Deletion of RD1 from *Mycobacterium tuberculosis* Mimics Bacille Calmette-Guérin Attenuation

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The tuberculosis (TB) vaccine bacille Calmette-Guérin (BCG) is a live attenuated organism, but the mutation responsible for its attenuation has never been defined. Recent genetic studies identified a single DNA region of difference, RD1, which is absent in all BCG strains and present in all *Mycobacterium tuberculosis* (MTB) strains. The 9 open-reading frames predicted within this 9.5-kb region are of unknown function, although they include the TB-specific immunodominant antigens ESAT-6 and CFP-10. In this study, RD1 was deleted from MTB strain H37Rv, and virulence of H37Rv:ΔRD1 was assessed after infections of the human macrophage–like cell line THP-1, human peripheral blood monocyte–derived macrophages, and C57BL/6 mice. In each of these systems, the H37Rv:ΔRD1 strain was strikingly less virulent than MTB and was very similar to BCG controls. Therefore, it was concluded that genes within or controlled by RD1 are essential for MTB virulence and that loss of RD1 was important in BCG attenuation.

Bacille Calmette-Guérin (BCG), the world's tuberculosis (TB) vaccine, has been controversial since its first use in 1921 [1]. Since then, BCG has been administered to >3 billion people [2], with an excellent safety record [3]. Currently, an estimated 118 million doses are delivered each year [2]. However, in controlled clinical trials, the efficacy of BCG for preventing pulmonary TB has ranged widely, with some studies reporting no efficacy at all [4]. Despite high vaccination rates in much of the world, the ongoing death toll of TB remains ~2 million deaths per year [5]. BCG is a live attenuated derivative of virulent *Mycobacterium bovis,* a close relative of *M. tuberculosis* (MTB). BCG was the result of more than a decade of continuous passage in vitro, but the genetic event responsible for its loss of virulence has never been defined. Understanding the molecular basis of BCG attenuation not only will provide insight into mechanisms of MTB pathogenesis but also will provide the impetus for much needed improvements in the TB vaccine.

Recent work has identified several regions of the MTB chromosome that are missing from various BCG substrains [6–9]. Of these, only 1 region of difference, RD1, is missing from every BCG strain analyzed to date but is present in every MTB [10, 11]. RD1 is the only known BCG mutation that may have occurred before clinical use of BCG in 1921 [8]. By annotation [12], the ~9.5-kb RD1 deletion completely removes 7 genes *(Rv3872–Rv3878)* and truncates 2 others *(Rv3871* and *Rv3879c)*. None of the affected genes has any known function, although 2 encode the MTB-specific secreted

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antigens ESAT-6 and CFP-10. In the present study, we assessed the role of the RD1 region in virulence of MTB and attenuation of BCG.

MATERIALS AND METHODS

Bacteria. H37Rv (ATCC 27294), H37Rv:ΔRD1, and *M. bovis* BCG-Russia (ATCC 35740) were grown to mid-log phase in 7H9 media with 0.05% Tween 80 and albumin, dextrose, catalase (ADC) supplement (Becton Dickinson) and were stored as 1-mL aliquots in 15% glycerol (final concentration) at −80°C. For individual experiments, bacilli were grown in roller bottles in Middlebrook 7H9 medium (Becton Dickinson) with 0.05% Tween 80 and ADC supplement or on Middlebrook 7H10 plates at 37°C, as described elsewhere [13]. When needed, 30 μg/mL kanamycin (50 μg/mL for *Escherichia coli*) and 50 μg/mL hygromycin (200 μg/mL for *E. coli*) were used. Routine DNA manipulations were performed in *E. coli* DH5α (Invitrogen).

Deletion of RD1. To delete the RD1 region, genomic regions (~800 bp each) flanking the RD1 region were amplified by polymerase chain reaction (PCR) and were cloned into the knockout plasmid pKO, to flank the *kanR* determinant. Electroporation into mycobacteria, initial selection on 7H10 plates with hygromycin, and secondary selection on 7H10 plates containing 10% sucrose were performed as described elsewhere [8, 14]. In the first screening step, each colony was tested by PCR with 2 primer pairs: one specific for integration upstream of the gene of interest and the other specific for integration downstream. Colonies determined to be positive at either end by means of PCR were grown to an OD_{600} of ~1.0 and were plated onto 7H10 plates containing 10% sucrose. Bacilli that grow on sucrose generally either have mutated copies of *sacB* or have lost the integrated plasmid. A portion of those in which the plasmid was lost also will lose the gene of interest. Colonies appearing on sucrose plates were placed into medium and were patched separately onto 7H10 plates with kanamycin and hygromycin. Sucrose-resistant, hygromycin-sensitive, and kanamycin-resistant colonies (which indicates loss of the integrated plasmid) were screened by use of PCR for loss of the gene of interest, and the deletion was confirmed by means of Southern blot.

Preparation of macrophages. To prepare the human macrophage–derived THP-1 cell line for infection, cells were pelleted by centrifugation, resuspended in RPMI 1640 medium plus 10% fetal calf serum (FCS) with 100 n*M* phorbol 12-myristate 13-acetate (PMA; Sigma), and delivered (5×10^5 cells in 0.5 mL) into 24-well tissue culture plates. Cells were incubated at 37°C with 5% CO_2 for 48 h. PMA-containing medium then was removed from the wells, cells were washed with warm RPMI 1640 medium, given fresh RPMI 1640 medium plus FCS, and reincubated for 24 h before infection. Peripheral blood mononuclear cells (PBMC) were isolated from healthy volunteers by centrifugation of fresh whole blood diluted 2:1 with RPMI 1640 medium on Histopaque-1077 (Sigma) for 30 min at 600 *g* at 18°C. PBMC were washed 3 times with cold RPMI 1640 medium, resuspended in RPMI 1640 medium with 10% human serum at 6×10^6 cells/mL, and plated at 0.5 mL volume into 24-well tissue culture plates. After 2 h of incubation at 37°C in 5% CO_2, nonadherent cells were removed with 3 washes of warm RPMI 1640 medium, and remaining cells were reincubated with RPMI 1640 medium plus 10% human serum for 4–6 days before infection. Adherent cells were counted in 3 wells to determine the MOI.

Macrophage infections. For each infection, bacteria stocks were grown to mid-log phase in 7H9 media, diluted in warm RPMI 1640 medium plus 10% FCS (THP-1) or human serum (PBMC), and added to each well containing ~3×10^5 cells at an MOI of 1. Cells were incubated at 37°C in 5% CO_2. After 4 h, extracellular bacteria were removed by 3 washes with warm RPMI, and cells received fresh media (RPMI 1640 medium plus 10% serum). At each time point, triplicate wells for each bacterial strain were processed. Supernatants were removed, adherent cells were lysed with 1% Triton X-100, and bacterial numbers were determined for each by plating for colony-forming units and/or by means of luciferase assay, which was performed as described elsewhere [14, 15]. Relative light units (rlu) from luciferase assays were converted to the number of bacilli by applying a strain-specific conversion factor derived from several plating experiments: H37Rv, 0.000283 rlu/bacillus; H37Rv:ΔRD1, 0.000442 rlu/bacillus; and BCG-Russia, 0.000206 rlu/bacillus. Macrophage metabolism was measured by metabolism of an oxidation-reduction dye (AlamarBlue; Biosource International), as an indication of cytotoxicity. AlamarBlue reagent at 1× in RPMI was added to wells, and, after 6 h incubation, results were measured at OD_{600} and OD_{570}. Percentage of reduction of AlamarBlue was calculated according to the manufacturer's instructions.

Aerosol infection of mice. Aerosol infections of mice were performed as described elsewhere [16]. C57BL/6 mice, aged 6–8 weeks, were obtained from Jackson Laboratories and were maintained in a biosafety–level 3 animal facility. For each infection, frozen bacteria stocks were thawed, sonicated and diluted to ~10^6 bacteria/mL, and nebulized in an aerosol infection chamber (Salter Labs) containing the mice. Infectious dose was determined by plating whole lung homogenates on day 1. At each time point, 5 mice/group were killed by cervical dislocation. Spleen and left lung were homogenized in PBS/0.05% Nonidet P40 and were plated into serial dilutions on 7H10 agar. Colonies were counted after 2–3 weeks of incubation at 37°C. In addition, the right lung was removed, inflated, fixed in 10% buffered formalin, embedded in paraffin, sectioned, and stained with hemotoxylin-eosin for evaluation of histopathology. For survival experiments, mice were infected as

above and were monitored 2–3 times per week for signs of advancing disease. To prevent needless suffering, mice were killed when death was imminent, as predicted by any of the following: weight loss of >20%, trembling, extreme lethargy, or labored breathing.

RESULTS

Targeted replacement of the RD1 region and initial strain characterization. To test the role of the RD1 region in MTB virulence and BCG attenuation, we generated precise deletions of RD1 from the MTB genome. We used homologous recombination and sucrose counterselection to replace the RD1 region of MTB strain H37Rv with the *aph* gene, which confers resistance to kanamycin. Gene replacement was confirmed by use of both PCR across the DNA junctions and Southern blot (data not shown). Two independent isolates of H37Rv:ΔRD1 generated at different times were chosen for further analysis. H37Rv:ΔRD1 clones were compared with virulent parent strain H37Rv and with attenuated BCG-Russia (ATCC 35740), which was chosen because it is potentially more similar to the original BCG than other extant BCG strains [8]. During 3 weeks in 7H9 broth in vitro, growth and survival of the 3 strains were indistinguishable (data not shown).

Analysis of infected macrophages in culture. The macrophage is the primary cell type to interact directly with MTB in vivo [17], and growth in cultured macrophages has been used to assess virulence of different mycobacterial strains [18, 19]. As an initial test of the role of the RD1 region in virulence, the ability of H37Rv, H37Rv:ΔRD1, and BCG-Russia to infect and grow within the human macrophage–like THP-1 cell line was assessed (figure 1*A*). Consistent with published data [15], H37Rv infected and multiplied well within THP-1 cells, reaching >10 times their original number in 4 days. Reduced replication of H37Rv at later time points correlated with damage to the infected macrophages. By day 7, the H37Rv-infected THP-1 cell monolayer was destroyed >80%, as judged by both microscopy (data not shown) and reduction of an indicator dye (figure 1*B*). In comparison, BCG-Russia failed to grow inside THP-1 cells over the course of these experiments, and, after 7 days, the BCG-Russia–infected monolayer was intact >90% (figure 1*A* and 1*B*). The growth of H37Rv:ΔRD1 was intermediate, increasing 3-fold in 7 days, with essentially no damage to the THP-1 monolayer (figure 1*A* and 1*B*). We also infected PBMC from human donors (figure 1*C*). Again, H37Rv grew well, BCG-Russia grew minimally, and H37Rv:ΔRD1's growth was strikingly similar to BCG-Russia.

Analysis of murine infections. To characterize further the effect of the RD1 region on growth and virulence of MTB, we introduced H37Rv:ΔRD1 and control strains into the lungs of C57BL/6 mice. These mice are relatively resistant to infection

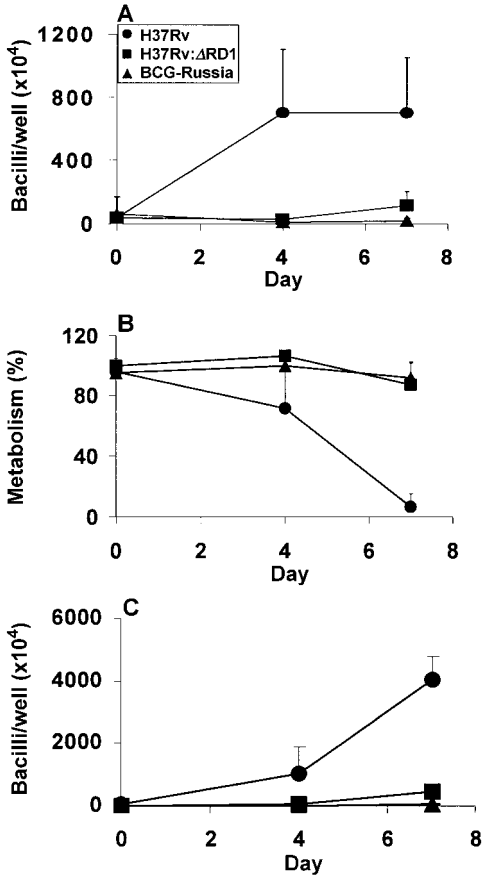

Figure 1. Bacterial growth and cytotoxicity in a human macrophage THP-1 cell line *(A* and *B)* and growth in human peripheral blood mononuclear cell (PBMC)–derived macrophages *(C)* after infection by H37Rv (●), H37Rv:ΔRD1 (■), or BCG-Russia (▲). Data for each time point are the mean and SD of 4 infections in panels *A* and *B* and of a representative infection (of 4) in panel *C*. Bacteria were quantitated by luciferase assay *(A)* or by plating for colony-forming units *(C)*. Cytotoxicity is indicated by the decline in macrophage metabolism over the course of infection *(B)*.

by MTB, although they carry the *Nramp1* allele that confers heightened susceptibility to BCG [20]. Aerosol infection was used because it mimics the most common transmission mode for human TB and because MTB is more virulent when delivered by this route [21]. In addition, we were able to evaluate each strain's ability to disseminate after aerosol inoculation of the lungs. Hematogenous seeding of the lung and the spleen is considered to be a critical step in the pathogenesis of human TB [22] and has been used to assess the relative virulence of different mycobacterial strains [23]. After infection, H37Rv numbers increased rapidly in the lungs, reaching peak levels by week 4 after infection, before decreasing to a plateau of $\sim 1 \times 10^6$ bacilli per lung (figure 2*A* and 2*B*). Dissemination to the spleen and subsequent growth also were rapid, with bacilli numbers reaching a steady level by 2–4 weeks (figure 2*C* and 2*D*). This pattern of rapid bacterial replication, which was fol-

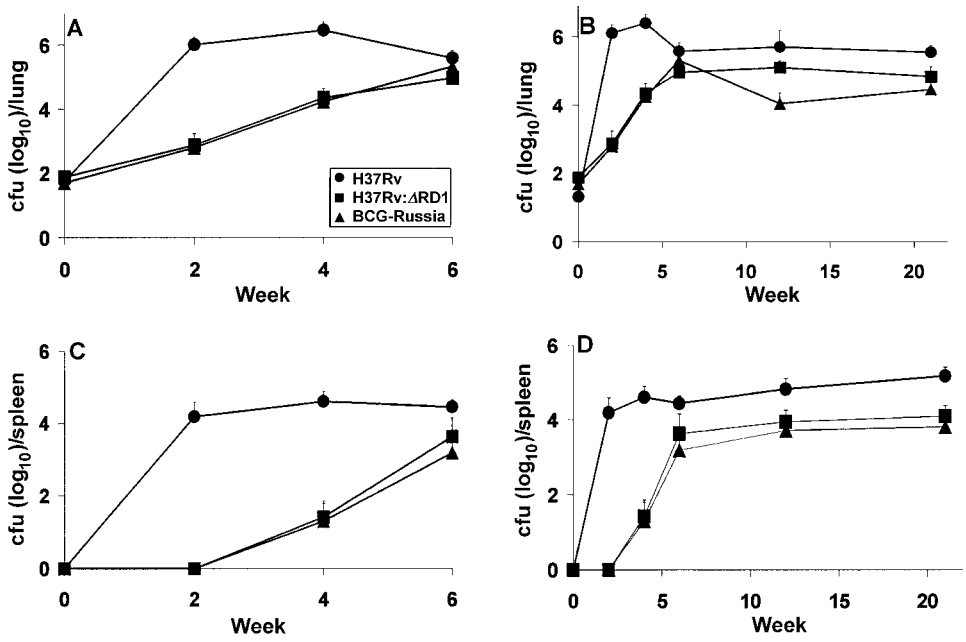

Figure 2. Bacterial growth in lungs *(A* and *B)* and spleens *(C* and *D)* after low-dose aerosol infection of 6–8-week-old C57BL/6 mice with wild-type H37Rv (●), H37Rv:ΔRD1 (■), or BCG-Russia (▲). *A* and *C,* Early-phase infection and dissemination. *B* and *D,* Progression of infection through 21 weeks. Data at each time point are the mean and SD of 15 mice per strain from 3 separate infections.

lowed by containment at the time that adaptive immunity normally begins to manifest, is typical of aerosol infection of TB in the mouse [24]. In striking contrast, infection with H37Rv:ΔRD1 or BCG-Russia bacilli resulted in slow growth in the lungs (figure 2*A* and 2*B*) and delayed dissemination to the spleen (figure 2*C* and 2*D*). By week 2 after infection, levels of H37Rv:ΔRD1 and BCG-Russia were ~4 log lower than that of H37Rv in each organ. The containment phase of the infection also was altered. H37Rv:ΔRD1 and BCG-Russia numbers increased steadily for 6 weeks, a full month beyond the point when replication of H37Rv had slowed. By week 6 after infection, levels of H37Rv:ΔRD1 and BCG-Russia were only ~10-fold lower than levels of H37Rv. This pattern was maintained throughout the 21-week experiment.

Although the bacterial burden in vivo was ultimately similar with all 3 strains, marked differences in lung histopathology were revealed. By week 6 after infection, lungs of mice infected with H37Rv showed widespread inflammation involving ~50% of the lung tissue, with organized multifocal granulomas (figure 3*A* and 3*D*). In contrast, lungs from H37Rv:ΔRD1 or BCG-Russia–infected mice were predominantly normal with only early perivascular lymphocytic infiltration and no granuloma formation (figure 3*B,* 3*C,* 3*E,* and 3*F*). Lungs from mice infected with H37Rv:ΔRD1 or BCG-Russia were indistinguishable from each other. By week 21 after infection, lungs from H37Rv-infected mice were characterized by diffuse inflammation that involved >75% of the lung tissue (figure 3*G*). In contrast, although numbers of H37Rv:ΔRD1 and BCG-Russia continued to increase in lungs for several weeks beyond when growth of H37Rv was controlled, lung pathology was not simply delayed. Damage in H37Rv:ΔRD1- and BCG-Russia–infected lungs remained extremely mild throughout the 21 weeks, with only modest perivascular collections of relatively unorganized lymphocytes and macrophages that were slightly larger than those appearing at 6 weeks after infection but still involved <10% of lung tissue (figure 3*H* and 3*I*). Inflammation remained perivascular and never developed into parenchymal or granulomatous disease, as was seen by week 6 after infection with H37Rv.

One of the clearest laboratory tests of mycobacterial virulence is to assess survival of infected animals [25]. Accordingly, mice were infected by aerosol with high doses of H37Rv or H37Rv:ΔRD1 and were monitored for signs of advancing TB. Mice were killed when any of the following criteria were met: >20% weight loss, trembling, extreme lethargy, or labored breathing. Mice infected with H37Rv bacilli began to fall gravely ill by week 19 after infection (figure 4). By week 39 after infection, only 2 of the 15 mice infected with H37Rv were still alive. In contrast, the 18 mice infected with H37Rv:ΔRD1 were still alive and appeared to be healthy when the experiment was terminated at 39 weeks. Again, the phenotype of H37Rv:ΔRD1 was strikingly different from its virulent parent and was indistinguishable from BCG.

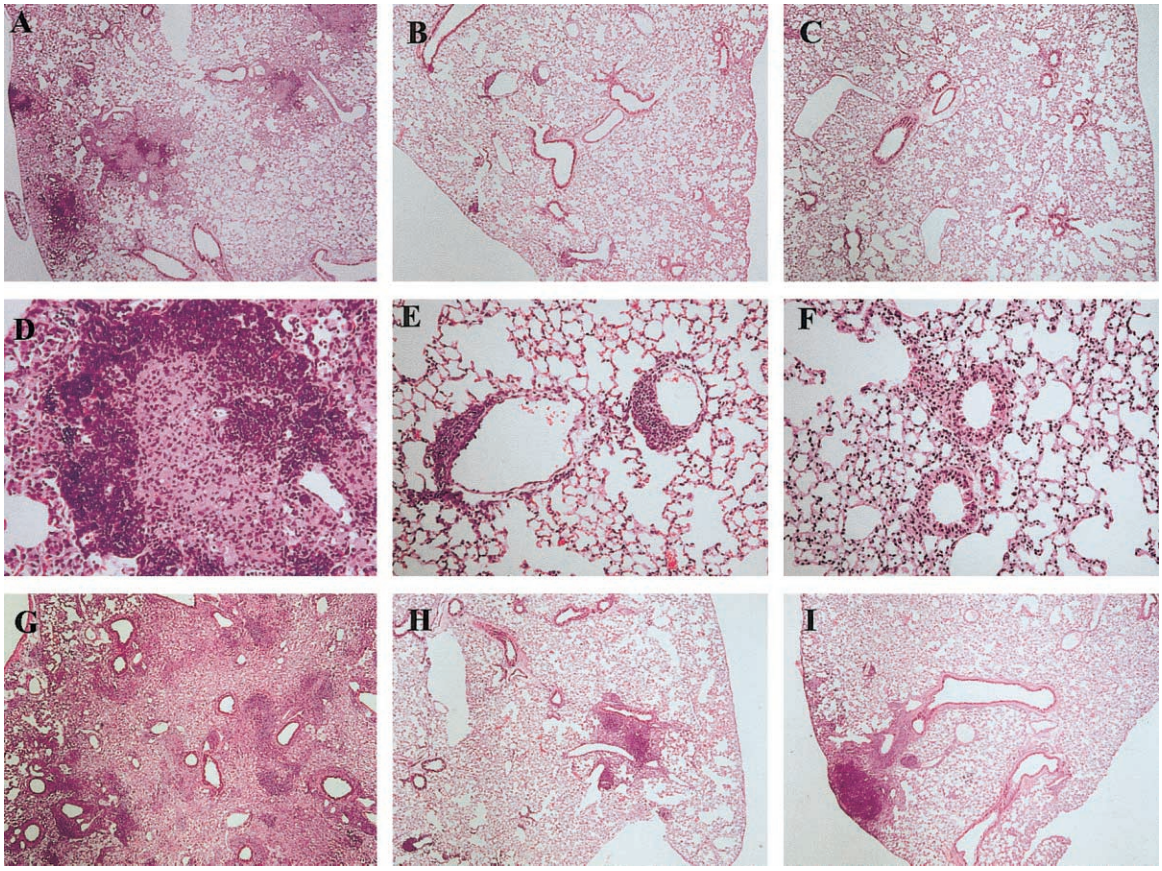

Figure 3. Lung histopathology after low-dose aerosol infection by H37Rv *(A, D,* and *G),* H37Rv:ΔRD1 *(B, E,* and *H),* or BCG-Russia *(C, F,* and *I).* At each time point, the left lung was removed, inflated, fixed in 10% buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin-eosin. Photographs shown are representative of at least 15 mice/strain. *A, B,* and *C,* Differences in overall lung involvement (original magnification, ×10) at week 6 after infection. *D, E,* and *F,* Differences in granuloma formation (original magnification, ×50; at week 6 after infection). *D,* Well-organized granuloma with tight rim of lymphocytes surrounding central core of histiocytes in a mouse infected with wild-type MTB. *E* and *F,* Areas of greatest inflammation in the H37:ΔRD1-infected *(E)* or BCG-Russia–infected *(F)* lung, consisting only of mild perivascular lymphocyte cuffs. *G, H,* and *I,* Differences in lung inflammation at week 21 after infection (original magnification, ×10).

DISCUSSION

The nature of the mutation underlying attenuation of BCG has been a mystery since Calmette's first observations of his attenuated strain [26]. During the last decade, associations have been made regarding the origin of BCG, mycobacterial virulence, and the RD1 region or gene(s) within it [6, 8, 27]. Our data demonstrate that loss of RD1 affects MTB growth and survival in cultured macrophages and greatly alters mycobacterial growth and dissemination, as well as the resultant histopathology and survival of the mouse. In every analysis, the phenotype of H37Rv:ΔRD1 was distinctly different from that of virulent H37Rv and remarkably similar to that of BCG. These studies indicate that sequences within or controlled by the RD1 region are needed for MTB virulence and argue for the hypothesis that loss of RD1 was an important event in the creation of BCG.

The role of RD1 in virulence of MTB and attenuation of BCG is substantiated by the fact that completely independent H37Rv:ΔRD1 strains yielded the same results in cultured macrophage and mouse infection assays (data not shown). We also have deleted the RD1 region from MTB strain Erdman (ATCC 35801). In cultured macrophages, this strain is as attenuated as H37Rv:ΔRD1 (data not shown). However, thus far we have been unable to complement deletion of RD1 in the traditional fashion. For reasons that are unclear, the genes in the RD1 region expressed only very poorly after we restored the entire region into the phage L5 attachment site [28] of H37Rv:ΔRD1 (data not shown). Complementation in *trans* of a 9-gene deletion with a 12-kb DNA fragment is not straightforward. Efforts to reinstall the RD1 DNA in situ and to complement the ΔRD1 phenotypes with smaller portions of the RD1 region are underway.

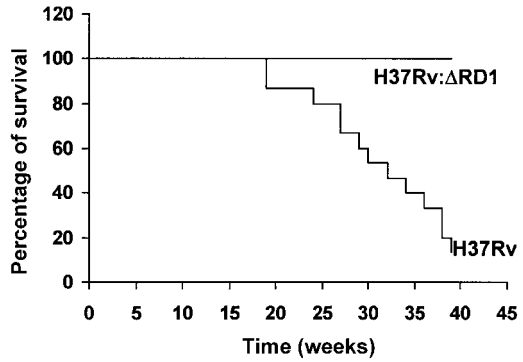

Figure 4. Survival of C57BL/6 mice after high-dose aerosol infection with H37Rv or H37Rv:ΔRD1. Results are from 15 mice infected with an average dose of 316 H37Rv bacilli and from 18 mice infected with an average of 730 H37Rv:ΔRD1 bacilli.

Although clearly attenuated, BCG and H37Rv:ΔRD1 were able to both grow and persist in C57BL/6 mice. Although these mice are relatively resistant to MTB, they carry the *Nramp1* allele, which confers sensitivity to BCG [20]. Previously, BCG has been shown to grow in an *Nramp*-susceptible mouse strain after aerosol infection [29]. Furthermore, although the BCG-Russia strain that we used may be a more aggressive BCG [30–32], we saw essentially identical growth in vivo after aerosol infection with BCG Pasteur (ATCC 35734; data not shown). As noted elsewhere [33], growth and persistence in vivo and the ability to induce pathology are separate manifestations of mycobacterial virulence that can be dissociated under the proper conditions.

The mechanism by which the RD1 region exerts its effects remains to be elucidated. At present, our data highlight 2 broad areas for further study. First, impaired replication in cultured macrophages and in the first weeks after aerosol challenge of mice implies that H37Rv:ΔRD1 and BCG are defective in some aspect of metabolism or response to innate host defenses that is revealed by these environments. At the same time, continued replication of these strains in mice beyond the time when H37Rv is contained and the lack of histopathology in vivo suggest that H37Rv:ΔRD1 and BCG elicit a very different adaptive immune response than virulent MTB. Perhaps expression of immunogens encoded by RD1 [34–37] at early times in vivo is necessary for induction of a powerful adaptive immune response.

It has been noted recently that ESAT-6 and CFP-10 form a tight 1:1 complex [38] and that homologues are widespread among gram-positive bacteria [39, 40]. It was suggested that these proteins may contribute to a novel bacterial secretion system, with the energy for protein export supplied by a conserved, membrane-bound ATPase encoded nearby (*Rv3870* and *Rv3871* in the RD1 region). This idea is attractive for 2 reasons. First, it provides a mechanism by which ESAT-6 and CFP-10, which lack signal sequences, may be exported from bacilli. Second, various secretion systems are essential for virulence of many other bacterial pathogens [41]. Among other options, we are currently testing the hypothesis that RD1 deletion has interrupted a novel secretion system that is necessary for MTB virulence.

Regardless of its mechanism of action, analysis of RD1-deficient MTB could contribute to development of a better TB vaccine. The ability to multiply and persist in vivo may be necessary for a vaccine to stimulate lasting protective immunity against MTB [32, 42, 43]. Because it derives from MTB and not *M. bovis,* H37Rv:ΔRD1 should share more antigenic determinants with MTB, may persist in vivo longer than BCG, and may provoke a more protective immune response. Thus, continued focus on the RD1 region should provide novel insight into mechanisms of MTB pathogenesis, help elucidate the nature of a productive immune response, and may help stimulate much needed improvements in the TB vaccine.

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