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Interaction of virulent and non-virulent *Rhodococcus equi* human isolates with phagocytes, fibroblast- and epithelial-derived cells

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Abstract: Rhodococcus equi is a facultative, intracellular, Gram-positive coccobacillus, increasingly reported in pneumonia of AIDS-infected patients. We investigated killing resistance properties of human *R. equi* virulent and avirulent human strains. Avirulent β-lactam-susceptible strains had lower intracellular colony forming units after 45 min incubation in murine macrophages J774 and human monocyte-macrophage TPH-1 than those of virulent strains. Only virulent β-lactam-resistant strains persisted within macrophages for at least 18 min only. A β-lactam-resistant mutant was obtained from a β-lactam-susceptible strain after selection in a penicillin G-containing culture medium. This mutant strain, like the natural virulent strains, persisted within macrophages, harboured cell-associated appendages, produced phage-like particles and induced, after its intravenous inoculation, a chronic infection in BALB/c nude mice. Supernatant culture of virulent strains transferred partial macrophage-killing resistance properties to avirulent strains. The same supernatant was toxic for L-929, HeLa and Vero cell cultures. These supernatant effects were heat-inactivated, trypsin-inactivated and did not seem to be linked to phage-like particle presence. These data argue that virulence, β-lactam-resistance, and macrophage-killing resistance are associated in human *R. equi* isolates. Moreover, only virulent strains produced uncharacterized toxic factors.

Key words: Rhodococcus equi; Virulence; Macrophage

Introduction

Rhodococcus equi, formerly referred to as *Corynebacterium equi*, is a Gram-positive coccobacillus. Primarily known as a pulmonary pathogen in the foal, it is mainly associated with pneumonia, lung abscesses, and systemic infections in humans [1]. Recently, this bacterium has been increasingly identified as an opportunistic pathogen in AIDS-infected patients [2–4]. *R. equi* is a facultative intracellular organism which may prevent the phagosome-lysosome fusion within phagocytic cells [5]. It persists in macrophages, which may explain its ability to escape normal pulmonary defense mechanisms [5]. Cell-mediated immunity plays a major role in immunity to

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R. equi. Both $CD4^+$ and $CD8^+$ T cell subsets are required for acquired resistance to *R. equi* but $CD8^+$ T cell subset plays the major role [6].

We have recently found that, within human clinical isolates, two antibiotic-resistance phenotypes co-exist: one β-lactam-resistant and the other β-lactam-susceptible [7]. Electron microscopy revealed that β-lactam-resistant strains possess cell-surface-associated appendages and produce phage-like particles. Testing for virulence in mice reveals that: (i) phage-like particle-producing strains have a 50% lethal dose by the intravenous (i.v.) route in euthymic and nude mice that is lower than those of the phage-like particle non-producers; (ii) inoculation of a sublethal dose by the i.v. route to nude mice led to a chronic infection with the phage-like particle-producing bacteria only; and finally (iii) in vitro growth curves indicate that the phage-like particle producing strains possess an ecological advantage. These results show that, in *R. equi* human isolates, the antibiotic-resistance phenotype is associated with virulence in mice and may be phage-mediated.

As interaction between human *R. equi* isolates and their target cells, i.e. monocyte-macrophages, has not yet been studied, the aim of this work was to analyze, in vitro, killing properties of human and mouse macrophages towards virulent and non-virulent *R. equi* strains. Moreover, the effects of the phage-particles isolated from virulent strains and bacterial culture supernatants were analyzed on various fibroblast- and epithelial-derived cell cultures.

Materials and Methods

Bacterial strains

R. equi clinical strains were isolated from the blood of AIDS patients with pulmonary abscesses and identified according to the API Corynebacteria test results (Biomérieux, Marcy l'Etoile, France). Two pairs of β-lactam-resistant (R)/β-lactam-susceptible (S) (PN1003 S and R, PN1004 S and R) were studied. β-Lactam-resistant strains only were virulent for nude mice [7]. Bacterial cultures were stored at −80°C and thawed before their appropriate dilutions for animal inoculation or in vitro infections. From *R. equi* PN1003 S strain, an in vitro β-lactam-resistant mutant was obtained as follows. J774 macrophage culture (see below) was cultured with *R. equi* PN1003 S in the presence of 10 μg/ml of penicillin G (Diamant, Paris) (a concentration which was 100-fold higher than penicillin G MIC towards *R. equi* PN1003 S). After 45 min incubation at 37°C, bacterium-containing culture medium was removed and J774 macrophages were further cultured in a gentamicin (50 μg/ml) containing medium for 48 h for inhibiting extracellular bacterial growth. Culture supernatant was then discarded and phagocytic cells were lysed in distilled water. Serial dilutions of the lysate were plated onto tryptic soy (TS) agar plates (Biomérieux, Marcy l'Etoile, France). *R. equi* strains were isolated and β-lactam MICs determined as previously described [8]. One of the isolates, *R. equi* PN1003 M11, was retained for further analysis.

Virulence test

Six- to 8-week-old female congenitally athymic nude (*nu/nu*) BALB/c mice were purchased from Iffa Credo (L'Arbresle, France). As previously described, athymic nude mice were intravenously inoculated with a sublethal dose (5×10^6 cfu) of *R. equi* [9]. Groups of three mice were killed at various intervals. Spleens, livers and lungs were homogenized in 2 ml of distilled water. Samples of the homogenates and 10-fold serial dilutions in saline were plated onto TS agar. Colonies were counted after 48 h of incubation at 37°C.

Killing rate

Two monocyte-macrophage cell lines were used; J774 (ATCC TIB 70) which derive from BALB/c mouse and TPH-1 (ATCC TIB 202) which derive from human acute leukemia. Cells were cultured in 24-well microplates (Falcon, Beckton Dickinson, Grenoble, France). J774 cells were seeded at a concentration of 8×10^6 cells per ml under 1 ml volume of RPMI 1640 (Gibco, BRL) supplemented with 5% fetal calf serum (Gibco), 2 mM glutamine (Gibco), and incubated for 2 h at 37°C in an atmosphere of 5% CO_2,

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95% air. Non-adherent cells were removed by washing three times with pre-warmed RPMI 1640 medium, then discarded, and fresh culture medium (as above but without antibiotics) was added to adherent cells. The Trypan blue exclusion test was performed to check for cell viability. TPH-1 cells which are non-adherent monocyte-macrophages were cultured in the same conditions as J774 cells but without the adherence period.

For early killing rate experiments, monocytes were infected with *R. equi* strains at a final concentration of either 8×10^7 or 8×10^8 bacteria per ml (multiplicity of infection of either 10 or 100). After 45 min incubation, the supernatant was removed, J774 cell cultures were washed three times with cold PBS (Gibco) and then lysed with 1 ml distilled water. For TPH-1 cells, a centrifugation step ($3000 \times g$ for 15 min at 4°C) gave a cell pellet which was submitted to lysis by water. The lysate was diluted in sterile water for bacterial numeration.

For late killing rate experiments, after the 45 min incubation, J774 supernatant was removed and discarded, and a fresh pre-warmed culture medium was added which contained gentamicin 50 μg/ml to ensure absence of extracellular *R. equi* growth. For TPH-1, same procedure was used, but a centrifugation step removed supernatant. After either 6 h or 18 h, macrophage cultures incubated at 37°C in the presence of 5% CO_2-95% air were lysed for bacterial numeration.

Phage preparation

From *R. equi* PN1003 R and PN1003 M11 virulent strains, 72 h cultures were grown onto TS agar at 37°C. Each plate was thoroughly rinsed with 1 ml of phosphate buffer supplemented with 10^{-3} M $MgSO_4$ and cells were examined by electronic microscope as previously described [7]. The suspension was then centrifuged at $15\,000 \times g$ for 30 min. Supernatant was then poured in a fresh tube and the pellet was discarded. DNase and RNase (Boehringer Mannheim) were added to the supernatant at a concentration of 1 μg/ml and the mixture was incubated for 30 min at room temperature. Solid NaCl was added to a final concentration of 1 M and the mixture was put on ice for 1 h. The mixture was centrifuged for 10 min at $15\,000 \times g$ at 4°C, then solid PEG 6000 (Sigma Chemicals) was added to the supernatant at a final concentration of 10% w/v [10]. After 1 h incubation on ice, the mixture was centrifuged at $15\,000 \times g$ for 20 min at 4°C and the supernatant was removed by aspiration. The phage particle containing pellet was resuspended in an appropriate volume of phosphate buffer supplemented with 10^{-3} M $MgSO_4$ and analyzed under the electronic microscope as previously described [7]. Phage particle number was estimated visibly as no indicator strains are available [7].

In vitro transfer of killing resistance properties from virulent to avirulent R. equi strains

Killing rate experiments were performed as described above. Avirulent strains (PN1003 S and PN1004 S) were added to J774 macrophage culture (MOI:10) together with 48 h bacterial culture filtrated (0.22 μm, Millipore) supernatant (diluted 1:2 or 1:10) of respectively *R. equi* PN1003 R or PN1004 R virulent strains. Similar experiments were performed after heat inactivation (90°C for 30 min) or trypsin inactivation (1.5 mg/ml for 30 min at 37°C followed by trypsin inhibition with soy inhibitor). After 45 min incubation, supernatant was removed and macrophage cells were either lysed or incubated for a further 6 h or 18 h period before being lysed.

Toxic effect of supernatant of R. equi culture

L-929, Vero and HeLa cells were seeded into 96-well microtiter Falcon plates at a concentration of 1×10^5 cells per well in a RPMI 1640 supplemented with L-glutamine (2 mM), and 10% heat-inactivated calf serum. Dilutions of filtrated supernatants from *R. equi* PN1003 S, PN1003 R, PN1003 M11 cultures were performed. These supernatants came from 37°C or 30°C bacterial cultures. After various incubation periods in 5% CO_2-95% air at 37°C, supernatants were removed and cytotoxicity was assessed as follows [11]. Adherent cells were stained for 10 min with crystal violet (0.2% in 2% ethanol solution). After washing three times in distilled water, stained solutions were dissolved in 1% SDS and 1% PBS.

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Table 1

Killing rates of *R. equi* human isolates of PN1003 series in J774 murine macrophages

Strains	Incubation time		
	45 min	6 h	18 h
PN1003 S [a]	3.52 ± 0.01 [b]	0	0
PN1003 R [c]	5.97 ± 0.02	5.23 ± 0.15	5.05 ± 0.05
PN1003 M11 [d]	5.92 ± 0.11	5.07 ± 0.06	5.28 ± 0.02

[a] *R. equi* PN 1003 S was a β-lactam-susceptible avirulent clinical isolate.
[b] Mean (± S.D.) of *R. equi* cfu in macrophage lysate (expressed in log 10).
[c] *R. equi* PN1003 R was a β-lactam-resistant virulent clinical isolate.
[d] *R. equi* PN1003 M11 was a β-lactam-resistant, avirulent, and in vitro-obtained mutant.
Experiments were performed in quadriplicate.

Optimal density was measured at 545 nm on an UV spectrophotometer. Percent cytotoxicity was defined as the relative absorbance of the test sample versus that of the control wells as follows: % cytotoxicity = 1 − (absorbance of sample/absorbance of control) × 100.

Statistical analysis

Student's unpaired *t*-test was used to determine the significance of cfu differences in killing rates, of the supernatant effects on bacterial persistence, and of cytotoxicity percentage. $P < 0.05$ was considered significant.

Results

R. equi killing rates

After 45 min incubation, the avirulent β-lactam-susceptible *R. equi* PN1003 S strain has a significant reduced number within macrophages as compared to virulent β-lactam-resistant PN1003 R (Table 1). In contrast to virulent strains, after 6 or 18 h incubation, avirulent strains did not persist within macrophages (Table 1). Similar results were obtained with human-derived monocytes TPH-1 and with another human *R. equi* strain couple, PN1004 S and PN1004 R (data not shown).

A β-lactam-resistant mutant strain *R. equi* PN1003 M11 was obtained when β-lactam-susceptible strain PN1003 S was incubated for 45 min with penicillin G and selection was performed within macrophages after a further 18 h incubation. MICs (μg/ml) of β-lactams for this strain was identical to this of β-lactam-resistant mutant PN1003 R (amoxicillin, 4; penicillin G, 4; cephalothin, 64). This in vitro-obtained mutant harboured cell-associated appendages and produced phage-like particles as previously described for natural β-lactam-resistant *R. equi* strains [7].

For any of the β-lactam-susceptible or resistant strains, no toxicity was found for macrophage culture after 18 h incubation (data not shown).

Virulence test

As previously demonstrated, *R. equi* PN1003 R and PN1004 R led to a chronic infection in nude mice whereas *R. equi* PN1003 S and PN1004 S β-lactam-susceptible strains were eliminated within three days (Fig. 1). The in vitro selected β-lactam-resistant mutant *R. equi* PN1003 M11 inoculated to nude mice induced a chronic infection as described for the natural β-lactam-resistant virulent strains (Fig. 1).

In vitro transfer of killing resistance properties

Filtrated supernatant (diluted 1:2) of *R. equi* PN1003 R added to J774 culture medium incubated with *R. equi* PN1003 S avirulent strain, led to an increase in killing resistance of PN1003 S (Table 2). However, the intramacrophagic PN1003 S cfu count in these conditions was not identical to that of *R. equi* PN1003 R virulent strain (Tables 1 and 2). Similar results were obtained with *R. equi* PN1004 series. No effect was noted when supernatant of virulent strains was added after its heat inactivation or trypsin treatment. Phage-like particles added at a concentration of 5×10^8 particles per ml to avirulent strain did not modify their killing resistance properties (data not shown).

Virulent strains produced a toxic factor

R. equi human isolates and the in vitro obtained *R. equi* PN1003 M11 were cultured for 48 h in TS broth. Filtrated supernatant was incubated pure or diluted onto L-929, Vero or HeLa

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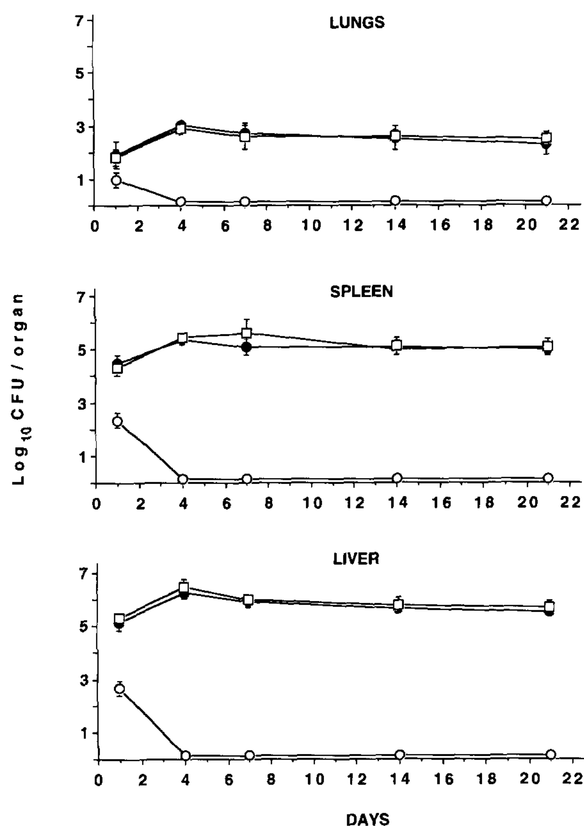

Fig. 1. Disseminated infections in nude mice following i.v. inoculation with a sublethal dose of 5×10^6 cfu of *R. equi* PN1003 series strains. The data are expressed as geometric means of cfu (±standard deviations, four mice per point) in the liver, lungs and spleens. The i.v. challenge was made of the following *R. equi* strains: β-lactam-resistant PN1003 R clinical isolate (●), β-lactam-susceptible PN1003 S isolate (○), and in vitro obtained β-lactam-resistant PN1003 M11 mutant (□).

cell cultures. Virulent strain supernatants from 37°C (and not the 30°C culture) were toxic for these cells. L-929 cell cultures were the most susceptible. In all cases, the cytotoxicity was time-dependent (Table 3). These toxic effects were inhibited by supernatant incubation at 90°C for 30 min or by trypsin treatment (Table 3).

Discussion

Although it has been reported for some *R. equi* animal isolates [12], the in vitro properties of *R. equi* human isolates had not yet been studied. Virulence of *R. equi* animal isolates is correlated to resistance to intracellular killing [12]. *R. equi*

Table 2

Effect of *R. equi* PN1003 R virulent strain culture supernatant on intracellular persistence of *R. equi* PN1003 S avirulent strain in macrophages J774

Supernatant [a] and treatment	Incubation time		
	45 min	6 h	18 h
No supernatant	3.55±0.21 [b]	0	0
Supernatant	4.20±0.01	1.33±0.10	0.84±0.15
Heat-inactivated supernatant [c]	3.58+0.07	0	0
Trypsin-treated supernatant [d]	3.46±0.16	0	0

[a] *R. equi* PN1003 R supernatant culture was filtered and diluted 1:2 in J774 macrophage culture medium. *R. equi* PN1003 S avirulent cultures (cfu at a MOI of 10) was incubated for 45 min in macrophage medium with supernatant media. Culture medium was thereafter removed and macrophages were maintained for either 6 or 18 h in a gentamicin-containing cell culture.
[b] Mean log 10 cfu ±S.D.
[c] Heat inactivation for 30 min at 90°C.
[d] Trypsin treatment (1.5 mg/ml) for 30 min at 37°C followed by soy inhibitor treatment.
Experiments were performed in quadriplicate.

Table 3

Toxic effects of *R. equi* culture supernatants on L-929 culture

Strain supernatant [a]	Cytotoxicity (%)		
	Incubation time		
	18 h	36 h	72 h
PN1003 S [b]	0	0	4±1
PN100 3 R [c]	80±2	95±1	100±1
PN1003 R Heat-inactivated supernatant [d]	0	0	3±1
PN1003 R Trypsin-treated supernatant [e]	0	0	4±1
PN1003 M11 [f]	81±3	94±2	100±1

[a] Supernatant of 48 h bacterial culture was filtrated and diluted 1:2 in L-929 cell culture medium.
[b] *R. equi* PN1003 S avirulent clinical strain.
[c] *R. equi* PN1003 R virulent clinical strain.
[d] Heat inactivation for 30 min at 90°C.
[e] Trypsin inactivation for 30 min at 37°C followed by soy inhibitor treatment.
[f] *R. equi* PN1003 M11 in vitro obtained virulent strain.
Experiments were performed in quadriplicate.

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human isolates are interesting since virulence factors do not seem to be the same as those of animal isolates [13]. In the animal isolates only, the presence of 15- to 17-kDa antigens is correlated with an 85-kb plasmid, and with virulence for mice [14]. However, in animal and human isolates, β-lactam-resistance phenotype, virulence and phage-like particle presence is correlated [7].

Our report showed that, in *R. equi* human isolates, there is a correlation between intramacrophagic persistence, virulence and β-lactam resistance. As measured after 45 min incubation, intramacrophagic cfu was lower for avirulent strains than for virulent strains. Early decrease of intramacrophagic cfu of avirulent strains may have resulted from either a decrease in bacterial uptake or from their rapid killing in macrophages. Higher intramacrophagic persistence of β-lactam-resistant strains as compared to those of β-lactam-susceptible strains cannot result only from their in vitro growth advantage [7]. The behaviour of the in vitro-obtained β-lactam-resistant *R. equi* mutant was similar to that of the natural β-lactam-resistant virulent strains. In vitro β-lactam selection from a β-lactam-susceptible strain led to the isolation of a β-lactam-resistant strain which persisted within macrophages and which was virulent after inoculation to nude mice. This result may have clinical implications, since β-lactam is often the first choice of therapy for treating pneumonia when the pathogen has not yet been identified. Blind β-lactam therapy may therefore select for virulent strains. Moreover, the development of this in vitro macrophage test will be helpful in precisely determining the effects of macrophage activation by $CD4^+$ and $CD8^+$ T cell subsets and by the cytokines whose role has been described in vivo [15].

The second interesting result was that transfer of killing resistance properties was obtained with avirulent strains incubated with supernatant of virulent strain culture. The filtrated supernatant was no longer effective after its heat inactivation or its trypsin treatment. Therefore, *R. equi* toxic factors from virulent strains may be at least partially protein based. In animal isolates, virulence is associated with 15- to 17-kDa antigens which are trypsin-digestable and temperature-regulated [14]. However, using a monoclonal antibody directed against animal isolate 15- to 17-kDa antigens (kind gift from S. Takai) with virulent *R. equi* human strains, we did not obtain any positive signal in an immunoblot study (data not shown). In virulent human strains, as well as in the β-lactam-resistant mutant PN1003 M11, the presence of phage-like particles was detected. It is therefore likely that β-lactam treatment may induce a stress effect for phage synthesis and/or expression of a phage-associated toxin gene. Phage by themselves did not seem to have any effect on bacterial persistence within macrophages. However, one may not rule out that phage components were partially denatured when isolated or/and their number was insufficient to modify bacterial persistence of avirulent strains. The absence of indicator strains for large scale phage purification may render further study difficult on the phage effects.

The third interesting finding was that virulent strains only produced toxic factors for fibroblast- and epithelial-derived cells. Surprisingly, the toxic factor production was noticeable after bacterial growth at 37°C and not 30°C. When protein content of the supernatant of virulent and avirulent strains cultured either at 37°C or 30°C were compared by SDS-PAGE after ethanol or ammonium sulphate precipitation, no protein difference was observed (data not shown). As reported, it is possible that other *R. equi* cell wall components such as mycolic acids may induce the observed effects [16]. L-929 cell cultures may be used for testing the effect of potential *R. equi* human toxic factors. Further work will be directed towards cloning and testing recombinant plasmids for toxic factor production as recently described in a *R. equi*-related bacterium, *Mycobacterium tuberculosis* [17]. Identification of such virulence factors may help to develop a *R. equi* vaccine.

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