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Vaccine Efficacy of a Lysine Auxotroph of *Mycobacterium tuberculosis*

Martin S. Pavelka, Jr.,[1]† Bing Chen,[2] Cynthia L. Kelley,[3] Frank M. Collins,[3] and William R. Jacobs, Jr.[2]*

Howard Hughes Medical Institute[2] and Department of Microbiology and Immunology, Albert Einstein College of Medicine,[1] Bronx, New York 10461, and Laboratory of Mycobacterial Diseases, Center for Biologics Evaluation and Research, Food and Drug Administration, Bethesda, Maryland 20892[3]

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The in vivo growth phenotype and vaccine efficacy of a lysine auxotrophic mutant of *Mycobacterium tuberculosis* strain H37Rv are described. An immunization experiment using a mouse model with an aerosol challenge showed that two doses of the *M. tuberculosis* mutant were required to generate protection equivalent to that of the *Mycobacterium bovis* BCG vaccine.

Despite the existence of antimicrobial drugs and a widely used vaccine, *Mycobacterium tuberculosis* remains the primary cause of adult death due to a bacterial agent (3). The emergence of multidrug-resistant strains of *M. tuberculosis*, the variable efficacy of the current vaccine, *Mycobacterium bovis* bacillus Calmette-Guérin (BCG), and the human immunodeficiency virus pandemic have all contributed to a growing global tuberculosis problem.

Several studies have described the development of attenuated auxotrophic strains of BCG and/or *M. tuberculosis* (4, 6, 7, 9). All of these studies utilized single immunization protocols and demonstrated differences in the protective responses thus elicited. In this study, we describe the in vivo growth characteristics of a previously described lysine auxotroph of *M. tuberculosis* H37Rv and evaluate the vaccine potential of this mutant by a multiple immunization protocol in a mouse model of the human disease followed by an aerosol challenge.

The *M. tuberculosis* strains Erdman and mc^{2}3026 (Δ*lysA*::*res*) and *M. bovis* BCG were grown in Middlebrook 7H9 broth (Difco) supplemented with 0.05% Tween 80, 0.2% glycerol, and 1× ADS (0.5% bovine serum albumin, fraction V [Roche]; 0.2% dextrose; and 0.85% NaCl) or on Middlebrook 7H10 or 7H11 solid medium (Difco) supplemented with 0.2% glycerol and 10% oleic acid-albumin-dextrose-catalase (Becton Dickinson). Culture media for the lysine auxotroph were supplemented with 1 mg of L-lysine (for both liquid and solid media) per ml, and 0.05% Tween 80 was also added to the solid medium (8). Liquid cultures were grown in 490-cm^2 roller bottles (Corning) at 4 to 6 rpm. Plates were incubated for 3 to 6 weeks. All cultures were incubated at 37°C. Titrated stocks of the bacteria were stored at −80°C until needed and then were thawed and diluted appropriately in phosphate-buffered saline containing 0.05% Tween 80 (PBST). The bacterial suspensions were plated at the time of injection to confirm the number of viable bacteria. Mice were sacrificed at 24 h postinjection in order to compare the bacterial CFU recovered from the mice with the CFU in the suspensions at the time of injection. Thus, the bacterial counts reported at time zero actually represent the viable bacteria recovered from the mice at 24 h postinjection.

We tested the vaccine potential of the *M. tuberculosis* lysine auxotroph mc^{2}3026 in the mouse model by means of a virulent aerosol challenge. Female pathogen-free C57BL/6 mice (Jackson Laboratories, Bar Harbor, Maine) were vaccinated intravenously with ca. 10^6 CFU of the *M. tuberculosis* lysine auxotroph or BCG-Pasteur suspended in 0.2 ml of PBST. Mice vaccinated with mc^{2}3026 were revaccinated at 4-week intervals, and the numbers of viable organisms in the lungs and spleens were determined weekly throughout the vaccination period. Five mice were examined at each time point.

Immunized mice were challenged 3 months after the initial vaccination. A frozen aliquot of an *M. tuberculosis* Erdman stock was thawed and diluted in PBST to ca. 10^6 CFU/ml, and 10 ml was introduced into the nebulizer of a Middlebrook aerosol chamber (Glas-Col, Terre Haute, Ind.). The mice were exposed to the infectious aerosol for 30 min, inhaling 50 to 100 CFU into their lungs over this period. Five mice were sacrificed immediately after the challenge period and the lung homogenates were plated to check the amount of the challenge inoculum actually reaching the lungs. Groups of vaccinated and control mice were sacrificed 14, 28, and 42 days later, and the lung and spleen homogenates were plated to determine the number of viable CFU of *M. tuberculosis* Erdman present. Data were analyzed using Student's *t* test and an analysis of variance among several independent means with the In Stat Statistics program (GraphPad Software, San Diego, Calif.).

A preliminary experiment demonstrated that a single intravenous immunization of immunocompetent C57BL/6 mice with the *M. tuberculosis* mutant did not generate a significant protective response to the subsequent aerosol challenge with virulent *M. tuberculosis* Erdman. In this experiment, the *M. tuberculosis* auxotroph was rapidly cleared from the mice (Fig. 1A), and the single immunization with the auxotroph was insufficient for reducing the bacterial burden in the lungs and spleens relative to a single immunization with BCG (Fig. 1B).

The failure of the auxotroph to confer protection might have been due to the inability of the mutant to persist long enough

* Corresponding author. Mailing address: Department of Microbiology and Immunology, Albert Einstein College of Medicine, 1300 Morris Park Ave., Bronx, NY 10461. Phone: (718) 430-2888. Fax: (718) 518-0366. E-mail: jacobs@aecom.yu.edu.

† Present address: Department of Microbiology and Immunology, University of Rochester School of Medicine and Dentistry, Rochester, NY 14642.

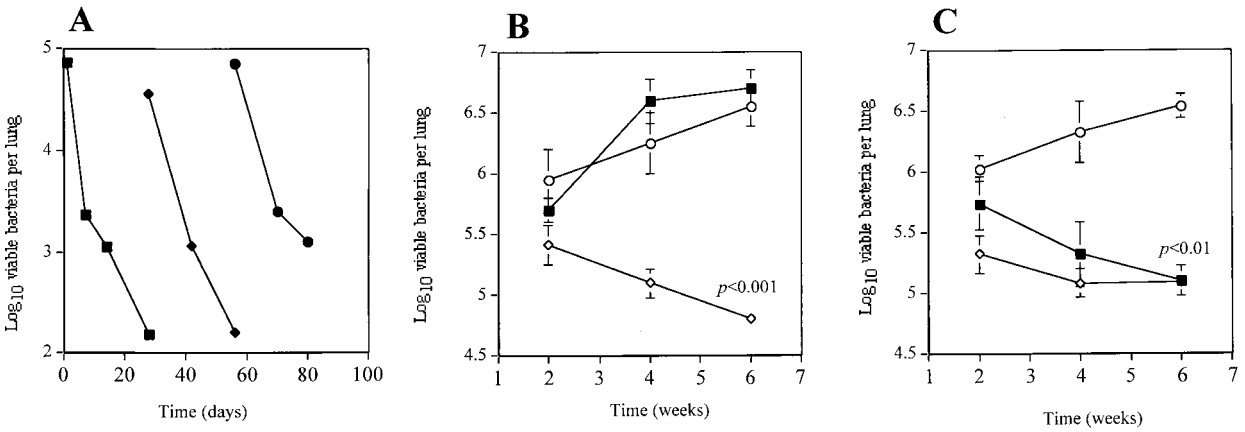

FIG. 1. Vaccine efficacy of the *M. tuberculosis* lysine auxotroph mc^{2}3026. C57BL/6 mice were injected intravenously with 10^6 CFU of the *M. tuberculosis* lysine auxotroph mc^{2}3026, followed by one or two additional injections at 4-week intervals. Five mice were sacrificed weekly after each immunization, and the viable bacteria counts of the auxotroph were determined for the lungs and spleens. Control mice were given a similar amount of BCG-Pasteur or PBST only. (A) Clearance of the auxotroph from the lungs of mice after each immunization period. Closed squares, one injection; closed diamonds, two injections; closed circles, three injections. Three months after the initial immunization the vaccinated and control mice were challenged with virulent *M. tuberculosis* Erdman by the aerosol route. Five mice were sacrificed following the challenge period, and the lung homogenates were plated to check the viable counts of the challenge inoculum. Groups of vaccinated and control mice were sacrificed 14, 28, and 42 days later, and the lung and spleen homogenates were plated to determine viable CFU. (B) Viable challenge bacteria per lung in mice given one dose of the *M. tuberculosis* lysine auxotroph. (C) Viable challenge bacteria per lung in mice given two doses of the auxotroph. Closed squares, viable challenge bacteria per lung in mice given the *M. tuberculosis* lysine auxotroph mc^{2}3026; open diamonds, viable challenge bacteria per lung in mice given BCG-Pasteur; open circles, viable challenge bacteria per lung in mice given PBST. *P* values are indicated in the figure. Note that the results shown here are for the lungs. Similar results (not shown) were obtained for the spleens in all the experiments.

or to synthesize enough antigen to induce an immune response that could significantly restrict the growth of the challenge organisms. One way to circumvent this problem is to give multiple doses of vaccine (2, 5). To this end, mice were intravenously immunized two or three times at 4-week intervals with the *M. tuberculosis* lysine auxotroph. In both cases, the vaccine strain was cleared from the lungs and spleens of all the mice at rates similar to that seen with the single immunization experiment (Fig. 1A). Three months after the first immunization, the mice were challenged with *M. tuberculosis* Erdman by the aerosol route and the bacterial counts in the lungs and spleens were determined and compared to a BCG-Pasteur-immunized control as well as to sham-immunized controls. As seen in Fig. 1C, double immunization with the *M. tuberculosis* lysine auxotroph induced a protective response that was equivalent to that of the BCG control. The reduction in counts in the lungs and spleens was equivalent to a 100-fold reduction in bacterial counts compared to the unvaccinated control (Fig. 1C). The results from the triple immunization experiment were essentially similar to those from the double immunization experiment described above (data not shown). Furthermore, mice that were immunized with three doses of the *M. tuberculosis* lysine auxotroph and challenged with virulent *M. tuberculosis* Erdman survived at least as long as the BCG-immunized control mice (Fig. 2).

Several studies have described the development and vaccine efficacy of attenuated mutant strains of *M. tuberculosis* (6, 7, 9). The first study reported that a purine auxotroph of *M. tuberculosis* was unable to grow in macrophages and was attenuated for growth in both mice and guinea pigs (7). A guinea pig vaccination experiment determined that a single immunization with the auxotroph allowed the animals to restrict the growth of virulent *M. tuberculosis* in the lungs as well as a single immunization with wild-type BCG after aerosol challenge. However, the reduction in growth of the challenge organism in the spleen afforded by vaccination with the auxotroph was not as extensive as that afforded by vaccination with BCG. Another

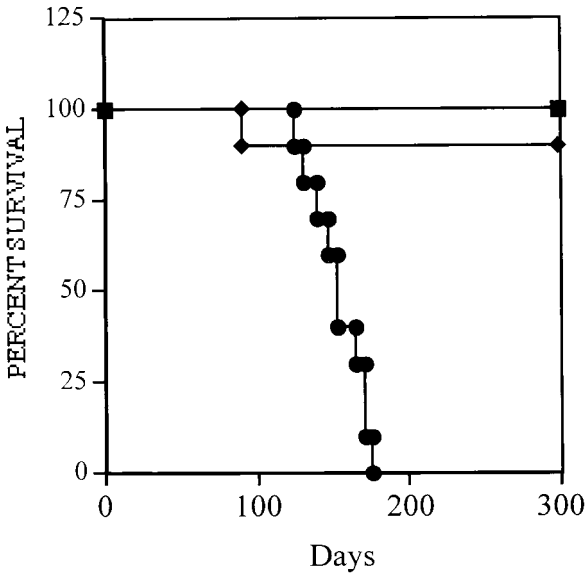

FIG. 2. Survival curves for mice immunized three times with the *M. tuberculosis* lysine auxotroph mc^{2}3026. C57BL/6 mice were injected intravenously with 10^6 CFU of the *M. tuberculosis* lysine auxotroph mc^{2}3026, followed by two more injections at 4-week intervals, and challenged as described in the text. The percent survival is shown for mice immunized thrice with the *M. tuberculosis* lysine auxotroph mc^{2}3026 (closed squares; 5 mice total), mice immunized once with BCG-Pasteur (closed diamonds; 5 mice), and PBST controls (closed circles; 10 mice).

study reported that a leucine auxotroph of *M. tuberculosis* Erdman cannot grow in macrophages and is avirulent in immunocompromised SCID mice (6). Immunocompetent mice vaccinated once with an *M. tuberculosis* leucine mutant did not significantly restrict the growth of the virulent challenge organism in the lungs or spleen as much as the control mice vaccinated with BCG (6). However, the mice immunized with the leucine auxotroph survived as long as the BCG-immunized controls and exhibited decreased histopathology relative to that seen in the nonimmunized controls (6). A third study showed that *M. tuberculosis* proline and tryptophan auxotrophs were attenuated and that a single immunization of mice with either of these mutants afforded protection against an intravenous challenge with virulent *M. tuberculosis* that was comparable to that afforded by vaccination with BCG, as indicated by the mean survival times (9). In those experiments, mice immunized with *pro* or *trp* mutants could restrict the growth of the challenge organisms to the same extent as mice immunized with BCG, although the magnitude of protection in either case (*M. tuberculosis* auxotrophs or BCG) was not as extensive as that seen in the other studies (9).

In the present study we have demonstrated that a single immunization of mice with the avirulent *M. tuberculosis* lysine auxotroph did not generate an immune response capable of significantly restricting the growth of virulent *M. tuberculosis* Erdman following an aerogenic challenge. However, administration of a second or a third dose of this vaccine increased protection substantially, as measured by the number of viable bacteria per organ, to a level similar to that achieved with a single dose of BCG-Pasteur. This level of protection did not seem to be greatly increased by a third dose of vaccine, although the triply immunized mice survived as long as the control mice immunized with a single dose of BCG-Pasteur. Mice that were immunized twice were not monitored to determine mean survival time, but upon comparing the growth curves of the challenge bacteria following the double and triple immunizations, it seems likely that the survival time for the doubly immunized mice would be much the same as that for the triply immunized mice.

We observed that the lysine auxotroph was cleared from the vaccinated C57BL/6 mice at the same rate, regardless of the number of times the mice had been vaccinated. One might expect that mice immunized three times might clear the auxotroph more quickly after the third injection than mice immunized only once. We believe that clearance in the C57BL/6 mice is as fast as possible, because we have observed the same clearance rate for the lysine auxotroph after injection into SCID mice (data not shown). If an immunocompetent C57BL/6 mouse cannot clear the lysine auxtroph any faster than an immunocompromised SCID mouse, then the rate of clearance in the C57BL/6 mouse must not be dependent on an immune function per se, but rather the result of the inability of the auxotroph to survive in the absence of lysine. It might be possible to increase the persistence of the *M. tuberculosis lysA* mutant by alteration of its metabolism with additional mutations.

The previous studies using *M. tuberculosis* auxotrophs as vaccine strains showed substantial variations in their effectiveness. This variability is likely to be due to a number of factors, including the different *M. tuberculosis* background strains used to construct the mutants, different mouse strains used in the various protection studies, and the different challenge organisms and challenge routes used. There was also considerable variation in the protective efficacy of the different vaccines compared to that observed in controls using BCG immunization. These differences pose a number of questions concerning the best indicators of protection, especially in the long term. Should viable bacterial counts or survival be the primary indicator of protection or should both be given equal weight? The results of this study indicate that more than one immunization with an *M. tuberculosis* lysine auxotroph did generate a significant protective response as indicated by both criteria. We believe it is important that multiple immunization protocols be considered in the further development of attenuated *M. tuberculosis* strains as potential human vaccines.

It is known that the effectiveness of BCG immunization decreases with time and that there is no booster effect with BCG. This may be due to the inability of the booster BCG vaccines to replicate in an immunized host. It has been suggested that this inability to boost could be overcome by using a booster vaccine that does not depend upon replication (10). This has recently been successfully tested in the mouse model, using BCG as the primary immunization and a protein subunit booster vaccine containing mycolyl transferase A (Ag85A) of *M. tuberculosis* (1). Since the *M. tuberculosis lysA* mutant does not replicate in the mouse, it could be used as a booster vaccine with which to augment childhood BCG immunization.

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REFERENCES

1. **Brooks, J. V., A. A. Frank, M. A. Keen, J. T. Belisle, and I. M. Orme.** 2001. Boosting vaccine for tuberculosis. Infect. Immun. **69:**2714–2717.
2. **Collins, F. M.** 1991. Antituberculous immunity: new solutions to an old problem. Rev. Infect. Dis. **13:**940–950.
3. **Dolin, P. J., M. C. Raviglione, and A. Kochi.** 1994. Global tuberculosis incidence and mortality during 1990–2000. Bull. W. H. O. **72:**213–220.
4. **Guleria, I., R. Teitelbaum, R. A. McAdam, G. Kalpana, W. R. Jacobs, Jr., and B. R. Bloom.** 1996. Auxotrophic vaccines for tuberculosis. Nat. Med. **2:** 334–337.
5. **Homchampa, P., R. A. Strugnell, and B. Adler.** 1992. Molecular analysis of the *aroA* gene of *Pasteurella multocida* and vaccine potential of a constructed *aroA* mutant. Mol. Microbiol. **6:**3585–3593.
6. **Hondalus, M. K., S. Bardarov, R. Russell, J. Chan, W. R. Jacobs, Jr., and B. R. Bloom.** 2000. Attenuation of and protection induced by a leucine auxotroph of *Mycobacterium tuberculosis*. Infect. Immun. **68:**2888–2898.
7. **Jackson, M., S. W. Phalen, M. Lagranderie, D. Ensergueix, P. Chavarot, G. Marchal, D. N. McMurray, B. Gicquel, and C. Guilhot.** 1999. Persistence and protective efficacy of a *Mycobacterium tuberculosis* auxotroph vaccine. Infect. Immun. **67:**2867–2873.
8. **Pavelka, M. S., Jr., and W. R. Jacobs, Jr.** 1999. Comparison of the construction of unmarked deletion mutations in *Mycobacterium smegmatis*, *Mycobacterium bovis* bacillus Calmette-Guerin, and *Mycobacterium tuberculosis* H37Rv by allelic exchange. J. Bacteriol. **181:**4780–4789.
9. **Smith, D. A., T. Parish, N. G. Stoker, and G. J. Bancroft.** 2001. Characterization of auxotrophic mutants of *Mycobacterium tuberculosis* and their potential as vaccine strains. Infect. Immun. **69:**1142–1150.
10. **von Reyn, C. F., and J. M. Vuola.** 2002. New vaccines for the prevention of tuberculosis. Clin. Infect. Dis. **35:**465–474.

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