Antibody Response of Horses to *Rhodococcus equi* Antigens

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ABSTRACT

The antigens extracted from strains belonging to seven capsular serotypes of *Rhodococcus equi*, as well as from two wild strains isolated from pneumonic foals, were examined. Whole-cell antigens and soluble products present in broth culture supernatants were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, electroblotted onto nitrocellulose, and stained with serum from hyperimmunized rabbits or foals. Foal sera used included sera from pneumonic animals with known titer to *equi* factors; from animals bled monthly on a farm with enzootic pneumonia, and from animals bled monthly on a farm with no history of *R. equi* pneumonia.

The humoral response of foals to somatic antigen preparations was negligible, with few differences noted between sera from healthy, subclinically affected, and sick foals. The humoral response to *R. equi* broth culture supernatant products appeared more marked and was related to *equi* factor antibody titer. These findings suggest that the humoral response to *R. equi* whole-cell antigens is unimportant in protection against disease, which is consistent with the behavior of the organism as a facultative intracellular pathogen.

Key words: *Rhodococcus equi*, *Corynebacterium equi*, horses, antibody response, antigens.

RÉSUMÉ

Cette expérience consistait à examiner les antigènes extraits de souches appartenant à sept sérotypes capsulaires de *Rhodococcus equi* et de deux autres, isolées de poulains atteints de pneumonie. On sépara les antigènes des bactéries entières et les produits solubles présents dans le surnageant des bouillons de culture, au moyen de l'électrophorèse sur gel de polyacrylamide-dodécylsulfate de sodium; on les asséchа ensuite à l'électricité, sur de la nitrocellulose, et on les colora avec du sérum hyperimmun de lapins ou de poulains. Le sérum de poulains qu'on utilisa provenait de sujets atteints de pneumonie et on en connaissait le titre à l'endroit des facteurs *equi*; il provenait aussi d'échantillons prélevés mensuellement chez des poulains qui vivaient sur une ferme où sévissait la pneumonie à *R. equi* et chez d'autres qui vivaient sur une ferme exempte de cette forme de pneumonie.

La réponse humorale des poulains aux préparations d'antigène somatique s'avéra négligeable, puisqu'on enregistra seulement quelques différences entre le sérum des poulains sains et celui de ceux qui présentaient une atteinte subclinique ou clinique. La réponse humorale aux produits du surnageant du bouillon de culture de *R. equi* sembla plus marquée et elle était reliée au titre d'anticorps contre les facteurs *equi*. Ces constatations suggèrent que la réponse humorale aux antigènes des cellules bactériennes intactes de *R. equi* ne revêt que peu d'importance dans la protection contre la maladie; cette constatation est compatible avec le comportement de l'organisme comme pathogène intracellulaire facultatif.

Mots clés: *Rhodococcus equi*, *Corynebacterium equi*, développement d'anticorps, antigènes.

INTRODUCTION

Rhodococcus (Corynebacterium) equi is an important cause of suppurative bronchopneumonia in foals (1). In a recent study in Ontario 10% of foal mortality between one and six months was attributed to this disease (2). The disease is often not detected clinically until it is far advanced and is then expensive to treat (3). A recent report suggested that antibody detection using enzyme-linked immunosorbent assays (ELISA) may be useful for early detection of infection (4) but the nature of the antigens detected was undefined.

Rhodococcus equi is thought to be a typical facultative intracellular parasite, in which immunity to infection is primarily cell mediated (1). Recent *in vitro* studies have shown that alveolar macrophages from foals are only capable of significant ingestion of *R. equi* in the presence of immune serum (2). In addition there is the suggestion that foals with low rather than high levels of antibody, detected by ELISA, are more likely to develop *R. equi* pneumonia (4). Recent studies of antibody in foals and their dams to *equi* factor(s) by Skalka (5) have also suggested that passive humoral protection against disease may occur. There are many aspects of the pathogenesis of *R. equi* pneumonia and of immunity to the disease which are unclear. There has been no systematic study of the effect of vaccination against disease and the protective antigens have not been defined.

The purpose of the work described here was to define which of the antigens of *R. equi* were important in the humoral immune response of foals to infection, with the aim of defining antigens useful in early serodiagnosis of the disease and in immunoprophylaxis.

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MATERIALS AND METHODS

BACTERIAL STRAINS

One strain of each of the seven capsular serotypes of *R. equi* (ATCC 33701-33707) as well as the type strain ATCC 6939 (6) were used in this study. In addition strains 3610/85 and 3857/85, isolated from the lungs of pneumonic foals, were used. After primary isolation on blood agar 3610 and 3857 were stored in aliquots at -70°C. In all studies, bacteria were recovered from frozen stocks and passaged at most three times, apart from 3610 and 3857 in which strains were also stored following 50th and 100th passage *in vitro* under the conditions described (7). Strains were cultured on different media (nutrient broth, blood agar, brain heart infusion broth, trypticase soy broth, minca broth) at 30°C and 37°C (7).

RABBIT HYPERIMMUNE SERA

Hyperimmune sera were raised in individual rabbits by the intravenous inoculation of live cultures of ATCC 33701 and 33702 (capsular serotype 1 and 2, respectively) (6). Once precipitins were detected (6) sera were collected and stored at -70°C. For immunoblotting procedures, rabbit sera were diluted 1:100 to 1:500 in 0.01 M phosphate buffered saline, pH 7.2, with 3% bovine serum albumin and 0.05% Tween 20 (8).

HORSE SERA

Horse sera examined came from several sources: 1) Sera from foals sick with *R. equi* pneumonia of known antibody titer to *equi* factors (9), 2) Sera collected at monthly intervals, from one month of age, from foals on a farm with enzootic *R. equi* pneumonia, 3) Sera from foals collected at monthly intervals, from the first day of age, from foals on a farm with no history of *R. equi* pneumonia. For immunoblotting procedures, horse sera were diluted 1:5 in the buffer described.

PREPARATION OF R. EQUI ANTIGENS

Whole cell, mainly protein, antigens were prepared for analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by the method described (7). Briefly, whole bacterial cells were harvested from the different media described after incubation at 30°C or 37°C by centrifugation, washed twice in 0.15 M NaCl, and stored at -70°C in distilled water. Supernatant preparations containing *equi* factor(s), the exotoxins of *R. equi*, were made as described (9) by ammonium sulfate precipitation of broth culture supernatants of *R. equi*. Lyophilates were stored at -70°C.

SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS

Proteins of *R. equi* were analyzed as described (7). The method of Laemmli (10) was followed, using a 5% stacking gel and a 8-15% gradient gel. Gels were 1.5 mm thick. Samples were solubilized in SDS-solubilizing solution containing 2-mercaptoethanol, boiled for 5 min, then applied to the gel at about 60 μg total protein: (Bio-Rad Protein Assay, Bio-Rad Laboratories) per lane. Protein and carbohydrate constituents were electrophoresed using a Bio-Rad Protein II slab apparatus (Bio-Rad Laboratories, Mississauga, Ontario), run at a constant current of 20 mA per gel until the entrance of the tracking dye into the resolving gel, when the current was increased to 25 mA. Temperature was maintained at 10-12°C by circulating cold water. Proteins were visualized with Coomassie blue stain. Molecular weight standards (Bio-Rad) were run with all gels.

WESTERN BLOTTING

Following Coomassie blue staining, protein profiles were photographed and the gels then destained using the method of Phelps (11), by soaking for 12-16 h in several changes of 50% methanol and 7% acetic acid at 50°C. After destaining, gels were equilibrated for 2 h in three or four changes of a transfer buffer. This consisted of 192 mM glycine, 25 mM Tris (pH 8.3), 20% (v/v) methanol, and 0.1% SDS, and is a modification of the buffer described by Towbin *et al* (8). Antigens were then transferred from the gels to nitrocellulose paper (Bio-Rad) by electroblotting at 210 mA for 6 h at 4°C in the transfer buffer. Immunological detection of antigens on the nitrocellulose was done by the method described by Hohmann and Faulkner (12). Briefly, nitrocellulose was washed in PBS (pH 7.4), and nonspecific binding was blocked by incubation for 2 h (for horse sera) or 6 h (for rabbit sera) in PBS-BSA 3%, Tween 20 0.05%, at 37°C. The nitrocellulose was then incubated with diluted horse (1:5) or rabbit (1:100) sera overnight at 4°C, before washing with agitation in five changes of PBS-Tween 20 of 4 min each. The nitrocellulose strips were then incubated with dilute (1:1000) affinity purified antihorse or antirabbit IgG (H+L), conjugated to alkaline phosphatase (Dimension Laboratories, Mississauga, Ontario) for 2 h at room temperature, washed with agitation in five changes of PBS-Tween 20 of 4 min each, then for 1 min in PBS (pH 7.4), then twice for 1 min each in 0.1 M Tris (pH 9.2), before staining with naphthol ASBI sodium phosphate (Sigma Chemicals, Saint Louis, Missouri), diluted 1 mg/mL in 0.002 M $MgCl_2$ 0.01 M Tris (pH 10.0) with 1 mg/mL Fast Red TR salt (Sigma). The reaction was allowed to proceed until the stain developed, when it was stopped by dilution in distilled water.

RESULTS

IMMUNOBLOTTING USING HYPERIMMUNE RABBIT SERA

Immunoblots of protein profiles of representatives of seven capsular serotypes and the type strain of *R. equi* are shown in Fig. 1. Approximately 20 antigens were detected using hyperimmune sera raised against the two different serotypes of *R. equi*. There were minor but inconsistent differences, which were mainly quantitative, between the profiles of the different strains grown under identical conditions. There were also generally minor differences in the immunogenic pattern of four different strains when they were grown in different media (brain heart infusion broth, blood agar, minca broth and nutrient broth).

One marked difference was the presence of two prominent proteins, a diffuse protein of MW approximately 17.5 kd and a more discrete protein of MW 15.1 kd (7), which were present in passaged strains of 3857 (Fig. 2) but were absent in the 50th and 100th passage of strain 3610 (Fig. 3). Both proteins appeared to be well detected by

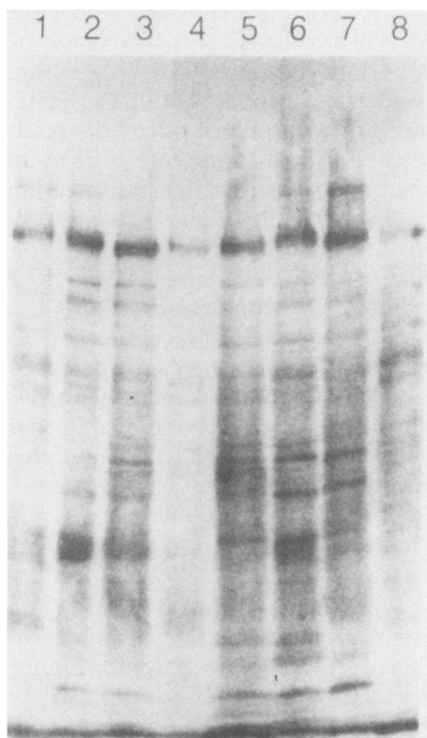

Fig. 1. Immunoblot of SDS-PAGE of whole cell proteins of capsular serotypes 1-7 (Lanes 1-7) and the type strain of *R. equi* (Lane 8), stained with rabbit hyperimmune serum to serotype 1. Strains grown in brain heart infusion broth for 48 h at 37° C (see Fig. 1, reference 7).

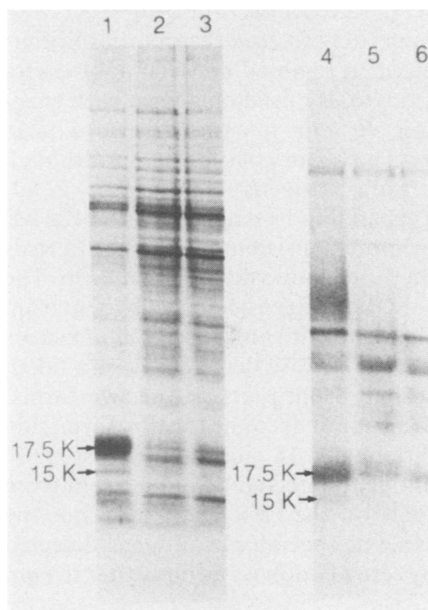

Fig. 3. SDS-PAGE of whole cell protein profile of *R. equi* strain 3610 on left (primary culture lane 1, 50th passage lane 2, 100th passage lane 3). On right (Lanes 4-6) is immunoblot of the same gel using rabbit hyperimmune serum to serotype 1. Strains grown in nutrient broth for 48 h at 37° C. Arrows indicate 15 kd and diffuse 17.5 kd protein band in lane 1, but not in lanes 2 and 3. Immunoblotting (lanes 4-6) shows transfer of these bands to lane 4, but their absence from lanes 5 and 6.

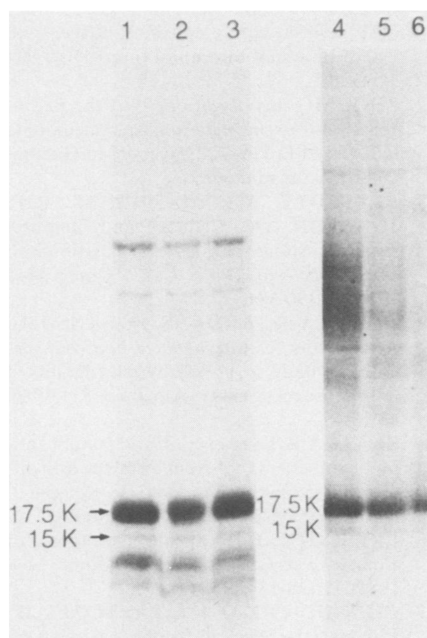

Fig. 2. SDS-PAGE of whole cell protein profile of *R. equi* strain 3857 on left (primary culture lane 1, 50th passage lane 2, 100th passage lane 3). On right (Lanes 4-6) immunoblot of the same gel, using rabbit hyperimmune serum to serotype 1. Strains grown in nutrient broth for 48 h at 37° C. Arrows indicate 17.5 kd and 15 kd proteins.

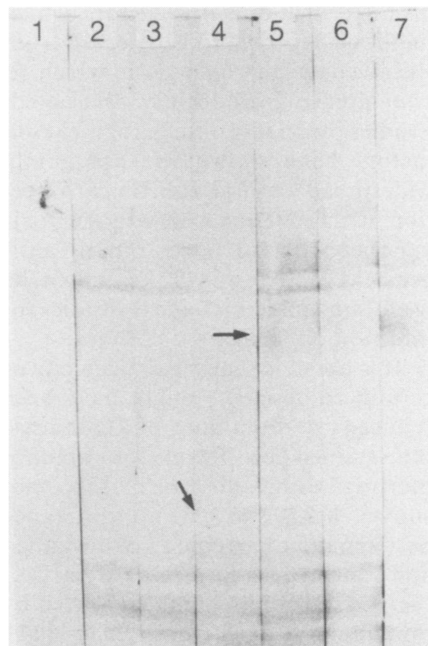

Fig. 4. Immunoblot of SDS-PAGE lyophilized supernatant of *R. equi* serotype 1 broth culture, stained with pooled foal sera of increasing antibody to *equi* factors. Lane 1, no antibody titer; lane 2-7 titers of 8, 16, 32, 64, 256 and 512 respectively. Arrows show faint antigens identified with increasing antibody titer.

hyperimmune rabbit sera (Figs. 2 and 3), although the presence of other proteins of about 17 kd appeared to mask the absence of the large diffuse protein in immunoblots of the 50th and 100th passaged strains (Fig. 3). However it is possible that the large diffuse protein was produced in small quantity.

IMMUNOBLOTTING USING HORSE SERA

Immunoblotting of a crude preparation of the soluble products of *R. equi*, including the *equi* factor(s), showed that foal sera with known anti-*equi* factor antibody detected up to eight well defined antigens (Fig. 4). Sera with high titers to *equi* factors generally detected more antigens than those with low titers. Sera with no antibody titer did not detect any antigens.

For immunoblotting of whole-cell antigens, pooled sera from foals on a farm with a history of endemic *R. equi* pneumonia were compared to pooled sera from foals on a farm with no similar history, using sera taken monthly from the same foals as they aged (one day to six months). Five antigens were detected with this sera (results not shown), two well defined narrow bands of MW approximately 105 kd and 122 kd, and three fainter bands of MW 19.5, 22 and 46 kd. The two higher MW bands were detected by all sera, but the 46 kd bands were detected only in sera from foals aged one day (farm with no history) or one month (farm with enzootic disease). The faint 22 kd band was detected in older foals on the farm with enzootic disease (foals aged four, five, and six months) and in six month old foals on the farm with no history of enzootic disease. The 19.5 kd band was detected inconsistently in sera from foals from both farms, four times in foals of various ages from the enzootically diseased farm and twice in sera from foals of different ages from the farm with no history of disease.

Immunoblotting of whole-cell antigen profiles were done using sera of known antibody titer to *equi* factors, obtained from foals sick with *R. equi* pneumonia. Seven faint, barely visible antigenic bands were detected inconsistently using this sera (results not shown). These bands did not correspond to those detected by sera collected serially from healthy foals from the two farms.

DISCUSSION

Hyperimmune rabbit sera, which contained precipitating antibodies to capsular polysaccharide antigens (6), was used at 1:100 dilution. Serum raised against two different serotypes detected at least 20 antigens of *R. equi* which were common to the seven capsular serotypes tested. Previous studies (7) showed that the SDS-PAGE profiles of different serotypes were almost identical, and this study has shown that SDS-PAGE profiles of *R. equi* are antigenically homogenous. Only minor differences were apparent when bacteria were cultured in different media.

Certain strains of *R. equi* subcultured repeatedly *in vitro* lack two proteins MW 15.1 and 17.5 kd (7). Strain 3857 retained these proteins but 3610 lost them on passage (Figs. 2 and 3). Both proteins were antigenic. The presence of other proteins of similar molecular weight appeared to mask the lack of the diffuse protein MW 17.5 kd in passaged strains of 3610 when immunoblotting was done (Fig. 3). The importance of these proteins in bacterial virulence remains to be determined.

The *equi* factor(s) preparation used in this study was a crude preparation from the same batch described in an earlier study of antibodies to these factors in the sera from foals sick with *R. equi* pneumonia (9). The material represented soluble products of *R. equi* released into a broth culture supernatant. It consisted of 11 distinct protein bands but which of these wer the two *equi* factors, the cholesterol oxidase and ceramide phosphate-active phospholipase C (13), was not determined. As expected, a humoral response to these soluble antigens was detected by immunoblotting in foals with known antibody titers to these proteins (Fig. 4), even in foals with low titers to *equi* factors. As antiserum titer rose an increased range of soluble antigens were detected (Fig. 4).

In marked contrast to the results obtained with soluble products, the humoral response of foals to somatic antigen preparations of *R. equi* was poor. Whereas rabbit hyperimmune serum was diluted 1:100 or 1:500, foal serum was diluted 1:5 for immunoblotting. Higher dilutions often failed to detect antibody. This apparent lack of response to somatic antigens was observed even in serum from foals with high titers to *equi* factors. Few differences were noted between pooled sera from foals raised on a farm with enzootic *R. equi* pneumonia and a farm with no history of the disease, although certain antigens (MW 19.5, 22 kd) appeared to be detected earlier (22 kd) or more consistently (19.5 kd) in foals on the enzootically diseased farm. The somatic antigens detected by sera from sick foals with antibody to *equi* factors were not those detected by sera taken serially from foals on the two farms. We cannot account for this surprising difference. In addition the somatic antigens detected by pooled sera from foals on the two farms were not the same as the soluble antigens detected by sera of known antibody titer to *equi* factors.

The observation that the humoral response to *R. equi* somatic protein antigens is negligible, even in sera of foals with chronic pneumonia, is consistent with the observations of others that *R. equi* pneumonia elicits a limited antibody response (2,3,14) and with the finding that, of the immunological tests used to detect humoral response (4,9,16), those tests which use supernatants have been the most successful. Foals responded best to soluble protein products including *equi* factors, which might be expected to be released from the alveolar macrophages in which *R. equi* are so prominently associated. Studies of the response of foals to *equi* factors have shown the apparently widespread natural subclinical infection with *R. equi* which many foals experience (5,16). Two somatic antigens, MW 19.5 and 22 kd, might be useful in epidemiological studies of infection on farms.

It is possible that the antigenicity of certain components might have been reduced by destaining of Coomassie blue stained gels. Results of immunoblotting using unstained gels, not shown here, showed no difference between immunogenicity of unstained and stained gels. Some minor parts of the *R. equi* components visualized by immunoblotting (Fig. 1) may have represented carbohydrate antigens not identified on Coomassie blue staining, but the majority of bands identified by immunoblotting were identifiable on Coomassie blue stained gels (Figs. 2 and 3).

The organism possesses mycolic acids in its cell wall (17), which may be important in ensuring survival within alveolar macrophages (15). The bacterium is a facultative intracellular parasite and cellular immunity is likely to be of major significance in protection against infection. The role in cell-mediated immunity of some of the soluble protein antigens to which a humoral response develops, as well as of the few somatic antigens, requires to be defined. This study has identified several soluble and somatic antigens which might usefully be studied further for their importance in the immune response to infection.

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