Posttreatment Reactivation of Tuberculosis in Mice Caused by Mycobacterium tuberculosis Disrupted in mce1R

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Background. The reactivation of tuberculosis arises in persons who are latently infected and in those who have been previously treated. The mechanism of the reactivation of tuberculosis in either situation is not well understood. A 13-gene mce1 operon of Mycobacterium tuberculosis was previously shown to be associated with latent infection in mice and may also play a role in reactivation.

Methods. We tested mce1 operon M. tuberculosis mutants in a Cornell mouse model to examine disease progression and reactivation.

Results. In BALB/c mice, the wild-type, mce1 operon mutant, and mce1R (negative transcriptional regulator of the mce1 operon) mutant M. tuberculosis strains were equally susceptible to orally administered isoniazid and pyrazinamide. However, after cessation of the treatment, the mce1R mutant rapidly and progressively proliferated in mouse lungs and spleens, whereas the other strains remained latent. The reactivation of the mce1R mutant was associated with disease progression in the mouse lungs.

Conclusion. This observation demonstrates that the constitutive expression of the mce1 genes by M. tuberculosis in the latent state can cause a reactivation of tuberculosis. The constitutive expression of the mce1 genes in the mce1R mutant may allow this mutant to maintain its lipid metabolism, enabling it to survive long-term and proliferate inside granulomas.

A large proportion of the ~8 million new cases of tuberculosis each year arise in individuals who have latent tuberculosis [1]. The lifetime risk of reactivation of tuberculosis in those who have no underlying medical conditions is 2%–23%, but the risk increases to 5%–15% annually in persons coinfected with human immunodeficiency virus (HIV) [2, 3]. In addition, many of those who develop a reactivation of tuberculosis have a history of previous treatment of tuberculosis, which is often incomplete [4]. Efforts to prevent reactivation of tuberculosis are hampered by our limited understanding of the mechanism of latent tuberculosis and the progression to active disease.

One major obstacle to studying latent tuberculosis and reactivation of tuberculosis has been the lack of an ideal animal model. Two murine models of latent Mycobacterium tuberculosis infection are recognized. Although neither of these models can be said to truly represent latent or reactivation of tuberculosis in humans, they have provided useful information [5–8]. In the first model, sometimes referred to as the low-dose model, mice are infected aerogenically with 5–10 colony forming units (CFUs) of M. tuberculosis. The pulmonary bacterial burden stabilizes at a value of 3–4 log_{10} CFUs, which is maintained for 15–18 months [5, 8]. After this point, the infection reactivates and the mice die. Flynn et al [6] and others have used this model to show that reactive nitrogen intermediates as well as tumor necrosis factor α play a role in preventing reactivation of disease [7, 9]. There are variations of this model that use different aerogenic inoculum doses to accelerate the disease outcome [10].

The other widely used mouse model to study latent
M. tuberculosis infection is the Cornell model [11–14]. Scanga et al [8] reported on the utility of several versions of the Cornell model and concluded that it is highly dependent on the parameters used to establish latency and that each version has certain limitations. The Cornell model has the advantage of achieving very low or undetectable numbers of bacilli and maintaining those low levels for many weeks; however, it has the disadvantage of artificially inducing latency with antibiotics [8]. This model may be more akin to those human patients who remain latently infected after completing treatment of active disease.

The genome of M. tuberculosis contains 4 related copies of a cluster of genes called mce operons (mce1–mce4) [15]. A phylogenomic analysis of the operons has shown that they encode possible adenosine triphosphate (ATP)–binding cassette (ABC) transporters [16]. We previously reported that the disruption of mce1 renders the M. tuberculosis mutant hypervirulent in BALB/c mice and unable to stimulate a T helper 1–type immune response [17]. The mce1 operon genes are negatively regulated by Mce1R in intracellular M. tuberculosis [18]. An M. tuberculosis mutant disrupted in mce1R constitutively expresses the mce1 genes in vivo and causes accelerated immunopathological response in the infected animal [19].

On the basis of the above-mentioned observations, we used the Cornell murine model of latent infection to examine the outcome of latent infection with the mce1 operon and mce1R mutants. Surprisingly, each of these mutants caused an unexpected course of infection in this model and provided new insights into the mechanism of reactivation of disease that had not been previously considered.

MATERIALS AND METHODS

Animals. Female BALB/c mice (Charles River, Rockland, MA) aged 8–10 weeks were maintained and studied in a Biosafety Level 3 animal facility at the University of California, Berkeley.

Bacterial strains and growth conditions. The M. tuberculosis H37Rv wild type (American Type Culture Collection strain 25618), M. tuberculosis H37Rv mce1 operon mutant, M. tuberculosis H37Rv mce1R mutant, complemented M. tuberculosis H37Rv mce1R mutant, and Erdman wild type were grown in Middlebrook 7H9 broth supplemented with 10% albumin-dextrose-catalase (Becton Dickinson), 0.2% glycerol, and 0.05% Tween 80 or on Middlebrook 7H11 agar containing oleic acid–albumin-dextrose-catalase supplement (Becton Dickinson), 0.5% glycerol, and the antifungal agent cycloheximide (100 mg/mL; Sigma-Aldrich). The 2 mutant strains (mce1 operon mutant and mce1R mutant) were generated from the M. tuberculosis H37Rv background strain by means of homologous recombination with a 2-step counterselection strategy, as described in detail elsewhere [17, 18, 20].

Infection and antibiotic treatment in mice. In the initial set of experiments, mice were infected via the tail vein with ~5 × 10^5 CFUs of mce1 operon and mce1R mutant bacilli in accordance with the traditional Cornell model. At 4 weeks after infection, mice were given isoniazid (100 μg/mL) and pyrazinamide (15 mg/mL) in drinking water ad libitum for 4 weeks. After a period of 4 weeks, some of the mice were treated with 0.08 mg of dexamethasone per day intraperitoneally for 6 d a week for 20 weeks. In the second set of experiments, mice were infected via the tail vein with a lower dose (5 × 10^2 CFUs per animal). They were otherwise treated similarly to those of the first set of experiments. In both experiments, mice were followed up for clinical symptoms, organ CFU counts, and histological analysis after cessation of the antibiotics.

When we observed an unexpected response with the mce1R mutant in the initial set of experiments, we studied this mutant further with the aerosol infection route. Mice were infected with 100–200 CFUs of the mce1R mutant, complemented mce1R mutant, or wild-type H37Rv strain per mouse with an aerosol generation device (Inhalation Exposure System; Glas-Col). The inoculum doses in each group were assessed as described below by harvesting the right lung of 4 mice (from each group of mice) 24 h after infection. At 4 weeks after infection, the mice initiated treatment with isoniazid (100 μg/mL) and pyrazinamide (15 mg/mL) delivered ad libitum in drinking water. The duration of treatment was 4 and 8 weeks, and the mice were followed up without administration of cortisone or dexamethasone for clinical symptoms, organ CFU counts, and histological analysis after cessation of the antibiotics.

Microbiological and histopathological examination. At designated time points, 4 or 5 animals from each group were killed and the lungs and spleen were harvested and examined grossly, histologically, and microbiologically. The number of tubercle bacilli in the corresponding organ homogenates in phosphate-buffered saline with Tween 80 (0.05%) was assessed by plating part of the homogenate on Middlebrook 7H11 agar (Difco) plates. The CFU counts were enumerated 3–4 weeks later. Data are presented as the mean value (± standard deviation) of 4 or 5 mice per group, and the experiment was performed twice with similar results. Differences between the mean of the CFU counts obtained from each treatment group were analyzed by the Student t test and were considered significant at P < .05.

Tissue samples for histopathological studies were prepared as described elsewhere [21, 22]. Briefly, the left lung was fixed in 10% buffered formalin and sectioned. Slides for histopathological analysis were made commercially (Histology Consultation Service), and lung sections were stained either with hematoxylin and eosin or by the Ziehl-Neelsen method for acid-fast bacilli. Lung sections from 4 mice from each group
per time point were analyzed by a veterinary pathologist specializing in mouse pathology, who was blinded to the sources of the specimens. The lung sections were evaluated according to the following set of criteria: (1) percentage of lung displaying parenchymal lesion, (2) severity of the organization of the lesions (in order of severity: diffuse, coalescing nodular, nodular, or alveolar septal thickening), (3) type of macrophages present (foamy or epithelioid), (4) lymphocyte distribution (mild, moderate, marked, or severe), (5) areas of necrosis (focal, multiple focal, diffuse, or coalescing), (6) involvement of airways (small to large numbers of inflammatory cells), and (7) number of neutrophils present.

RESULTS

High-dose tail vein infection in mice treated for 4 weeks. At a dose of $5 \times 10^6$ CFUs, all mice infected with the mce1R mutant rapidly progressed to death, and hence this dose could not be used with this mutant in the traditional Cornell model. Detailed results of the BALB/c infection with the mce1R mutant are published elsewhere [20]. However, with the wild-type and mce1 operon mutant M. tuberculosis, bacilli were recovered from organs during the course of dexamethasone treatment as follows: 1 of 3 mice infected with the mutant and killed at 12 weeks of treatment with dexamethasone (or 24 weeks after tail vein infection) showed 470 CFUs from the spleen homogenate and 17 CFUs from the lung homogenate. No CFUs were recovered from any other mice killed at 4, 8, or 20 weeks of treatment with dexamethasone. Each of 2 of 3 mice infected with the wild type and killed at 20 weeks of treatment had 17 CFUs from the spleen homogenate and no CFUs from the lung homogenate. No CFUs were recovered from mice killed at earlier time points (4, 8, and 12 weeks).

Low-dose tail vein infection in mice treated for 4 weeks. Because of the high pathogenicity of the mce1R mutant at an inoculum dose of $5 \times 10^3$ CFUs, we repeated the tail vein infection with a lower dose. Surprisingly, at a dose of $5 \times 10^2$ CFUs, a mean of $\sim 10^4$ mce1R mutant bacilli were recovered from the lungs of 3 mice killed 4 weeks after the antibiotics were stopped and before the dexamethasone was initiated (Figure 1). The number of bacilli continued to increase until it reached $\sim 10^6$ bacilli per lung after 12 weeks of dexamethasone treatment. No wild type or mce1 operon mutants were recovered at any time point from the lungs of mice given dexamethasone, but 4 weeks after cessation of the drugs, we did see recovery of both strains in mice not given dexamethasone (Figure 1B).

Aerosol infection in mice treated for 4 weeks. The rapid reactivation of the mce1R mutant after cessation of the antibiotics was unexpected. We therefore studied mce1R in more detail, using the aerosol infection model, to compare 2 different durations of antibiotic administration (4 and 8 weeks). BALB/c mice were infected with the M. tuberculosis H37Rv wild type, mce1R mutant, and complemented mce1R strain via the inhalation route. At 24 h after infection, 4 mice were killed from each infected group for CFU recovery in the lungs and spleen. The initial inoculum doses were $1.3–1.4 \times 10^2$ CFUs per mouse for each group (Figure 2). At 4 weeks after infection, no significant difference in the number of CFUs recovered from the lungs ($1.0–2.5 \times 10^5$ CFUs) was observed in mice infected with any of the M. tuberculosis stains (Figure 2A). The number of CFUs recovered from the spleens of mice infected with the mce1R mutant ($9.5 \pm 3.16 \times 10^4$ CFUs) was similar to that recovered.
bacilli in the spleen and in low numbers (CFUs) and pyrazinamide resulted in undetectable numbers of viable isoniazid and pyrazinamide. The 4-week course of isoniazid A and 2 of bacilli in the lungs (Figures 2 A). However, the number of CFUs recovered from the spleens of mice infected with the mce1R mutant (1.7 ± 0.5 × 10^4 CFUs) became progressively greater than that of mice infected with the H37Rv wild type (2.4 ± 0.9 × 10^4 CFUs) (P<.005) or the complemented mce1R strain ([7.5 ± 2.5] × 10^2 CFUs) (P<.001) (Figure 2B).

Most importantly, by 25 weeks after infection, the number of CFUs recovered from the lungs and spleens of mice infected with the mce1R mutant continued to increase to (1.5 ± 0.4) × 10^6 and (1.3 ± 0.3) × 10^4 CFUs, respectively, whereas the number of CFUs recovered from the organs of mice infected with the H37Rv wild type and complemented mce1R strain remained unchanged at (1.0 ± 0.3) × 10^4 and (4.4 ± 1.6) × 10^3 CFUs in the lungs and (1.7 ± 0.7) × 10^3 and (5.8 ± 1.5) × 10^3 CFUs in the spleen, respectively.

**Lung histopathology in aerosol-infected mice treated for 4 weeks.** Histopathological examination of lungs of mice was performed prospectively for all mice aerosol-infected with wild-type M. tuberculosis H37Rv, the mce1R mutant, and the complemented mce1R strain. First, mice were killed 4 weeks after infection just before the antibiotic treatment started. A large proportion of the lung parenchyma (25%–75%) in all of the mice contained multifocal and coalescing nodular lesions comprising a mixed population of foamy and epithelioid macrophages (Figure 3, row 1). The histology of these lung sections was, for the most part, indistinguishable. There was no obvious change in the proportion of lung parenchymal lesions after 4 weeks of treatment in any of the infected mice. One of the lung sections from a mouse infected with the mce1R mutant showed small numbers of degenerate neutrophils in the parenchyma and mild intraluminal degenerative neutrophils.

Mice treated for 4 weeks were examined 17 weeks after cessation of the treatment (25 weeks after the initial infection) (Figure 4). The lung parenchymal involvement in mice infected with the H37Rv strain and the complemented mutant was 25%–50%, whereas that of mice infected with the mce1R mutant was <25%. In all mice, the distribution of the lung lesions was multifocal and nodular, containing a mixed population of foamy and epithelioid macrophages. No significant difference in the number of acid-fast bacilli was observed between the lung sections of mice infected with the wild-type H37Rv strain, complemented mce1R strain, or mce1R mutant (data not shown).

**Aerosol infection in mice treated for 8 weeks.** Because the wild type and complemented mutant could still be recovered from mice treated for 4 weeks, we extended the treatment to 8 weeks. The numbers of bacterial CFUs recovered at time from the spleens of mice infected with the wild-type H37Rv strain ([1.3 ± 0.4] × 10^4 CFUs), complemented mce1R strain ([6.0 ± 1.8] × 10^4 CFUs), or mce1R mutant ([2.6 ± 0.5] × 10^4 CFUs) (Figure 2A). However, the number of CFUs recovered from the spleens of mice infected with the mce1R mutant ([1.7 ± 0.5] × 10^4 CFUs) became progressively greater than that of mice infected with the H37Rv wild type ([2.4 ± 0.9] × 10^4 CFUs) (P<.005) or the complemented mce1R strain ([7.5 ± 2.5] × 10^2 CFUs) (P<.001) (Figure 2B).

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points before the 8-week medication course were similar to those recovered before the 4-week medication course.

The 8-week course of isoniazid and pyrazinamide resulted in undetectable numbers of viable bacilli in the lungs and spleens of mice in all infected groups at the end of the treatment course (Figure 5). At 6 weeks after the 8-week treatment course, (18 weeks after infection), \((1.3 \pm 1.0) \times 10^7\) CFUs from the lungs and \((2.0 \pm 1.8) \times 10^7\) CFUs from the spleens of mice infected with the \(mce1R\) mutant were recovered, whereas no CFUs were recovered from any of the organs of mice infected with the H37Rv wild type or complemented \(mce1R\) strain (Figures 5A and 5B).

At 24 and 32 weeks after infection, the number of CFUs recovered from mice infected with the \(mce1R\) mutant continued to increase from \((7.5 \pm 5.5) \times 10^2\) to \((5.3 \pm 4.0) \times 10^2\) CFUs in the lungs and from \((1.1 \pm 0.9) \times 10^2\) to \((4.4 \pm 3.8) \times 10^2\) CFUs in the spleen, whereas the number of CFUs recovered from the organs of mice infected with H37Rv wild type and complemented \(mce1R\) strain remained undetectable. Interestingly, no \(mce1R\) mutant was detected in the lungs or spleens of any mice at 3 weeks after cessation of the 8-week antibiotic course, which suggests that during this period, this mutant was indeed in a latent state.

Lung histopathology in aerosol-infected mice treated for 8 weeks. At the end of 8 weeks of treatment, the proportion of the parenchymal lung involvement in all mice decreased from that during the period just before the treatment was started (Figure 3). In those infected with the H37Rv strain or the complemented \(mce1R\) mutant, the proportion of parenchymal involvement decreased to <25% in most of the lung sections (Figure 3, row 2). In mice infected with the \(mce1R\) mutant, the parenchymal involvement also decreased to 25%–50% (Figure 3, row 3).
Figure 4. Histopathology of lung sections from BALB/c mice infected with wild-type *Mycobacterium tuberculosis* strain H37Rv (A), complemented mce1R mutant (B), or mce1R mutant (C). The mice were infected via the inhalation route; after 4 weeks, they were given a 4-week course of antibiotics. Lung sections from 4 mice in each group were harvested at 17 weeks after the end of treatment (25 weeks after infection) and stained with hematoxylin and eosin. Lung tissues in all groups show granulomatous interstitial pneumonia. Original magnifications, ×100 (row 1) and ×400 (row 2); scale bars, 80 μm (row 1) and 20 μm (row 2).

3C, row 2). Granulomatous lesions were rare and small in mice infected with the mce1R mutant, and no such lesions were observed in mice infected with the other strains.

Lung sections of the remaining mice were harvested at 20 weeks after cessation of the 8-week treatment (32 weeks after infection) (Figure 3, row 3). At this time point, the lung lesions were much milder than those in the lungs of mice observed at 17 weeks after 4 weeks of treatment (Figure 4). Nodular lung lesions were observed only in mice infected with mce1R mutant (Figure 3C, row 3). Lung sections of mice infected with mce1R mutant showed mild granulomatous pneumonia with multifocal nodular lesions that were characterized by scattered foci of alveolar macrophages, with occasional foamy alveolar macrophages, and large focal aggregates of lymphocytes within the nodule (Figure 3C, row 3).

**DISCUSSION**

In this study, we used a variant of the Cornell mouse model of latent tuberculosis to study latent infection with *M. tuberculosis* mce1 operon and mce1R mutants. The unexpected observation was the rapid posttreatment proliferation of the mce1R mutant. At similar infection inoculum doses, the mce1R mutant rapidly proliferated in mouse lungs and spleens after the cessation of antibiotics, regardless of whether the mice were infected via the tail vein or by the aerosol route. The mce1R mutant, as well as the other strains, exhibited a similar level of susceptibility to the drugs during the treatment course, as evidenced by the low or absent CFU counts at the end of each treatment course (Figures 1, 2, and 5). All infected mice initially responded clinically to the treatment, as evidenced by progressive resolution of the lung lesions (Figure 3). However, among mice treated for 8 weeks, only those infected with the mce1R mutant showed recrudescence of the lung lesions after treatment cessation. This recrudescence correlated with increasing numbers of CFUs recovered from the lungs (Figure 5). Thus, the mce1R mutant appears to cause a true posttreatment reactivation of tuberculosis in mice. The fact that the mce1R mutant is regulated in vivo [18, 19] suggests that this response can occur in a natural course of infection with wild-type *M. tuberculosis* in humans. Indeed, posttreatment reactivation of tuberculosis in humans is not uncommon among those who do not complete a standard 6-month course of treatment, even with drug-susceptible tuberculosis.

We showed that at 3 weeks after cessation of the drugs, the mce1R mutant could not be cultured from lungs (Figure 5). Thus, this phase of infection was consistent with this organism...
Figure 5. Results of a modified Cornell model of infection with wild-type Mycobacterium tuberculosis strain H37Rv (squares), mce1R mutant (circles), and complemented mce1R mutant (triangles). BALB/c mice were infected via the inhalation route, and antibiotics were given to mice in each group for 8 weeks after 4 weeks of infection. The number of colony-forming units (CFUs) recovered from the lungs (A) and spleens (B) is shown as the mean value (± standard deviation) of 4 or 5 mice per group at the indicated periods of infection.

being a latent state of infection. Here, the drugs may have reduced the bacterial count to a value below the threshold recoverable on the Middlebrook 7H11 agar plate. We note that it is possible that the treatment that resulted in no recovery of the wild type represents complete sterilization and not latent infection. However, at least in 1 experiment, we did observe later recovery of wild-type bacteria from a subset of infected mice, as depicted in Figure 1B.

Six of the mce1 genes (mce1A–mce1AF) encode cell wall proteins that resemble substrate proteins of ABC transporters, whereas the upstream genes yrbE1A and yrbE1B encode proteins that resemble ABC transporter permeases [16]. ABC transporters use an adenosine triphosphatase (ATPase) to supply energy for translocation of a substrate across a membrane [23]. Dassa and Bouige [23] have suggested that RvO655, an ATPase called Mkl, may serve this function for M. tuberculosis. Joshi et al [24] have shown that RvO655 and mce1 are functionally linked and that the mce1 operon may encode an ABC transporter involved in lipid import. Interestingly, the mce1 operon carries a gene, fadD5, that encodes a fatty acyl co-A synthetase, which is putatively involved in fatty acid catabolism [15]. Dunphy et al [25] recently suggested that FadD5 may be involved in recycling mycolic acids from dying M. tuberculosis inside granulomas. Mce1R belongs to a family of GntR-negative transcriptional regulators involved in lipid transport and metabolism in other bacterial organisms [18, 26]. Another member of the mce family of operons, mce4, was recently shown to serve as a cholesterol importer that is necessary for persistent infection in mice [27]. These observations together suggest that the mce operon family is involved in lipid transport across the M. tuberculosis cell wall and membrane, possibly for the organism to gain nutrients (carbon as an energy source) during its long-term survival inside granulomas.

Beste et al [28] recently reported that in Mycobacterium bovis bacille Calmette-Guérin, the mce1 operon may be involved in the bacillus switching to a slow growth rate, which supports the idea that the regulation of the mce1 operon by mce1R can affect M. tuberculosis replication in vivo. The mce1 genes in the mce1R mutant are constitutively expressed, whereas in the wild type, these genes are repressed during the early phase of infection in vivo [18, 19]. The expression of the mce1 genes may allow M. tuberculosis to import and metabolize lipids for its survival during the persistent state. When the number of organisms is reduced by treatment to a level that is undetectable in culture, wild-type strains that do not express the mce1 genes may become restricted for replication by the host immune response, whereas the mce1R mutant strains expressing the mce1 genes would continue to take up and metabolize lipids and replicate. We have shown elsewhere that wild-type M. tuberculosis expresses mce1 genes after ~8 weeks into infection in mice [19]. We do not know at this time what triggers expression or repression of these genes in vivo. In a course of persistent infection, an infected host may harbor a mixed population of strains that express these genes and strains that do not express these genes. The progression to reactivation of disease, as opposed to remaining in latent state, may depend on the balance of these 2 bacterial populations. Factors that disrupt this balance, such as immunosuppression, old age, large initial infectious inoculum doses, or certain M. tuberculosis strain-related factors, could lead to the reactivation of tuberculosis.
References


