Vaccine efficacy of an attenuated but persistent *Mycobacterium tuberculosis* *cysH* mutant

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The emergence of drug-resistant *Mycobacterium tuberculosis* strains and the widespread occurrence of AIDS demand newer and more efficient control of tuberculosis. The protective efficacy of the current *Mycobacterium bovis* bacille Calmette–Guérin (BCG) vaccine is highly variable. Therefore, development of an effective new vaccine has gained momentum in recent years. Recently, several *M. tuberculosis* mutants were tested as potential vaccine candidates in the mouse model of tuberculosis. However, only some of these mutants were able to generate protection equivalent to that of BCG in mice. This study reports the vaccine potential of an attenuated 5'-adenosine phosphosulfate reductase mutant ($\Delta cysH$) of *M. tuberculosis*. Immunization of mice with either BCG or $\Delta cysH$ followed by infection with the virulent *M. tuberculosis* Erdman strain demonstrated that $\Delta cysH$ can generate protection equivalent to that of the BCG vaccine.

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INTRODUCTION

It is estimated that two billion people worldwide are latently infected with *Mycobacterium tuberculosis*, the aetiological agent of tuberculosis (TB) (Dye *et al.*, 2002b), and that 2–23% of these latently infected persons will develop active disease at some point in their lifetime (Parrish *et al.*, 1998). A new vaccine against TB is needed, especially in the context of the lengthy drug treatment requirement, the proliferation of multidrug-resistant strains and the burgeoning HIV/AIDS pandemic (Dye *et al.*, 2002a). Currently, an attenuated strain of *Mycobacterium bovis* (bacille Calmette–Guérin; BCG) is used as a vaccine in most parts of the world. The BCG vaccine has a number of drawbacks, including the waning of protective immunity over time (Sambandamurthy & Jacobs, 2005), highly variable efficacy in adults (Fine, 1995) and poor safety in immunocompromised individuals (Armbruster *et al.*, 1990; Besnard *et al.*, 1993). Therefore, a new vaccine with improved efficacy and a greater margin of safety is urgently required.

Recently, several studies have described the vaccine potential of attenuated *M. tuberculosis* strains in the mouse model of tuberculosis. Most of these attenuated strains were gene-deleted mutants that were auxotrophic for various substrates and only some of these strains were able to generate protection equivalent to that of the BCG vaccine (Sambandamurthy & Jacobs, 2005). For example, the pantothenate ($\Delta panCD$), tryptophan ($\Delta trpD$) and proline ($\Delta proC$) auxotrophic strains generated an overall protection comparable to that generated by BCG (Sambandamurthy *et al.*, 2002; Smith *et al.*, 2001). However, when mice were immunized once with either the purine ($\Delta purC$), leucine ($\Delta leuD$) or lysine ($\Delta lysA$) auxotrophic strains, they did not confer protection comparable to that conferred by BCG (Hondalus *et al.*, 2000; Jackson *et al.*, 1999; Pavelka *et al.*, 2003). Here, we report an auxotrophic (for cysteine and methionine) *M. tuberculosis* strain ($\Delta cysH$), with a limited replicating capacity in mouse tissues, that generated protection equivalent to or slightly better than that generated by the BCG vaccine. Attenuation of the $\Delta cysH$ mutant in the mouse model of tuberculosis has been described previously (Senaratne *et al.*, 2006).

METHODS

Bacterial strains and culture conditions. *M. bovis* BCG–Pasteur (BCG-P), $\Delta cysH$ and *M. tuberculosis* Erdman were grown in

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Abbreviations: BCG, bacille Calmette–Guérin; H&E, haematoxylin and eosin; p.i., post-infection; TB, tuberculosis.

Middlebrook 7H9 broth containing 10% ADC (Becton Dickinson), 0.2% glycerol and 0.05% Tween 80 or on Middlebrook 7H10 agar containing OADC (Becton Dickinson), 0.5% glycerol and the antifungal agent cycloheximide ($100 \mu\text{g ml}^{-1}$) (Sigma-Aldrich). For the growth of ΔcysH , all of the above media were supplemented with 2 mM methionine. Our previous experiments have shown that the minimum concentration of methionine necessary for the replication of ΔcysH is 2 μM . The presence or absence of the above amounts (2 mM) of methionine on 7H10 agar plates made no difference to the recovery of c.f.u. of *M. tuberculosis* Erdman. Construction and Southern blot confirmation of the ΔcysH mutant have been described previously (Senaratne *et al.*, 2006).

Infection and vaccination of mice. Female C57BL/6 mice (Jackson Laboratories) were vaccinated subcutaneously with approximately 5×10^5 c.f.u. ΔcysH or BCG-P suspended in 0.2 ml PBS containing 0.05% Tween 80 (PBST). A control group of mice was mock-vaccinated with 0.2 ml PBST. Twelve weeks after vaccination, three mice from each of the vaccinated groups were sacrificed, and lungs, spleen and liver were harvested and homogenized in PBST. The number of tubercle bacilli in the corresponding organ homogenates was assessed by plating part of the homogenate on 7H11 agar (Difco) plates and c.f.u. were enumerated 21 days later. Within the limits of the level of detection (10 bacilli per organ), no bacteria were detected in the lungs or liver of either group of mice. However, on average, approximately 100 c.f.u. per spleen (SD <47) were detected in both groups of mice.

Sixteen weeks after vaccination, all of the mice (vaccinated or mock-vaccinated) were infected with *M. tuberculosis* Erdman via the inhalation route using the Inhalation Exposure System (Glas-Col). The inoculum doses in each group were assessed as described above by harvesting the right lung of three mice (from each group of mice) 24 h post-infection (p.i.). The dose of infection was approximately 1000 bacilli per lung for each infection. At 27 and 56 days p.i., the bacterial load of each organ (right lung, spleen and liver) was determined by c.f.u. enumeration as described above. Student's *t*-test was used to analyse the differences between c.f.u. counts obtained from different mouse groups. Differences were considered significant at the given *P* values.

Histopathology. Left lungs were fixed in formaldehyde and sectioned for histopathological analysis. Lung sections were either stained with haematoxylin and eosin (H&E) or stained for acid-fast bacilli (Histology Consultation Service, Everson, WA, USA). Lung sections from three mice per each group per time point were analysed by two veterinary pathologists at the University of California at Davis, USA.

RESULTS

Bacterial burden is significantly lower in ΔcysH - and BCG-vaccinated mice

After infection with *M. tuberculosis* Erdman, six mice were sacrificed from each infected group at two different time points (27 and 55 days) to analyse the bacterial burden in the mouse tissues. The number of c.f.u. recovered from all three organs of mock-vaccinated mice at both time points p.i. was significantly higher ($P < 0.02$) than the number of c.f.u. recovered from ΔcysH - or BCG-vaccinated mouse organs (Fig. 1). The mean number of c.f.u. recovered from BCG-vaccinated mouse livers at day 55 p.i. was 5825 (± 3412) and from the livers of ΔcysH -vaccinated mice was 4400 (± 787) ($P < 0.026$). However, all other c.f.u. recovery from ΔcysH -vaccinated and BCG-vaccinated mouse organs was not significantly different ($P > 0.075$). In summary, as seen in Fig. 1, the c.f.u. counts in the lungs, spleens and livers of the vaccinated mice were reduced by more than tenfold compared with the c.f.u. counts in mock-vaccinated control mice at 27 days p.i.

Lung histopathology in ΔcysH - and BCG-vaccinated mice

In addition to the bacterial burden, we used another virulence parameter, lung histopathology, to assess the

Fig. 1. Vaccine efficacy of ΔcysH assessed by bacterial burden in mouse organs. Results are shown as the bacterial burden of the right lung (a), spleen (b) and liver (c) of ΔcysH -, BCG- or mock-vaccinated mice infected with *M. tuberculosis* Erdman 16 weeks after vaccination. Mice were infected with aerosolized bacteria (~ 1000 bacilli); $n = 3$ mice per group per time point.

protective effect of $\Delta cysH$. Two pathologists independently analysed *M. tuberculosis* Erdman-infected lung sections of vaccinated and mock-vaccinated mice. All mice developed a granulomatous interstitial pneumonia, which progressed from areas of interstitial expansion and well-demarcated nodules to coalescing nodules and diffuse lesions over time. At 27 and 55 days, both pathologists found that the extent and severity of the inflammation in the lung sections from $\Delta cysH$ -vaccinated mice was similar to or less than the inflammation in the BCG-vaccinated mice. In contrast, they found that the lesions in the mock-vaccinated mice progressed more quickly. At both 27 and 55 days, the inflammation was more extensive and severe than in either group of vaccinated mice (Fig. 2). A Fites acid-fast stain on the lung sections demonstrated very low numbers of acid-fast bacilli in the $\Delta cysH$ - and BCG-vaccinated groups at both 27 and 55 days, and at 27 days, the numbers of acid-fast bacilli in the mock-vaccinated groups were much higher than those in the $\Delta cysH$ - and BCG-vaccinated groups (Table 1).

Thus vaccination with $\Delta cysH$ resulted in pulmonary lesions and a bacterial burden similar to or slightly less severe than

those of BCG-vaccinated mice and markedly less severe than those of mock-vaccinated animals (Figs 1 and 2; Table 1). Therefore, we concluded that immunization with $\Delta cysH$ induced a protective response that was equivalent to that of the BCG vaccine.

DISCUSSION

It is interesting to note that, in contrast to vaccinated mice, c.f.u. counts in the lungs and spleens of mock-vaccinated mice showed a considerable reduction in c.f.u. from 27 to 55 days p.i. (Fig. 1). This reduction in the number of bacteria may be due to the induction of a stronger immune reaction in the later phase of the infection in response to the large number of bacteria present in the early stage of the infection in mock-vaccinated mice. However, the pathological injury to lung tissues at 55 days p.i. was much higher in mock-vaccinated mice than in vaccinated mice (Fig. 2 and Table 1). The more pronounced pathological injury in mock-vaccinated mice may also be explained by the stronger immune reaction in the later stage of the infection in mock-vaccinated mice.

Fig. 2. Vaccination of mice with $\Delta cysH$ decreases lung pathology. Lung sections were harvested at 55 days p.i. and stained with H&E. (a) All groups had a granulomatous interstitial pneumonia. At 55 days p.i., the mock-vaccinated group had lesions that were predominantly diffuse, whereas the lesions in the BCG- and $\Delta cysH$ -vaccinated groups were more contained, with nodules, coalescing nodules and rare diffuse areas of inflammation. A larger percentage of the parenchyma was involved in the mock-vaccinated group compared with the BCG- and $\Delta cysH$ -vaccinated groups. (b) The inflammatory infiltrate in all groups was predominantly foamy, with epithelioid macrophages intermingled with minimal numbers of neutrophils. There were small amounts of necrosis at 55 days p.i. and the mock-vaccinated group had greater neutrophil infiltration and necrosis compared with the BCG- and $\Delta cysH$ -vaccinated groups. All of the mice were infected as in Fig. 1. Photographs were taken by a veterinary pathologist and are representative of each group at the given time point. Bars, 300 μ m (a); 15 μ m (b).

Table 1. Summary of pathology report of mouse lung histology at 27 and 55 days p.i.

Mouse lungs used for pathology analysis were from the same experiment as shown in Figs 1 and 2; values are the mean of measurements from three mice in each group. Four H&E-stained and two acid-fast stained sections from each mouse were analysed.

	Percentage of lung involved*		Necrosis†		Lesion distribution‡	
	27 days p.i.	55 days p.i.	27 days p.i.	55 days p.i.	27 days p.i.	55 days p.i.
BCG-vaccinated	20–35	40–60	0	0–0.5	1	2
$\Delta cysH$ -vaccinated	25–30	30–45	0	0	1	2
Mock-vaccinated	45–60	80–85	0.5–1.5	3	3	4

*Mean percentage of pulmonary parenchyma affected by a granulomatous pneumonia.

†Arbitrary values given by pathologists with increasing severity from 0 to 4.

‡The distribution of the lesions for each section of mouse lung was categorized as follows: 1, nodular with some coalescing nodules; 2, predominantly nodular and coalescing nodular and rarely diffuse; 3, nodular and coalescing nodular with some diffuse areas; 4, predominantly diffuse.

$\Delta cysH$ is defective in the production of 5'-adenosine phosphosulfate reductase, which is needed for the synthesis of cysteine and methionine (Senaratne *et al.*, 2006). As reported previously, $\Delta cysH$ remains virulent in immunocompromised $Rag1^{-/-}$ mice (Senaratne *et al.*, 2006); therefore, the use of the single deletion mutant $\Delta cysH$ as a vaccine candidate in its present form may not be warranted, due to its potential pathogenicity in immunocompromised individuals. However, the ability of $\Delta cysH$ to generate protection similar to that of BCG remains of immunological interest.

As mentioned above, several deletion mutant strains of *M. tuberculosis* that were auxotrophic for one substrate were tested previously as vaccine strains (Sambandamurthy & Jacobs, 2005); of all the auxotrophic strains evaluated, only *panCD*, *proC* and *trpD* mutants were able to confer protection similar to that of BCG (Sambandamurthy *et al.*, 2002; Smith *et al.*, 2001). It is interesting to note that the *panCD*, *proC* and *trpD* mutants had limited ability to replicate in mouse tissues (Sambandamurthy *et al.*, 2002; Smith *et al.*, 2001), whereas the replication of other auxotrophic mutants in mouse tissues was either negligible or nonexistent (Hondalus *et al.*, 2000; Jackson *et al.*, 1999; Pavelka *et al.*, 2003). Our previously published data demonstrated that $\Delta cysH$ has the ability to replicate in mouse tissues (Senaratne *et al.*, 2006), and in this study, we have shown that the level of protection provided by $\Delta cysH$ is similar to that of BCG. Therefore, the outcome of our $\Delta cysH$ vaccine experiment supports the hypothesis of Sambandamurthy & Jacobs (2005) that the capacity of live vaccine strains to generate long-term immunity in mice is related to their ability to undergo limited replication *in vivo*. Additionally, in a previous study, the persistence of several BCG substrains in mouse spleens was assessed to acquire information about their vaccine efficacy (Lugosi, 1992). In this study, we were able to detect both BCG and $\Delta cysH$ in mouse spleens 12 weeks after vaccination. In summary, it is possible that persistence of vaccine strains

through limited replication *in vivo* generates better protective immunity against *M. tuberculosis*.

In contrast, a recent finding showed that $\Delta lysA$ $\Delta panCD$ and $\Delta leuD$ $\Delta panCD$ mutants auxotrophic for two substrates did not replicate in mouse tissues, yet provided protection similar to that of BCG as a vaccine (Sambandamurthy *et al.*, 2005; Sampson *et al.*, 2004). It is not clear why vaccination with the $\Delta lysA$ and $\Delta leuD$ single mutants failed to reduce tissue pathology and organ burden relative to BCG (Hondalus *et al.*, 2000; Pavelka *et al.*, 2003), whereas the $\Delta lysA$ $\Delta panCD$ and $\Delta leuD$ $\Delta panCD$ triple mutant strains, generated by creating two additional mutations in *panCD* in the $\Delta lysA$ and $\Delta leuD$ single mutants, respectively, provided protection similar to that of BCG (Sambandamurthy *et al.*, 2005; Sampson *et al.*, 2004). One explanation may be that only certain types of *M. tuberculosis* mutants can induce a protective immune response. In this respect, the $\Delta cysH$ mutant falls into the category of $\Delta panCD$ and suggests that deletion of *lysA* or *leuD* in $\Delta cysH$ may generate a potential vaccine strain similar to the $\Delta lysA$ $\Delta panCD$ and $\Delta leuD$ $\Delta panCD$ strains. In addition, combination of these mutations may improve the overall safety profile of the resultant mutant, providing potential for use in immunocompromised individuals and newborns. These systematic approaches to TB vaccination studies may contribute to the knowledge necessary to generate an efficient vaccine against *M. tuberculosis*.

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