Enhanced Protection against Tuberculosis by Vaccination with Recombinant Mycobacterium microti Vaccine That Induces T Cell Immunity against Region of Difference 1 Antigens

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Mycobacterium microti, the vole bacillus, which was used as a live vaccine against tuberculosis until the 1970s, confers the same protection in humans as does Mycobacterium bovis bacille Calmette-Guérin (BCG). However, because the efficacy of the BCG vaccine varies considerably, we have tried to develop a better vaccine by reintroducing into M. microti the complete region of difference 1 (RD1), which is required for secretion of the potent T cell antigens early secreted antigen target (ESAT)–6 and culture filtrate protein (CFP)–10. The resultant recombinant strain, M. microti OV254::RD1-2F9, induced specific ESAT-6 and CFP-10 immune responses in mice with CD8+ T lymphocytes that had strong expression of the CD44hi activation marker. This vaccine also displayed better efficacy against disseminated disease in the mouse and the guinea pig models of tuberculosis than was seen in animals vaccinated with M. microti alone or with BCG. The M. microti OV254::RD1-2F9 vaccine was less virulent and persistent in mice and than was BCG::RD1-2F9 may represent a safer alternative to BCG::RD1-2F9.

Mycobacterium tuberculosis is now considered to be responsible for more adult deaths than any other pathogen. Better measures to prevent tuberculosis are urgently needed. Even if antibiotic treatments are very efficient, long-term administration is required, and drug resistance can arise when drugs are not taken appropriately. Two live attenuated vaccines confer some protection against tuberculosis: Mycobacterium bovis bacille Calmette-Guérin (BCG) and Mycobacterium microti. The most widely used vaccine is BCG, which was attenuated after passages of M. bovis in the 1920s [1]. M. microti was initially isolated in the 1930s from voles (Microtus agrestis), in which it causes a tuberculosis-like disease, but it is harmless to humans and most other mammals [2–4]. In the British Medical Research Council trial in the 1950s, which involved ∼10,000 adolescents, the vole bacillus proved to be safe and, in addition, was found to protect with the same efficacy as BCG, averaging 77% over a 20-year follow-up period [5]. At the same time, between 1950 and 1969, the vole bacillus substrate OV166 was given to ∼500,000 newborns in the Czech Republic without causing any complications and induced the same immunogenicity against tuberculosis as, but fewer allergic reactions than, BCG [6].

For still unknown reasons, BCG exhibits a protective efficacy of 0%–80% against adult pulmonary disease [7]. One hypothesis for its relative inefficacy is that the vaccine is eliminated from the host too rapidly, which, in turn, prevents the generation of long-term immune
memory. Another hypothesis is that BCG and *M. microti* lack important *M. tuberculosis* antigens. Recently, comparative genomic studies identified some antigens that are missing from both these vaccines [8, 9] and that are encoded by genes localized in region of difference (RD) 1, RD3, RD5, RD8, RD9, and RD10 of the *M. tuberculosis* genome. Of particular interest, the RD1 is missing from all strains that have been used on a large scale in the history of prevention of human tuberculosis [8, 10]. The deletion of the RD1 locus is not identical in *M. microti* or BCG, but, in both cases, the genes for 2 strong T cell antigens, early secreted antigen target (ESAT)-6 and culture filtrate protein (CFP)-10, have been removed [9]. Restoring the RD1 to BCG Pasteur (BCG::RD1-2F9) led to stronger immunogenicity and enhanced vaccine efficacy [11]. Along with these potent antituberculosis activities, this BCG knock-in strain also exhibited an increase in virulence in immunodeficient mice, which raised concern about its future use as a vaccine [12]. In addition, BCG::RD1-2F9 was found to be more persistent in immunocompetent hosts. Although virulence is determined by bacterial proliferation and the number of lesions, persistence is monitored by the ability to recover bacteria from the target organs (i.e., the spleen and lungs) at much later time points. To circumvent this problem, we investigated the expression of RD1 antigens in *M. microti*, a mycobacterium that grows more slowly than does BCG.

Here, we reintroduced the whole RD1 into *M. microti* strain OV254 to yield strain OV254::RD1-2F9 and compared its efficacy with that of BCG::RD1-2F9 by measuring levels of virulence, immunogenicity, and protection against *M. tuberculosis*. Virulence of the *M. microti* recombinant vaccine was first compared with that of *M. microti* OV254 and BCG::RD1-2F9. We then studied general T cell activation induced in vivo by OV254::RD1-2F9, relative to its parental strain, and investigated specific T cell responses to RD1 immunogens after vaccination. Subsequently, we performed protection studies in 2 different animal models. These results showed that *M. microti* OV254 is an effective delivery system for *M. tuberculosis* antigens and generates protective immune responses.

**MATERIALS AND METHODS**

**Genetic constructs and mycobacterial strains.** Electrocompetent cells for OV254::RD1-2F9 were prepared from 400 mL of a 20-day-old Middlebrook 7H9 culture (Difco) supplemented with albumin-dextrose-catalase (ADC; Difco), 2 mg/mL (-) sodium pyruvate, and 0.05% Tween 80. Bacilli were harvested by centrifugation at 3000 × g for 20 min, washed twice with H2O at room temperature, and resuspended in 1–2 mL of 10% glycerol at room temperature after recentrifugation. Two hundred fifty microliters of bacilli was mixed with cosmid clone RD1-2F9 or the vector control pYUB412, as described elsewhere [12], and was electroporated by use of a Biorad Gene Pulser (Biorad). After electroporation, bacilli were resuspended in medium and left for 1 day at 37°C. Transformants were selected on Middlebrook 7H11 medium (Difco) supplemented with oleic acid–ADC (Difco) and 50 µg/mL streptomycin (Invitrogen). Hygromycin-resistant colonies appearing after 3 weeks were analyzed for the presence of the integrated vector by use of polymerase chain reaction using primers specific for RD1-encoded genes, as described elsewhere [9, 11].

**Survival and virulence studies.** Fifty-milliliter cultures of the individual mycobacterial strains were grown in parallel in Middlebrook 7H9-ADC medium supplemented with 0.05% Tween 80 and 50 µg/mL hygromycin. Bacteria were harvested, washed, and resuspended in 50 mmol/L sodium phosphate buffer (pH 7.0). The bacteria were then sonicated briefly and allowed to stand for 1 h, to allow residual aggregates to settle. The bacterial suspensions were then aliquoted and frozen at −80°C. A single defrosted aliquot was used to quantify the colony-forming units before vaccination. Female CB-17/Icr severe combined immunodeficient (SCID) mice (8–10 weeks old) were initially obtained from C. M. Hetherington (National Institute for Medical Research Mill Hill, London, England) and were bred as described elsewhere [13] before intravenous (iv) infection with 10^6 cfu. Six-week-old female BALB/c or C57BL/6 mice (Charles River) were vaccinated iv via the lateral tail vein (10^6 cfu) or via the aerosol route. Aerosol challenge was performed as described elsewhere [11], with a suspension containing 5 × 10^8 organisms/mL, to obtain an inhaled dose of 100 ± 10 cfu in lungs. Organs from killed mice were homogenized by use of an MM300 apparatus (Qiagen) and 2.5-mm-mm diameter glass beads. Serial 5-fold dilutions in medium were plated on 7H11 agar with 50 µg/mL hygromycin when appropriate, and colony-forming unit counts were ascertained at 37°C, after 4 weeks of growth for *M. microti* recombinant strains and after 3 weeks of growth for *M. bovis* BCG recombinant strains.

Animal studies were approved by the Institut Pasteur Safety Committee in accordance with French and European guidelines.

**Immunological analyses.** Adult C57BL/6 (H-2b) mice were vaccinated subcutaneously (sc) with 10^6 cfu of recombinant *M. microti* vaccines prepared as described for virulence studies. Three weeks after vaccination, splenocytes were cultured (1 × 10^6 cells/well) in 96-well, flat-bottom plates, in synthetic H-1 medium (BioWhittaker) supplemented with 2 mmol/L L-glutamine, 100 IU/mL penicillin, 100 µg/mL streptomycin, and 50 µmol/L β-mercaptoethanol, in the presence of various concentrations of appropriate antigens. After 3 days of incubation at 37°C with 5% CO2, cultures were pulsed with 1 µCi [methyl-3H]-thymidine (ICN) for 16 h. Cells were then harvested, and the amount of [methyl-3H]-thymidine incorporated was counted by use of an LKB β-plate counter. The phenotype of proliferating T cells was determined by adding to the cultures 1 µg/mL anti-CD4 (GK1.5) or anti-CD8 (H35-17-2) mono-
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clonal antibodies (MAbs). Production of interferon (IFN)-γ was assessed after in vitro stimulation of splenocytes with 10 μg/mL concanavalin A (Sigma); purified protein derivative (Serum Institute); recombinant ESAT-6, CFP-10, or MalE; or synthetic peptides from ESAT-6 (1–20) [14], antigen 85A (241–260) [15], or MalE (100–114) [16]. After 3 days of incubation, amounts of IFN-γ were quantified in culture supernatants by use of an ELISA with a detection limit of 500 pg/mL, using R4-6A2 and biotin-conjugated XMG1.2 MAb (BD PharMingen), and then were detected with horseradish peroxidase–coupled streptavidin followed by o-phenylenediamine as substrate (Invitrogen).

For fluorescence-activated cell sorter (FACS) analysis, splenocytes from 3 individual mice were stained with phycoerythrin-conjugated anti-CD4 (RM4-5) or anti-CD8 (53–6.7) MABs and fluorescein isothiocyanate–conjugated MABs specific to CD25 (7D4), CD69 (HL2F), or CD44 (IM7) (all obtained from BD PharMingen). Cells were then fixed in 2% paraformaldehyde for 16 h at 4°C and were analyzed, after setting gates on forward versus side light scatter, in a FACScan flow cytometer using CellQuest software (both from Becton Dickinson).

**Protection studies in mice.** M. tuberculosis H37Rv–titrated aliquots were obtained from a 20-day-old culture in Dubos medium supplemented with ADC and 0.05% Tween 80 and were prepared as described for the other mycobacterial strains.

Six-week-old female C57BL/6 mice were vaccinated either sc (105 cfu) or iv (106 cfu) via the lateral tail vein. Where appropriate, 2 months after vaccination, mice were treated for 3 weeks with antibiotics by adding isoniazid (20 μg/mL; Sigma) and rifampicin (10 μg/mL; Euromedex) to their drinking water. Mice were then either challenged by the aerosol route (inhaled dose, 106 cfu) or iv (106 cfu). Colony-forming unit counts were ascertained after 2 weeks growth at 37°C, as described above.

**Protection studies in guinea pigs.** Groups of 6 female Dunkin-Hartley guinea pigs weighing ~250 g were vaccinated sc in the nape with 105–106 cfu of recombinant OV254::RD1-2F9 or M. microti or with saline alone. Aerosol challenge was performed 10 weeks after vaccination by use of a contained Henderson apparatus, as described elsewhere [17, 18]. Fine-particle aerosols of M. tuberculosis, with a mean diameter of 2 μm, were generated in a Collison nebulizer and delivered directly to the guinea pig’s snout. The challenge strain M. tuberculosis H37Rv (NCTC 7416) suspension contained 75 organisms/mL, to obtain an estimated retained, inhaled dose of ~500 cfu/lung [19].

**RESULTS**

**Comparative virulence of OV254::RD1-2F9 with BCG::RD1-2F9 in mice.** We recently described the stable complemen-
tation of BCG and *M. microti* OV254 with the complete RD1 by an integrative shuttle cosmid [12]. These 2 strains express RD1-encoded antigens and, notably, secrete large amounts of the immunodominant ESAT-6 and CFP-10 antigens in their culture supernatants [11]. First, we investigated the virulence of OV254::RD1-2F9, relative to that of *M. microti* OV254 complemented with the vector alone, OV254::pYUB412, and to the analogous construct in BCG, BCG::RD1-2F9. When injected into SCID mice, which lack B and T lymphocytes, median survival time was reduced by 100 days by the 2 RD1-expressing constructs, relative to parental strains *M. microti* OV254 and BCG (*P < .001 and *P < .006, respectively, Student’s *t* test) (figure 1A). However, OV254::RD1-2F9 remained less virulent, compared with BCG::RD1-2F9 (*P < .0001), confirming our previous analysis of bacterial loads [12]. Second, when injected iv into immunocompetent BALB/c mice, OV254::RD1-2F9 did not proliferate more than OV254::pYUB412 and was even cleared from the spleen more rapidly (figure 1B). Moreover, after administration of OV254::RD1-2F9 to the more-resistant C57BL/6 mice, the infection cleared, and neither bacterial growth nor lesions were detected in the lungs, whereas BCG::RD1-2F9 multiplied (data not shown). Taken together, these data demonstrate that, in the immunocompetent host, the OV254::RD1-2F9 vaccine is no more persistent than its parental strain and is far less virulent than BCG::RD1-2F9.

**Analysis of peripheral lymphoid organs of mice vaccinated with OV254::RD1-2F9.** To characterize the immune response induced by *M. microti* strains, we first analyzed the peripheral lymphoid organs of C57BL/6 mice vaccinated with OV254::RD1-2F9 or OV254::pYUB412. The number of cells in the draining lymph nodes of mice 3 weeks after sc vaccination with OV254::RD1-2F9 was almost twice that of their counterparts vaccinated with OV254::pYUB412 (data not shown). Because memory immune responses to *M. tuberculosis* have been shown to involve CD4+ and CD8+ T cells expressing the phenotype CD44hi CD69lo and CD25lo [20, 21], we performed flow cytometric analysis of these lymphocyte activation markers on mouse T cells. Splenic T lymphocytes from OV254::RD1-2F9–vaccinated mice reproducibly showed a statistically significant increase in the percentages of CD44hi CD8+ T cells, compared with mice vaccinated with OV254::pYUB412 (*P < .02, Student’s *t* test) or with control mice (*P < .01) (figure 2). Compared with control mice, the percentages of CD25+ or CD69+ cells in vaccinated mice remained unchanged within CD4+ or CD8+ T subsets. This preferential CD44 up-regulation suggests that vaccination with *M. microti* complemented with the entire RD1 generates activated/memory CD8+ T cells, which could increase protective immunity.

**T cell immunity to RD1-encoded antigens in mice vaccinated with OV254::RD1-2F9.** RD1-specific immune responses in mice vaccinated with either OV254::RD1-2F9 or OV254::pYUB412 were further characterized. As shown in figure 3A, 3 weeks after vaccination, C57BL/6 mice vaccinated with the 2 *M. microti* strains developed significant proliferative responses to Ag85A, a powerful mycobacterial immunogen expressed by both these strains, whereas no response was detected against MalE, which was used as a negative control antigen (data not shown). CD4+ splenic T lymphocytes of mice vaccinated with OV254::RD1-2F9 had marked proliferative responses after in vitro restimulation with the immunodominant ESAT-6:1–20 peptide (figure 3A). Moreover, splenocytes from mice vaccinated with OV254::RD1-2F9 produced substantial levels of IFN-γ after in vitro restimulation with recombinant ESAT-6 or CFP-10 proteins or with ESAT-6:1–20 peptide, but not with control proteins or peptides (figure 3B). As a further control, animals vaccinated with *M. microti* carrying the vector alone displayed neither proliferation of RD1 antigen–specific splenocytes nor production of IFN-γ. These results show that OV254::RD1-2F9 is a potent vaccine candidate able to induce marked T cell immunity to immunodominant RD1 antigens.

**Protection in mice after challenge with *M. tuberculosis.*** To assess the protective effect of these recombinants, groups of C57BL/6 mice were vaccinated either iv or sc with OV254::

![Figure 2](image-url)  
**Figure 2.** Expression of T cell activation markers by splenocytes of mice, untreated or vaccinated subcutaneously with 10⁶ cfu of *Mycobacterium microti* OV254::pYUB412 or OV254::RD1-2F9. Percentages of cells positive for activation markers within CD4+-splenic (white bars) or CD8+-splenic (black bars) T cells are expressed as the mean ± SD of 3 mice/group. *P < .02, vs. OV254::pYUB412-vaccinated mice; **P < .01, vs. untreated control (Student’s *t* test for both comparisons). Data are representative of 4 independent experiments.
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**Figure 3.** Induction of T cell immunity to region of difference (RD) 1–encoded antigens in mice vaccinated with *Mycobacterium microti* OV254::RD1-2F9. **A,** Proliferative response of splenocytes of C57BL/6 mice vaccinated subcutaneously with 10^6 cfu of OV254::RD1-2F9 (●) or OV254::pYUB412 (○) subsequent to in vitro stimulation with Ag85A:241-260 or early secreted antigen target (ESAT)–6:1–20 immunodominant peptides. **B,** Concentration of interferon (IFN)–γ in culture supernatants of splenocytes from OV254::RD1-2F9–vaccinated (black bars) or OV254::pYUB412-vaccinated (white bars) mice that were stimulated with concanavalin A (ConA), purified protein derivative (PPD), Mal-E:100-114 (negative control), ESAT-6, or culture filtrate protein–10 (rCFP-10) proteins. Results are of at least 3 independent experiments and are expressed as mean ± SD concentrations from duplicate culture wells.

RD1-2F9 or the respective controls (BCG and *M. microti* OV254). Two months after vaccination, mice were challenged and, as described above, *M. microti* OV254 behaved exactly like BCG in mice, conferring 1-log protection in both the lungs and the spleen, relative to the nonvaccinated mice. A marked reduction of the colony-forming unit counts was further observed in the spleens of the OV254::RD1-2F9–vaccinated mice 19 and 36 days after aerosol infection, compared with BCG- or *M. microti* OV254–vaccinated mice (figure 4A). At these time points, a few recombinant hygromycin-resistant myco-

bacteria could still be recovered from the spleen in some of the mice. To investigate whether the presence of the recombinants is required for the protective effect, mice were treated with antibiotics before challenge, to clear the vaccine strains from the organs. Under these conditions, no recombinant *M. microti* could be isolated from the lungs and the spleen at the first day of the challenge. Again, we observed the same specific increased protection at early time points in the spleens of mice vaccinated with OV254::RD1-2F9 (data not shown). Histological studies did not show a difference in the number of lesions in the lungs from mice vaccinated with OV254::RD1-2F9, compared with the lungs from BCG- or *M. microti* OV254–vaccinated mice, although a clear difference could be seen between vaccinated and nonvaccinated mice (figure 4B). The reproducible protective effect in the spleen led us to investigate the protective efficacy of OV254::RD1-2F9 in the guinea pig, which is a more suitable animal model for tuberculosis.

**Reduced splenic bacterial load in guinea pigs after an aerosol challenge.** In this experiment, OV254::RD1-2F9 was directly compared with the BCG standard. Guinea pigs were vaccinated sc with either OV254::RD1-2F9, BCG, or saline, and were challenged 10 weeks later with a high dose of *M. tuberculosis* via the aerosol route. The 6 guinea pigs from the saline group were killed when they developed signs of severe tuberculosis, at days 43, 85, 107, 107, 118, and 149 after infection. Guinea pigs in both the BCG- and the OV254::RD1-2F9–vaccinated groups gained, on average, 120 g throughout the whole infection period. By the end of the experiment, 6 months after challenge, 4 of 6 guinea pigs from the OV254::RD1-2F9–vaccinated group and 2 of 6 guinea pigs from the BCG-vaccinated group remained clinically well, as monitored by weight gain. In terms of survival, both of the vaccines prolonged survival significantly longer than did the saline control (*P* < .005 and *P* < .033, respectively, Student’s *t* test), although there was no statistical difference between the BCG and the OV254::RD1-2F9 groups. Bacterial loads in the lungs and the spleen were then quantified. Colony-forming unit counts in the spleens of the OV254::RD1-2F9–vaccinated guinea pigs were significantly lower than those in the saline group, whereas those from the BCG-vaccinated group were not significantly different from the saline group (figure 5A). Despite this, bacterial load in the lungs of OV254::RD1-2F9–vaccinated guinea pigs remained higher than that in the lungs of BCG-vaccinated guinea pigs and was not statistically different from that in the saline group (figure 5B). Thus, this experiment suggests that expression of RD1-encoded genes induced a reduction of splenic bacterial load independently of what took place in the lungs.

**DISCUSSION**

The protective efficacy of *M. microti* against tuberculosis has been well established, but the vaccine was abandoned in the
1970s [5, 6, 22]. Since then, a few studies have reported that M. microti is as efficient as BCG Pasteur and even imparts better protection than sc BCG when administered orally [23, 24]. Because of reports of adverse reactions to BCG, we have investigated here both the effects on virulence and the protective efficacy of M. microti strains that express the RD1 antigens. First, although complementation with RD1 increased virulence and persistence of BCG [12], reintroduction of RD1 into M. microti OV254 did not result in higher bacterial proliferation in normal mice and guinea pigs. OV254::RD1-2F9 is therefore truly attenuated, demonstrating that this recipient strain represents a safer alternative than BCG for the development of an RD1-based vaccine. Second, this result shows that, unlike M. bovis and M. tuberculosis [25, 26], loss of the RD1 does not entirely account for the attenuation of M. microti. In addition, the M. microti isolates from immunocompromised patients with pulmonary tuberculosis also lack the RD1 [9, 27], which suggests that attenuation of M. microti reference strain OV254 may be due to the absence of other regions of difference and due to point mutations. Deeper genomic analysis, along with the delineation of the full sequence of BCG and M. microti, will surely bring more insights into this field.

Moreover, this is the first report of a mutant strain showing divergent effects in the 2 commonly used models for phenotypic
screening, with the recombinant strain being more virulent than the parent strain in the SCID model but more attenuated in the immunocompetent host. These results thus suggest that, in the context of M. microti, the effect of RD1 on increased bacterial fitness, as indicated by the SCID mouse experiment, was overcome by stronger host immune responses. Indeed, strong T cell immune responses were generated against ESAT-6 and CFP-10. In spite of M. microti being the slowest growing member of the M. tuberculosis complex, the present study has proved that it is as efficient at secreting the immunogens as BCG [11], highlighting its potential as an antigen delivery system. In addition, the present study has shown that induction of ESAT-6-specific Th1 responses can be achieved without the need for an additional adjuvant, as has been suggested elsewhere [29]. Although we have focused on the immune responses directed toward ESAT-6 and CFP-10, other proteins from the RD1, such as PPE68, are likely to be responsible for enhanced immunogenicity as well [30].

It has been reported that CD44hi CD4+ or CD8+ T cells accumulate in the lungs of mice after infection with BCG or M. tuberculosis [20, 28] and that there is a correlation between protection against M. tuberculosis and the presence of prominent IFN-γ-producing CD8+CD44hi splenic T cells [21]. In accordance with these observations, we detected better recruitment of CD8+CD44hi memory lymphocytes in mice vaccinated with OV254::RD1-2F9 than in control mice. It remains to be seen why the same effect was not observed for BCG and what exact role this T cell subset plays on protection. Furthermore, we have demonstrated that the combination of RD1-encoded antigens and live M. microti act in synergy, leading to better protection against tuberculosis than that conferred by M. microti, by BCG that expresses ESAT-6, or by ESAT-6 alone, when used as a subunit or a combined DNA vaccine with mycobacterial antigens 85B and MPT64 [29, 31, 32]. The increased protective ability of OV254::RD1-2F9 in the spleen is similar to that observed elsewhere for BCG::RD1-2F9 [11]. One explanation for this particular effect in the spleen could be that expression of RD1 antigens varies in the organs, preventing generation of specific immune responses. Another possibility is that these recombinant RD1 vaccine strains are able to induce better immune responses in the spleen than in the lungs, where the immune response required to protect against disease may be different. In-depth immunological studies should clarify this phenomenon. Nevertheless, the present study clearly has shown that generation of a memory type immune response and protection in animals does not rely on increased in vivo growth and persistence of the vaccine strain. This observation argues for a major contribution of RD1 antigens in lowering the bacterial load in the spleen. It is now necessary to determine the exact contribution of each of these antigens to protection, since they should be considered as truly important immunogens and not just be reserved for diagnostic purposes [33].

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References


