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A novel live recombinant mycobacterial vaccine against bovine tuberculosis more potent than BCG

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Abstract

Mycobacterium bovis infection of cattle and other domesticated animals exacts a significant economic toll in both economically developing and industrialized countries. Vaccination of herds and/or wild animals that share their grazing land and serve as reservoirs of infection has been proposed as a strategy to combat bovine tuberculosis. However, the only currently available vaccine, *M. bovis* BCG, is not highly efficacious. Here we show that a live recombinant vaccine, rBCG30, which expresses large amounts of the *Mycobacterium tuberculosis* 30 kDa major secretory protein, is more efficacious against bovine tuberculosis than BCG in the highly demanding guinea pig model of pulmonary tuberculosis. Compared with the parental wild-type BCG strain, rBCG30 administered intradermally induced significantly greater cell-mediated and humoral immune responses against the 30 kDa protein, as determined by measuring cutaneous delayed-type hypersensitivity and antibody titers. As for potency, in three independent experiments, rBCG30 induced greater protective immunity than BCG against aerosol challenge with a highly virulent strain of *M. bovis*, reducing the burden of *M. bovis* by 0.4 ± 0.2 log colony-forming units (CFU) in the lung ($P < 0.05$) and by 1.1 ± 0.4 log CFU in the spleen ($P = 0.0005$) below the level in BCG-immunized animals. A recombinant BCG vaccine overexpressing the identical *M. bovis* 30 kDa protein, rBCG30Mb, also induced greater cell-mediated and humoral immunity against the 30 kDa protein than BCG and greater protective immunity against *M. bovis* challenge; however, its potency was not significantly different from rBCG30. As rBCG30 is significantly more potent than BCG against *M. bovis* challenge, it has potential as a vaccine against bovine tuberculosis in domesticated animals and in wild animal reservoirs.

1.   Introduction

Tuberculosis in humans caused by *Mycobacterium bovis*, the agent of tuberculosis in cattle, has been reduced to low levels in industrialized countries as a result of milk pasteurization and animal tuberculosis control programs. However, in economically developing countries, where pasteurization is only sporadically practiced and animal tuberculosis control programs are frequently suboptimal or nonexistent, bovine (i.e. *M. bovis* - caused) tuberculosis in humans remains a substantial public health problem [1].

M. bovis infection of domesticated animals, especially cattle, exacts a significant economic toll in both developing and industrialized countries. *M. bovis* has one of the broadest host ranges of all pathogens; it has been found in over 20 animal species [2]. In cattle, the infection reduces milk production, the food value of the meat, and fertility; the economic losses have been conservatively estimated to exceed $3 billion annually [3]. Control measures, where they can be afforded, require extensive testing of herds and slaughter of infected animals to prevent bovine-to-human transmission [3]. Complicating the control of *M. bovis* infection, wild animals infected with *M. bovis* frequently serve as reservoirs for infecting the domesticated animals. Such wild animals include the badger in the United Kingdom and Ireland, the bushtail possum in New Zealand, the white-tailed deer in the United States, the marsh antelope in Zambia, and the African buffalo in South Africa [2, 4-7].

Vaccination of herds and/or wild animals that share their grazing land has been proposed as a viable strategy for augmenting the control of tuberculosis in cattle [1,8]. Vaccination of herds may be a particularly attractive option in settings where the culling of animals presents an economic hardship. Vaccination of wild animal reservoirs may be a more effective control measure, and one more acceptable to animal conservationists, than culling these animals to control tuberculosis in cattle. Culling wild animals to control cattle tuberculosis has met with mixed success [9]; in Great Britain, where the incidence of cattle

tuberculosis has soared in recent decades, culling of badgers may actually have increased the incidence of bovine tuberculosis in cattle [10].

The attenuated *M. bovis* strain Bacille Calmette-Guérin or BCG, widely used as a vaccine against *Mycobacterium tuberculosis* in humans, has been tested as a vaccine against bovine tuberculosis. Its efficacy against *M. bovis* infection in cattle in field trials has been inconsistent and generally poor (<50%), not unlike its efficacy against human tuberculosis, primarily caused by *M. tuberculosis* [11]. Hence, a more potent vaccine against *M. bovis* infection is needed.

We have previously described a live recombinant BCG vaccine, rBCG30, that is significantly more potent than BCG in protecting against *M. tuberculosis* aerosol challenge in the guinea pig model of pulmonary tuberculosis [12, 13]. This animal model is noteworthy among small animal models for its high susceptibility to both *M. tuberculosis* and *M. bovis* infection and for the close clinical, immunological and pathological resemblance of tuberculosis in this species to tuberculosis in humans and cattle. rBCG30 expresses large amounts of the *M. tuberculosis* 30 kDa major secretory protein, a mycolyl transferase [12]. In each of eight consecutive experiments, guinea pigs vaccinated with rBCG30 and challenged 10 weeks later with *M. tuberculosis* by aerosol had significantly fewer CFU in their lungs and spleens 10 weeks after challenge than guinea pigs immunized with the parental strain of BCG (on average 0.75 ± 0.1 log fewer CFU in the lungs and 1.1 ± 0.1 log fewer CFU in the spleens; n = 178 BCG-immunized animals and n = 179 rBCG30-immunized animals; *P*<<0.0001). Moreover, compared with BCG-immunized animals, who were highly protected compared with sham-immunized animals, rBCG30-immunized animals had ~10-fold fewer lung lesions, significantly less lung pathology, and significantly longer survival after *M. tuberculosis* aerosol challenge [12, 13]. rBCG30 is currently in human clinical trials.

In this study, we have explored the potential for rBCG30 as a vaccine against bovine tuberculosis by studying its efficacy in protecting against *M. bovis* aerosol challenge in the outbred guinea pig model of pulmonary tuberculosis.

2.   Materials and methods

2.1.   Vaccines and challenge strain

The vaccines tested were the parental *Mycobacterium bovis* BCG Tice (BCG), rBCG30 (pSMT3-MTB30 or pMTB30) Tice [12], and rBCG30Mb (pSMT3-MB30) Tice, a recombinant BCG Tice expressing large amounts of the homologous *M. bovis* Type strain (ATCC 19210) 30 kDa major secretory protein. rBCG30Mb was generated by electroporating the recombinant plasmid pSMT3, consisting of the vector backbone and a ~1.5 kb piece of *M. bovis* Type strain DNA flanked by *Cla*I and *Bam*HI restriction sites and containing the coding region of the 30 kDa major secretory protein and the promoter region immediately upstream of the coding region, into BCG Tice bacteria. The strain stably maintained the recombinant plasmid, and the level of recombinant *M. bovis* 30 kDa protein expression remained almost constant over a 12-month period in the absence of antibiotics, as confirmed by immunoblotting with 30 kDa protein specific antisera (7.1-fold more protein than the wild-type BCG Tice background level).

The challenge strain was the *Mycobacterium bovis* Type strain (ATCC 19210), prepared as described for *M. tuberculosis* in previous studies [12].

2.2.   Immunization of guinea pigs

Specific-pathogen free 250- to 300-g outbred male Hartley strain guinea pigs from Charles Rivers Breeding Laboratories were injected intradermally with 10^3 CFU of parental BCG, rBCG30, or rBCG30Mb prepared as previously described [12, 13]. The preparation of the vaccines entailed washing the bacteria by centrifugation, enumerating them in a Petroff-Hausser chamber, and resuspending them in phosphate-buffered saline (PBS); such bacteria had a very high (>90%) CFU:particle ratio. Control animals were sham-immunized with buffer (PBS) only. For studies of cutaneous delayed-type hypersensitivity (DTH), guinea pigs were immunized in groups of 6 animals each. For studies of

protective immunity, separate guinea pigs were immunized in groups of 15 animals each except for sham-immunized animals (9 per group). Separate groups of animals were studied in the protection experiments to eliminate the possibility that skin-testing might influence the results by boosting immunoprotection [14]. All animal experiments were approved by the Chancellor's Animal Research Committee at UCLA.

2.3. Cutaneous DTH and antibody titer

Guinea pigs were shaved over the back and injected intradermally with 10 µg of the *M. tuberculosis* 30 kDa major secretory protein (r30) in 100 µl PBS. r30 was purified as described from culture filtrates of recombinant *Mycobacterium smegmatis* 1-2c containing plasmid pMTB30 and demonstrated to be indistinguishable from the native protein [15, 16]. The extent (diameter) of induration was assessed 24 hr later by palpation and inspection in direct and oblique light.

Immediately after the skin test was read, we euthanized a subset of 4 of the 6 animals and assayed their serum for antibody titer to r30 by ELISA, using Costar (Corning, N.Y.) 96-well EIA/RIA High Binding Plates, r30 at 1 µg/well, guinea pig serum diluted 1:250 to 1:1,024,000, alkaline phosphatase-conjugated goat anti-guinea pig IgG (Sigma) at a dilution of 1:1,000, and Alkaline Phosphatase Substrate Kit (BioRad, Hercules, CA).

2.4. Protective immunity to aerosol challenge

Ten weeks after the guinea pigs were immunized, the animals were challenged with an aerosol generated from a single cell suspension of *M. bovis*. In Experiments 1, 2, and 3, the animals received a high (~20 live bacteria), medium (~10 live bacteria), and low (~5 live bacteria) dose, respectively, of bacteria into the lungs, based on counting primary lesions in the lungs of animals euthanized three weeks after exposure to aerosols generated from bacterial

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suspensions in the range of concentrations used in this study. Afterwards, guinea pigs were individually housed in stainless steel cages contained within a laminar flow biohazard safety enclosure and allowed free access to standard laboratory food and water. The animals were observed for illness and weighed weekly for 10 weeks and then euthanized. The lungs, spleen, and liver of each animal were removed aseptically, and the right lung and spleen were cultured for CFU of *M. bovis* as described for culturing *M. tuberculosis* in previous studies [12, 13].   The liver was inspected visually and the extent of involvement with tuberculosis was graded using a scale of 0-4 where 0, 0.5, 1, 1.5, 2, 2.5, and 3 were assigned livers with 0, 1-9, 10-19, 20-39, 40-59, 60-79, and 80-120 surface lesions, respectively; 3.5 was assigned livers with 100-200 surface lesions including coalescent lesions; and 4 was assigned livers with >200 lesions.

2.5.   Statistics

Parametric analysis of variance (ANOVA) and non-parametric Kruskal-Wallis (K-W) methods were used to compare the mean and median induration, reciprocal antibody titers, net weight gain or loss, and log CFU across immunization groups. For the mean comparisons by ANOVA, post hoc mean comparisons were judged statistically significant using the Fisher-Tukey LSD criterion. To combine CFU data across experiments, we first normalized individual animal log CFU values to the mean of the sham group in each experiment using the formula: [Log CFU Standardized = (Log CFU – Mean Log CFU for Sham)/Mean Log CFU for Sham], thereby obtaining the proportional change from the sham mean. Similarly, to combine liver score data across experiments, we first normalized individual animal scores to the mean of the sham group in each experiment using the formula: [Liver Grade Standardized = (Liver Grade – Mean Liver Grade for Sham)/Mean Liver Grade for Sham]. For the combined data, the Wilcoxon rank sum test was used to compute non-parametric *P* values.

3. Results

3.1. Animals immunized with recombinant BCG expressing high amounts of the M. tuberculosis or M. bovis 30 kDa major secretory protein exhibit enhanced cell-mediated and humoral immune responses to the 30 kDa protein

We sham-immunized animals or immunized them with BCG, rBCG30, or rBCG30Mb, and 10 weeks later, we assessed their immune responses to the recombinant *M. tuberculosis* major secretory protein (r30). Animals immunized with either rBCG30 or rBCG30Mb exhibited stronger cutaneous delayed-type hypersensitivity (DTH) responses to r30 than animals immunized with the parental BCG strain or sham-immunized animals (Fig. 1). In Experiment 1, animals immunized with BCG, as with sham-immunized animals, had very little or no induration to r30, a result similar to that obtained in all previous experiments [12-14]. However, in Experiment 2, BCG-immunized animals had significantly greater induration than sham-immunized controls, an exceptional outcome. In any case, animals immunized with either rBCG30 or with rBCG30Mb had significantly greater induration than BCG-immunized animals ($P < 0.02$ by ANOVA or K-W for animals immunized with BCG vs. either rBCG30 or rBCG30Mb; Table 1).

rBCG30 and rBCG30Mb-immunized animals also had greater antibody responses to the 30 kDa protein than BCG-immunized animals (Fig. 2), differences that were statistically significant ($P \leq 0.03$ by ANOVA or K-W for animals immunized with BCG vs. either rBCG30 or rBCG30Mb; Table 1). In both experiments, the humoral immune response of the BCG-immunized animals to r30 was not significantly greater than that of the sham-immunized animals, as previously observed [14].

3.2.   rBCG30 and rBCG30Mb-immunized animals exhibit greater protective immunity to M. bovis aerosol challenge than BCG-immunized animals

To assess the capacity of rBCG30 and rBCG30Mb to induce immunoprotection against *M. bovis,* 10 weeks after immunization, we challenged immunized and control animals by aerosol with the highly virulent *M. bovis* Type strain and monitored the subsequent course of infection for 10 weeks. We immunized animals as in the studies of cutaneous DTH and antibody responses described above, except that we used different animals to eliminate the possibility that the skin test itself might influence the result. In addition, we studied much larger numbers of animals per group so as to obtain more reliable data on the burden of *M. bovis* in animal organs, which preliminary studies showed can vary over a relatively wide range in outbred guinea pigs. We performed three experiments in which animals received a high (Experiment 1), medium (Experiment 2), or low (Experiment 3) challenge dose.

In Experiments 1 and 2, in which animals received a high or medium challenge dose of *M. bovis*, respectively, sham-immunized animals began losing weight beginning three weeks after challenge, as in previous experiments in which animals were challenged with *M. tuberculosis*. Differences in weight gain between sham-immunized animals and each of the immunized groups of animals were statistically significant in these experiments ($P < 0.01$, 0.001, and 0.0001 by ANOVA and $P = 0.05$, 0.008, and 0.0002 by K-W for sham vs. BCG, rBCG30Mb, or rBCG30, resp., in Experiment 1; $P < 0.0001$ by ANOVA and $P \leq 0.0007$ by K-W for sham vs. BCG, rBCG30Mb, or rBCG30, resp., in Experiment 2). However, in Experiment 3, in which animals received a low challenge dose, and consequently had a much lower organ burden of *M. bovis* (see below), sham-immunized animals did not lose weight during the 10 week observation period (except during week 9 when there was an accidental water outage). In Experiments 2 and 3, all immunized animals gained weight normally after challenge, i.e. there was no significant difference in weight gain among the different immunized groups after challenge or between immunized animals and

uninfected controls (Fig. 3). However, in Experiment 1, in which animals were challenged with a high dose of *M. bovis*, even immunized animals failed to gain weight normally and these animals lost weight during the final three weeks of the experiment. BCG-immunized animals fared worse in this respect than animals immunized with either rBCG30 or rBCG30Mb. Differences in weight gain between BCG and rBCG30 were significant during the final three weeks (P = 0.03 by ANOVA or K-W) and at the end of the experiment (P = 0.02 by ANOVA and P = 0.04 by K-W). In Experiment 1, nearly half (4 of 9) of the sham-immunized animals died by the end of the observation period; 1 animal in each of the larger BCG- and rBCG30-immunized groups also died.

To assess the capacity of the vaccines to induce protective immunity, we euthanized the animals 10 weeks after challenge and assayed CFU of *M. bovis* in their lungs and spleens (Fig. 4). Sham-immunized animals had the highest burden of *M. bovis* in their lungs and spleens; the burden was much higher in Experiments 1 and 2, in which animals received the highest challenge doses, than in Experiment 3, in which animals received the lowest dose. Compared with sham-immunized animals, BCG-immunized animals had a markedly lower organ burden, a reduction averaging 0.9 ± 0.1 logs in the lung and 1.25 ± 0.03 logs in the spleen, differences that were significant in the lung and spleen in Experiments 1 and 2 and borderline significant in the spleen in Experiment 3; Table 2).

Most importantly, rBCG30- or rBCG30Mb-immunized animals showed a substantial further reduction in the lung and spleen burden. Taking all three experiments together, the reduction in CFU for rBCG30 averaged 0.4 ± 0.2 logs in the lung and 1.1 ± 0.4 logs in the spleen (P < 0.05 in the lung and P = 0.0005 in the spleen). Differences between BCG and rBCG30 were small in Experiment 1, in which animals were challenged with a very high dose; possibly this high a dose of *M. bovis* partially overwhelmed the immunoprotective capacity of the immune system, as these animals had very severe disease, reflected by weight loss in both immunized and sham-immunized animals and by a very high death rate in sham-immunized animals before the end of the observation period. In

Experiments 2 and 3, in which animals received lower challenge doses and the course of disease was less severe after challenge, akin to that in animals challenged with *M. tuberculosis* in previous studies, differences between BCG and rBCG30 averaged 0.6 ± 0.1 logs in the lung and 1.5 ± 0.3 logs in the spleen ($P < 0.04$ in the lung and $P = 0.0004$ in the spleen). Similarly, rBCG30Mb gave superior immunoprotection to BCG in both Experiments 1 and 2, the only ones in which it was tested, but the differences were much greater in the lower challenge dose Experiment 2, where rBCG30Mb-immunized animals had 0.5 logs fewer CFU in the lungs and 1.1 logs fewer CFU in the spleens than BCG-immunized animals ($P = 0.02$ by ANOVA and 0.04 by K-W in the lung and $P = 0.01$ by ANOVA and 0.001 by K-W in the spleen; Table 2).

Differences between rBCG30-immunized animals and sham-immunized animals were of course even greater than between rBCG30- and BCG-immunized animals, averaging 1.3 ± 0.2 logs in the lung and 2.4 ± 0.4 logs in the spleen in the three experiments, differences which were highly significant (Table 2).

At necropsy, in addition to culturing the lung and spleen for *M. bovis*, we graded the extent of liver pathology using a scale of 0-4, as described in Methods. Paralleling the culture results in the lung and spleen, animals immunized with rBCG30 had fewer macroscopic tuberculous lesions in their livers than BCG-immunized animals. In Experiments 1, 2, and 3, the mean grades for sham-immunized animals were 1.67, 2.00, and 0.17, respectively; the mean grades for BCG-immunized animals were 0.68, 0.64, and 0.29, respectively; and the mean grades for rBCG30-immunized animals were 0.50, 0.41, and 0.00, respectively. Differences in the liver pathology grades between BCG- and rBCG30-immunized animals were statistically significant ($P < 0.02$ for the three experiments combined).

4.   Discussion

Our study demonstrates that rBCG30 is a more potent vaccine than BCG in protecting against aerosol challenge with virulent *M. bovis* in the stringent guinea pig model. Animals immunized with rBCG30 had significantly fewer *M. bovis* organisms in the lung and spleen and significantly fewer tuberculous lesions in the liver 10 weeks after challenge than animals immunized with BCG. The recombinant BCG overexpressing the native *M. bovis* 30 kDa major secretory protein (rBCG30Mb) was also more potent than BCG but not more potent than rBCG30 expressing the *M. tuberculosis* 30 kDa protein. This result was not surprising given that the mature forms of the two proteins are identical.

The *M. bovis* Type strain used to challenge animals in this study appeared more virulent for guinea pigs than the *M. tuberculosis* Erdman strain used in previous studies. In Experiment 1, in which animals were challenged with 20 *M. bovis* delivered to the lung, as determined by the number of primary lung lesions three weeks after challenge in a separate group of animals, nearly half of the sham-immunized animals died before the end of the 10-week observation period, and even immunized animals lost weight. In contrast, in previous experiments in which animals were challenged with a comparable or even greater dose of *M. tuberculosis*, sham-immunized animals nearly always survived the 10-week observation period and BCG or rBCG30-immunized animals never lost weight [12]. The apparent greater virulence of *M. bovis* than *M. tuberculosis* for guinea pigs is consistent with previous observations of several investigators that *M. bovis* strains generally are more virulent for guinea pigs than the human-adapted *M. tuberculosis* strains [17-19]. Because of the severity of the *M. bovis* challenge in Experiment 1, we lowered the challenge doses in Experiments 2 and 3 with the result that the immunized animals were better protected by the vaccines administered.   This suggests that the high challenge dose of *M. bovis* in Experiment 1 was so severe that it partially overwhelmed the capacity of the immune system to defend the host. We observed a similar phenomenon in a previous study of immunoprotection against the intracellular bacterial lung

pathogen *Legionella pneumophila.* In that study, guinea pigs immunized by sublethal infection with *L. pneumophila* and then challenged by aerosol with a 100% lethal dose (LD_{100}) of wild-type *L. pneumophila* or 2-fold the LD_{100} were highly protected from death whereas guinea pigs challenged with 10-fold the LD_{100} were either not protected or weakly protected [20].

As a live mycobacterial vaccine against bovine tuberculosis in animals, rBCG30 has several advantages. First, the vaccine is inexpensive to manufacture, an important consideration in the animal husbandry industry. Second, the vaccine is relatively easy to administer, requiring only a single injection. Third, the vaccine has been found safe both in small animals (guinea pigs and mice) and in humans in a Phase 1 study. In three animal species, the safety of rBCG30 appears comparable to BCG, which has a very well established safety profile, as over 4 billion doses have been administered to humans since the vaccine was developed nearly a century ago.

rBCG30 also has a noteworthy disadvantage in that it would interfere with the diagnostic tests in current use to assess whether cattle have been infected with *M. bovis*, which rely on the detection of an immune reaction to tuberculin. However, newer diagnostic tests in development that rely on the detection of immune responses to proteins that are absent from BCG or rBCG30, but still present in *M. bovis* and thus capable of reliably distinguishing *M. bovis*-infected cattle from BCG or rBCG30-vaccinated cattle [21-23], would alleviate this problem. The development of such a diagnostic test for use in conjunction with a cattle vaccine was recommended as a high priority by the Independent Scientific Review Group in its report to the British government [8].

Several new generation vaccines have been developed and tested for their capacity to induce protective immunity against *M. bovis* challenge in various animal models. These include a leucine auxotroph of BCG [24], a nutritionally impaired avirulent mutant of *M. bovis* [25], DNA vaccines [26, 27] and prime-boost vaccines utilizing DNA vaccines as the prime and BCG, attenuated *M. bovis*, or a protein vaccine as a boost [28-30]. None of these vaccines were significantly more potent than BCG and some were less potent, including a DNA

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vaccine that was found to be equivalent to BCG in an inbred mouse model but clearly inferior to BCG in the more stringent guinea pig model [26, 27]. The most impressive vaccine regimens reported have utilized a DNA prime or a prime comprising *M. bovis* culture filtrate in a CpG oligodeoxynucleotide-containing adjuvant and a BCG boost; these prime-boost vaccine regimens showed a trend toward improvement over BCG in several parameters studied in cattle [29, 31]. The leucine auxotroph, while inferior to BCG in efficacy, offered the advantage of not sensitizing guinea pigs to tuberculin [24]. Finally, in an experiment in which guinea pigs were presensitized with a *M. avium* strain containing the DNA insertion element IS901, a newly attenuated strain of *M. bovis* demonstrated significantly better protection in the lung than BCG; however, in the same experiment, the newly attenuated strain of *M. bovis* was not superior to BCG in either nonsensitized animals or animals presensitized with an *M. avium* strain lacking the IS901 insertion element [32]. With the exception of this newly attenuated strain of *M. bovis* in the setting of presensitization, of all the new generation vaccines against *M. bovis* infection studied, only rBCG30 has been found significantly more potent than BCG. That it has been demonstrated more potent in the stringent outbred guinea pig model bodes well for future studies of efficacy in cattle and in wild animals that serve as reservoirs for *M. bovis* infection of herds.

Acknowledgments

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Table 1. Key Statistical Analyses of DTH and Antibody titers

Expt	Comparison	DTH		Antibody Titer	
		P(ANOVA)	*P*(K-W)	*P*(ANOVA)	*P*(K-W)
1	Sham vs. BCG	NS*	NS	NS	NS
	BCG vs. rBCG30	0.008	0.02	0.0008	0.02
	BCG vs. rBCG30Mb	0.004	0.02	0.003	0.03
2	Sham vs. BCG	0.0005	0.002	NS	NS
	BCG vs. rBCG30	<0.0001	0.02	0.01	0.03
	BCG vs. rBCG30Mb	0.0005	0.004	0.02	0.03

Table 2. Key Statistical Analyses of CFU in the lung and spleen

Expt	Comparison	Lung		Spleen	
		P(ANOVA)	*P*(K-W)	*P*(ANOVA)	*P*(K-W)
1	Sham vs. BCG	0.01	0.03	0.005	0.006
	Sham vs. rBCG30	0.005	0.03	0.0007	0.002
	Sham vs. rBCG30Mb	0.004	0.04	0.002	0.007
	BCG vs. rBCG30	NS	NS	NS	NS
	BCG vs. rBCG30Mb	NS	NS	NS	NS
2	Sham vs. BCG	<0.0001	0.0005	0.02	0.003
	Sham vs. rBCG30	<0.0001	<0.0001	<0.0001	<0.0001
	Sham vs. rBCG30 Mb	<0.0001	<0.0001	<0.0001	<0.0001
	BCG vs. rBCG30	0.0007	0.002	0.0002	0.0003
	BCG vs. rBCG30Mb	0.02	0.04	0.01	0.001
3	Sham vs. BCG	NS	NS	0.06	0.05
	Sham vs. rBCG30	0.04	0.02	0.0003	0.0007
	BCG vs. rBCG30	NS	NS	0.02	0.08

* NS, Not significant

Figure Legends

Fig. 1. Cutaneous DTH after immunization.

Guinea pigs in groups of six were sham-immunized (Sham) or immunized with BCG, rBCG30, or rBCG30Mb. Ten weeks later, the animals were skin-tested with an intradermal injection of r30, and the extent of induration was measured after 24 hours. Data are the mean diameter ± SE.

Fig. 2. Antibody titer after immunization.

Immediately after the skin test was read in the animals described in **Fig. 1**, three or four of the animals in each group were euthanized and their serum assayed for antibody titer to r30 by ELISA. Data are the reciprocal antibody titer for each individual animal (closed circles) and the geometric mean titer (bar) for each group. For statistical purposes, titers of ≤125 are scored as 125.

Fig. 3. Weight loss after *M. bovis* challenge.

Guinea pigs were sham-immunized (Sham) or immunized with BCG, rBCG30, or rBCG30Mb. Ten weeks later, the animals were challenged by aerosol with a high (Experiment 1), medium (Experiment 2) or low (Experiment 3) dose of a highly virulent strain of *M. bovis* and weighed weekly for 10 weeks. An additional group of control animals was not challenged but weighed weekly (Uninfected controls). Data are the mean net weight gain or loss ± SE for each group of animals compared with their weight immediately before challenge. In Experiment 1, daggers (†) indicate deaths of animals. After the death of an animal in a group, data are the mean net weight change for the surviving animals in the group.

Fig. 4. Organ burden of bacteria after *M. bovis* challenge.

At the end of the 10-week observation period, the challenged animals described in Fig. 3 were euthanized and CFU of *M. bovis* in the lung and spleen were assayed. Data are the mean ± SE for all animals in a group. In Experiment 3, in which the lower limit of detection was 1.0 log/organ, some animals in the BCG- and rBCG30-immunized groups had no detectable CFU. In the lung, 2 BCG-

immunized and 4 rBCG30-immunized animals, and in the spleen, 4 BCG-immunized and 9 rBCG30-immunized animals had zero CFU detected and were scored at the lower limit of detection for statistical purposes.
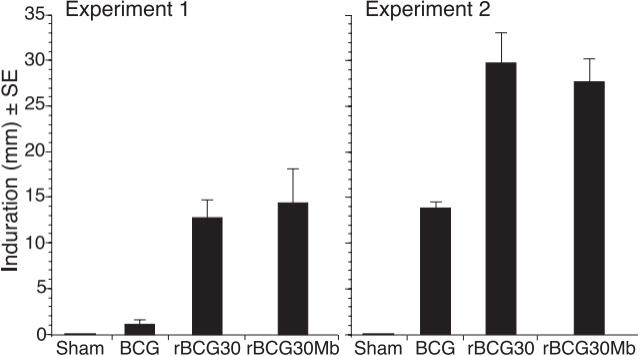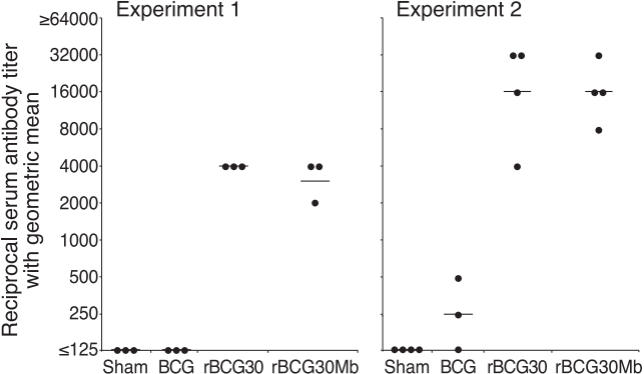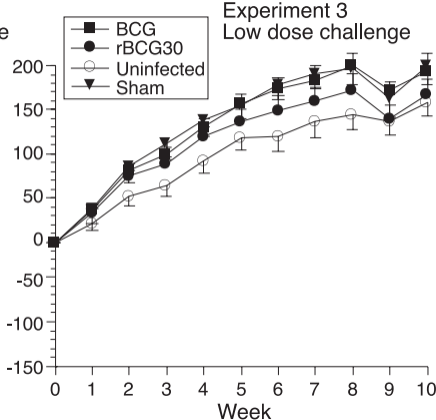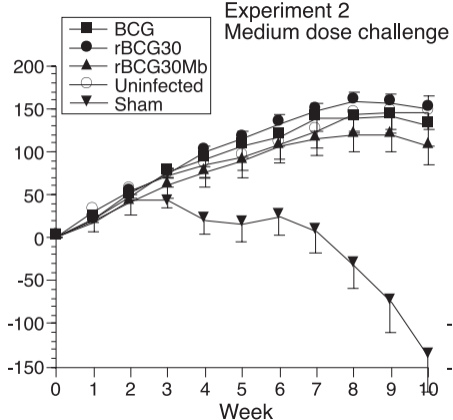
Figure 1

Figure 2

Figure 3

Experiment 1
High dose challenge

Experiment 2
Medium dose challenge

Experiment 3
Low dose challenge

Legend:
- BCG
- rBCG30
- rBCG30Mb
- Uninfected
- Sham

Y-axis: Net weight gain or loss (g) ± SE

X-axis: Week

Figure 4

Experiment 1
High dose challenge

Experiment 2
Medium dose challenge

Experiment 3
Low dose challenge