neutrophil function assays, lymphocyte blastogenesis assays, and enzyme-linked immunosorbent assays (ELISAs) were used to test the R. equi-induced response of the equine natural immune system, cell-mediated immune system, and humoral immune system, respectively.

Phagocytosis and killing of bacteria by polymorphonuclear leukocytes (PMNs) plays an important role in the defense of the lung.[143,171,189] R. equi infection results in massive infiltration of

PMNs into the lung,[112] but these phagocytes are frequently incapable of eliminating infection. The PMNs may instead serve as a cellular host for R. equi dissemination and multiplication.[21,95,121] Resistance to phagocytosis and killing by PMNs exhibited by other species of bacteria can be related to surface structures of the bacterial cell wall.[40,68,100] The presence of PMN-suppressing component on the surface of bacteria can be detected with PMN function tests.[106,172] Modification of the PMN isolation procedures allowed recovery of large numbers of purified PMNs from the peripheral blood of normal horses. The phagocytic, metabolic, and degradative activities of equine PMNs were evaluated in the presence of R. equi components. Decreased bactericidal activities of PMNs in the presence of R. equi provided evidence that immunosuppression of natural immune mechanisms plays a role in the pathogenesis of R. equi infection.

The second phase of experimentation utilized lymphocyte blastogenesis as an in vitro correlate of the cell-mediated immune response to R. equi to evaluate the status of experimentally or naturally exposed horses.[157,158] Antigens capable of stimulating blastogenesis of sensitized lymphocytes were prepared from R. equi cultures. Addition of these antigens to cell cultures containing lymphocytes from horses which are sensitized to R. equi stimulated the multiplication of R. equi-sensitized cells. R. equi antigen-induced lymphocyte blastogenesis was correlated with the presence of a cell-mediated immune response in the horse from which the cells were collected. This assay was used to evaluate the cell-mediated immune

responses of infected and clinically normal horses following exposure to R. equi.

The third and final phase of this project was an attempt to resolve the unknown status of humoral immunity to R. equi. Antibodies which react with R. equi have been identified in the serum of infected or experimentally inoculated horses, but the antibody detection systems which were used indicated low quantities of this antibody.[139,158] Using similar techniques, other workers failed to detect antibody to R. equi in horse serum.[20,42,115] The bulk of the serologic investigation of R. equi has been performed with rabbit antisera.[12,18,19,32,91,124,198] Discrepancies in the published evaluations of serum antibody levels probably reflect inadequacies in these tests. Rather than attempting to optimize existing antibody test systems for R. equi, efforts were concentrated on the development of a new method for evaluating equine serum for the presence of anti-R. equi antibody. The enzyme-linked immunosorbent assay (ELISA) incorporates high sensitivity and great adaptability into a practical system.[138,192,196] The basic principles of the ELISA were adapted for use with R. equi. R. equi antigen was bound to the plastic wells of a microtiter plate. Serum from animals of unknown antibody status was added to the wells, and anti-R. equi antibody, if present, bound to the antigen. Non-binding antibodies were washed away, leaving only the R. equi antigen and its corresponding antibody in the well. Anti-equine serum was used to label the equine antibodies which were attached to the plate antigen. Prior conjugation of an enzyme to the

anti-equine serum provided reactivity of this molecule in a color-producing system when the appropriate substrate and indicator were added. The intensity of the color reaction indicated the amount of anti-*R. equi* antibody bound to the *R. equi* plate antigen. The ELISA was used to quantitate anti-*R. equi* antibody in the serum of experimentally and naturally exposed horses.

The three-fold approach of this project was used to study interactions of *R. equi* with the three immunologic defense systems of the horse. The study of a single aspect of natural, cell-mediated, or humoral immunity did not explain all the phenomena occurring in these systems, but the described approach provided a sound basis for speculations on the interactions of *R. equi* and equine host defense mechanisms.

SECTION I.

EFFECT OF *RHODOCOCCUS EQUI* ON EQUINE POLYMORPHONUCLEAR LEUKOCYTE FUNCTION

Summary

A procedure was developed for isolating large numbers of purified polymorphonuclear leukocytes (PMNs) from the peripheral blood of horses. Equine PMN function was evaluated by three procedures: i) *Staphylococcus aureus* ingestion, ii) nitroblue tetrazolium reduction, and iii) iodination. Four preparations of *R. equi* were added to polymorphonuclear leukocytes (PMNs) in each test system. Live bacteria, heat-killed bacteria, the washed pellet from heat-killed bacteria, and the supernatant fluid from heat-killed bacteria were evaluated for effects on equine PMN function. None of the *R. equi* preparations had an effect on *S. aureus* ingestion by equine PMNs. Nitroblue tetrazolium reduction by PMNs, a measure of oxidative metabolism, was suppressed by pellet and supernatant fractions. Values for the iodination reaction were depressed by all *R. equi* preparations, indicating decreased activity of the myeloperoxidase-hydrogen peroxide-halide system of the PMN. Further evaluation of the supernatant from heat-killed *R. equi* showed that it retained its inhibitory effect on iodination following autoclaving and/or passage

through a 10,000 MW filter. R. equi fractions did not alter the enzymatic conversion of ^{125}I to a protein-bound form in a PMN-free assay developed to evaluate this reaction. R. equi probably inhibits the fusion of lysosomes with phagosomes during degranulation. The presence of a surface component capable of inhibiting bactericidal mechanisms of the PMN may play an important role in intracellular survival of R. equi.

Introduction

Rhodococcus (Corynebacterium) equi is a pleomorphic, rod-shaped, aerobic Gram-positive bacterium which causes infections in several animal species and man.[1,24] The most common syndrome associated with R. equi is purulent bronchopneumonia with abscessation in foals less than 6 months of age. The pathogenesis of infection and the route of transmission are unknown.[28] Onset of the disease is insidious, and lesions are usually well-established before diagnosis is confirmed and treatment is initiated.[20] The nature of the lesions in R. equi pneumonia indicates that the bacterium is able to establish infection despite the presence of large numbers of polymorphonuclear leukocytes (PMNs).[17]

R. equi has been described as an intracellular pathogen capable of living and multiplying within phagocytic cells.[12] Lung lesions produced by R. equi resemble lesions produced by known intracellular

pathogens such as mycobacteria. The main feature which distinguishes the lesions of R.equi from those of tuberculosis in cattle is the heavy infiltration of neutrophils associated with R. equi infection.[15] Numerous bacteria can be found inside phagocytic cells on histologic examination of lesions from R. equi-infected animals.[5,16,18] The intracellular survival of R. equi may play an important role in the pathogenesis of disease. Bacteria which can withstand the extreme environment of the phagosome may be sheltered from the effects of antimicrobial substances and from detection and elimination by the immune system.

The activities of phagocytic cells in the lung provide an important means of defense against invasion by pathogenic microorganisms.[19] Polymorphonuclear leukocytes are among the first cells to respond to infectious bacterial agents.[25] The purpose of this experimentation was to study the in vitro functions of equine peripheral blood PMNs and to determine whether the phagocytic or bactericidal mechanisms of the PMNs were altered by the presence of R. equi.

Materials and Methods

Animals and blood collection

Eight apparently healthy adult horses of varied breeding were used for blood collection. Jugular venipuncture was used to

aseptically draw 100 ml of peripheral blood into 10 ml of anticoagulant solution containing 0.15 M sodium citrate, 0.04 M citric acid, and 0.14 M dextrose.

PMN preparation

Anticoagulated blood was diluted with an equal volume of phosphate-buffered saline solution, pH 7.2 (PBSS) and centrifuged at 1000 x g for 20 min. The plasma and buffy coat layers were aspirated and discarded. Erythrocytes were lysed with two volumes of phosphate-buffered distilled water (0.0132 M, pH 7.2) for 50 seconds, and the solution was restored to isotonicity with one volume of phosphate-buffered (0.0132 M, pH 7.2) 2.7% NaCl. Centrifugation at 500 x g for 10 min resulted in formation of a pellet containing predominantly neutrophils and eosinophils. The pelleted cells were counted, washed, and resuspended in PBSS at a concentration of 5.0×10^7 PMNs per ml.

Bacterial preparation

R. equi isolated from a tracheal wash from a foal with pneumonia was identified by Gram-positive staining reaction, pleomorphic rod-shaped morphology, mucoid colonial appearance, lack of carbohydrate fermentation, and catalase production[7]. The bacteria were passaged once from the primary isolation plate, grown in trypticase soy broth (TSB) (BBL Microbiology Systems, Cockeysville, MD) containing 10% bovine serum, and stored in this medium at -70°C until use. Following 24 hr growth in TSB, the bacterial suspension was used to inoculate

Roux flasks containing trypticase soy agar (BBL). The cultures were incubated for 72 hr at 37°C in an environment of 5% carbon dioxide in air. Bacteria were harvested from the agar surface after addition of 5 ml of sterile PBSS to each flask. The cells were washed 3 times by centrifugation in PBSS at 10^4 x g and resuspended in PBSS to a concentration which, when diluted 1 to 10, had an optical density of 0.2 at a wavelength of 600 nm. A portion of the suspension containing live bacteria was utilized without further treatment. The remainder was heated to 65°C for 60 min to kill the cells. Centrifugation of the heat-killed R. equi at 10^4 x g for 20 min yielded a clear, colorless supernatant fluid. Portions of this supernatant were subjected to: i) autoclaving at 121°C and 15 lbs pressure for 15 min, ii) passage through a 10,000 MW ultrafiltration membrane (Amicon Corp., Lexington, MA), or iii) a combination of autoclaving and then filtration. The pellet of heat-killed cells was washed 3 times by centrifugation at 10^4 x g in PBSS and resuspended to give an optical density of 0.2 at a 1 to 10 dilution.

PMN function tests

The procedures used to evaluate equine PMN function were adapted from procedures previously described for use with bovine PMNs. The [^{125}I] iododeoxyuridine-labeled Staphylococcus aureus, opsonized zymosan, lysostaphin, NBT solution, NaI, and [^{125}I]-NaI were prepared as previously described.[22] All PMN function tests were conducted in duplicate. Each pair of tubes received one of the R. equi bacterial

fractions or an equal volume of PBSS just prior to the addition of the PMNs. Ingestion was measured by uptake of heat-killed [^{125}I] iododeoxyuridine-labeled Staphylococcus aureus. The reaction was initiated when 2.5 x 10^6 equine PMNs were added to test tubes containing 100 ul [^{125}I]-labeled S. aureus, 50 ul of a 1 to 10 dilution of bovine serum containing anti-S. aureus antibody, 0.30 ml Earles' balanced salt solution with Ca^{++} and Mg^{++} (EBSS) (Grand Island Biological Co., Grand Island, NY), and 100 ul of either a R. equi fraction or PBSS. The S. aureus-to-PMN ratio was approximately 60 to 1. Following a 10 min incubation in a shaker water bath at 37°C, 0.5 ml of lysostaphin solution was added to disrupt extracellular S. aureus. Incubation was continued for another 30 min, after which time the PMNs were washed twice by centrifugation at 400 x g in PBSS. The percent of [^{125}I]-labeled S. aureus which was ingested was determined by measuring PMN-associated radioactivity in a gamma counter. Standard and background tubes were included in each run, and results were expressed as the percent of [^{125}I]-labeled S. aureus ingested as previously described.[22] Nitroblue tetrazolium (NBT) reduction was performed by adding 5.0 x 10^6 PMNs to tubes containing 200 ul of NBT solution, 100 ul of opsonized zymosan, 0.6 ml of EBSS, and 200 ul of a bacterial fraction or PBSS. Tubes were incubated for 10 min at 37°C in a shaker water bath. Addition of 5.0 ml of cold 1 mM N-ethylmaleimide in saline stopped the reaction. Centrifugation at 500 x g for 10 min pelleted the cells and the formazan precipitate which resulted from NBT reduction. The supernatant fluid was discarded, and

5.0 ml of pyridine was added to the pellet. Extraction of the formazan was accomplished by sonication for 3 sec and heating in a boiling water bath for 10 min. Following centrifugation at 500 x g for 10 min, 2 ml of supernatant fluid were removed. The optical density (OD) of the supernatant fluid at 580 nm was determined in a spectrophotometer. Results were expressed as OD per 5 x 10^6 PMNs per 10 min in 5.0 ml pyridine. The reaction tubes for determination of iodination contained 0.5 mg of opsonized zymosan, 40 nm NaI, and 0.05 uCi [^{125}I]-NaI in a total volume of 0.45 ml of EBSS. Each tube received 100 ul of a bacterial fraction or PBSS. The reaction was initiated by adding 2.5 x 10^6 PMNs. Tubes were capped and incubated for 20 min at 37°C in an end-over-end tumbler revolving 20 times per minute. Addition of 2 ml of cold 10% trichloroacetic acid (TCA) terminated the reaction and precipitated the protein present. The precipitate was washed twice with 2 ml of cold TCA by centrifugation at 1000 x g for 5 min at 4°C. Radioactivity remaining in the precipitate was measured in counts per minute (cpm) with a gamma counter. Standard and background tubes were included in each run, and results were expressed as nmole NaI per 10^7 PMNs per hour as previously described.[22]

Enzymatic iodination

Test tubes for enzymatic iodination contained 40 nm of NaI, 0.05 uCi of [^{125}I]-NaI, 0.05 U horseradish peroxidase (Sigma Chemical Co., St. Louis, MO), 0.05 mg of xanthine (Sigma), and 100 ul of R. equi

fraction or PBSS in 0.30 ml of EBSS containing 0.1% bovine serum albumin. Paired samples included 1 set of standard and background tubes and 4 sets of treated tubes. Reactions were initiated by addition of 0.02 U of xanthine oxidase (Sigma) to all tubes except backgrounds. Tubes were incubated and harvested in the manner described for the PMN iodination assay. Results are expressed in counts per minute.

Statistical analysis

Data were collected from duplicates of control PMNs and bacterial fraction-treated PMNs from each animal. The statistical significance of the effect of bacterial fraction treatment was determined by an analysis of variance procedure. To facilitate comparisons between PMN function test procedures, the treatment means obtained from this analysis were converted to a percent control form as follows: (treatment mean/control mean) x 100 = % control. Means and standard errors of the means were calculated for results of enzymatic iodination.

Results

The PMN isolation procedure described yielded an average of 3.4 x 10^6 PMNs per ml of equine blood, with recovery of an average of 70% of the PMNs present. Final cell preparations contained an average of 87% neutrophils, 7% eosinophils, and 6% mononuclear cells.

The mean (± SEM) for S. aureus ingestion by equine PMNs in the presence of PBSS was 18 (± 1.6) percent ingestion. This represented the uptake of approximately 11 S. aureus cells per PMN during the 10-min incubation period. None of the R. equi fractions affected the ability of equine PMNs to ingest radiolabeled S. aureus (Table 1).

Table 1: Effect of R. equi bacterial fractions on equine PMN function. Values are means from 8 animals, ± standard error of means.

Fraction	Percent of control value		
	S. aureus ingestion	NBT reduction	Iodination
Live R. equi	109.5 ± 3.1	117.1 ± 11.7[a]	66.7 ± 2.7[b]
Heat-killed R.equi	94.7 ± 8.0	94.6 ± 3.1	56.3 ± 2.0[b]
Pellet from heat-killed R. equi	94.9 ± 10.8	94.7 ± 1.9[a]	88.5 ± 3.1[b]
Supernatant fluid	99.0 ± 6.2	86.4 ± 3.0[b]	72.1 ± 4.6[b]
Autoclaved supernatant fluid	ND[c]	ND	74.5 ± 3.0[b]
<10,000 MW filtrate	ND	ND	77.5 ± 4.0[b]
Autoclaved <10,000 MW filtrate	ND	ND	77.1 ± 3.3[b]

[a] $P < 0.05$.
[b] $P < 0.01$.
[c] Not determined.

Reduction of NBT by equine PMNs in the presence of PBSS gave a mean (± SEM) control value of 0.27 (± 0.01) optical density per 5 x 10^6 PMNs per 10 min in 5.0 ml pyridine. Addition of live R. equi to this test enhanced NBT reduction, while pellet and supernatant fractions of the bacterium resulted in suppressed values (Table 1).

The mean (± SEM) value for iodination in control tubes was 46 (± 6.1) nmole NaI per 10^7 PMNs per hour. All of the R. equi fractions which were tested suppressed iodination by equine PMNs (Table 1).

Enzymatic iodination gave a mean (± SEM) control value of 694 (± 20) cpm. Mean values (± SEM) from tubes treated with live R. equi, heat-killed R. equi, pellet fraction, and supernatant fluid were 672 (± 16), 671 (± 21), 719 (± 19), and 689 (± 11) cpm, respectively. The standard contained 32220 (± 218) cpm, and the background level was 243 (± 8) cpm. There was no significant difference in results from tubes containing PBSS and from those treated with R. equi fractions.

Discussion

The procedure developed for isolating PMNs from the peripheral blood of horses facilitated collection of a large number of purified PMNs. Extensive manipulation of the cells was not required, and no special chemicals were employed. Dilution of the whole blood and speed of centrifugation were found to markedly affect the percent recovery. The function of equine PMNs in the described assays was

comparable to results obtained with other species.[14,22]

Results of the S. aureus ingestion assay indicate that R. equi does not inhibit the phagocytic capacity of equine PMNs. Although this assay did not directly measure phagocytosis of R. equi, there was no indication of any inhibitory effect of R. equi on ingestion of bacteria by equine PMNs. The apparent lack of anti-phagocytic properties associated with R. equi may be a factor in the pathogenesis of infection. If the bacterium can resist the bactericidal activity of the phagocyte, ingestion by PMNs is likely to be important in the establishment, dissemination, and persistence of R. equi pneumonia. The intracellular environment may temporarily provide an excellent means of escaping immune surveillance and humoral and cellular defense mechanisms.

The NBT reduction assay was used to evaluate the burst of oxidative metabolism which accompanies phagocytosis by PMNs. Reactions of the oxidative pathway result in formation of superoxide anion, hydrogen peroxide, the hydroxyl radical, and perhaps singlet oxygen.[21] These highly reactive compounds are formed inside the phagocytic vacuole and at the external cell membrane and are important in the killing of phagocytized microorganisms. Superoxide anion causes the reduction of NBT to an insoluble purple formazan.[29] The amount of formazan produced can be measured spectrophotometrically and used to quantitate the burst of oxidative metabolism associated with phagocytosis by the PMN.

Addition of live R. equi to the PMNs in the assay resulted in increased values for NBT reduction. This increase in NBT reduction can be explained by our observation that live R. equi is capable of reducing NBT in the absence of PMNs. Heat-killed R. equi does not reduce NBT in the absence of PMNs. A decrease in NBT reduction values was observed following addition of the washed, heat-killed R. equi and the supernatant fluid from heat-killed R. equi. Suppression of NBT reduction by these fractions indicates that R. equi can inhibit the burst of oxidative metabolism which is associated with phagocytosis. This suppression may result in decreased bactericidal activity by the PMN.

The iodination reaction measures one of the most potent antimicrobial mechanisms of the PMN.[3,23] In a complex chain of events, inorganic iodide is converted to a protein-bound form through the action of hydrogen peroxide and myeloperoxidase.[11] Hydrogen peroxide is formed as a result of oxidative metabolism by the PMN. Myeloperoxidase is present in the primary granules of the PMN and is delivered to the phagosome by degranulation. A failure of degranulation, a deficiency of myeloperoxidase in the primary granule, or inhibition of the myeloperoxidase-catalyzed reaction will result in a decreased value for iodination. A deficiency of hydrogen peroxide may limit the iodination reaction when oxidative metabolism has been depressed or when hydrogen peroxide is destroyed.

All the R. equi fractions which were tested caused a significant decrease in iodination values. The lack of any effect of the

fractions on ingestion of S.aureus by PMNs indicates that inhibition of phagocytosis does not contribute to the effect of R. equi on iodination. Suppression of oxidative metabolism, as evaluated by NBT reduction, may contribute to the decrease in iodination caused by the bacteria. Oxidative metabolism, however, is probably not the limiting factor in the iodination reaction by normal PMNs when conducted under the conditions used in this experiment.[22] This conclusion would be supported by the magnitude of the suppression of iodination compared to the level of the effects on NBT reduction. Lack of significant effects of the R. equi fractions on enzymatic iodination in the absence of neutrophils indicates that R. equi does not act by destroying hydrogen peroxide. A possible explanation for the inhibition of iodination is that R. equi inhibits degranulation of the primary lysosomes.[9,10]

The supernatant fluid from heat-killed R. equi retained most of the suppressive effect on iodination exhibited by the live or heat-killed whole organisms. R. equi has a capsule, thought to be composed of polysaccharide, which has been shown to be extractable with heat.[4,27] The supernatant fraction from R. equi probably contained portions of the bacterial capsule along with other surface components. Further studies with this fraction showed that autoclaving and filtration through a 10,000 MW filter did not affect the ability of the supernatant fluid to suppress iodination. These results indicate that R. equi possesses a heat-stable surface component of less than 10,000 MW which is capable of inhibiting the bactericidal mechanisms

of equine PMNs. The presence of this component may contribute to intracellular survival and establishment of infection by R. equi.

These data are compatible with the classification of R. equi as an intracellular pathogen. Bacteria which are capable of intracellular survival, such as mycobacteria and brucellae, are readily ingested by PMNs.[8,13] Presence within the phagosome protects these bacteria from components of the host immune system such as complement and antibody. The effect of R. equi on the function of PMNs isolated from adult horses indicates that the pathogen is able to inhibit the bactericidal activities of normal PMNs. Adult horses, however, rarely become infected with R. equi despite the prevalence of this bacterium in equine environments.[2,26] The suppressive effect of R. equi on equine PMN function is only one factor in a complex etiology. Recent work has demonstrated that PMNs from foals do not function as well as those from mares.[6] Immunosuppression caused by viral infection, malnutrition, and stress-inducing environmental factors may lead to further inhibition of the phagocytic cell system in young foals. Inhibition of other components of the immune system by similar mechanisms may contribute to the pathogenesis of R. equi infection.

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SECTION II.

EQUINE CELL-MEDIATED IMMUNE RESPONSE TO *RHODOCOCCUS EQUI*

Summary

A lymphocyte blastogenesis assay was developed to serve as an in vitro correlate of cell-mediated immunity to *Rhodococcus (Corynebacterium) equi (R. equi)* in the equine. Lymphocytes obtained from a group of experimental ponies showed no response in cell culture to *R. equi* heat extract or lysosome extract antigens. Ponies were assigned to groups for experimental inoculation. Three ponies received subcutaneous inoculation with live *R. equi*, three were given live *R. equi* by intranasal and intratracheal routes, and four ponies were untreated. Lymphocytes from all inoculated ponies exhibited a mitogenic response to *R. equi* antigens in lymphocyte blastogenic assays performed between the 7th and 40th days post-inoculation. Lymphocytes from uninoculated control ponies remained unresponsive to *R. equi* antigens. Delayed-type hypersensitivity reactions developed in all experimentally exposed ponies following intradermal administration of the *R. equi* antigen preparations. In a second phase of experimentation, blastogenesis assays were performed on lymphocytes from horses in herds with endemic *R. equi* infections. Results

indicated that many of the animals had significant cell-mediated responses to the bacterium, but there was no distinct correlation between the immune response and clinical condition. These data indicate that cell-mediated immunity is involved in the interaction of the equine immune system with R. equi.

Introduction

Rhodococcus (Corynebacterium) equi was first described in 1923 as the cause of purulent bronchopneumonia in young foals.[15,21] The bacterium has subsequently been characterized as an opportunistic intracellular pathogen which is carried and shed by most adult horses.[1,7,27] Although the organism is prevalent in the environment, clinical disease caused by the bacterium is sporadic and primarily affects foals between the ages of 2 and 6 months.[2]

There is little information on the mechanisms of immunity to R. equi to explain the apparent protection of the majority of the horse population from clinical infection. Cell-mediated and humoral immune responses to R. equi are generally not detected in normal animals. Cell-mediated immunity has been detected in experimentally and naturally-infected foals and in adult horses on farms where R. equi infection was endemic.[18,19,25] The development of a cellular response is believed to be important in resistance to a facultative intracellular pathogen such as R. equi.[1,6,7,10,14,17]

This experimentation was undertaken to evaluate R. equi-induced responses of lymphocytes from ponies prior to and after exposure to R. equi. A lymphocyte blastogenic assay was evaluated for use as an in vitro correlate of cell-mediated immunity by comparison with R. equi-induced delayed-type hypersensitivity responses in vivo. The in vitro assay was also used to assess cell-mediated immunity in horses which were naturally exposed to R. equi infection.

Materials and Methods

Experimental animals

The 10 mares and geldings used were of predominantly Shetland breeding, ranged in age from 2 to 6 years, and were in fair to good condition. All animals were housed together prior to experimental inoculation. Ponies were randomly assigned to a group, and each group was housed separately. The control group (n = 4) and the subcutaneously inoculated group (n = 3) were housed in outdoor pens with shelter. The group which received respiratory tract exposure (n = 3) was held in an isolation facility. All animals were observed daily for signs of clinical disease and animals in the intranasal-intratracheal group were necropsied on day 45.

Experimental inoculation

R. equi was isolated from tracheal wash fluid of a foal with a naturally-occurring case of pneumonia and was identified by accepted

methods.[9] Following one passage on 5% blood agar, the organisms were inoculated into trypticase soy broth (TSB) (BBL Microbiology Systems, Cockeysville, MD) containing 10% horse serum, incubated for 24 hr at 37°C, and stored in aliquots at -70°C until use. A flask of TSB was inoculated with an aliquot of bacteria and incubated at 37°C for 24 hr. This bacterial suspension was used to inoculate Roux flasks containing trypticase soy agar (TSA) (BBL). After 72 hr growth at 37°C in an atmosphere of 5% carbon dioxide in air, bacteria were harvested from the agar surface. Following 3 washes by centrifugation at 10^4 x g in phosphate-buffered saline solution (PBSS), the bacteria were resuspended in PBSS. When diluted 1 in 10, the bacterial suspension had an optical density (OD) of 0.2 at a wavelength of 600 nm and contained 3.4×10^8 live bacteria per ml as determined by plate counts. Animal inoculations were given on day 0 of the experiment. Ponies in one group received 1.0 ml of the suspension of live bacteria subcutaneously at one site on the neck. Intranasal and intratracheal inoculation of the other group utilized 1.0 ml of the bacterial suspension diluted with 9.0 ml of PBSS. A syringe fitted with an intranasal tip was used to spray 1.0 ml of the suspension into each nostril. Catheterization of the trachea was accomplished through a 14 ga hypodermic needle placed into the tracheal lumen near the base of the neck. A sterile 3.5 French polypropylene catheter was inserted through the lumen of the needle and advanced until a cough reflex was elicited. A syringe was attached to the catheter and the remaining 8.0 ml of diluted bacterial suspension was inoculated intratracheally.

Naturally exposed animals

Horses and ponies which were studied following natural exposure came from breeding farms having a high incidence of R. equi pneumonia in the foals. Infected foals were defined as animals less than 4 months of age in which infection was confirmed by tracheal wash isolation of R. equi. The dams of these unweaned foals were classified as adults. Animals in the weanling category had been raised together and had experienced an outbreak of respiratory disease following weaning. Approximately half of the weanlings had exhibited signs of respiratory infection but were clinically normal at the time of sample collection.

Lymphocyte blastogenesis assay

Jugular venipuncture was used to aseptically collect 25 ml of peripheral blood from each animal into 250 U sodium heparin (Nutritional Biochemicals Corp., Cleveland, OH). Erythrocytes were allowed to settle for 20 min in a 37°C water bath. Ten ml of plasma were removed and diluted with 10 ml of 37°C PBSS. Diluted plasma was layered over 6 ml of Ficoll-Hypaque (Histopaque-1077, Sigma Chemical Co., St. Louis, MO) and centrifuged at 300 x g for 30 min. Mononuclear cells at the plasma-Histopaque interface were removed and washed once with 40 ml of warm Hanks' balanced salt solution without Ca^{++} and Mg^{++} (HBSS) by centrifugation at 150 x g for 15 min. Total and differential cell counts were performed after resuspension of the cells in 1 ml of HBSS. Lymphocytes were cultured in Medium 199 with

Earles' salts (Flow Laboratories, McLean, Va.) which contained 15% heat inactivated horse serum, 1% antibiotic-antimycotic solution (Grand Island Biological Co., Grand Island, NY), and 21 mmoles Hepes buffer (Flow). The cells (4×10^5) in 0.2 ml were added to each well of a flat-bottomed microtiter plate (Costar, Cambridge, MA). A volume of 0.05 ml of mitogen or antigen was added to indicated wells. Cultures were incubated for 5 days at 37°C in a humidified atmosphere of 5% carbon dioxide in air. Eighteen hours prior to harvest, 0.25 uCi of tritiated thymidine (Amersham Corp., Arlington Heights, IL) was added to all wells. Lymphocytes were harvested on glass-fiber filter pads with an automatic cell harvester. Each pad was placed in a vial containing 15 ml of a toluene-based scintillation cocktail and counted for 4 min in a liquid scintillation counter.

Mitogen and antigen preparations

Phytohemagglutinin (PHA) (PHA-p, Difco, Detroit, MI) was diluted 1 to 200 (predetermined optimal concentration) in HBSS. *R. equi* antigens were prepared from a live bacterial suspension (10 X a 0.2 OD preparation). Heat extract was prepared by autoclaving the bacterial suspension for 15 min at 121°C and 15 pounds of pressure. The clear, colorless supernatant fluid obtained following centrifugation at 10^4 x g for 30 min was used on lymphocyte cultures at a 1 in 4 dilution in HBSS. A lysozyme extract of *R. equi* was obtained by mixing the live bacterial suspension with an equal volume of lysozyme solution (pH 7.5) containing 0.06 M sodium chloride, 0.03 M sodium citrate, 0.05 M

potassium monophosphate, and 150 ug/ml lysozyme (38.5 U/ug) (Sigma). The mixture was incubated at 37°C for 16 hr. The supernatant fluid was harvested following centrifugation at 10^4 x g for 30 min and used in lymphocyte blastogenesis cultures at a 1 in 10 dilution in HBSS.

Skin testing

On day 40 following experimental exposure, all infected and control ponies were injected intradermally with PBSS and the two R. equi antigens at the same concentration used for lymphocyte cultures. The left side of the neck was clipped, and 0.10 ml of each preparation was injected into a separate site. At 24, 48, and 72 hr following injection, the size of the reaction at each of the three sites was measured with a calipers, and the diameter in mm was recorded.

Data analysis

Counts per minute (cpm) were averaged from sets of triplicate control, mitogen-, and antigen-stimulated cultures from each animal. Statistical significance of the cpm data was determined with analysis of variance. Stimulation indices were calculated as follows: stimulation index = (cpm of stimulated culture) / (cpm of unstimulated control culture). A stimulation index greater than 2 was considered to indicate a positive blastogenic response. Lesion diameter data from the skin tests were averaged for each group and an analysis of variance was used to determine statistical significance.

Results

All animals experimentally exposed to R. equi developed clinical signs of infection. Ponies injected subcutaneously developed abscesses at the inoculation site in 3 to 5 days. The abscesses broke open and drained a purulent exudate for approximately 4 days. Healing occurred without complications in two to three weeks. Animals in the group administered live R. equi intranasally and intratracheally exhibited an increase in body temperature (up to 39.5°C) during one or more of the days in the week following inoculation. In the second week after inoculation, these animals showed increased respiratory rates and coughing. Respiratory signs abated over the next 2 weeks and the animals exhibited no further clinical signs. Necropsies performed on day 45 revealed purulent lung lesions in two of the ponies, but pathologic changes were more limited in the third. R. equi was cultured from tracheal wash fluid and from cut sections of lung tissue from all animals in this group.

Lymphocyte isolation procedures yielded an average of 1.5×10^6 lymphocytes per ml of whole blood. Cell preparations contained an average of 95% lymphocytes. Counts on unstimulated control cultures ranged from 450 cpm to 1100 cpm. Some variations in the PHA response among the 3 groups of ponies during experimentation were noted but differences were not significant (Table 1). The PHA responses of lymphocytes from naturally exposed animals were comparable to those of experimentally exposed ponies.

Table 1: Blastogenic responses of cultured lymphocytes to PHA and *R. equi* antigens before and after inoculation of ponies with live *R. equi*. Results expressed as counts per minute (± SEM) of ^{3}H thymidine.

Animal group	Unstimulated control culture	*R. equi* heat extract	*R. equi* lysozyme extract	PHA
Period 1: pre-inoculation				
Control	658 ± 123	692 ± 199	603 ± 158	45997 ± 2106
Intranasal/ intratracheal inoculation	648 ± 179	561 ± 168	452 ± 85	47367 ± 3359
Subcutaneous inoculation	473 ± 101	457 ± 112	452 ± 120	41204 ± 5721
Period 2: post-inoculation				
Control	1058 ± 167	1300 ± 221	929 ± 99	31514 ± 3057
Intranasal/ intratracheal inoculation	880 ± 115	3656 ± 552[a]	2760 ± 526[a]	27952 ± 2902
Subcutaneous inoculation	807 ± 96	3483 ± 496[a]	2466 ± 303[a]	30128 ± 3164

[a] $P < 0.01$ when compared to control group results.

Blastogenic responses of lymphocytes from experimental ponies in response to R. equi extracts were not significant when compared to control cultures on days -2 and 0 of the experiment (Table 1). The lymphocytes of ponies in the control group remained unresponsive to R. equi extracts throughout the experimental period (Figure 1). Lymphocytes from ponies which were subcutaneously or intranasally/ intratracheally exposed to R. equi developed significant blastogenic responses to both R. equi extracts. Sensitization of lymphocytes in the two exposed groups of ponies was evident at day 8 post-inoculation and persisted for the duration of the experiment. There were no significant differences in results obtained using antigens prepared by heat treatment or lysozyme treatment of R. equi. Animals exposed by either route had equivalent lymphocyte blastogenic responses. All exposed ponies developed concurrent antibody responses to R. equi (see Section III).

Experimental ponies in the inoculated groups exhibited positive skin test reactivity at 48 and 72 hours following intradermal inoculation with R. equi extracts (Table 2). Two animals in the control group responded to the heat extract of R. equi, but none of the control animals reacted to the lysozyme extract.

There was no identifiable trend in the response of lymphocytes from naturally exposed animals to R. equi extracts (Figure 2). Significant cell-mediated sensitization, as indicated by lymphocyte blastogenic responses, was detected in a relatively small number of infected foals. A wide range of responses were observed in animals

from the weanling and adult categories. Lymphocytes from several adults and from weanlings which had no evidence of clinical disease exhibited blastogenic responses to the R. equi antigens.

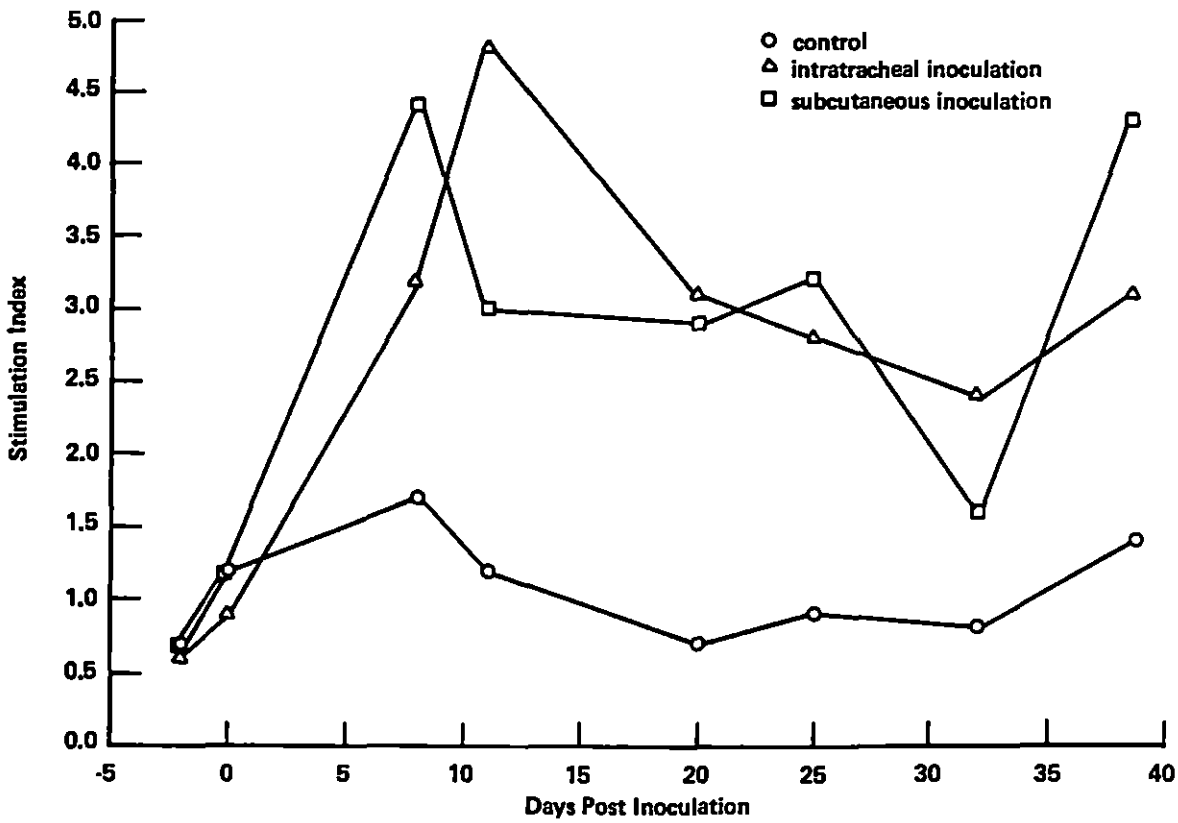

Figure 1: Mean stimulation indices of 5-day lymphocyte cultures from experimental ponies in response to R. equi lysozyme extract. Animals were inoculated as indicated on day 0. Symbols represent the group mean.

Table 2: Mean diameters of the skin reactions of ponies at 48 and 72 hours after intradermal inoculation of heat- and lysozyme-extracts of R. equi

Group (n)	Mean Diameter of Skin Reaction (mm)			
	48 hr		72 hr	
	R. equi heat extract	R. equi lysozyme extract	R. equi heat extract	R. equi lysozyme extract
Control (4)	13.5	0	10.7	0
Intratracheal/ intranasal inoculation (3)	28.0	13.3	21.7	18.3[a]
Subcutaneous inoculation (3)	14.0	10.3	20.0	13.7[a]

[a] $P < 0.01$ when compared to control group.

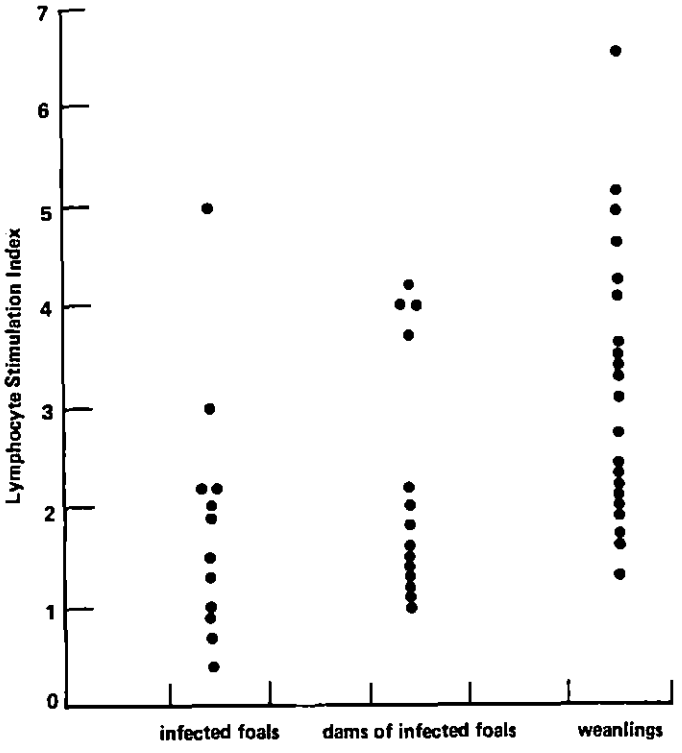

Figure 2: Stimulation indices of cultured lymphocytes from horses on farms with high incidences of R. equi pneumonia. Points represent blastogenic responses of 5-day lymphocyte cultures to lysozyme extract of R. equi. Infected foals were defined as animals less than 4 months of age which had confirmed R. equi infection. Weanlings ranged in age from 4 to 6 months. Adults were dams of infected foals.

Discussion

Experimental induction of infection in ponies and the availability of naturally infected and cohort animals provided an opportunity to evaluate cell-mediated reactivity of horses to R. equi. Experimentally infected ponies were reactive when skin tested and these and some of the naturally exposed animals provided lymphocytes that were reactive in lymphocyte blastogenic assays. The lymphocyte blastogenesis procedure developed for this study provided an in vitro model for the study of cell-mediated immunity to R. equi. Lymphocytes from all animals underwent extensive mitosis to give high stimulation indices in response to PHA administration. The uniform responses of the PHA cultures were a reflection of favorable culture conditions and good blastogenic potential of the lymphocytes in the test system. This response to a polyclonal T cell and possibly B cell activator indicated that specifically sensitized lymphocytes in the cultures should respond to their corresponding antigens.

Treatment of R. equi with heat or lysozyme provided suitable antigens for stimulating sensitized lymphocytes in blastogenesis assays. Autoclaving presumably extracted capsular and cell wall materials similar to those prepared by other workers utilizing hot acid treatment.[4,8,13,22,26] Lysozyme treatment was selected for its hydrolytic activity on glycosidic bonds of peptidoglycan in the bacterial cell wall.[23] Supernatant fluid collected after this treatment probably also contained components of the capsule and cell

wall of R. equi. These materials were probably carbohydrate and/or glycolipid in character since protein could not be detected in the preparations.

Skin tests for hypersensitivity performed on the experimental ponies provided a second assessment of cell-mediated reactivity for comparison with lymphocyte blastogenesis as a measure of cell-mediated immunity. Reactions to the lysozyme extract of R. equi showed direct correlation with the results of lymphocyte blastogenesis assays. However, skin reactivity of two of three control animals to the heat extract of R. equi did not correlate with the in vitro assays. Perhaps the in vivo test is more sensitive, the reaction was nonspecific, or toxic components were present in the antigen preparation.

Lymphocyte blastogenic assays performed following the inoculation of experimental ponies with R. equi demonstrated reactivity to the bacterium. All ponies were negative for lymphocyte blastogenic responses prior to experimental exposure, although these animals belonged to an age group considered to be resistant to infection. The possibility exists that these animals were previously exposed to R. equi and the response observed was secondary or anamnestic in character. A long-term study on the reactivity of lymphocytes from these animals to R. equi antigens may have demonstrated a gradual decline in blastogenic activity and an eventual return of the 2 exposed groups to a level of reactivity indistinguishable from that of the controls.

Data collected from animals on farms with high incidences of R. equi infection indicated that cell-mediated immune responses occur following natural exposure. The variable responses of the animals surveyed suggest that various factors associated with the host-parasite relationship determine the immunologic reactivity of any one animal. Immunosuppression has been identified as an important contributing factor to establishment of R. equi infection in man.[3,5,11,12,16,20,24] In the clinically affected foals studied, immunologic status associated with age, levels of maternal antibody, viral infection, or environmental stress may have contributed to both the susceptibility to infection and to the variations in immunologic reactivity. The R. equi-induced lymphocyte blastogenesis exhibited by lymphocytes from a significant number of naturally-exposed adult animals indicated a potential role for the cell-mediated immune system in resistance to R. equi.

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SECTION III.

EQUINE HUMORAL IMMUNE RESPONSE TO *RHODOCOCCUS EQUI*

Summary

An enzyme-linked immunosorbent assay (ELISA) was developed to test equine serum for the presence of antibodies to *Rhodococcus equi*. Experimental ponies had no detectable antibody to *R. equi* prior to exposure to the bacterium. Following experimental inoculation, animals in groups which received live *R. equi* subcutaneously or intranasally/intratracheally developed high titers to *R. equi*. Uninoculated controls remained seronegative. Serum was also collected from animals of various ages that were naturally exposed to *R. equi*. There was a wide range of anti-*R. equi* titers in these animals. Since experimentally infected animals seroconverted while some naturally infected foals failed to seroconvert, the role for antibody in resistance to *R. equi* infection remains unknown.

Introduction

R. equi is a common inhabitant of the equine gastrointestinal tract,[23] and foals between 2 and 6 months of age can develop severe purulent bronchopneumonia due to infection with *R. equi*.[9,11,12,13]

The disease has high mortality and occurs sporadically throughout the world.[2] Up to 15% of a foal crop on affected farms may succumb without any sign of disease in older horses.[1,8,15,19] The immunologic mechanisms which protect adult horses from R. equi have not been characterized. Cell-mediated immunity has been proposed as an important mediator of protection against infection.[17,18,21] Humoral responses have not been consistently demonstrated probably due to lack of a sensitive and specific test system. Bacterial agglutination, passive hemadsorption, complement fixation, precipitation, and agar gel diffusion tests have been used with varying degrees of success over the years.[3,5,6,10,14] Some investigators report having detected little or no antibody to R. equi,[4,7,14] while others have found low-titered antibody in sera collected from infected animals.[16,18]

The enzyme-linked immunosorbent assay (ELISA) has gained much favor as a sensitive and practical test for detection of antibodies.[20,22] Therefore, ELISA was selected and adapted for detection of R. equi antibody in equine serum. The test was used to evaluate anti-R. equi antibody levels in horses which were experimentally or naturally exposed to the bacterium.

Materials and Methods

Challenge-exposed animals

The 10 mares and geldings used for experimental induction of R. equi infection were of predominantly Shetland breeding, ranged in age

from 2 to 6 years, and were in fair to good condition. All animals were housed together prior to experimental inoculation. Ponies were randomly assigned to a group, and each group was housed separately. The control group (n = 4) and the subcutaneously inoculated group (n = 3) were housed in outdoor pens with shelter. The group which received respiratory tract exposure (n = 3) was kept in an indoor isolation room.

Experimental inoculation

R. equi isolated from tracheal wash fluid from a foal with a naturally-occurring case of pneumonia was identified by accepted methods.[9] Following one passage on 5% blood agar, the organisms were inoculated into trypticase soy broth (TSB) (BBL Microbiology Systems, Cockeysville, MD) containing 10% horse serum, incubated for 24 hr at 37°C, and stored in aliquots at -70°C. A flask of TSB was inoculated with an aliquot of bacteria and incubated at 37°C for 24 hr. This bacterial suspension was used to inoculate Roux flasks containing trypticase soy agar (TSA) (BBL). After 72 hr growth at 37°C in an atmosphere of 5% carbon dioxide in air bacteria were harvested by suspension from the agar surface. Following 3 washes by centrifugation at 10^4 x g in phosphate-buffered saline solution (PBSS), the bacteria were resuspended in PBSS. A 1 in 10 dilution of the final bacterial suspension had an optical density (OD) of 0.2 at a wavelength of 600 nm and contained 3.4×10^8 live bacteria per ml. Animal inoculations were given on day 0 of the experiment. Ponies in

one group received 1.0 ml of the suspension of 10 x 0.2 OD live bacteria subcutaneously at one site on the neck. Intranasal and intratracheal inoculation of the other group utilized 1.0 ml of the bacterial suspension diluted with 9.0 ml of PBSS. A syringe fitted with an intranasal tip was used to spray 1.0 ml of the suspension into each nostril. Catheterization of the trachea was accomplished through a 14 ga hypodermic needle placed into the tracheal lumen near the base of the neck. A sterile 3.5 French polypropylene catheter was inserted through the lumen of the needle and advanced until a cough reflex was elicited. A syringe was attached to the catheter and the remaining 8.0 ml of diluted bacterial suspension was inoculated intratracheally.

Naturally-exposed animals

Horses and ponies from breeding farms having high incidences of R. equi pneumonia in the foals were bled for serum. Foals were defined as animals less than 4 months old which were diagnosed by tracheal wash isolation of R. equi to be infected with the organism. The dams of these unweaned foals were classified as adults. Animals in the weanling category had been raised together and had experienced an outbreak of respiratory disease following weaning. Approximately half of the weanlings had exhibited signs of respiratory infection during this outbreak. These animals were clinically normal at the time of sample collection.

ELISA

Preparation of the R. equi antigen used in the ELISA was identical to a previously described antigen extraction procedure (see Section II). R. equi isolated from a case of foal pneumonia was prepared to a concentration which, when diluted 1 in 10, gave an optical density of 0.20. This suspension was autoclaved at 121°C and 15 pounds of pressure for 15 min, and the supernatant fluid was harvested by centrifugation at 10^4 x g for 30 min. The autoclaved R. equi extract was diluted 1 in 100 in buffer solution (0.125 M sodium carbonate-bicarbonate, pH 9.6). Each well of an Immulon I flat bottom polystyrene microtitration plate (Dynatech Laboratories, Inc., Alexandria, VA) received 100 ul of diluted antigen. Plates were sealed in a humidified chamber, incubated for 4 hr at 37°C, and then stored up to 2 weeks at 4°C. Unadsorbed antigen was removed and the wells washed with a pH 7.2 wash solution containing 0.5 M sodium chloride, 0.012 M monobasic sodium phosphate, 0.03 M dibasic sodium phosphate, and 0.5% Tween 80 (Sigma Chemical Co., St. Louis, MO) in deionized water. The wash solution was delivered to and removed from wells with a 12-well microtitration plate washer (Costar, Cambridge, MA). Wells were washed 6 times and then plates were inverted and tapped on absorbent paper to remove remaining fluid. Following removal of unadsorbed antigen by washing, 2-fold dilutions of equine test and control sera in wash solution were added to all wells except the first well in each row. Wells received 100 ul of diluted serum. Plates were covered and incubated at 37°C for 20 min. Unadsorbed

serum components were removed by washing, and 50 ul of horseradish peroxidase-conjugated rabbit anti-equine IgG (Miles Laboratories, Inc., Elkhart, IN; diluted 1:1000 in phosphate buffered saline solution, pH 7.4) were added. Plates were covered, incubated for 15 min at 37°C, and washed. Substrate solution (100 ul) containing 0.80 uM hydrogen peroxide and 0.40 mM 2,2'-azino-di-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) (Sigma) in a pH 4.0 diluent (0.05 M citric acid in deionized water) was added to each well. Substrate was allowed to react for 20 min at 37°C, and color development was stopped by addition of 50 ul of 0.5% hydrofluoric acid to all wells. Optical density readings were obtained with an automated micro-ELISA reader set at 508 nm wavelength.

Positive and negative serum standards were included on each ELISA plate. The positive serum standard was produced by repeated subcutaneous inoculation of an adult pony with live R. equi. Serum used as the negative control for anti-R. equi antibodies was collected from a gnotobiotically-derived pony foal which had been conventionalized in the absence of other horses.

Serum titers were determined on the basis of differential color reactions between the negative control serum values and corresponding readings for the test serums. Wells which received only substrate and hydrofluoric acid were used to zero the optical density reader. The averages of daily readings on dilutions of the negative serum standard were used to determine the standard deviation of these values. A dilution of unknown serum was considered positive for anti-R. equi

antibodies if it had an optical density reading greater than 3 standard deviations above the reading for the negative serum standard of corresponding dilution. The last positive dilution within a series of positive values was selected as the titer and converted to log base 2 of the serum dilution.

Results

The ELISA was standardized with negative and positive sera that were used as standards for all subsequent assays. Optical density means and standard errors of means for 20 determinations at a serum dilution of 1 in 32 were 0.73 $\pm$ 0.06 for the positive serum standard and 0.10 $\pm$ 0.006 for the negative serum standard.

Anti-*R. equi* antibodies were not detected in the sera of challenged ponies prior to inoculation (Figure 1). At day 11, serum from animals in both groups exposed to *R. equi* contained antibodies which reacted with *R. equi* antigen in the ELISA to give titers ranging from 1:32 to 1:2048. Antibody titers of inoculated ponies remained high throughout the experiment. Serum from control animals did not contain detectable levels of antibody. Titers of sera collected from animals which were naturally exposed to *R. equi* were extremely variable and had no apparent association with clinical history (Figure 2). Lack of a detectable antibody response to *R. equi* was common in foals and weanlings which had symptoms of the disease. Many normal adult animals had high antibody titers to *R. equi*.

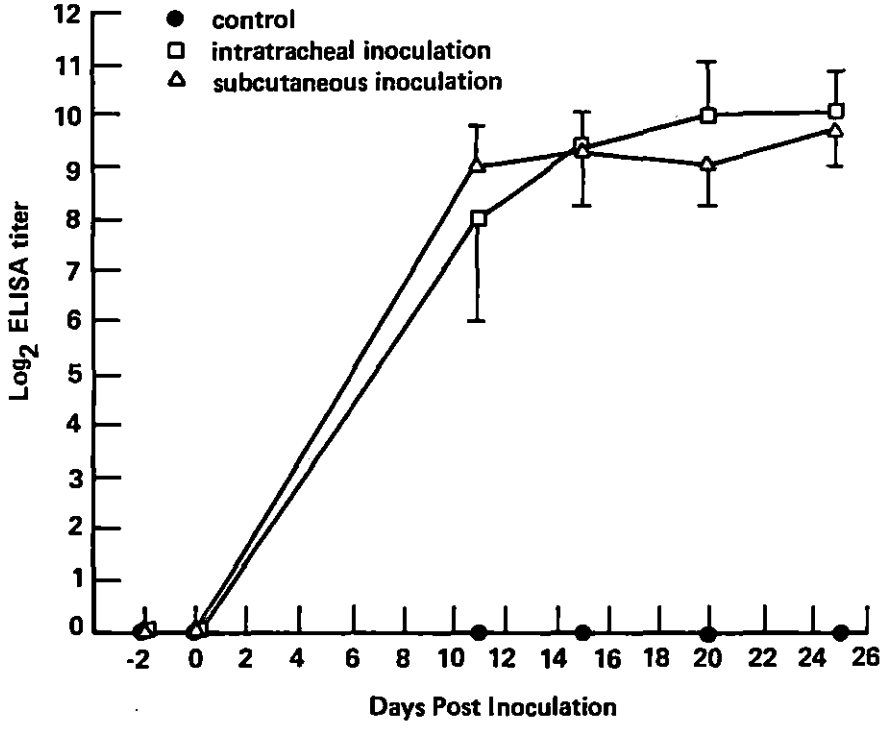

Figure 1: Log base 2 of mean anti-R. equi ELISA titers of serum from experimental ponies. Indicated groups were inoculated with live R. equi on day 0.

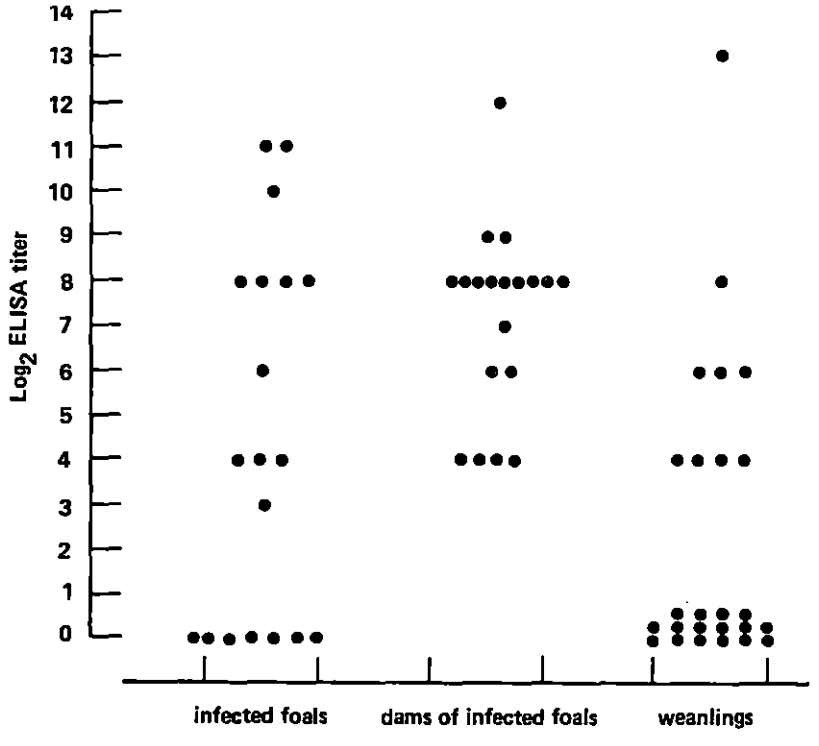

Figure 2: Log base 2 of anti-R. equi ELISA titers of serum from horses on farms with high incidences of R. equi pneumonia.

Discussion

The indirect ELISA proved to be a sensitive and specific assay for antibodies to *R. equi* in equine serum. As with most ELISA procedures that utilize serial dilutions of serum, some background color reaction was observed in wells containing the highest concentration of serum. However, the availability of a known negative serum permitted establishment of a baseline for evaluation of test serums. The use of a positive standard served as a control for test reactivity on any given day. The specificity of the ELISA for anti-*R. equi* antibodies was further evaluated with serum obtained before and after experimental inoculation with *R. equi*. Significant ELISA reactivity occurred with all sera obtained from experimental ponies exposed to *R. equi* but no reactivity was present in control animal sera.

The heat extract of *R. equi* used as antigen in the ELISA probably contained polysaccharides from the capsule of *R. equi*. These materials have been shown to be extractable with heat and to participate in immunologic reactions.[3,10,16] Extracts prepared by treatment of *R. equi* with hot acid or lysozyme exhibited similar reactivity in the ELISA.

Rapid development of antibodies in high concentration following experimental *R. equi* exposure indicated that the responses of the ponies may have been secondary rather than primary in nature. There was no detectable difference in the kinetics of the responses or

levels of anti-*R. equi* antibody in animals in the groups which received mucosal exposure or *R. equi* parenterally. If the test had utilized conjugates that would specifically detect IgM, IgA, IgG, and IgG(T) antibodies, kinetic analysis of antibody classes produced in response to *R. equi* might have indicated the primary or secondary nature of the response.

Groups of animals from farms with high incidences of *R. equi* pneumonia showed no distinct patterns in humoral response to *R. equi*. Variations in the level of antibodies produced by individuals in a group were probably influenced by a number of factors. Levels of antibody may have been affected by the age and immunologic status of the animal, the route and dose of *R. equi* exposure, and the time of exposure relative to serologic testing. However, from the number of animals evaluated it is clear that the humoral response to natural exposure to *R. equi* must be extremely variable. Whether or not humoral immunity contributes to resistance to infection by this agent remains an open question. The ELISA procedure proved to be a sensitive method for detection of antibodies but will need to be utilized in controlled experiments for the evaluation of the significance of humoral immunity to this agent.

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SUMMARY

The objectives of this research project were fulfilled by the findings of the experimentation. Interactions of R. equi with the natural, cell-mediated, and humoral immune systems of the equine were characterized by equine polymorphonuclear leukocyte (PMN) function assays, equine lymphocyte blastogenic assays, and equine enzyme-linked immunosorbent assays (ELISAs). A surface component of R. equi exhibited immunosuppressive activities against equine PMNs. Strong cell-mediated and humoral immune responses against R. equi were detected in some horses, but other animals showed little response to the bacterium. Conclusions based on the experimental observations support two long-held speculations about R. equi infection while contesting another idea that is prevalent in the literature.

The immunosuppressive activites of R. equi and its surface components exhibited in equine PMN function assays support the contention that R. equi acts as an intracellular pathogen. Reduced effectiveness of bactericidal mechanisms in the presence of R. equi may promote intracellular survival of this bacterium. Suppression of PMNs may act in concert with other immunosuppressive factors to allow establishment of infection. The presence of a PMN-suppressive component on R. equi is not sufficient to account for pathogenesis, but the factor probably affects the nature of the infection once it is established.

The second prevalent speculation supported by the research concerns the cell-mediated response of horses to R. equi. Significant and consistent lymphocyte blastogenic responses by cells from infected horses provide evidence that a cell-mediated immune response to R. equi develops in many animals. The data gathered in this experiment, however, demonstrate the lack of this response in most foals which were infected with the bacterium. Correlation of immune status to other events in the life of these foals may reveal underlying causes of susceptibility to R. equi infection.

Unexpected results from the study of the antibody response to R. equi provide previously undocumented information about this immune response. High concentrations of antibody to R. equi seem incompatible with the cell-mediated response that is also present in many horses. Many infected foals demonstrated weak cell-mediated and humoral immune responses to R. equi. A role for antibody in resistance to R. equi infection is unrecognized, but the strong humoral response to R. equi in some horses may indicate that the humoral immune response contributes to resistance to infection.

Research creates far more questions than it answers, and this project was faithful to that principle. Three basic questions addressed in the objectives of the project have been answered, but other aspects of the host-parasite relationship were not resolved. Future studies should be directed towards further characterization of the mechanism of resistance to R. equi infection.

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