A New Vaccine against Tuberculosis Affords Greater Survival after Challenge than the Current Vaccine in the Guinea Pig Model of Pulmonary Tuberculosis

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Tuberculosis (TB) remains an enormous global health problem, and a new vaccine against TB more potent than the current inadequate vaccine, Mycobacterium bovis BCG, is urgently needed. We describe a recombinant BCG vaccine (rBCG30) expressing and secreting the 30-kDa major secretory protein of Mycobacterium tuberculosis, the primary causative agent of tuberculosis, compound the problem (8, 14). A better vaccine against tuberculosis is urgently needed (11, 23). The current vaccine, Mycobacterium bovis BCG, a live attenuated vaccine derived from the bovine tuberculosis bacillus in the early 1900s by Calmette and Guérin, while protective against disseminated forms of tuberculosis such as meningitis and miliary tuberculosis, is of inconsequential efficacy against pulmonary tuberculosis, the dominant form (9, 12).

In a previous study (20), two recombinant BCG vaccines (rBCG30) overexpressing the major secretory protein of M. tuberculosis, a 30-kDa mycolyl transferase (2, 27), were described. This protein is not only the major secretory protein of M. tuberculosis in broth culture (19) but it is also among the major proteins of all M. tuberculosis proteins expressed in human macrophages (15, 21). Derived from the commercially available Connaught (Conn) and Tice strains of BCG, the recombinant vaccines were tested in the demanding guinea pig model of pulmonary tuberculosis. In contrast to other small animals used for models of tuberculosis, guinea pigs are much more susceptible to tuberculosis than humans, yet they develop disease that closely mimics human disease clinically, immuno-logicaly, and pathologically. Guinea pigs immunized with the recombinant vaccines and then challenged by aerosol with a high dose of the highly virulent Erdman strain of M. tuberculosis had 0.5 log fewer M. tuberculosis bacteria in their lungs and 1 log fewer bacteria in their spleens 10 weeks after challenge than guinea pigs immunized with the parental BCG vaccines. Differences that statistically were highly significant. Moreover, the rBCG30-immunized animals had significantly less lung pathology and fewer tubercles in their lungs, livers, and spleens than the animals immunized with the parental BCG vaccines. However, aside from these laboratory and pathological findings, a superior effect of the recombinant vaccines on the well-being of the animals was not demonstrated.

In the present study, we examine the impact of recombinant vaccines on the survival of guinea pigs challenged with M. tuberculosis. We demonstrate that animals immunized with the recombinant rBCG30 vaccines survive significantly longer than animals immunized with the parental conventional BCG vaccines after aerosol challenge with virulent M. tuberculosis. We also show that the recombinant and parental vaccines initially multiply in guinea pig lungs, spleens, and regional lymph nodes and are subsequently cleared from these tissues at the same rate, although, interestingly, low levels of organisms persist for at least a half of a year after immunization.

MATERIALS AND METHODS

Strains. The M. bovis BCG strains Conn (Connaught Laboratories), Tice (Organon), Copenhagen (ATCC 27290), Glaxo (ATCC 35741), Japanese (ATCC 35737), and Pasteur (ATCC 35734) and the M. tuberculosis Erdman strain (ATCC 35801) were used.

The following strains were used for bacterial mating experiments: Escherichia coli strains DH5α [recA1 endA1 gyrA96 thi-1 hsdR17 (mcrB mcrA) supE44 relA1 lacZΔM15 lacY1 Δ(lac-proA169 smg-6 araD1399 strA802 mcrA trpC2 Δ(102A) lacZΔM15 teeA169 Δ(gif61) prom1)]. They were maintained by transformation with pMC9 Amp' Tet'. pMC9 Amp' Tet' was constructed by ligating the BglII–HindIII fragment containing the pMC9 promoter and the Tetramycin resistance gene amplified from Tetramycin-resistant E. coli strain. The fragment was ligated into pUC18 and then transformed into E. coli DH5α. The transforming DNA was isolated and maintained in E. coli strains DH5α and XL1-Blue [recA1 endA1 gyrA96 thi-1 hsdR17 (mcrB mcrA) supE44 relA1 lacZΔM15 lacY1 Δ(lac-proA169 smg-6 araD1399 strA802 mcrA trpC2 Δ(102A) lacZΔM15 teeA169 Δ(gif61) prom1)].

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FIG. 1. Plasmid pMTB30. Derived from the mycobacterium E. coli shuttle vector pSMT3 (13), the plasmid contains a full-length copy of the M. tuberculosis 30-kDa major secretory protein gene and flanking regions, including the 5' promoter region. The insert is placed into the HindIII and BamHI restriction enzyme sites in the pSMT3 vector in an orientation opposite that of the vector-encoded promoter of heat shock protein 60 (15).

Guinea pig immunization. Specific-pathogen-free 250- to 300-g outbred male Hartley strain guinea pigs from Charles River Breeding Laboratories were injected intradermally with 10^7 CFU of parental BCG or rBCG30 Conn or Tice Hartley strain guinea pigs from Charles River Breeding Laboratories were infected intradermally with 10^7 CFU of parental BCG or rBCG30 Conn or Tice Hartley strain guinea pigs from Charles River Breeding Laboratories were infected intradermally with 10^7 CFU of parental BCG or rBCG30 Conn or Tice Hartley strain guinea pigs from Charles River Breeding Laboratories were infected intradermally with 10^7 CFU of parental BCG or rBCG30 Conn or Tice Hartley strain guinea pigs from Charles River Breeding Laboratories were infected intradermally with 10^7 CFU of parental BCG or rBCG30 Conn or Tice Hartley strain guinea pigs from Charles River Breeding Laboratories were infected intradermally with 10^7 CFU of parental BCG or rBCG30 Conn or Tice Hartley strain guinea pigs from Charles River Breeding Laboratories were infected intradermally with 10^7 CFU of parental BCG or rBCG30 Conn or Tice.
tested by the UCLA Clinical Microbiology Laboratory (Microbiology Reference Laboratory, Cypress, Calif.) for sensitivity to the following antibiotics (concentrations are given in parentheses): ethambutol (2.5 mg/ml), isoniazid (0.1 and 0.4 mg/ml), pyrazinamide (100 mg/ml), rifampin (2.0 mg/ml), streptomycin (2.0 mg/ml), ethionamide (5.0 mg/ml), capreomycin (5.0 mg/ml), ciprofloxacin (2.0 mg/ml), amikacin (5.0 mg/ml), and p-aminosalicylic acid (4.0 or 5.0 mg/ml).
rBCG30 Conn strain ($P = 0.007$). By 41 weeks after challenge, by which time all of the sham- and parental BCG-immunized animals had died, only 4.8% of the rBCG30 Conn-immunized animals remained alive, whereas 35% of the rBCG30 Tice-immunized animals remained alive. Beginning at 42 weeks after challenge, the uninfected control animals began to die off. At this time point, 35% of the rBCG30 Tice-immunized animals remained alive. By the time all of the rBCG30 Tice-immunized animals had died, half of the uninfected controls had also died. Although the death rate after 41 weeks of the cohort of rBCG30 Tice-immunized animals that survived to this point was somewhat greater than that of the uninfected controls, the recombinant vaccine allowed this cohort of infected animals to nearly reach full life expectancy under laboratory conditions.

Virulence of the vaccine. Preliminary to human trials of the rBCG30 Tice strain, we examined the virulence of this strain in the guinea pig model in comparison with the parental BCG Tice vaccine, which has a well-established safety profile in humans. First, we examined the effect of the vaccine on the general health status of the animals, including weight gain. In guinea pigs immunized intradermally with $10^7$ CFU of the recombinant and parental strains, no adverse health effects were observed for either vaccine, and animals in both groups gained weight at the normal rate, including during the first 10 weeks after immunization, when bacterial levels in animal organs were highest (Fig. 4a). Second, we investigated the capacity of guinea pigs to clear the vaccine strains. Guinea pigs immunized with either the parental or recombinant vaccine were euthanized at various intervals after challenge, after which the lungs, spleens, and regional (inguinal) lymph nodes were assayed for CFU of BCG or rBCG30. In animals immunized with either strain, CFU peaked at 3 weeks after immunization in all three tissue sites and then rapidly declined to
levels near zero by 10 weeks (Fig. 4b). Both the parental and recombinant vaccines were cleared at the same rate. Extensive culturing of specimens (50% of the organ homogenate on 20 agar plates) obtained 10 weeks or later after immunization revealed low levels of persistent organisms at all three sites (Fig. 4b, inserts). rBCG30 isolated from the three sites (50 colonies per site) at 6 weeks after immunization were hygromycin resistant, indicating that the recombinant bacteria retain the pMTB30 plasmid in vivo in the absence of selective pressure just as they do when subcultured monthly for at least 1 year in broth medium lacking hygromycin. These studies demonstrated that the recombinant and parental Tice strains are comparably avirulent in an animal that is highly susceptible to mycobacterial infection.

**Additional safety studies.** In preparation for human trials of the rBCG30 Tice vaccine, we have performed several additional studies of potential relevance to the safety of the vaccine in humans, as follows.

(i) Assay for self-transmissibility of plasmid pMTB30. We first investigated whether the plasmid pMTB30 was capable of self-transmissibility from one bacterium to another and especially from the vaccine strain to other bacteria. We tested the self-transmissibility of plasmid pMTB30 (Hygr) from (i) the recombinant *E. coli* strain DH5α to *E. coli* XL1-Blue, (ii) the recombinant *M. bovis* rBCG30 Tice strain to the *E. coli* strains DH5α and Y1090 (Tet r), and (iii) the recombinant *M. bovis* rBCG30 Tice strain to the kanamycin-resistant mycobacteria *M. smegmatis* 1-2c [sodA::Tn5(Kanr)] and *M. tuberculosis* Erdman [glnA1::Tn5(Kanr)]. We observed no hygromycin (250 μg/ml)- or hygromycin (250 μg/ml)- and tetracycline (12.5 μg/ml)-resistant *E. coli* colonies, no hygromycin (50 μg/ml)- and kanamycin (20 μg/ml)-resistant *M. smegmatis*, and no hygromycin (50 μg/ml)- and kanamycin (20 μg/ml)-resistant *M. tuberculosis* (M. tuberculosis plates were also supplemented with 20 mM L-glutamine to compensate for the glutamine synthetase deficiency). In a parallel series of experiments, we tested the self-transmissibility of the *E. coli*-mycobacterium shuttle plasmid pVK173T. We replicated the mating experiments described for pMTB30, and again we observed no apramycin (50 μg/ml)- or apramycin (50 μg/ml)- and tetracycline (12.5 μg/ml)-resistant *E. coli* colonies and no apramycin (30 μg/ml)- and kanamycin (20 μg/ml)-resistant *M. tuberculosis* (M. tuberculosis plates were also supplemented with 20 mM L-glutamine to compensate for the glutamine synthetase deficiency). In a parallel series of experiments, we tested the self-transmissibility of the *E. coli*-mycobacterium shuttle plasmid pVK173T. We replicated the mating experiments described for pMTB30, and again we observed no apramycin (50 μg/ml)- or apramycin (50 μg/ml)- and tetracycline (12.5 μg/ml)-resistant *E. coli* colonies and no apramycin (30 μg/ml)- and kanamycin (20 μg/ml)-resistant mycobacterial colonies.

In a set of control experiments, we assayed the transfer of a conjugative plasmid (pLC28StrpCamr) between *E. coli* strains under the same conditions. We mixed *E. coli* S17 (harboring the plasmid) with *E. coli* SM10 (Strp Camr) and screened for streptomycin- and chloramphenicol (both at 30 μg/ml)-resistant exconjugants. Exconjugates were obtained at a frequency of 9.5 × 10⁻⁶.

These studies demonstrated that plasmid pMTB30 is not self-transmissible to *E. coli* or mycobacteria.

(ii) Assay for mobilization of plasmid pMTB30. Although the above studies showed that plasmid pMTB30 was not self-transmissible, it remained possible that the plasmid could be

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**FIG. 4. Parental and recombinant BCG Tice strains are comparably avirulent in guinea pigs.** (a) Weight gain after immunization. Guinea pigs were immunized intradermally with 10⁵ CFU of BCG or rBCG30 Tice and weighed weekly. Both groups (*n* = 12/group) gained weight normally after immunization. (b) Clearance of vaccine strains. Guinea pigs were immunized intradermally as described for panel a and then euthanized 2 to 26 weeks later (*n* = 3/group/timepoint), as indicated, and CFUs per organ were assayed in the right lung, spleen, and inguinal lymph nodes. The insets show total CFU per organ at 10 to 26 weeks after immunization plotted against a vertical scale appropriate for low bacterial counts. SE, standard error.
mobilizable by a helper plasmid and thus transferred to another bacterial species. We tested this possibility by first introducing pMTB30 into E. coli S17 harboring the conjugal plasmid pLC28Strp’Cam’ and then mating this strain with E. coli SM10 (Strp’ Cam’). No streptomycin (30 µg/ml)- and hygromycin (250 µg/ml)-resistant exconjugants were obtained. More importantly, when this same E. coli S17 strain was mated with the mycobacterial strains M. smegmatis 1-2c [sodA::Tn5 (Kan’)] and M. tuberculosis Erdman [glnA1::Tn5(Kan’)], no hygromycin (50 µg/ml)- and kanamycin (20 µg/ml)-resistant mycobacterial colonies were obtained (M. tuberculosis Erdman plates were again supplemented with 20 mM l-glutamine). As a positive control, we introduced the mobilizable E. coli-mycobacterium shuttle plasmid pVK173T into E. coli S17 (containing pLC28Strp’Cam’) and then mated this strain with the mycobacterial strains M. smegmatis 1-2c [sodA::Tn5(Kan’)] and M. tuberculosis Erdman [glnA1::Tn5(Kan’)]. These matings resulted in hygromycin (50 µg/ml)-, apramycin (30 µg/ml)-, and kanamycin (20 µg/ml)-resistant M. smegmatis colonies at a frequency of 7.7 × 10^{-7} and M. tuberculosis colonies with the same resistance markers at a frequency of 1.3 × 10^{-7}, demonstrating that this plasmid can be mobilized to other mycobacteria.

Although these studies showed that pMTB30 was not mobilizable with an E. coli-based helper plasmid, it remained possible that the plasmid was mobilizable to other mycobacteria with a mycobacterial helper plasmid. We investigated this possibility by introducing plasmid pGB9(Kan’) into the wild-type strains M. smegmatis 1-2c and M. tuberculosis Erdman and then mating these strains with the recombinant rBCG30 Tice[pMTB30(Hyg’)]. In both cases, hygromycin (50 µg/ml)- and kanamycin (20 µg/ml)-resistant exconjugant BCG Tice colonies were obtained at frequencies of ~8.0 × 10^{-7}. However, when recombinant BCG Tice[pMTB30(Hyg’), pGB9 (Kan’)] bacteria were mixed with M. smegmatis 1-2c or M. tuberculosis Erdman wild-type bacteria, no hygromycin (50 µg/ml)- and kanamycin (20 µg/ml)-resistant colonies were obtained, except, of course, for the donor bacteria. (M. smegmatis is differentiated from BCG and M. tuberculosis by its much faster growth [visible colonies after an incubation period of 3 to 4 days versus 10 to 12 days for BCG and M. tuberculosis], and BCG is differentiated from M. tuberculosis by replating the bacteria on plates containing 50 µg of 2-thiophene carboxylic acid hydrazide/ml, which allows growth of only M. tuberculosis.) Thus, pGB9 is self-transmissible but cannot mobilize pMTB30 into mycobacteria.

These studies showed that pMTB30 cannot be mobilized with the help of a conjugal plasmid, a not-unexpected result, since the sequence of pMTB30 lacks both a mob locus and an oriT element.

(iii) Assay for pMTB30 expression in a nonmycobacterium.

To determine if the plasmid pMTB30 could be expressed in a nonmycobacterium, we transformed the plasmid by electroporation into E. coli DH5α, cultured the transformed organism in the presence of hygromycin to maintain the plasmid, and assayed expression of the 30-kDa protein in both the bacterial pellet and the culture medium by immunoblot analysis by using polyvalent mycolyl transferase-specific antibodies. The antibodies did not react with either of the E. coli-derived protein fractions, demonstrating that E. coli cannot express the 30-kDa protein gene as contained in the plasmid pMTB30. In contrast, the antibodies reacted strongly with both the recombinant protein expressed in rBCG30 Tice and the purified 30-kDa protein (r30). This analysis showed that even when pMTB30 is forced into E. coli by electroporation, it does not express the M. tuberculosis 30-kDa major secretory protein, a result consistent with nonrecognition of the mycobacterial promoter by E. coli.

(iv) Antibiotic susceptibility. Both parent BCG strains and recombinant BCG strains were resistant to pyrazinamide, as is typical of M. bovis. All were sensitive to the other nine antibiotics tested. However, the recombinant rBCG30 Tice strain was slightly less sensitive to isoniazid (resistant to a concentration of 0.1 µg/ml but sensitive to a concentration of 0.4 µg/ml) than the parental BCG Tice, the recombinant rBCG30 Conn, and the parental BCG Conn (all sensitive to concentrations of both 0.1 and 0.4 µg/ml). Possibly, the rBCG30 Tice strain’s much greater expression of the 30-kDa mycolyl transferase, a protein involved in the synthesis of the cell wall component trehalose dimycolate, rendered this strain slightly more resistant to isoniazid, an inhibitor of mycolic acid synthesis.

DISCUSSION

This study demonstrates that the recombinant rBCG30 vaccines enhance survival after M. tuberculosis challenge. The magnitude of both protection and the cutaneous DTH response to r30 was correlated with the amount of the 30-kDa protein produced by the vaccine strain, i.e., animals immunized with the higher-producing rBCG30 Tice strain survived longer and had a greater DTH response than animals immunized with the lower-producing rBCG30 Conn strain, and the rBCG30 vaccines induced greater survival and DTH than the parental BCG strains, which produced the least amount of 30-kDa major secretory protein.

The clear superiority of the higher-producing rBCG30 Tice strain compared with the lower-producing rBCG30 Conn strain in this study was not demonstrated in the predecessor study in which the two recombinant vaccines induced comparable reductions in bacterial load in the lungs and spleen (20). Hence, the assessment of survival but not bacterial load after immunization and challenge allowed for differentiation between the vaccines in potency. However, even in the predecessor study, the rBCG30 Tice-immunized animals had less organ pathology than the rBCG30 Conn-immunized animals.

Many different strains of BCG have been used as vaccines throughout the world, reflecting a lack of evidence that any one of them is superior to any other. Colditz et al. (9), in their meta-analysis of the published literature on BCG trials, investigated the impact of BCG pedigree on between-study variability and found no impact. They additionally concluded that trials with Mycobacterium microti (vole bacillus), a strain less closely related to M. tuberculosis than M. bovis BCG, gave protection equal to that of BCG. Recently, the Tice and Conn BCG strains were compared for immunogenicity and reactogenicity in a human clinical trial, and no difference was detected between them (18). Similarly, in this and our previous study, no significant difference in efficacy was observed between the parental BCG Conn and Tice strains. These and four other BCG strains secreted comparably low amounts of the 30-kDa protein.
This study lends further support to the extracellular protein hypothesis for vaccines against intracellular pathogens, which holds that proteins secreted or otherwise released into the intracellular compartment of such pathogens are potentially potent immunoprotective molecules (3–6, 16, 19, 20, 25). Such proteins are available for processing and presentation to the immune system as major histocompatibility complex-peptide complexes on host cells. These complexes serve to alert the host immune system to the presence of a pathogen sequestered within the host cell, allowing it to mount an appropriate antimi-
brobial response. As in previous studies (3–5), in which immunization with either of two major extracellular proteins of Legionella pneumophila, the agent of Legionnaires’ disease, was demonstrated to enhance the survival of guinea pigs after challenge by aerosol with L. pneumophila, this study shows that immunization with a major extracellular protein of M. tuberculosis, albeit via a live vector rather than as a protein in an adjuvant, enhances survival after challenge by aerosol with M. tuberculosis in a highly susceptible animal model.

Prior to this study, an alternative explanation for the enhanced efficacy of the recombinant BCG vaccine compared with that of the parental BCG vaccine was that the recombi-
nant vaccine was more virulent in the guinea pig model and, for this reason, it induced a stronger immune response. How-
ever, the parental and recombinant BCG vaccines were found to be equally avirulent for guinea pigs, ruling out this possi-
bility.

Of the small-animal models of tuberculosis, the guinea pig model most closely resembles human disease. Like humans, but unlike mice and rats, guinea pigs are susceptible to low doses of aerosolized M. tuberculosis, exhibit a high sensitivity to tuberculosis, develop a cutaneous DTH response characterized by a dense mononuclear cell infiltrate, and exhibit Langhans giant cells and ccaseation in their lung lesions. A major differ-
ence between humans and guinea pigs, however, is the suscep-
tibility to disease after infection with M. tuberculosis. Whereas only about 10% of humans develop active disease after infection, some soon after and some after a period of latency, 100% of guinea pigs develop active rapidly progressive disease to which they quickly succumb. The high susceptibility of guinea pigs to the development of active rapidly fatal disease renders this model especially suitable for assessing the capacity of a vaccine against tuberculosis to enhance survival after M. tuber-
culosus challenge.

Assessing survival in guinea pigs after respiratory challenge with M. tuberculosis is a time-consuming and expensive under-
taking. While not often done, our study is not the first to do so. In a study conducted over three decades ago, Wiegshaes et al. (26), using experimental conditions very different from ours (lower challenge dose, less-virulent challenge strain, shorter immunization-challenge interval, and different housing condi-
tions, etc.), found that guinea pigs immunized with the BCG vaccine survived significantly longer than sham-immunized an-
imals after aerosol challenge with M. tuberculosis, which was similar to our results with the parental BCG vaccines. Coincidingly, the BCG vaccine studied by Wiegshaes et al. origi-
nated at Tice Laboratory at the University of Illinois, as did one of our parental vaccines.

Thus far, our studies of the rBCG30 Tice vaccine have dem-
strated that, in the highly susceptible guinea pig model of pulmonary tuberculosis, immunization with the vaccine results in a marked reduction in bacterial burden, number of tuber-
cles, and pathology in the lungs and other organs, and in enhanced survival—all to an extent greater than that achieved by immunization with parental BCG Tice. To what extent this effect of the rBCG30 vaccine will translate to humans and whether the vaccine will have an impact on reactivation tuber-
culosus remains to be determined in forthcoming clinical stud-
ies. However, given the relatively high innate resistance of even unvaccinated humans to M. tuberculosis, it seems reasonable to postulate that an immunologic intervention such as the rBCG30 Tice vaccine that is likely to shift the balance of power between the host and pathogen further toward the host will have a beneficial impact on human disease incidence. Even a modest reduction in disease incidence would translate into hundreds of thousands of lives saved.

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tory protein of Legionella induces humoral and cell-mediated immune re-


