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Failure of Pulmonary Clearance of *Rhodococcus equi* Infection in CD4$^+$ T-Lymphocyte-Deficient Transgenic Mice

SUZANNE T. KANALY,* STEPHEN A. HINES, AND GUY H. PALMER

Department of Veterinary Microbiology and Pathology, Washington State University, Pullman, Washington 99164-7040

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Pulmonary clearance of *Rhodococcus equi* requires functional T lymphocytes. In this study, CD8$^+$ T-lymphocyte-deficient transgenic mice cleared virulent *R. equi* from the lungs while infection in CD4$^+$ T-lymphocyte-deficient transgenic mice persisted. Although both CD4$^+$ and CD8$^+$ T cells function early in pulmonary defense against *R. equi*, clearance is dependent on CD4$^+$ T lymphocytes.

Rhodococcus equi is a gram-positive, facultative intracellular respiratory pathogen which causes severe pneumonia in young horses and immunodeficient humans (3, 12). Pulmonary disease is characterized by chronic pyogranulomatous inflammation and progressive loss of respiratory function due to the inability of alveolar macrophages to kill phagocytized *R. equi* (23, 24). In immunodeficient animals and humans, antibiotics can reduce bacterial numbers but not effect clearance of the organism (1, 4, 6, 9, 11, 14, 15, 18). Specifically, human immunodeficiency virus-infected patients with opportunistic *R. equi* infections frequently have relapses requiring lifelong antibiotic therapy (1, 4, 6, 14, 18). Identification of the immune mechanisms that mediate clearance of *R. equi* from the lungs would provide a basis for appropriate adjunct immunotherapy.

Immunodeficient mice are susceptible to *R. equi* and provide a model to test the role of T-lymphocyte subsets in pulmonary immunity against *R. equi* (2, 8, 10, 20). Previous studies have shown that severe combined immunodeficient (SCID) mice which lack T and B lymphocytes and athymic nude mice which lack T lymphocytes are unable to clear pulmonary *R. equi* infection (9, 20). In contrast, immunocompetent mice clear the infection by 21 days (8, 10, 20). These findings, in combination with the failure of human immunodeficiency virus-positive humans with low numbers of CD4$^+$ T cells to control *R. equi* infection, support the hypothesis that T lymphocytes function in the clearance of pulmonary *R. equi*. We investigated, in two independent experiments, the role of CD4$^+$ T lymphocytes and CD8$^+$ T lymphocytes in bacterial clearance from murine lungs.

R. equi ATCC 33701 is a virulent strain that possesses the 82-kb plasmid and expresses the 15- to 17-kDa proteins associated with *R. equi* virulence (16, 17). Avirulent strain 6939 lacks the plasmid and does not express the 15- to 17-kDa proteins (16, 17). Each strain was characterized, prior to inoculation, for the presence or lack of the virulence-associated plasmid by agarose gel electrophoresis as previously described (data not shown) (17). In addition, presence or absence of the virulence-associated proteins was confirmed by immunoblotting (Fig. 1). Briefly, bacterial proteins were electrophoretically separated, transferred to nitrocellulose, and reacted with a 1:1,000 dilution of either normal horse serum or serum from an *R. equi*-infected foal. Antibody binding was detected with horseradish peroxidase-labeled goat anti-equine immunoglobulin G antibody and enhanced chemiluminescence.

The role of CD8$^+$ T lymphocytes in the clearance of pulmonary *R. equi* was investigated with transgenic mice deficient in CD8$^+$ T lymphocytes (GenPharm International, Mountain View, Calif.) and C.B-17 Icr Tac DF SCID mice (Taconic, Germantown, N.Y.). CD8$^+$ T-lymphocyte-deficient mice (β_2m$-/-$) were produced by inactivation of the β_2 microglobulin gene, leading to failure to express major histocompatibility complex class I surface antigens and severe deficiency of functional CD8$^+$ T lymphocytes (21, 22). These mice will be referred to as β_2m$-/-$ CD8$^+$ T-lymphocyte-deficient mice. Mice heterozygous for the β_2 microglobulin gene disruption (β_2m$+/-$) express major histocompatibility complex class I antigens, have functional CD8$^+$ T lymphocytes, and were used as immunocompetent controls for the β_2m$-/-$ CD8$^+$ T-lymphocyte-deficient mice. Immunocompetent BALB/c mice served as controls for SCID mice. Prior to each experiment, the specific T-lymphocyte subset deficiency was confirmed, in three mice per group, by flow cytometric analysis of peripheral blood mononuclear cells or splenocytes. Surface-bound monoclonal antibodies 2.43, GK1.5, and B220, which recognize CD8$^+$ T lymphocytes, CD4$^+$ T lymphocytes, and B lymphocytes, respectively, were detected with fluorescein isothiocyanate-labeled mouse anti-rat immunoglobulin G (Table 1). Twenty mice of each strain were anesthetized with intraperitoneal ketamine and xylazine and inoculated intratracheally with 2×10^7 either virulent or avirulent *R. equi* bacteria. Five mice from each group were euthanized at 3 and 21 days postchallenge. Lungs were collected and weighed aseptically, minced in 1.0 ml of sterile phosphate-buffered saline, and homogenized in a laboratory stomacher for 45 s. Tissue homogenates were serially diluted on modified selective media and incubated for 48 h at 37°C to determine the number of CFU (19). Lung weights were not significantly different between mouse strains (data not shown). The means and standard deviations for CFUs were determined for each group. A Kruskal-Wallis nonparametric one-way analysis of variance and least significant difference were used to determine significant differences. The virulence-associated phenotype of bacteria isolated from the lungs of infected mice was compared to that of the inoculated bacteria by immunoblotting. In all mice, the phenotype of the isolated bacteria was identical to that of the inoculated bacteria. Immunoblots of representative isolates from SCID mice are shown in Fig. 1.

* Corresponding author.

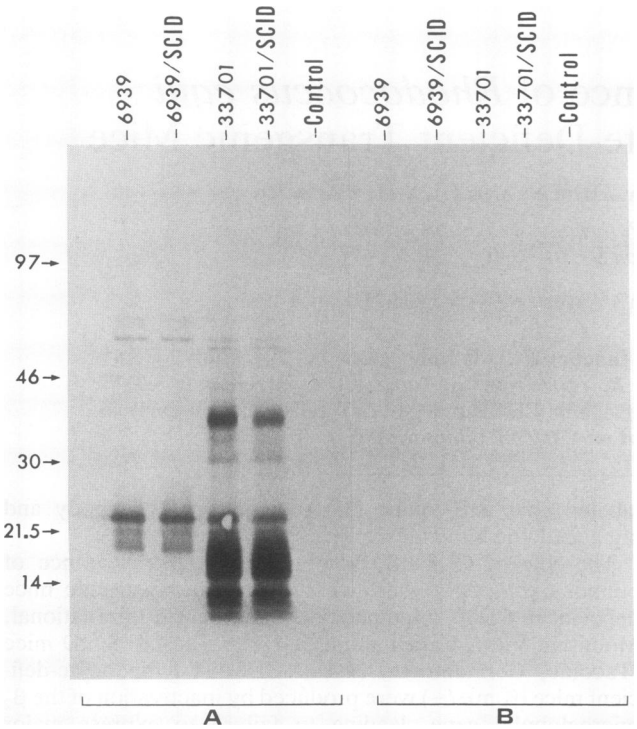

FIG. 1. Detection of virulence-associated 15- to 17-kDa polypeptides by immunoblotting. Avirulent strain 6939 and virulent strain 33701 were used to inoculate mice. 6939/SCID and 33701/SCID were bacteria recovered at 21 days postinfection from SCID mice. *Anaplasma marginale* was used as a negative antigen control. In panel A, the blot was reacted with serum from an *R. equi*-infected foal. In panel B, the blot was reacted with normal foal serum. The numbers on the left are molecular sizes in kilodaltons.

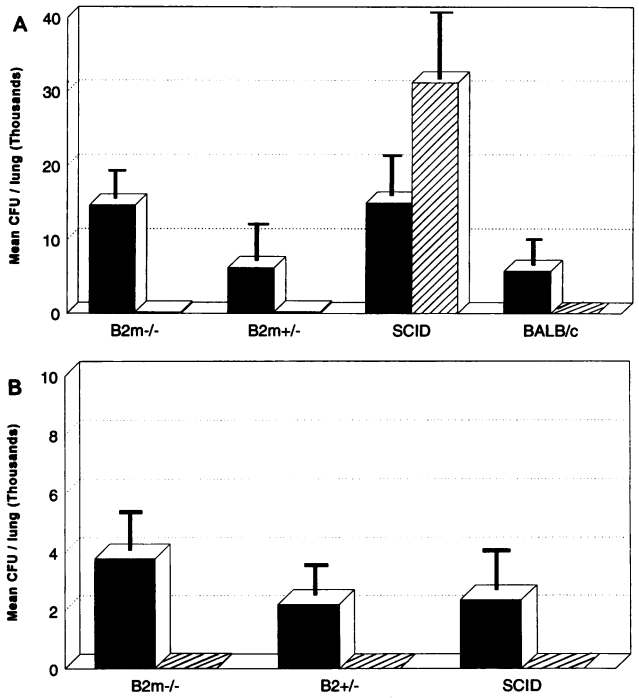

FIG. 2. (A) Pulmonary clearance of virulent *R. equi* 33701 in β_2m−/− CD8$^+$ T-lymphocyte-deficient mice, β_2m+/− immunocompetent controls, SCID mice, and immunocompetent BALB/c mice. (B) Pulmonary clearance of avirulent *R. equi* 6939 in β_2m−/− CD8$^+$ T-lymphocyte-deficient mice, β_2m+/− immunocompetent controls, and SCID mice. Results are mean CFU (± standard deviations) for individual lungs from five mice in each group at each time point. ■, 3 days; ▨, 21 days.

At day 3, the β_2m−/− CD8$^+$ T-lymphocyte-deficient mice resembled SCID mice in their decreased ability to contain acute pulmonary infection with virulent strain 33701 (Fig. 2A). In contrast, significantly ($P < 0.05$) fewer bacteria were recovered from the lungs of both immunocompetent BALB/c mice and β_2m+/− controls (Fig. 2A). At 21 days postinfection, SCID mice were still unable to clear the virulent *R. equi* strain from the lungs. However, β_2m−/− CD8$^+$ T-lymphocyte-deficient mice were identical to immunocompetent mice in the ability to clear virulent *R. equi* infection (Fig. 2A). There were no significant differences among mouse strains in the ability to control avirulent strain 6939 at 3 days postinfection (Fig. 2B). In contrast, all mice were able to clear avirulent bacteria by 21 days, including SCID and β_2m−/− CD8$^+$ T-lymphocyte-deficient mice (Fig. 2B).

TABLE 1. Lymphocyte subsets in mice prior to challenge

Mouse strain	% Total cells[a]		
	CD8$^+$	CD4$^+$	B
β_2m−/−	1.56 ± 0.49[b]	18.2 ± 0.72[b]	ND[c]
β_2m+/−	7.29 ± 0.44[b]	7.25 ± 0.29[b]	ND
BALB/c	15.71 ± 2.03[b]	33.29 ± 5.42[b]	10.22 ± 3.39[b]
SCID	1.05 ± 0.44[d]	1.13 ± 0.39[d]	3.31 ± 0.45[d]
CD4 deficient	22.40 ± 1.96[d]	2.60 ± 0.49[d]	35.96 ± 3.29[d]
C57BL/6	19.20 ± 2.77[d]	28.33 ± 2.14[d]	10.57 ± 2.83[d]

[a] Results are means (± standard deviations) for three mice per group.
[b] Peripheral blood cells.
[c] ND, not determined.
[d] Splenocytes.

To investigate the role of CD4$^+$ T lymphocytes in the clearance of pulmonary *R. equi*, we used transgenic mice deficient in CD4$^+$ T lymphocytes (Genpharm) and C.B-17 Icr Tac DF SCID mice. CD4$^+$ T-lymphocyte-deficient mice were generated by disruption of the major histocompatibility complex A^b_β gene (5). These mice do not express major histocompatibility complex class II molecules on cell surfaces and lack functional class II-restricted CD4$^+$ cells. Congenic C57BL/6 mice (Taconic) were used as immunocompetent controls. Fifteen mice of each strain were anesthetized and inoculated intratracheally with 2×10^7 virulent *R. equi* bacteria at 6 weeks of age. Five mice from each group were euthanized at 3, 21, and 28 days postchallenge. Lungs were collected and weighed aseptically, homogenized, and serially diluted on modified selective media to determine the numbers of CFUs as described for the previous experiment. At day 3, CD4$^+$ T-lymphocyte-deficient mice resembled SCID mice and had high numbers of bacteria in their lungs; however, bacterial numbers were not significantly different from those of immunocompetent C57BL/6 mice (Fig. 3). In contrast to immunocompetent strains and β_2m−/− CD8$^+$ T-lymphocyte-deficient mice, CD4$^+$ T-lymphocyte-deficient mice were unable to clear bacteria completely by 21 days. To determine whether clearance was prevented or merely delayed, mice were also examined at 28 days postinfection. *R. equi* persistence in CD4$^+$ T-lympho-

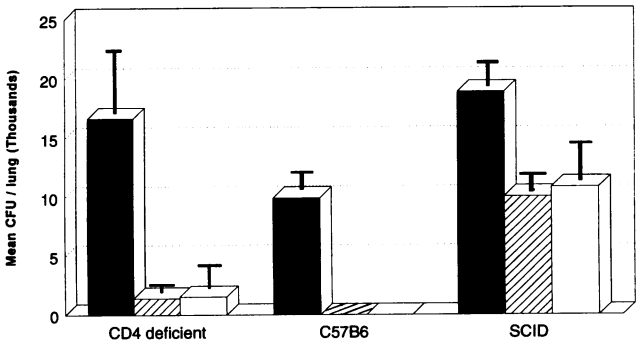

FIG. 3. Pulmonary clearance of virulent *R. equi* 33701 in $CD4^+$ T-cell-deficient mice, immunocompetent C57BL/6 mice, and SCID mice. Results are mean CFU (± standard deviations) for individual lungs from five mice in each group at each time point. ■, 3 days; ▨, 21 days; □, 28 days.

cyte-deficient mice was demonstrated by the lack of further reduction of bacterial numbers in the lungs by 28 days postinfection (Fig. 3).

The results of this study indicate that both $CD4^+$ T lymphocytes and $CD8^+$ T lymphocytes contribute early to the control of pulmonary *R. equi* infection. Similarly, in a previous study, partial in vivo depletion of $CD4^+$ or $CD8^+$ T lymphocytes decreased the ability of BALB/c mice to control bacterial numbers in the liver and spleen at 4 and 11 days following an intravenous challenge (10). Our work indicates that in the absence of $CD8^+$ T cells, mice remain capable of completely clearing bacteria from their lungs by 21 days. Therefore, despite their effects on bacterial numbers during acute infection, $CD8^+$ T lymphocytes are not required for pulmonary clearance.

In contrast, the failure of $CD4^+$ T-lymphocyte-deficient mice to clear *R. equi* infection indicated that class II-restricted $CD4^+$ T lymphocytes are required for pulmonary clearance. $CD4^+$ T-lymphocyte-deficient mice were unable to resolve infection for 28 days. Failure of $CD4^+$ T-lymphocyte-deficient mice to clear *R. equi* infection has important implications regarding therapy. Human patients with AIDS have low numbers of $CD4^+$ T lymphocytes. In these patients and in nude mice, antibiotics can reduce bacterial numbers but not clear *R. equi* from the lungs. Restoration of specific $CD4^+$ T-cell-derived mediators in combination with antibiotic therapy may facilitate clearance.

The ability of $CD4^+$ T-lymphocyte-deficient mice to reduce bacterial numbers compared with that of SCID controls indicates that other cell types are involved in immune protection but are insufficient to mediate clearance at days 21 and 28 postinfection. Transgenic $CD4^+$ T-lymphocyte-deficient mice may have upregulated $CD8^+$ T-lymphocyte activity relative to controls, or other cell types, such as $CD4^-$, $CD8^-$ γ/δ T cells, may be involved in reduction of bacterial numbers. In *Listeria monocytogenes* infection of mice, γ/δ T cells are important for containment of bacterial numbers at the early stage of infection but are not required for clearance (7). Although enhanced γ/δ or $CD8^+$ responses may also help to contain *R. equi* infection in mice, these compensatory mechanisms are not sufficient to mediate clearance in the absence of $CD4^+$ T cells (13).

The ability of all strains of mice, including SCID mice, to clear the avirulent strain of *R. equi* effectively by 21 days indicates that bacterial virulence factors are involved in the pathogenesis of *R. equi* pneumonia. These factors are encoded, at least in part, by the 82-kb plasmid, whose presence correlates closely with in vivo virulence for mice (17). Because the key event in the pathogenesis of *R. equi* infection is persistence within macrophages, it is likely that the plasmid encodes proteins that mediate intracellular survival.

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