Rhodococcus equi Virulence-Associated Antigens and Specific Antibody Response in AIDS Patients Infected with *R. equi**

Claudio Maria Mastroianni, Miriam Lichtner, Fabio Mengoni, Maria Teresa Mascellino, Vincenzo Vullo, Salvatore Delia

Objectives: To analyze the expression of the 15- to 17-kDa plasmid-encoded antigens from *Rhodococcus equi* isolates of 7 AIDS patients and determine the immunologic response to these proteins in the patients' sera.

Methods: The expression of the virulence proteins in *R. equi* isolates and the specific antibody response were investigated by immunoblotting. Plasmid DNA was analyzed by agarose gel electrophoresis.

Results: The only patient infected with a strain carrying the virulence 85-kb plasmid and expressing the 15- to 17-kDa antigens developed a fatal pneumonia and did not produce specific antibodies to the virulence proteins. Of the 6 patients infected with *R. equi* strains lacking both proteins and plasmid, only 1 subject had a pulmonary disease with poor clinical outcome and exhibited a negligible humoral immune response to *R. equi* antigenic components, whereas the other patients who produced a remarkable antibody response developed either an asymptomatic infection (1 case) or pneumonia (4 cases) which completely cleared up.

Conclusions: Our findings suggest that *R. equi* disease can be induced without the expression of the 15- to 17-kDa virulence-associated plasmid-encoded antigens in HIV-infected patients with very low CD4+ cell counts. Nevertheless, both the synthesis of the virulence proteins and a defective humoral immune response to *R. equi* may contribute to the severity of rhodococcal disease.

Key Words: *R. equi* infection, AIDS, virulence proteins, antibody response.

Rhodococcus equi is a facultative, intracellular, gram-positive coccobacillus that can cause severe pneumonia and lung abscesses in 1- to 3-month-old foals. Initially described as an animal pathogen, it is now recognized as an emerging opportunistic pathogen in immuno-compromised humans, in whom a similar pneumonia frequently occurs (1, 2). At present, *R. equi* is increasingly isolated from sputum or blood cultures from patients with AIDS. In these subjects, the pulmonary disease is characterized by frequent relapses and a high mortality rate, despite continuous antimicrobial therapy and surgical intervention.

Bacterial virulence factors and host immunity play an important role in the pathogenesis of *R. equi* infections. The virulence of *R. equi* in both mice and horses has been shown to be directly associated with the expression of 15- to 17-kDa surface protein antigens encoded by a large plasmid of approximately 85 kb (3–5). The virulence-associated plasmid-encoded antigens, which are temperature-regulated and are located on the external surface of cells (6), have also been used as markers for the identification of virulent isolates from horses and their environment. Moreover, immunoblotting experiments have demonstrated that foals naturally infected with *R. equi* produced a remarkable antibody response to these proteins (3). The importance of 15- to 17-kDa antigens as virulence factors in human *R. equi* infections as well

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as their immunogenicity in infected patients remain to be determined.

The aim of this study was to analyze the expression of the 15- to 17-kDa plasmid-encoded antigens in *R. equi* isolates from AIDS patients and to determine the immunologic response to these proteins in the patients' sera. The correlation between these data and the clinical outcome of rhodococcal disease was also evaluated.

Materials and Methods

Bacteria

R. equi reference strain ATCC 33701, which was previously found to be virulent in a mouse model(3), was obtained from American Type Culture Collection (Rockville, MD). Clinical strains (5683D, 212B, 239D, 2275B, 2633C, 210D, 605B) were isolated from the sputum of 7 different patients with AIDS and identified as *R. equi* by the API Corynebacterium test results (Biomérieux, Marcy l'Etoile, France). Strains were subcultured no more than 3 times and stored in aliquots with 15% glycerol at −80°C.

Sera

Serum samples were collected from a foal naturally infected with *R. equi* and from the 7 culture-positive AIDS patients.

Immunoblotting

Whole-cell antigens were prepared by harvesting bacteria grown at 38°C for 48 h in brain heart infusion broth. Samples were sonicated and solubilized in 3% SDS reducing buffer with 5% 2-mercaptoethanol. The lysates were boiled for 20 min. and centrifuged at 12,000 g for 5 min. to remove debris. Lysates containing approximately 2×10^8 cells per well were separated by SDS-PAGE[7] with a running gel and a stacking gel of 13% and 4% acrylamide respectively, and electro-transferred to nitrocellulose for immunoblot analysis. The blotted nitrocellulose sheets were blocked with 0.25% PBS-Tween for 60 min. at room temperature and incubated overnight at room temperature with serum samples diluted 1/100 in 0.05% PBS-Tween. The blots were then washed three times in 0.05% PBS-Tween and incubated for 60 min. at 37°C in a 1/1000 dilution of horseradish peroxidase-conjugated rabbit anti-horse IgG (ICN Biomedicals, Inc, Costa Mesa, CA) or anti-human IgG (Dakopatts, Glostrup, Denmark). After the sheets were washed, the substrate 4-chloro-1-naphthol (Bio-Rad, Laboratories Richmond, CA) was used for immunoenzymatic detection. The development of the reaction was stopped with distilled water.

Plasmid isolation

The 85-kb plasmid was isolated from *R. equi* by the alkaline lysis method as decribed by Takay et al (8). The bacteria were incubated at 37°C for 2 h in a buffer containing 0.05 M TRIS hydrochloride, 0.01 M EDTA, 0.05 M NaCl and 20% (wt/vol) sucrose (pH 8.0) plus 5 mg/ml lysozyme. Cells were lysed in 3.0% (wt/vol) SDS in 0.05 M TRIS hydrochloride buffer (pH 12.6) at 55°C for 2 h. Chromosomal DNA was precipitated with 5 M potassium acetate buffer (pH 4.8) and centrifuged at 10,000 g for 15 min. The aqueous upper phase was recovered and purified DNA was precipitated with 0.6 volumes of cold isopropanol for 15 min at room temperature and centrifuged at 10,000 g for 15 min. at 0°C. After washing with 70% ethanol, the DNA pellet was resuspended in 50 mM TRIS – 20 mM disodium EDTA, pH 8.0 (TE buffer) and analyzed by 0.8% agarose gel electrophoresis. Plasmids were visualized by ethidium bromide staining.

Results

Immunoblot and plasmid analysis

Immunoblots of the 7 *R. equi* clinical isolates and reference strain ATCC 33701 are shown in figure 1A. The foal-infected serum had an intense immunoreactivity with the 15- to 17-kDa antigens of the reference strain ATCC 33701. When whole-cell antigens of the *R. equi* strains isolated from the patients were evaluated for their immunoreactivity, a diffuse band, corresponding in molecular mass to the 15- to 17-kDa virulence-associated proteins, was strongly recognized by the infected-foal serum in only one clinical isolate (strain 239D). No differences were detected among the other components of the whole-cell antigens of all examined strains. The analysis of the plasmid profiles revealed that *R. equi* 239D also contained an approximately 85-kb plasmid, which corresponded in size to the virulence plasmid from ATCC 33701 (figure 1B).

The immunoblot analysis of the human antibody response showed that serum samples from all but one AIDS patient reacted with several components of *R. equi* antigens, but not with the diffuse 15- to 17-kDa proteins (figure 2). In addition, the AIDS patient infected with strain 239D, which showed the 15- to 17-kDa antigens in immunoblotting with infected-foal serum, exibited no serological reactivity with these proteins.

Correlation with clinical findings

The correlation between the *R. equi* isolates and the clinical outcome of *R. equi* infection in the 7 AIDS patients is reported in Table 1. Clinical isolate 239D,

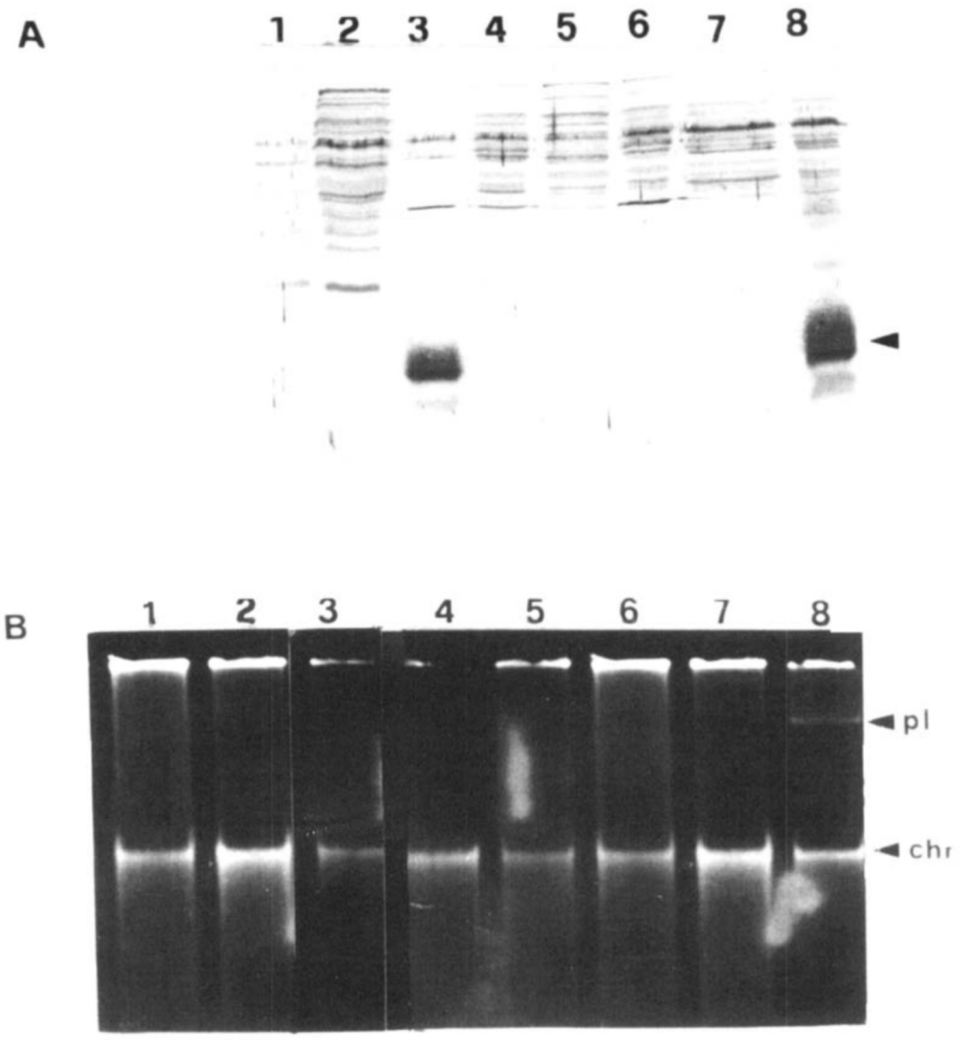

Figure 1 Immunoblot (A) and plasmid (B) profiles of *R. equi* strains. The virulence-associated 15- to 17-kDa antigens were detected by immunoblotting with naturally infected foal serum. Plasmid DNA was isolated, analyzed on a 0.8% agarose gel, and stained with ethidium bromide. Lanes: 1, strain 5683D; 2, strain 212B; 3, strain 239D; 4, strain 2275B; 5, strain 2633C; 6, strain 210D; 7, strain 605B; 8, strain ATCC 33701. Arrow at right indicates band for 15- to 17-kDa antigens. Abbreviations: pl=85-kb plasmid; chr=chromosomal DNA.

carrying the 85-kb plasmid and expressing the virulence-associated antigens, was obtained from a 28-year-old man who presented with a 3-week history of fever, malaise, weight loss (8 kg) and a non-productive cough. A chest X-ray showed a large consolidation with cavity in the lower lobe of the right lung. After isolation of *R. equi* from the induced sputum, antibiotic treatment was given without benefit, and a chest radiography revealed progressive enlargement of the area of cavitation and a pleural effusion. The patient died 4 weeks later.

As for the other clinical isolates which did not express virulence-associated antigens and plasmid, 4 (strains 5683D, 2275B, 210D, 605B) were from AIDS patients with cavitary pneumonia which completely cleared up with antibiotics. After a clinical follow-up of at least 14 months, 3 of these patients (No. 1, 6 and 7) were still free of pulmonary disease, while one

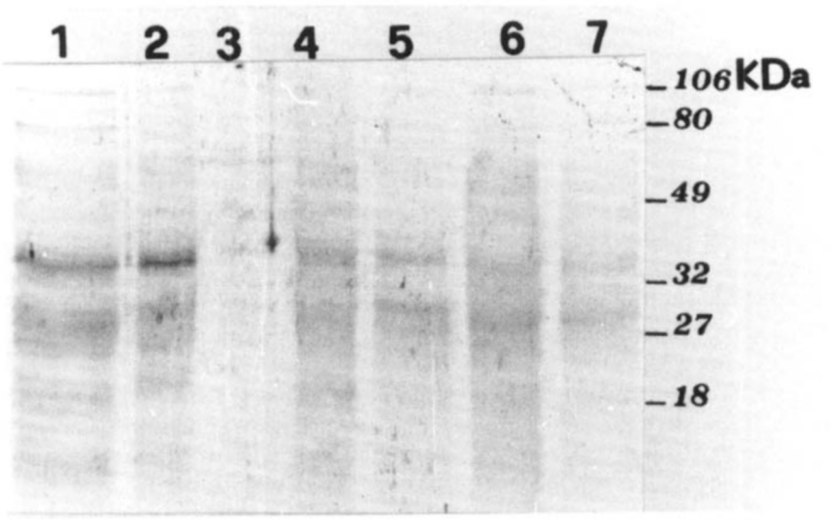

Figure 2 Immunoblot analysis of serological antibody response to *R. equi* in AIDS patients infected with avirulent (lanes 1-6) and virulent *R. equi* (lane 7). The ATCC 33701 reference strain was used for the preparation of whole-cell antigens. Lanes 1, 2, 4 and 5: recovered patients; lane 6: patient with asymptomatic *R. equi* infection; lanes 3 and 7: patients who died from *R. equi* pneumonia. Numbers at right indicate molecular mass.

patient (No. 4) showed evidence of a pulmonary relapse and *R. equi* was again cultured from the sputum. This isolate was still lacking expression of the virulence antigens and presence of the plasmid encoding them (data not shown). It had the same antibiotic susceptibility as the original strain. After 3 weeks of antibiotic therapy with erythromycin, rifampicin, imipenem/cilastatin and teicoplanin, the patient made a complete clinical and radiologic recovery.

Clinical isolate 212B was obtained from a 29-year-old female who was hospitalized because of fever, productive cough, moderate dyspnea and radiologic evidence of a cavitated infiltration in the lower lobe of the right lung. She responded to treatment during the initial 4 weeks of therapy with rifampicin and erythromycin, which produced a reduction of radiographic infiltration. Then, pulmonary disease progressed despite addition of imipenem/cilastatin and teicoplanin and the patient's clinical condition deteriorated with onset of respiratory failure followed by death. Interestingly, it was this patient who had weak seroreactivity with all the major antigenic determinants of *R. equi*.

Table 1 Correlation between the *R. equi* isolates and the clinical outcome of *R. equi* infection in the 7 AIDS patients.

Clinical isolate No.	*Patient No.*	*Age/sex*	*Animal exposure*	*CD4+ cells/μl*	*CD8+ cells/μl*	*Clinical/X-ray presentation*	*Antibiotics*	*Outcome*
5683D	1	37/M	None	18	456	Cavitating pneumonia	Ery, Rif	Recovered, survived
212B	2	29/F	None	9	126	Cavitating pneumonia	Ery, Rif, Imp, Tec	Died
239D	3	28/M	None	5	977	Cavitating pneumonia	Ery, Rif, Imp, Van	Died
2275B	4	38/M	Horses	7	108	Cavitating pneumonia	Ery, Rif, Imp, Tec,	Relapse, survived
2633C	5	34/M	None	15	500	Asymptomatic infection	None	Survived
210D	6	33/M	None	9	801	Cavitating pneumonia	Ery, Rif, Cip, Imp	Recovered, survived
605B	7	22/M	None	32	1561	Cavitating pneumonia	Ery, Rif, Cip, Imp	Recovered, survived

Abbreviations: Ery=erythromycin; Rif=rifampicin; Cip=ciprofloxacin; Imp=imipenem/cilastatin; Tec=teicoplanin; Van=vancomycin.

Finally, the sixth *R. equi* strain (2633C), lacking both virulence proteins and plasmid, was isolated from the sputum of an AIDS patient who did not show any clinical and radiologic evidence of either pulmonary or extrapulmonary rhodococcal disease.

Discussion

Previous investigations have demonstrated that rhodococcal disease in horses correlates with the isolation of *R. equi* strains which contain an 85-kb plasmid, express 15- to 17-kDa antigens and are virulent in mice, whereas strains lacking both proteins and plasmid are avirulent and do not cause disease (3, 4). The present paper provides evidence that the development of *R. equi* disease in AIDS patients doesn't correlate with the isolation of strains expressing the 15- to 17-kDa plasmid-encoded antigens from biological specimens and, furthermore, that plasmid-negative strains may induce clinical illness. Indeed, of the six patients infected with the 85-kb plasmid-negative strains, only one did not show any clinical evidence of rhodococcal disease, while the other 5 developed cavitary pneumonia. Similar findings were obtained by Takai and colleagues (9) who reported that opportunistic infections in immunocompromised patients with and without AIDS can be caused by both virulent and avirulent *R. equi* strains. However, these authors do not correlate the presence of bacterial virulence factors with the clinical course of rhodococcal disease. In the present study all but one patient infected with plasmid-negative *R. equi* strains recovered from the pulmonary disease, while the only patient infected with a strain expressing both virulence-associated 15- to 17-kDa antigens and the 85-kb plasmid had a severe and eventually fatal pneumonia. Our findings suggest that *R. equi* disease can be induced without the expression of the 15- to 17-kDa virulence-associated plasmid-encoded antigens in HIV-infected patients with very low CD4+ cell counts ($<35/\mu l$).

The 15- to 17-kDa plasmid-encoded antigens represent important virulence factors in *R. equi* infections in foals. These proteins are produced in vivo by virulent *R. equi*, are highly immunogenic in infected foals, and might be involved in the intracellular survival and replication of the bacterium within horse alveolar macrophages (10). The function and the immunologic properties of the 15- to 17-kDa antigens in human *R. equi* infections appear to be different from that reported in horses. Our work firstly suggests that the expression of virulence-associated plasmid-encoded antigens by *R. equi* does not seem to be essential for the development of clinical disease in AIDS patients. Secondly, although expressed by *R. equi* human isolates, the virulence proteins do not evoke a specific antibody response in infected patients. Indeed, our immunoblotting experiments demonstrated that AIDS patients infected with *R. equi* may produce serum antibodies that react with various antigenic determinants of the bacterium but do not recognize the diffuse band corresponding to the 15- to 17-kDa proteins. The importance of humoral immunity in protection against *R. equi* disease has been emphasized by experimental data concerning the immune response of horses (11-13). Moreover, we recently demonstrated that the clinical outcome of *R. equi* pneumonia was more severe in AIDS patients who exhibited a negligible humoral immune response to *R. equi* (14). The results of the present study showed that among the 6 AIDS patients infected with *R. equi* strains lacking both virulence proteins and plasmid, the only one who did not recover from pneumonia showed a decreased antibody reactivity to *R. equi* antigenic components.

The pathophysiology of *R. equi* infection in humans has yet to be elucidated. The role of animals in the transmission of the infection appears to be limited since the majority of patients have had no history of contact with farm animals or manure. Among the 7 patients described here, only one (14%) had had recent exposure to horses. Our recent data on the detection of *R. equi* antibodies in AIDS patients without *R. equi* disease and in healthy individuals suggest that exposure to this organism is widespread in the environment (14). In AIDS patients, the impairment of T-cell-mediated immunity is clearly the critical determinant for the development of *R. equi* disease, which can be induced by both virulent and avirulent strains. Both CD4+ and CD8+ lymphocytes are important in the resistance against *R. equi*, even though CD8+ cells seem to play the major role in host defence (15). The results reported here suggest that, once infection is established, both the synthesis of the virulence-associated antigens and a defective humoral immune response may contribute to the severity of the disease. If the patients are infected with avirulent strains, the humoral immunity seems to have an important function in the recovery from the opportunistic infection. Indeed, since the impaired production of specific antibodies reduces the opsonization of bacteria, the uptake, phagosome-lysosome fusion and killing of *R. equi* by macrophages and neutrophils might be altered. In the case of infection with virulent strains, the disease outcome may be affected by the production of virulence factors which enhance the replicative capacity and prolong the survival of the organism within macrophages. In this respect, the 15- to 17-kDa plasmid-encoded proteins, which are not recognized by antibody-mediated immunity, may mediate *R. equi* intramacrophage

persistence, even if the involvement of other cell wall components, such as mycolic acids or toxic factors related to β-lactam resistance (16–18), cannot be excluded.

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