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Role of T-Lymphocyte Subsets in *Rhodococcus equi* Infection

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***Rhodococcus equi*, a facultative intracellular gram-positive bacterium, can induce life-threatening infections in immunocompromised patients, especially those with AIDS. We have studied the mechanism of acquired immunity to this pathogen in a murine model. Protective immunity was induced by live but not killed bacteria. Adoptive transfer of resistance was obtained with spleen cells but not immune serum from mice immunized intravenously 30 days earlier with live bacteria. In normal mice, an intravenous challenge of 5×10^6 CFU of *R. equi* was cleared from the spleen, liver, and lungs within 3 weeks, whereas athymic nude mice were unable to clear the bacteria. In vivo depletion with monoclonal antibodies showed that both $CD4^+$ and $CD8^+$ T-cell subsets participate in the clearance of bacteria and that $CD8^+$ T cells play the major role.**

Rhodococcus equi is a gram-positive coccobacillary bacterium. It is a frank pathogen in foals, in which it principally causes subacute or chronic suppurative bronchopneumonia (28). The route of infection is most likely to be an aerosol inoculation (28). *R. equi* is increasingly reported as an opportunistic pathogen in immunocompromised patients, especially those with AIDS (9). Pneumonia and pulmonary abscesses are the most common human *R. equi* infections. However, osteomyelitis and brain and kidney abscesses resulting from blood dissemination have been also reported (9, 28, 33). *R. equi* infections are difficult to treat, as relapses occur frequently after antibiotic treatment (9, 28). A better understanding of the pathophysiology of *R. equi* infections may help to improve their treatment.

R. equi is a facultative intracellular pathogen with a mycolic acid-rich cell wall like that of *Mycobacteria* spp. (28). In infected foals it survives within macrophages, producing pyogranulomatous, macrophage-rich lesions (28, 29). In experimental infections in foals, the lethal dose varies from one strain to another and is approximately 10^8 to 10^{10} bacteria by the respiratory route (12, 18, 29). Death can be partially due to asphyxia caused by the inflammatory process in pulmonary abscesses (12, 28). One major pathogenic mechanism of *R. equi* may be its ability to prevent phagosome-lysosome fusion within macrophages (36). The relative contributions of cell-mediated and humoral immunity in the resistance against *R. equi* infections are controversial (7, 8, 28). Specific anti-*R. equi* antibodies in immune foals have been described (11, 17, 19, 29, 31, 34), but their protective role is uncertain (17, 19, 28). Martens et al. have reported that the intravenous (i.v.) administration of immune plasma prior to a challenge by aerosol decreases the severity of pneumonia in foals (19).

Cell-mediated immunity, reflected by lymphocyte blastogenesis and delayed hypersensitivity responses to *R. equi*, is present after infection and in virtually all horses over 6 months old which do not develop *R. equi* infections (7, 34). However, the protective role of cell-mediated immunity has not been demonstrated.

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Studies of the pathogenesis and immune mechanisms involved in *R. equi* infection are hampered by the difficulty of obtaining healthy foals in sufficient numbers to conduct statistically valid experiments, and murine models have therefore been proposed. When mice are infected by the i.v. route, the 50% lethal dose ranges between 2×10^6 and 1×10^9 organisms, depending on the bacterial strain (32). Bacteria multiply slowly during the first few days of infection, mainly in the spleen and liver and, to a lesser extent, in the lungs. The bacteria are then progressively cleared within a few weeks (23). Following respiratory challenge, the bacteria multiply mainly in the lungs and pneumonia can occur with inocula higher than 10^7 organisms (2, 3, 23); the course of the pneumonia is shorter than that in foals (2, 28). As in foals, *R. equi* is found within macrophages (3). Large numbers (10^8) of bacteria can be present in the organs of infected mice without causing death (23), indicating that *R. equi* is weakly pathogenic for mice. The bacterium behaves as an opportunistic pathogen, and the severity of experimental infections is increased in immunocompromised (i.e., athymic or cyclophosphamide-treated) mice (23, 35).

The purpose of this study was to determine the mechanism of acquired resistance to *R. equi* infection in a murine model. The i.v. route of inoculation was used to obtain reproducible numbers of bacteria in the organs.

We found that T cells are crucial for resistance to *R. equi* infection. Moreover, in vivo treatment with anti-CD4 and/or anti-CD8 monoclonal antibodies (MAb) showed that both $CD4^+$ and $CD8^+$ T cells are required for host defense against *R. equi* and that the $CD8^+$ T-cell subset plays the major role.

MATERIALS AND METHODS

Mice. Six- to 8-week-old female euthymic and congenitally athymic nude (*nu/nu*) BALB/c mice were purchased from Iffa Credo (L'Arbresle, France).

Bacteria. *R. equi* PN 1002 was isolated from the blood of an AIDS patient with pneumonia. Its 50% lethal dose in BALB/c mice was $>10^8$ bacteria by the i.v. route. *R. equi* was cultured in tryptic soy broth for 48 h at 30°C (the temperature for *R. equi* optimal growth) and then stored at −70°C. For each experiment a sample was thawed and appropriately diluted in saline for i.v. inoculation in a 0.2-ml volume. A virulent strain of *Listeria monocytogenes*, ob-

tained from G. Milon (Institut Pasteur, Paris, France) was cultured overnight at 37°C in tryptic soy broth.

Infection of mice and bacterial counts. Mice were inoculated i.v. with 5×10^6 CFU of *R. equi* and groups of three to four animals 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 tryptic soy agar. Colonies were counted after 48 h of incubation at 30°C. *L. monocytogenes* was injected i.v. (2×10^3 CFU), and the number of bacteria per spleen was determined by plating serial dilutions of spleen homogenates onto tryptic soy agar. Colonies were counted after 24 h of incubation at 37°C.

Immunization procedure. BALB/c mice were inoculated i.v. according to two protocols. One group received a killed *R. equi* vaccine consisting of 5×10^6 cells (heated to 65°C for 60 min), while another received 5×10^6 CFU of viable *R. equi*. Thirty days later, both groups, as well as nonimmunized controls, received an i.v. challenge with 5×10^6 CFU of live *R. equi*. Four days after the infectious challenge, the number of viable organisms in the spleen, liver, and lungs was determined.

To examine the specificity of acquired resistance, mice inoculated with viable *R. equi* either 30 or 6 days earlier, as well as control mice, were challenged i.v. with 2×10^3 CFU of *L. monocytogenes*. Viable *L. monocytogenes* in the spleens was enumerated 3 days after the challenge. *L. monocytogenes* and *R. equi* colonies were differentiated according to their morphology on tryptic soy agar (salmon pink *R. equi* colonies).

Adoptive transfer of resistance by immune serum. Normal mice and mice immunized with live *R. equi* were bled from the heart at day 30 postinfection, and their sera were injected intraperitoneally into normal mice (0.2 ml per mouse). The recipients were challenged 1 h later with 5×10^6 CFU of *R. equi* by the i.v. route, and viable bacteria in the spleen, liver, and lungs were enumerated 3 days later.

Adoptive transfer of resistance by immune spleen cells. Normal mice and mice immunized with viable *R. equi* 30 days earlier were sacrificed and the spleens were pressed through a stainless steel screen into Hanks' balanced salt solution (HBSS). Cells were washed with the same solution and erythrocytes were lysed with Tris-buffered NH_4Cl (pH 7.2) and then washed again in HBSS. Spleen cells (5×10^7 in 0.5 ml) were injected i.v. into sublethally irradiated (400 rads of gamma radiation given 24 h earlier) syngeneic mice. Recipient mice were challenged 1 h later with 5×10^6 CFU of *R. equi* i.v. and killed 3, 5, or 7 days later. Viable bacteria in the spleen, liver, and lungs were enumerated.

T-cell subset depletion in vivo. Hybridomas GK1.5 producing rat anti-CD4 immunoglobulin G2 subclass b (IgG2b) MAb and H35-17.2 producing rat anti-CD8 IgG2b MAb, kindly provided by G. Milon (Institut Pasteur), were grown intraperitoneally in pristane-primed Swiss nude mice. Antibodies were partially purified from ascites by 50% ammonium sulfate precipitation. After dialysis against phosphate-buffered saline, the rat IgG concentration was determined by means of radial immunodiffusion (Serotec, Bicester, United Kingdom). Infected BALB/c mice were injected intraperitoneally on days −1 and +1 with 1 mg of anti-CD4 MAb and/or 400 μg of anti-CD8 MAb i.v. Control mice received 0.2 ml of ascites containing an irrelevant (antipenicilloyl) MAb prepared as described above. The mice were then inoculated i.v. with 5×10^6 CFU of *R. equi* and killed 11 days later. Viable bacteria in the spleen and liver were enumerated.

Fluorocytometric analysis. Cell suspensions were prepared from spleens of mice treated with MAb. Erythrocytes were lysed with Tris-buffered NH_4Cl (pH 7.2), and the cell suspensions were washed in HBSS. Samples (2×10^6) of spleen cells were resuspended in HBSS containing 5% fetal calf serum and 0.2% sodium azide. The cells were treated successively, in the cold, with appropriate dilutions of GK1.5 MAb and a fluorescein isothiocyanate-labeled mouse anti-rat IgG(κ) (Mark-1; Biosys, Compiègne, France) for the detection of $CD4^+$ T cells or with biotinylated anti-Lyt-2 antibody and fluorescein isothiocyanate-labeled avidin (both from Becton Dickinson, Mountain View, Calif.) for the detection of $CD8^+$ T cells. After being washed, the cells were analyzed in a flow cytometer (FACScan; Becton Dickinson) gated to exclude nonviable cells.

Statistical analysis. Student's unpaired *t* test was used to determine the significance of differences between control and experimental groups.

TABLE 1. Effects of heat-killed and live *R. equi* on protective immunity

Treatment[a]	Log_{10} CFU (± SD) of *R. equi*[b]		
	Spleen	Liver	Lung
Control	6.42 ± 0.13	6.60 ± 0.13	3.21 ± 0.09
Heat-killed bacteria	6.10 ± 0.30	6.52 ± 0.12	3.15 ± 0.06
Live bacteria	3.50 ± 0.40[c]	4.23 ± 0.09[c]	1.0 ± 0.17[c]

[a] Mice were inoculated i.v. with either heat-killed or live *R. equi* (5×10^6 CFU) and challenged i.v. with *R. equi* (5×10^6 CFU) 30 days later. Controls were unimmunized mice.
[b] Bacteria were enumerated 4 days after the infectious challenge.
[c] Statistically different from the control: $P < 0.01$.

RESULTS

Induction of protective immunity. Groups of mice were inoculated with live or heat-killed bacteria and challenged 30 days later with 5×10^6 CFU of *R. equi*. The number of viable bacteria per organ was determined 4 days after the challenge. Only mice vaccinated with viable *R. equi* had a reduced number of bacteria in the spleen (Table 1). Similar results were obtained when liver or lung CFU counts were taken into account. Such protection was specific for *R. equi*, as indicated by the lack of increased resistance to challenge with *L. monocytogenes* at day 30 (data not shown). However, a degree of nonspecific resistance to *L. monocytogenes* was present 6 days after *R. equi* infection; 3 days after challenge at day 6, the log_{10} CFU (± standard deviation [SD]) of *L. monocytogenes* per spleen was 3.56 ± 0.39 in *R. equi*-infected mice compared with 5.09 ± 0.25 in controls ($P < 0.01$).

Transfer of acquired resistance. Serum from mice immunized with live bacteria did not transfer significant resistance to normal mice. In mice receiving immune and normal serum, the log_{10} CFU (± SD) values 3 days after challenge with *R. equi* were, respectively, 3.20 ± 0.25 and 3.34 ± 0.32 in lungs, 5.80 ± 0.20 and 5.94 ± 0.42 in spleen, and 6.38 ± 0.21 and 6.27 ± 0.42 in liver. In contrast, acquired resistance was transferred by spleen cells from mice inoculated with live bacteria 30 days earlier (Fig. 1). The adoptive protection was statistically significant ($P < 0.05$) in all the organs examined at days 5 and 7 and in the liver and spleen at day 3 after challenge.

Comparison of *R. equi* clearance in euthymic and congenitally athymic nude BALB/c mice. Both mouse strains were infected with 5×10^6 CFU of *R. equi*, and bacterial counts in

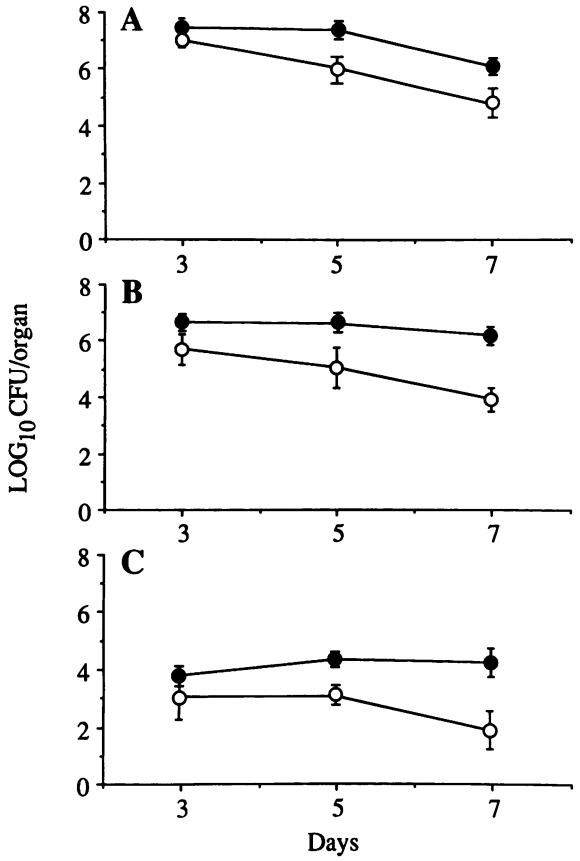

FIG. 1. Adoptive transfer of resistance to *R. equi* by spleen cells. Normal irradiated (400 rads) mice received either normal (●) or immune (○) spleen cells from mice immunized with live bacteria 30 days earlier. They were challenged 1 h later with 5×10^6 CFU of *R. equi*. Bacterial CFU (± SD) were enumerated in liver (A), spleen (B), and lungs (C) of groups of three recipient mice at days 3, 5, and 7 after challenge.

the organs were determined at various intervals. In both mouse strains, bacterial counts were higher in the liver and spleen than in the lungs, peaking at day 4 postinoculation (Fig. 2). However, the kinetics of CFU counts followed the same trend in all the organs examined. In immunocompetent mice, the bacterial counts progressively decreased during the 3-week experiment, reaching the limit of detectability within 21 days. In contrast, in infected nude mice, bacterial counts plateaued between days 4 and 21. Nude mice were unable to clear the bacterial inoculum. Despite a large inoculum (5×10^6 CFU of *R. equi*), no deaths occurred during the 3-week experiment.

TABLE 2. Residual $CD4^+$ and $CD8^+$ T cells after in vivo depletion with MAb

Treatment[a]	% Residual cells	
	$CD4^+$	$CD8^+$
Control	21.7 ± 4.8	19.0 ± 8.1
Anti-CD4	4.5 ± 1.2	19.3 ± 4.8
Anti-CD8	18.7 ± 2.1	6.8 ± 2.6
Anti-CD4 + anti-CD8	4.0 ± 1.6	3.2 ± 1.8

[a] Mice were treated in vivo on days −1 and +1 of *R. equi* challenge with the indicated MAb; the percentages of $CD4^+$ and $CD8^+$ T cells were determined on day 11 postinfection.

Effects of in vivo T-cell subset depletion. The administration of MAb induced a depletion of the corresponding T-cell subsets as indicated by flow cytometric analysis of spleen cells (Table 2). In vivo depletion of $CD4^+$ T cells slightly but significantly increased bacterial counts in the liver on day 6 and in the spleen and liver on day 11 postinfection (Table 3). In vivo depletion of the $CD8^+$ T-cell subset alone led to a significantly larger increase in CFU in the spleen ($P < 0.01$) and liver ($P < 0.05$) on day 11 postinfection. In vivo treatment with both anti-CD4 and anti-CD8 MAb induced higher bacterial counts in the spleen and liver than that with anti-CD8 MAb alone ($P < 0.05$), showing that both T-cell subsets participate in host defense against *R. equi* infection.

DISCUSSION

The increasing number of reported *R. equi* infections in AIDS patients suggests the involvement of T-cell-mediated immunity in acquired resistance against this pathogen. Our results in a murine model show that cell-mediated immunity is indeed crucial in host defense against *R. equi*, as it is against other intracellular organisms. Vaccination with live organisms conferred a degree of protection, while killed

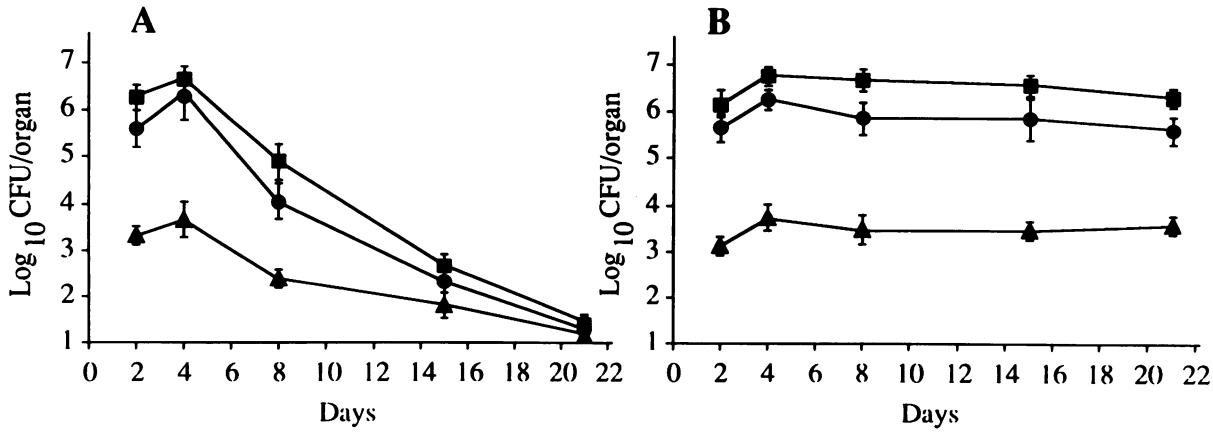

FIG. 2. Time course of *R. equi* infection in euthymic (A) and athymic nude (B) BALB/c mice. The data are expressed as geometric means of CFU (± SD) in the liver (■), spleen (●), and lungs (▲) after an i.v. inoculation with 5×10^6 CFU of *R. equi* (three mice per point).

TABLE 3. Effects of T-cell subset depletion in vivo on *R. equi* infection

Day postinfection and treatment[a]	Log_{10} CFU ± SD	
	Spleen	Liver
6		
Control	4.30 ± 0.14	5.06 ± 0.10
Anti-CD4	4.55 ± 0.22	5.64 ± 0.26[b]
Anti-CD8	5.04 ± 0.31[b]	5.72 ± 0.08[c]
11		
Control	1.50 ± 0.17	2.38 ± 0.24
Anti-CD4	1.90 ± 0.01[b]	2.87 ± 0.09[b]
Anti-CD8	3.19 ± 0.21[c]	3.39 ± 0.25[c]
Anti-CD4 + anti-CD8	3.59 ± 0.02[c]	4.12 ± 0.14[c]

[a] Mice were injected intraperitoneally on days −1 and +1 with the indicated MAb and challenged i.v. with *R. equi* (5×10^6 CFU) at day 0. Bacterial counts in the organs were carried out on the indicated days postinfection.
[b] Statistically different from controls: $P < 0.05$.
[c] $P < 0.01$.

vaccine was unable to do so, confirming previous reports (28, 29). This is in agreement with findings made with other facultative intracellular pathogens such as *L. monocytogenes* (16). The protection induced by live *R. equi* was specific when measured 30 days after immunization, although 6 days after infection a degree of resistance to *L. monocytogenes* was present, suggesting nonspecific macrophage activation at that time.

Adoptive transfer of resistance by immune serum was unsuccessful in our experimental conditions, suggesting that humoral immunity was not involved. Contrary to our results, it has been reported that immune plasma can protect foals against fatal *R. equi* disease (19). This discrepancy may result from differences in the animal species, i.e., foals and mice. In addition, the mechanisms of acquired resistance may be different when the bacteria are inoculated by the respiratory and i.v. routes.

Adoptive transfer of resistance was obtained with spleen cells from mice immunized with live bacteria, and nude mice were unable to clear *R. equi* from their organs, in accordance with a recent report (35). Taken together, the above data strongly suggest the role of T cells in host defense against *R. equi* infection.

In vivo depletion of $CD4^+$ and/or $CD8^+$ T-cell subsets indicated that both T-cell subsets play a role in acquired resistance to *R. equi* infection. However, it is striking that in vivo depletion of the $CD8^+$ T-cell subset alone led to a marked increase in CFU in liver and spleen.

It is generally assumed that $CD4^+$ T cells and, more specifically, the TH1 subset are involved in acquired resistance against facultative intracellular bacteria and that they increase the bactericidal activity of macrophages by producing gamma interferon (21, 22, 24, 26). However, $CD8^+$ T cells play a major role in *L. monocytogenes* infection (1, 15, 20, 30) and participate in resistance to *Mycobacterium tuberculosis*, especially when the challenge is given by aerosol (26, 27). The role of $CD8^+$ T cells in some bacterial infections is supported by the presence in immune animals of $CD8^+$ T cells which are cytolytic for infected target cells (6, 14), together with the role of class I molecules of the major histocompatibility complex in the genetic control of acquired resistance (5, 25), the passive transfer of resistance by $CD8^+$ T cells (20, 27), and finally, the effect of in vivo selective depletion of $CD8^+$ T cells by MAb on the course of infection (1, 20). The involvement of $CD8^+$ T cells in protective immunity implies that bacterial antigens are processed in a way which allows them to be presented to T cells in association with class I molecules of the major histocompatibility complex (4). The role of $CD8^+$ T cells in resistance to intracellular bacteria may be related to their capacity to lyse infected cells or to produce gamma interferon (13).

It has also been reported that supernatants from foal lymphocyte cultures stimulated with *R. equi* antigens can activate *R. equi*-infected macrophages (10). The role of lymphokines such as gamma interferon may therefore be important in host defense against *R. equi* infection. Further work is required to evaluate the role of these lymphokines, which are known to be involved in resistance to infection by other facultative intracellular pathogens (22).

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