Mycobacterium tuberculosis strains disrupted in *mce3* and *mce4* operons are attenuated in mice

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The *Mycobacterium tuberculosis* genome contains four copies of an operon called *mce* (*mce1–4*). Previously we reported that *M. tuberculosis* disrupted in the *mce1* operon is more virulent than wild-type *M. tuberculosis* in mice. We generated single deletion mutants in *mce3* ($\Delta mce3$) and *mce4* ($\Delta mce4$) operons and a double deletion mutant ($\Delta mce3/4$). Similar doubling times and growth characteristics were observed for all mutants and the wild-type (parent) *M. tuberculosis* H37Rv strain in culture and in macrophages. In addition, similar bacterial burdens were detected in organs from mice infected with $\Delta mce3$ and the parent strain. However, the bacterial burdens of mice infected with $\Delta mce4$ and $\Delta mce3/4$ were less than those of mice infected with the parent strain. The median survival times of mice infected with wild-type *M. tuberculosis*, $\Delta mce3$, $\Delta mce4$ and $\Delta mce3/4$ were 40.5, 46, 58 and 62 weeks, respectively. Histopathological examination of lungs at 15 weeks post-infection showed that the extent of the lung lesions was less prominent in mice infected with $\Delta mce4$ and $\Delta mce3/4$ mutants than in mice infected with the other two strains. These observations suggest that the *mce3* and *mce4* operons have a role distinct from that of *mce1* for *in vivo* survival of *M. tuberculosis*.

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INTRODUCTION

It is estimated that 2 billion people worldwide are latently infected with *Mycobacterium tuberculosis*, the aetiologic agent of tuberculosis (Dye *et al.*, 2002), and 2–23 % of these latently infected persons ultimately develop active disease in their lifetime (Parrish *et al.*, 1998). We previously showed that an *M. tuberculosis* strain disrupted in an operon called *mce1* ($\Delta mce1$) was more virulent in mice than its parent wild-type (WT) strain, H37Rv; the mutant was also diminished in its ability to induce a Th1-type immune response (Shimono *et al.*, 2003). We thus suggested that *mce1* genes may temper the ability of *M. tuberculosis* to cause overt disease and play a role in establishing latent infection in mice (Shimono *et al.*, 2003).

The *mce1* operon is a member of a family of related operons comprising *mce1*, 2, 3 and 4 containing

homologous genes arranged similarly (Cole *et al.*, 1998; Tekaia *et al.*, 1999). Six of the *mce1* genes (*mce1A–F*) encode proteins that localize to the cell wall (Chitale *et al.*, 2001; Shimono *et al.*, 2003; Tekaia *et al.*, 1999). One of the *mce1* proteins (Mce1A) was previously shown to confer upon a nonpathogenic *Escherichia coli* strain an ability to enter HeLa cells (Arruda *et al.*, 1993; Chitale *et al.*, 2001). The corresponding protein in the *mce2* operon (Mce2A), which is 67 % similar in amino acid sequence to Mce1A, did not exhibit this activity. Santangelo *et al.* (2002) have shown that *mce3* genes are negatively regulated by a gene (Rv1963) belonging to the *tetR* family. In contrast, *mce1* has a negative transcriptional regulator (*mce1R*) belonging to the GntR family at the corresponding position (Casali *et al.*, 2006; Cole *et al.*, 1998). Recently we have shown that the *M. tuberculosis* strain disrupted in *mce1R* exhibits enhanced virulence in mice, indicating that the level of expression of the *mce1* products has a profound effect on the clinical outcome of the infection (Uchida *et al.*, 2007). Gioffre *et al.* (2005) have studied $\Delta mce1$, $\Delta mce2$ and $\Delta mce3$ in BALB/c mice. The above authors used two methods of

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Abbreviations: H&E, haematoxylin and eosin; p.i., post-infection; WT, wild-type.

infection: intratracheal and intraperitoneal routes. When mice were infected via the intratracheal route, all three *mce* mutants showed reduced bacterial counts; when mice were infected via the intraperitoneal route, $\Delta mce1$ showed 50 % increased bacterial counts while the counts decreased for $\Delta mce2$ and remained unchanged for $\Delta mce3$. The mouse survival study was carried out only up to 20 weeks (Gioffre *et al.*, 2005). Joshi *et al.* (2006) studied $\Delta mce4$ in C57BL/6 mice using intravenous infection. They co-infected mice with $\Delta mce4$ and WT H37Rv and studied the infection up to 100 days. Thus these other studies have not assessed the long-term survival of *mce2–4* operon mutations in mice. The long-term effects of *mce* mutations on mice are important to assess the suggested role of the *mce* operons in latency. We therefore studied the long-term survival (>60 weeks) of mice infected with mutants disrupted in *mce3* and *mce4* using a low-dose aerosol infection model.

METHODS

Mycobacteria and culture conditions. WT *M. tuberculosis* H37Rv and its derivative strains (*mce* mutants) were grown in Middlebrook 7H9 broth containing 10 % ADC (Becton Dickinson), 0.2 % glycerol and 0.05 % Tween 80 (7H9-ADCT) or on Middlebrook 7H11 agar containing OADC (Becton Dickinson), 0.5 % glycerol and the antifungal agent cycloheximide ($100 \mu\text{g ml}^{-1}$) (Sigma-Aldrich). Bacteria were passed through a $5 \mu\text{m}$ pore filter to prepare single cell suspensions prior to mouse infection and measuring growth curves.

Generation of *mce* mutants. The *M. tuberculosis* H37Rv *mce* mutants were constructed by the method of Parish & Stoker (2000). Deleted alleles were created by amplifying (approx.) 1 kb regions upstream and downstream of each operon. Following digestion with the appropriate restriction enzymes, these PCR products were subcloned into the p2NIL vector in tandem. Mutant selection and additional vector information have been previously described by Parish & Stoker (2000). Deletion mutations were confirmed by Southern blot hybridization.

Mouse infections. Eight-week-old C57BL/6 mice (Jackson Laboratories) were infected with the *M. tuberculosis* strains via inhalation by the Inhalation Exposure System (Glas-col). The inoculum doses were assessed from harvest of the right lungs of three mice (per infection) 24 h post-infection (p.i.). The dose of infection was 72–108 bacilli per lung for all infections. Lungs were homogenized and plated onto 7H11 agar, followed by enumeration of c.f.u. 21 days later. At different time points p.i., the right lung, liver and spleen from three mice were collected, homogenized in PBS-Tween (0.05 %), appropriately diluted, and plated onto 7H11 agar plates (supplemented as above). At 21 days, the bacterial load of each organ was determined by c.f.u. enumeration.

Determination of mouse morbidity. Four groups of eight to nine mice each, infected with each of the above strains, were followed until they exhibited moribund features that occur just before death. At this point, mice were anaesthetized with a mixture of ketamine HCl, xylazine and acepromazine injected subcutaneously, and then euthanized by cervical dislocation. Loss of weight accompanied by failure to groom, ruffled fur and lethargy were used to assess morbidity, in addition (in some instances) to the recommendation of the veterinary staff of the North Animal Facility of UC Berkeley. The health of the mice was monitored daily by the above veterinary staff.

Determination of survival and induction of cytokines in macrophages by *mce* mutants. The RAW 264.7 murine macrophage-like cell line (ATCC) was cultured and maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 10 % fetal bovine serum (Omega Scientific) at 37°C in a 5 % CO_2 humidified incubator. Cells were plated at 2×10^5 cells per well in 24-well tissue culture plates. The macrophages were incubated (i.e. infected) with either 2×10^5 , 2×10^6 or 2×10^7 bacteria for 6 h. After 6 h of infection, macrophages were washed three times with DMEM to remove extracellular bacteria. To examine the intracellular invasion, growth and survival of bacteria, we lysed the macrophages with 1 ml PBS-0.5 % Triton X-100 and their serial dilutions were plated onto 7H11 agar. This was performed 6, 48 and 72 h p.i. c.f.u. on plates were enumerated 21 days after plating. Supernatants of the above cell cultures were used to measure TNF α , IL-12, IL-6, IL-10 and monocyte chemoattractant protein 1 (MCP-1) produced by macrophages in response to infection with WT or the *mce* mutant strains. Lipopolysaccharide ($1 \mu\text{g ml}^{-1}$) (Sigma) was used as a positive control and uninfected macrophages served as negative controls for each experiment. The above cytokines and MCP-1 were measured by ELISA with the reagents purchased from eBioscience.

Histology. The mouse left lung fixed in 10 % neutral (PBS) buffer formalin was embedded in paraffin, sectioned and stained for histology with either haematoxylin and eosin (H&E) or the Ziehl-Neelsen technique. Sectioning and staining were performed by Histology Consultation Services, Everson, Washington, USA. For comparative purposes, sections were obtained from the same regions of all lungs; three sections were obtained from each lung from three mice. Sections obtained from the top and the bottom parts of the lung were stained by H&E, while the section obtained from the middle region was stained by the Ziehl-Neelsen technique. The histopathology of each lung was assessed for two to four H&E-stained sections and one to two sections stained by the Ziehl-Neelsen technique. Histopathology of 6 weeks and 15 weeks p.i. lungs was analysed. Pathological analysis was done by a veterinary pathologist from the School of Veterinary Medicine UC Davis, USA.

Statistics. Mouse survival was compared by Kaplan-Meier curves. The mean c.f.u. counts of *M. tuberculosis* recovered from organs of each mouse group (three per group) were compared by the Student's *t*-test.

RESULTS

Generation of *mce* mutants

We generated three mutants by in-frame deletion of the *mce3*, *mce4* and both *mce3* and *mce4* operons in *M. tuberculosis* H37Rv ($\Delta mce3$, $\Delta mce4$ and $\Delta mce3/4$, respectively). The mutations were designed so that eight genes (*yrbE3A–mce3F*) within *mce3* (Tekaiia *et al.*, 1999) and eight genes and the first 250 bp of the ninth gene (*yrbE4A* to the first 250 bp of Rv3493c) within *mce4* (Tekaiia *et al.*, 1999) would be deleted (Figs 1 and 2). Deletions of the genes were confirmed by Southern blot analysis (Figs 1 and 2) and PCR (data not shown). The resulting mutants, $\Delta mce3$, $\Delta mce4$ and $\Delta mce3/4$, displayed *in vitro* growth characteristics similar to those of the WT parent strain (data not shown).

Survival and induction of cytokines in RAW macrophages by *mce* mutants

Previously we reported that, upon infection, in comparison to WT, the *mce1* mutant induces less TNF- α , IL-6 and

Fig. 1. Southern blot analysis of the *mce3* operon deletion. (a) Genomic organization of the region surrounding the *mce3* operon in the WT (*M. tuberculosis* H37Rv). Black arrows depict the deleted genes [*yrbE3A–mce3F*: TubercuList coordinates (<http://genolist.pasteur.fr/TubercuList/>) 2207697–2216573] of the *mce3* operon. Two black boxes (labelled 5' probe and 3' probe) indicate regions of DNA probes used in Southern blot analysis. (b) Genomic organization of the region surrounding the *mce3* deletion in $\Delta mce3$ and $\Delta mce3/4$. White arrows in (a) and (b) depict the genes neighbouring the region of the deletion: *mce3R* (5' end) and Rv1972 (3' end). Genomic DNA was digested with *PvuII* for Southern blot analysis. (c) Southern blot analysis of genomic DNA from WT (lane 2), $\Delta mce3$ (lane 3) and $\Delta mce3/4$ (lane 4). DIG-labelled molecular mass standards II (lane 1) and VII (lane 5) are from Roche Diagnostics. Arrows indicate the molecular mass standards in kbp.

Fig. 2. Southern blot analysis of the *mce4* operon deletion. (a) Genomic organization of the region surrounding the *mce4* operon in the WT (*M. tuberculosis* H37Rv). Black arrows depict the completely or partially deleted genes [first 250 bp of *Rv3493c* to *yrbE4A*: TubercuList (<http://genolist.pasteur.fr/TubercuList/>) coordinates 3911426–3920856] of the *mce4* operon. Two black boxes (labelled 5' probe and 3' probe) indicate the regions of DNA probes used in Southern blot analysis. (b) Genomic organization of the region surrounding the *mce4* deletion in $\Delta mce4$ and $\Delta mce3/4$. The black arrow depicts the partially deleted *Rv3493c* (479 bp of the 3' end). In (a) and (b), white arrows depict the genes neighbouring the region of the deletion: *Rv3492c* (5' end) and *Rv3502c* (3' end). Genomic DNA was digested with *PvuII* for Southern blot analysis. (c) Southern blot analysis of genomic DNA from WT (lane 2), $\Delta mce3/4$ (lane 3) and $\Delta mce4$ (lane 5). DIG-labelled molecular mass standards II (lane 1) and VII (lanes 4 and 6) are from Roche Diagnostics. Arrows indicate the molecular mass standards in kbp.

MCP-1 in RAW macrophages (Shimono *et al.*, 2003). Similarly, we investigated the induction of the above cytokines and MCP-1 by RAW cells infected with $\Delta mce3$, $\Delta mce4$ and $\Delta mce3/4$ in comparison to WT. In addition to the above two cytokines and chemokine, we also investigated the induction of two additional cytokines, IL-10 and IL-12. Macrophages were infected with three different inoculum doses (1 macrophage to either 1, 10 or 100 bacilli) of *M. tuberculosis* strains. In all three inoculum doses, we did not observe any reproducible difference in cytokine or chemokine induction by $\Delta mce3$, $\Delta mce4$ and $\Delta mce3/4$ compared to WT (data not shown). All three *mce* mutants replicated similarly to WT in RAW macrophages (data not shown).

Bacterial burden and survival of mice

Similar bacterial burdens were detected in organs from mice infected with $\Delta mce3$ or the WT at all time points (Fig. 3). However, at 15 weeks p.i., recovery of $\Delta mce4$ and $\Delta mce3/4$ c.f.u. from mouse lungs was significantly less than that of the WT ($P < 0.05$). All other c.f.u. recoveries (from all three organs) at 15 weeks p.i. from mouse organs infected with *mce* mutants were not significantly different compared to c.f.u. recoveries from mouse organs infected with the WT (Fig. 3).

The long-term survival time of mice infected with $\Delta mce3/4$ or $\Delta mce4$ was significantly longer than that of mice infected with the WT or $\Delta mce3$. The median survival time of WT-infected mice ($n=8$) was 40.5 weeks compared to 46 weeks ($P=0.02$) for $\Delta mce3$ -infected mice ($n=8$). By comparison with WT infected mice, the median survival times for $\Delta mce3/4$ -infected mice ($n=9$) and $\Delta mce4$ -infected mice ($n=9$) were 62 weeks ($P < 0.0001$) and 58 weeks ($P < 0.0001$), respectively. The statistical differences between the survival times of mice infected with $\Delta mce3$ versus $\Delta mce3/4$ and $\Delta mce3$ versus $\Delta mce4$ strains were also significant ($P=0.0003$ and 0.003 , respectively). Additionally, the differences between the survival times of mice infected with $\Delta mce4$ versus $\Delta mce3/4$ strains were significant ($P=0.029$) (Fig. 4).

Histopathological analysis

Histopathological examination of lungs at 15 weeks p.i. showed that the extent of the lung lesions (granulomatous interstitial pneumonia) was less prominent in $\Delta mce4$ - and $\Delta mce3/4$ -infected mice than in mice infected with the other two strains (Fig. 5a). At 15 weeks p.i., the average area of the lung parenchymal lesions in mice infected with WT, $\Delta mce3$, $\Delta mce4$ or $\Delta mce3/4$ was 53 %, 54 %, 19 % and 22 %, respectively. In all mouse groups, granulomatous interstitial pneumonia was observed, which initially (at 6 weeks p.i.) began as areas of interstitial expansion and demarcated nodules, sometimes progressing to coalescing nodules. In the WT- and $\Delta mce3$ -infected mouse groups, the lesions progressed over time to become more diffuse

Fig. 3. Bacterial burdens of mouse organs infected with the *mce* mutants. Recovery of c.f.u. from the right lung at different time points p.i. (a), and recovery of c.f.u. at 105 days p.i. from the right lung [re-representation of (a) at 105 p.i.] (b), spleen (c) and liver (d) for WT, $\Delta mce3$, $\Delta mce4$ or $\Delta mce3/4$ from C57BL/6 mice after aerosol infection ($n=3$ mice per group per time point).

and less well demarcated. In the $\Delta mce4$ - and $\Delta mce3/4$ -infected groups, the lesions remained mostly as nodules and did not progress significantly. The inflammatory infiltrate in all four groups was similar and was predominantly a mixture of foamy and epithelioid macrophages intermingled with minimal numbers of

Fig. 4. Survival of mice infected with *mce* mutants. Survival of C57BL/6 mice after aerosol infection with WT and *mce* mutants; $n=8$ for WT, $n=8$ for $\Delta mce3$, $n=9$ for $\Delta mce4$ and $n=9$ for $\Delta mce3/4$ -infected groups.

neutrophils (Fig. 5b). Although there was a small but significant difference in the survival times between the WT- and $\Delta mce3$ -infected groups, no differences could be detected in the histopathological lesions between them.

DISCUSSION

This study found that the *mce3* and *mce4* operon mutants were attenuated in mice compared to WT *M. tuberculosis* H37Rv, as evidenced by the longer survival times of mice infected with the mutants. This observation suggests that the *in vivo* function of these operons is distinct from that of the *mce1* operon, whose mutation was previously shown to cause increased mortality in mice (Shimono *et al.*, 2003). It should be noted that the current study used C57BL/6 mice instead of BALB/c mice used by Shimono *et al.* (2003). Since C57BL/6 mice are relatively more resistant than BALB/c mice to *M. tuberculosis* infection, the differences in outcome could have been influenced by mouse species differences. However, a recent study showed that the *mce1* operon mutant has the same virulence phenotype in C57BL/6 mice as it does in BALB/c mice (Lima *et al.*, 2007).

The longer survival times of mice infected with $\Delta mce4$ suggest that the attenuation of $\Delta mce4$ is greater than that of $\Delta mce3$. This is also indicated by the differences in c.f.u. recovery at 15 weeks p.i. from mouse lungs (Fig. 3) and the corresponding histopathology (Fig. 5). At this time point, the above two parameters did not indicate any attenuation

Fig. 5. Histology of pulmonary lesions in infected mice. Sections of C57BL/6 mouse lungs magnified $\times 20$ (a) and $\times 200$ (b) stained with H&E. Bars, 300 μm (a) and 30 μm (b). Mouse lungs from all groups were harvested at 15 weeks p.i. All the mice were infected as in Fig. 3. The photographs were taken by a veterinary pathologist so as to represent each group. $\Delta mce4$ - and $\Delta mce3/4$ -infected mice had pulmonary lesions that were nodular and more contained, involving a smaller area of pulmonary parenchyma, than those of $\Delta mce3$ - and WT-infected mice, where the lesions were more diffuse and involved a larger percentage of the parenchyma.

of $\Delta mce3$ compared to WT in mice. However, the survival times of mice infected with $\Delta mce3$ were significantly longer than those of the mice infected with WT. There was also a small but significant difference between the survival times of $\Delta mce3/4$ - versus $\Delta mce4$ -infected mice. Therefore, it is possible that the greater attenuation of the double $mce3/4$ mutant is due to the combined effect of mutations in both $mce3$ and $mce4$ operons. However, the mouse survival, lung pathology and bacterial burden data suggest that the attenuation of $\Delta mce4$ in mice is much more prominent than that of $\Delta mce3$. Nevertheless, all of the above observations indicate that $mce3$ and $mce4$ operon mutants behave differently from the $mce1$ operon mutant in mice, suggesting that, despite similarity in gene sequences and arrangement, the functions of the $mce3$ and $mce4$ operons are distinct from that of the $mce1$ operon.

The decreased lung bacterial burden in mice infected with $\Delta mce4$ or $\Delta mce3/4$ mutants compared to that in mice infected with WT and $\Delta mce3$ at 15 weeks p.i. may be because either (1) the disruption of $mce4$ causes bacteria to replicate more slowly in host cells or (2) the $mce4$ operon mutant is killed more rapidly when the bacteria first encounter the host adaptive immune response. Since $\Delta mce4$ can replicate similarly to other strains in RAW macrophages (data not shown), it is unlikely that $\Delta mce4$ is more susceptible to the antibacterial activity inside the host cell. $\Delta mce4$ -infected mouse lung has less extensive granulomatous pneumonia (i.e. fewer immune cells). One explanation is that the $mce4$ operon-related products attract proinflammatory cells to the lung. However, as observed in RAW cells, the absence of the $mce4$ operon did not have any effect on the ability of *M. tuberculosis* to induce or suppress TNF- α , IL-6, IL-10, IL-12 and MCP-1 in *ex vivo*-infected macrophages. Thus the diminished proinflammatory cell response in lungs of mice infected with $\Delta mce4$ and $\Delta mce3/4$ and their reduced c.f.u. counts may be due to a decreased replicative ability of these strains under the host adaptive immune response.

Recently, Kumar *et al.* (2003) detected the expression of $mce1$, 3 and 4 operons in tubercle material collected from infected animals (guinea pigs and rabbits). These observations support our findings that mce operons other than $mce1$ are expressed during the disease state. In addition, Ahmad *et al.* (2004) demonstrated the expression of several $mce3$ genes during natural infection of humans infected with *M. tuberculosis*.

Our results support the findings of a recent study by Gioffre *et al.* (2005) that reported that the $mce3$ operon mutant was attenuated in mice. They did not examine $\Delta mce4$. Their survival study, however, was carried out only up to 20 weeks. Hence the long-term effect of their $\Delta mce3$ cannot be compared with our results. However, in that study, attenuation was observed only when mice were infected via the intratracheal route, and not when the intraperitoneal route was used. They suggested that the route of infection may make a difference in infection outcome. This study used a more physiologically relevant

aerosol route of infection. The route of infection (aerosol vs intravenous) with another mutant ($\Delta mce1R$) in mice, however, did not make any difference to clinical outcome or survival (Uchida *et al.*, 2007). Additionally, Joshi *et al.* (2006) studied the mice infected with mce mutants up to 100 days only, while we studied the survival of mice infected with $\Delta mce3$ and $\Delta mce4$ for >60 weeks. As with the study of Gioffre *et al.* (2005), we were unable to complement the $mce2$ and 3 operon mutants due to the large size of the deleted region.

In conclusion, the studies described in this paper on $mce3$ and $mce4$ operons suggest that despite their similarity with the $mce1$ operon in gene organization and sequences, the $mce3$ and $mce4$ operon-encoded proteins have a role markedly distinct from $mce1$ operon-encoded products.

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