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## Enhancing the Protective Efficacy of *Mycobacterium bovis* BCG Vaccination against Tuberculosis by Boosting with the *Mycobacterium tuberculosis* Major Secretory Protein

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**Tuberculosis continues to ravage humanity, killing 2 million people yearly. Most cases occur in areas of the world to which the disease is endemic, where almost everyone is vaccinated early in life with *Mycobacterium bovis* BCG, the currently available vaccine against tuberculosis. Thus, while more-potent vaccines are needed to replace BCG, new vaccines are also needed to boost the immune protection of the 4 billion people already vaccinated with BCG. Until now, no booster vaccine has been shown capable of significantly enhancing the level of protective immunity induced by BCG in the stringent guinea pig model of pulmonary tuberculosis, the “gold standard” for testing tuberculosis vaccines. In this paper, we describe a booster vaccine for BCG comprising the purified recombinant *Mycobacterium tuberculosis* 30-kDa protein, the major secreted protein of this pathogen. In the guinea pig model of pulmonary tuberculosis, boosting BCG-immunized animals once with the 30-kDa protein greatly increased cell-mediated and humoral immune responses to the protein in three consecutive experiments. Most importantly, boosting BCG-immunized animals once with the 30-kDa protein significantly enhanced protective immunity against aerosol challenge with highly virulent *M. tuberculosis*, as evidenced by a significantly reduced lung and spleen burden of *M. tuberculosis* compared with those for nonboosted BCG-immunized animals (mean additional reduction in CFU of  $0.4 \pm 0.1$  log in the lung [ $P = 0.03$ ] and  $0.6 \pm 0.1$  log in the spleen [ $P = 0.002$ ]). This study suggests that administering BCG-immunized people a booster vaccine comprising the 30-kDa protein may enhance their level of immunoprotection against tuberculosis.**

Tuberculosis continues as a major scourge of mankind. Each year, approximately 8 million people develop active tuberculosis and 2 million people die of this disease (8). Most of these people are vaccine failures in that they have developed tuberculosis despite having been vaccinated previously with *Mycobacterium bovis* BCG or bacillus Calmette-Guérin. While not highly effective, BCG is the only vaccine against tuberculosis currently available, and it has been used widely. More than 4 billion doses of BCG have been administered worldwide.

Two major approaches to improving the immunoprotection of the world's population against tuberculosis may be considered. The first approach, the one that has received the greatest attention, is to develop a vaccine that provides greater immunoprotection than BCG. Several years ago, our laboratory described the first such vaccine, a recombinant BCG expressing the *Mycobacterium tuberculosis* 30-kDa major secretory protein (r30), also known as antigen 85B,  $\alpha$ -antigen, or FbpB (14). With the highly demanding guinea pig model of pulmonary tuberculosis, the most relevant small animal model of tuberculosis, this vaccine, named rBCG30, induces greater protection than BCG against aerosol challenge with a highly virulent strain of *M. tuberculosis*. The enhanced protective efficacy is

manifested by significantly fewer tuberculous lesions, significantly less lung pathology, significantly decreased numbers of *M. tuberculosis* bacteria in the lung and spleen, and significantly enhanced survival in rBCG30-immunized animals compared with BCG-immunized animals (14, 15). This vaccine is now in human clinical trials. More recently, a second vaccine superior to BCG was described that also expresses a major *M. tuberculosis* protein (27). Such new recombinant BCG vaccines are intended as replacements for the current BCG vaccine, which is typically administered in the neonatal period.

A second approach to improving the immune resistance of the human population to tuberculosis, one largely neglected, is to improve the immunity against *M. tuberculosis* of persons already vaccinated with BCG. Such individuals constitute the great majority of people living in tuberculosis-endemic areas of the world. Revaccinating BCG-immunized people (homologous boosting) has not proved particularly efficacious; studies have shown little, no, or decreased efficacy (17, 18, 20, 21, 29, 30). In animal studies, the results of revaccination also have been disappointing (5, 7); in some cases, immunoprotection is even reduced (5). Similarly, using the guinea pig model of pulmonary tuberculosis, we have found that immunizing with rBCG30 or BCG twice does not result in greater immunoprotection than is the case with a single immunization (unpublished data). However, a different tack is heterologous boosting of BCG, i.e., boosting BCG with a fundamentally different vaccine.

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In previous studies, we have provided evidence that extracellular proteins of intracellular pathogens are key immunoprotective determinants (2, 25). In the case of *M. tuberculosis*, we have demonstrated that immunization of naive guinea pigs with the *M. tuberculosis* 30-kDa major secretory protein induces protective immunity against aerosol challenge with virulent *M. tuberculosis* (13). This is the same protein overexpressed in rBCG30, the vaccine noted above that is more potent than BCG. In the present study, we have examined the capacity of the 30-kDa protein to boost the protective immunity of BCG-vaccinated animals. Using the stringent guinea pig model, we shall demonstrate that a prime-boost strategy involving priming with BCG and boosting once with r30 provides enhanced immunoprotection against aerosol challenge with *M. tuberculosis* compared with immunizing with only BCG.

#### MATERIALS AND METHODS

**Vaccines and challenge strain.** The live vaccines used were the parental *Mycobacterium bovis* BCG Tice (BCG) and a recombinant BCG Tice (rBCG30) overexpressing the *M. tuberculosis* 30-kDa major secretory protein (14, 15). The booster vaccine used was r30, purified as described from culture filtrates of recombinant *Mycobacterium smegmatis* 1-2c containing plasmid pMTB30 and demonstrated to be indistinguishable from the native protein (12, 13). The challenge strain was *M. tuberculosis* Erdman strain (ATCC 35801). The live vaccines and challenge strain were cultured and prepared for use as described previously (14).

**Immunization of guinea pigs.** Specific-pathogen-free 250- to 300-g outbred male Hartley strain guinea pigs from Charles River Breeding Laboratories were immunized intradermally with  $10^3$  CFU of BCG or rBCG30 only or immunized first with  $10^3$  CFU of BCG or rBCG30 and 6 weeks (experiment 2) or 7 weeks (experiments 1 and 3) later with r30. Control animals were sham immunized by intradermal administration of buffer (phosphate-buffered saline). In the first two experiments, r30 was administered in Syntex adjuvant formulation (1, 13, 14) at a dose of 100  $\mu$ g of protein in 100  $\mu$ l of adjuvant. In the third experiment, each dose of 20  $\mu$ g of r30 was (i) first diluted to a final volume of 100  $\mu$ l phosphate-buffered saline and then mixed with 150  $\mu$ l of AS02A adjuvant (3, 10, 26); (ii) first diluted to a final volume of 125  $\mu$ l phosphate-buffered saline and then mixed with 125  $\mu$ l of AS01B adjuvant (26); or (iii) diluted in 250  $\mu$ l phosphate-buffered saline and administered without adjuvant. For studies of cutaneous delayed-type hypersensitivity (DTH), guinea pigs were immunized in groups of six animals each. For the simultaneously conducted studies of protective immunity, separate guinea pigs were immunized in groups of 15 animals each except for sham-immunized animals (9 to 15 per group) and BCG-immunized animals boosted with r30 without adjuvant in the third experiment (6 animals per group).

**Cutaneous DTH.** Guinea pigs were shaved over the back and injected intradermally with 10  $\mu$ g of r30 in 100  $\mu$ l PBS. The extent of induration was measured 24 h later. In experiment 1, the diameter of hard induration was measured as in previous studies by determining the area impeding the movement of a blunt instrument pushed across the skin toward the lesion from four directions. In experiments 2 and 3, the diameter of induration was assessed by palpation and inspection in direct and oblique light, the method used to read tuberculin skin tests in humans.

**Antibody titer.** Immediately after the skin test was read for the skin-tested animals described in the previous paragraph, we euthanized a subset of three to five of the six animals and assayed their serum for antibody titer to r30 by enzyme-linked immunosorbent assay (ELISA), using Costar (Corning, NY) 96-well EIA/RIA High Binding plates, r30 at 1  $\mu$ g/well, guinea pig serum diluted 1:250 to 1:1,024,000, alkaline phosphatase-conjugated goat anti-guinea pig immunoglobulin G (IgG) (Sigma, St. Louis, MO) at a dilution of 1:1,000, and an alkaline phosphatase substrate kit (Bio-Rad, Hercules, CA).

**Protective immunity to aerosol challenge.** Ten weeks after the first immunization (if the animals were boosted) or only immunization (if the animals were not boosted), the guinea pigs were challenged with an aerosol generated from a 7.5-ml single-cell suspension containing a total of  $7.5 \times 10^4$  CFU of *M. tuberculosis* using a Collison 6-jet nebulizer at a pressure setting of 20 lb/in<sup>2</sup>; this dose delivered ~10 live bacteria to the lungs of each animal, based on counts of primary lesions in the lungs of animals euthanized 3 weeks after exposure to this dose. 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 inspected immediately for pathology, and the right lung and spleen were cultured for CFU of *M. tuberculosis* as described previously (14).

**Statistics.** Parametric analysis of variance (ANOVA) and nonparametric 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, posthoc mean comparisons were judged statistically significant using the Fisher-Tukey least-significant difference 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 following 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.

#### RESULTS

**Boosting BCG- or rBCG30-immunized animals with r30 enhances the cutaneous DTH response to r30.** To determine the impact of boosting with r30 on the development of a cell-mediated immune response to r30 in BCG- or rBCG30-immunized animals, we assessed the animals for cutaneous DTH response to r30. In three consecutive experiments, we sham immunized guinea pigs, immunized guinea pigs with only BCG or only rBCG30, or immunized guinea pigs with BCG or rBCG30 and then boosted them 6 or 7 weeks later with r30 ( $n = 6$  animals per group). Ten weeks after the first immunization or only immunization, we assayed the animals for their cutaneous DTH response to r30 (Fig. 1). In all three experiments, animals immunized with only BCG had negligible cutaneous DTH responses, equivalent to those of sham-immunized animals. Boosting BCG-immunized animals with r30 markedly increased the cutaneous DTH response by a minimum of fourfold, differences which were highly significant in experiments 1 and 3 ( $P \leq 0.0002$  by ANOVA;  $P \leq 0.007$  by K-W [Table 1]). As in previous studies, animals immunized with only rBCG30 had a significantly greater cutaneous DTH response than animals immunized with only BCG ( $P = 0.0002$  in experiments 1 and 3 and  $P = 0.05$  in experiment 2 by ANOVA;  $P < 0.007$  in experiments 1 and 3 by K-W). Boosting rBCG30-immunized animals with r30 increased the cutaneous DTH response to r30 nearly twofold in experiments 1 and 2 ( $P = 0.02$  by ANOVA, Experiment 1), but not in Experiment 3 where the response in animals immunized with rBCG30 alone was very high. Boosting with r30 in adjuvant did not result in a significantly greater cutaneous DTH response than boosting without adjuvant in either BCG- or rBCG30-immunized animals in experiment 3, the only experiment in which this comparison was made. The results with adjuvants AS02A and AS01B, evaluated only in experiment 3, were comparable.

**Boosting BCG- or rBCG30-immunized animals with r30 enhances the humoral immune response to r30.** Immediately after the skin test was read for the skin-tested animals that were described in the previous paragraph, we euthanized a subset of these animals and assayed their serum for antibody to r30 by ELISA (Fig. 2). In all three experiments, animals immunized with only BCG had baseline reciprocal antibody titers equivalent to that previously measured for unimmunized animals. In experiment 3, where a sham-immunized group was studied for antibody titer within the same experiment, titers for animals immunized with only BCG were not significantly dif-

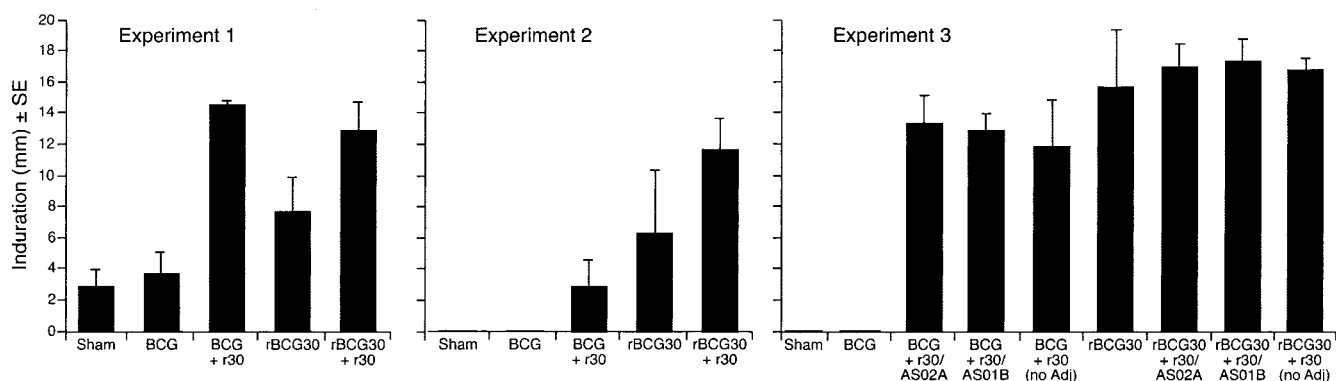


FIG. 1. Cutaneous DTH after immunization. Guinea pigs in groups of six were sham-immunized (Sham), immunized with only BCG or rBCG30, or immunized first with BCG or rBCG30 and then boosted with r30. Ten weeks later, the animals were skin tested with an intradermal injection of r30, and the extent of induration was measured after 24 h. One method was used to measure induration in experiment 1, and a different method was used in experiments 2 and 3, as described in the text. Data are the mean diameter  $\pm$  SE. In experiment 3, BCG- or rBCG30-immunized animals were boosted with r30 in the presence or absence of adjuvant (no Adj), as indicated.

ferent from titers for sham-immunized animals—both were negligible (Fig. 2, experiment 3). Boosting BCG-immunized animals with r30 markedly increased the antibody response; in the three experiments, the geometric mean reciprocal antibody titer (averaging the mean titers of AS02A, AS01B, and no-adjuvant groups in experiment 3) was increased by 27-fold, 147-fold, and 197-fold (mean  $\pm$  standard error [SE]  $n$ -fold increase,  $124 \pm 50$ ), differences which were statistically significant ( $P = 0.04$ ,  $P = 0.008$  and  $P \leq 0.007$  in experiments 1, 2, and 3, respectively, by K-W [Table 1]).

Animals immunized with only rBCG30 had significantly higher antibody titers than animals immunized with only BCG. In the three experiments, geometric mean titers for rBCG30-immunized animals were 2-fold to 32-fold higher (mean  $\pm$  SE  $n$ -fold increase,  $14 \pm 9$ ) than for BCG-immunized animals ( $P = 0.05$  in experiments 1 and 2 and  $P = 0.007$  in experiment 3 by K-W [Table 1]). Boosting rBCG30 with r30 also significantly increased antibody titers by 161-fold, 62-fold, and 40-fold in

the three experiments (mean  $\pm$  SE  $n$ -fold increase,  $88 \pm 37$ ;  $P \leq 0.02$ , experiments 1 to 3 by K-W [Table 1]).

Boosting BCG-immunized animals with r30 in adjuvant (AS02A or AS01B) resulted in higher mean antibody titers than boosting without adjuvant (Fig. 2, experiment 3), a difference that was statistically significant for r30 in AS01B ( $P = 0.02$  by K-W).

**Boosting BCG-immunized animals with r30 enhances protective immunity against aerosol challenge with *M. tuberculosis*.** To determine the impact of r30 boosting on protective immunity, we challenged immunized and control animals by aerosol with highly virulent *M. tuberculosis* and monitored the subsequent course of infection for 10 weeks. We immunized animals as in the study of cutaneous DTH 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.*

TABLE 1. Key statistical analyses of DTH and antibody titers

| Expt | Comparison                     | DTH                    |                      | Antibody titer         |                      |
|------|--------------------------------|------------------------|----------------------|------------------------|----------------------|
|      |                                | <i>P</i> value (ANOVA) | <i>P</i> value (K-W) | <i>P</i> value (ANOVA) | <i>P</i> value (K-W) |
| 1    | BCG vs. BCG + r30              | <0.0001                | 0.003                | NS                     | 0.04                 |
|      | BCG vs. rBCG30                 | 0.0002                 | 0.006                | NS                     | 0.05                 |
|      | rBCG30 vs. rBCG30 + r30        | 0.02                   | NS                   | 0.004                  | 0.02                 |
| 2    | BCG vs. BCG + r30              | NS <sup>a</sup>        | NS                   | 0.03                   | 0.008                |
|      | BCG vs. rBCG30                 | 0.05                   | NS                   | NS                     | 0.05                 |
|      | rBCG30 vs. rBCG30 + r30        | NS                     | NS                   | <0.0001                | 0.01                 |
| 3    | BCG vs. BCG + r30/AS02A        | <0.0001                | 0.002                | <0.0001                | 0.002                |
|      | BCG vs. BCG + r30/AS01B        | <0.0001                | 0.002                | NS                     | 0.006                |
|      | BCG vs. BCG + r30 No adj       | 0.0002                 | 0.007                | 0.0002                 | 0.007                |
|      | BCG vs. rBCG30                 | <0.0001                | 0.007                | <0.0001                | 0.007                |
|      | rBCG30 vs. rBCG30 + r30/AS02A  | NS                     | NS                   | 0.0003                 | 0.01                 |
|      | rBCG30 vs. rBCG30 + r30/AS01B  | NS                     | NS                   | 0.07                   | 0.009                |
|      | rBCG30 vs. rBCG30 + r30 No adj | NS                     | NS                   | NS                     | 0.009                |

<sup>a</sup> NS, not significant.

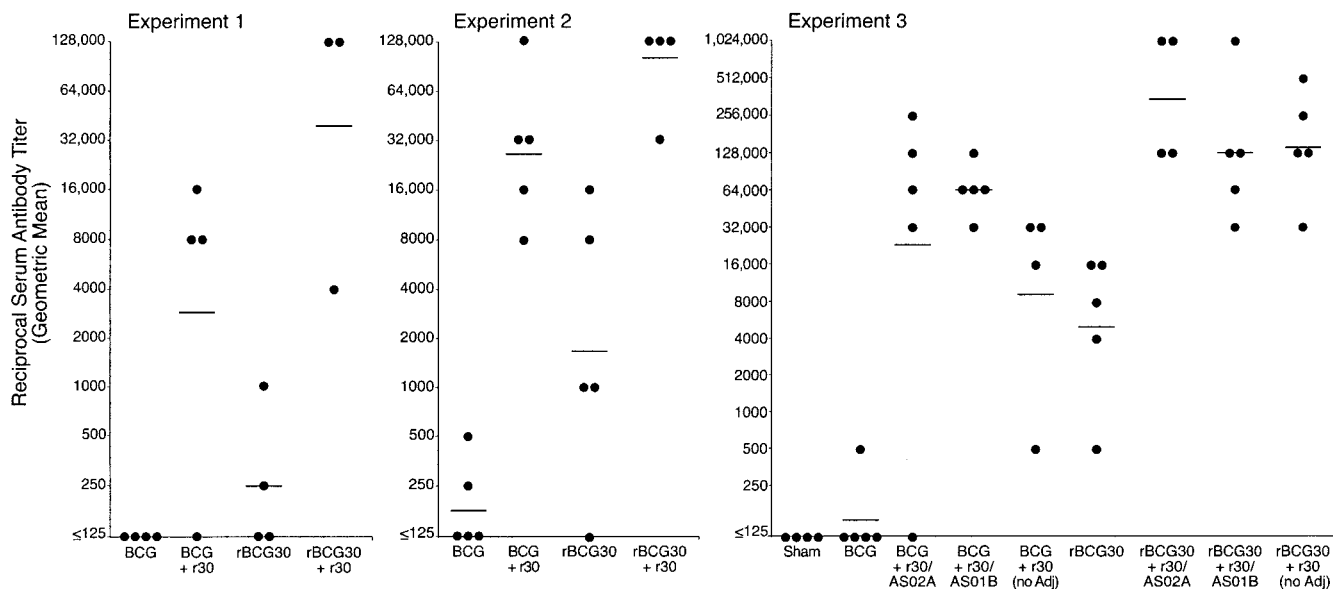


FIG. 2. Antibody titer after immunization. Immediately after the skin test was read in the animals described in Fig. 1, three to five of the animals in each group were euthanized and their serum assayed for titer of antibody to r30 by ELISA. Data are the reciprocal antibody titer for each individual animal (closed circles) and the geometric mean titer (bar) for each group. For statistical purposes, titers of  $\leq 125$  are scored as 125.

*tuberculosis* in animal organs, which can vary over a relatively wide range in outbred guinea pigs. In all three experiments, 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). Previous studies have similarly demonstrated that BCG immunization protects guinea pigs from weight loss associated with *M. tuberculosis* aerosol challenge; hence, this parameter is not useful for discriminating between BCG and more-potent vaccines (14). In contrast to immunized animals, sham-immunized animals failed to gain weight normally after challenge, most noticeably in experiment 1, where this group of animals lost weight beginning 2 weeks after challenge, a time typically coinciding with dissemination of *M. tuberculosis* from the initial site of infection in the lung. In experiments 2 and 3, weight gain in sham-immunized animals lagged that in immunized animals.

Differences in weight gain between sham-immunized animals and each of the immunized groups of animals were statistically significant in all three experiments.

To determine the impact of r30 boosting on the burden of *M. tuberculosis* in animal organs, we euthanized the animals 10 weeks after challenge and determined numbers of CFU of *M. tuberculosis* in their lungs and spleens (Fig. 4). Sham-immunized animals had a very high burden of *M. tuberculosis* in their lungs and spleens. Compared with sham-immunized animals, BCG-immunized animals had a markedly lower organ burden, a reduction averaging  $1.4 \pm 0.2$  logs in the lung and  $2.1 \pm 0.1$  logs in the spleen, differences that were highly significant ( $P \leq 0.001$  by ANOVA or K-W in lung and spleen in each experiment [Table 2]).

Most importantly, in all three experiments, boosting BCG-immunized animals with r30 resulted in a substantial further reduction in the lung and spleen burden. The reduction in

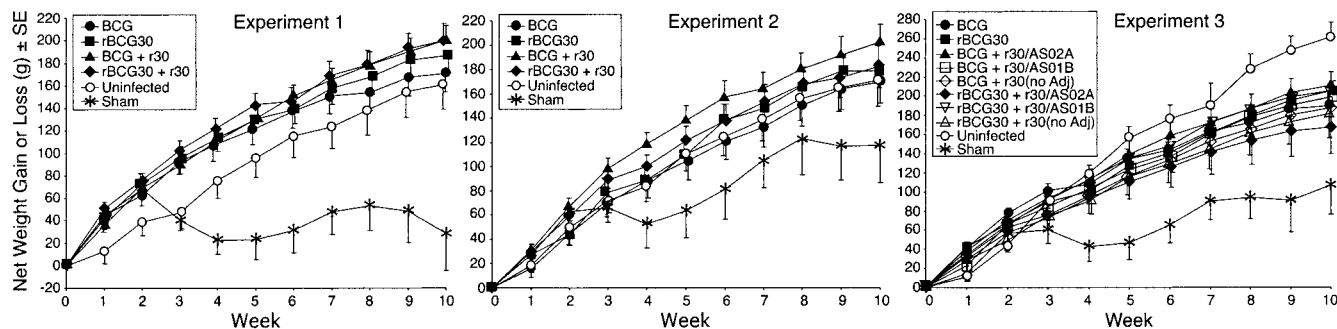


FIG. 3. Weight loss after *M. tuberculosis* challenge. Guinea pigs were sham immunized (Sham), immunized with only BCG or rBCG30, or immunized first with BCG or rBCG30 and then boosted with r30. Ten weeks later, the animals were challenged by aerosol with highly virulent *M. tuberculosis* and weighed weekly for 10 weeks. An additional group of control animals was not challenged but weighed weekly (Uninfected). Data are the mean net weight gain or loss  $\pm$  SE for each group of animals compared with their weight immediately before challenge.



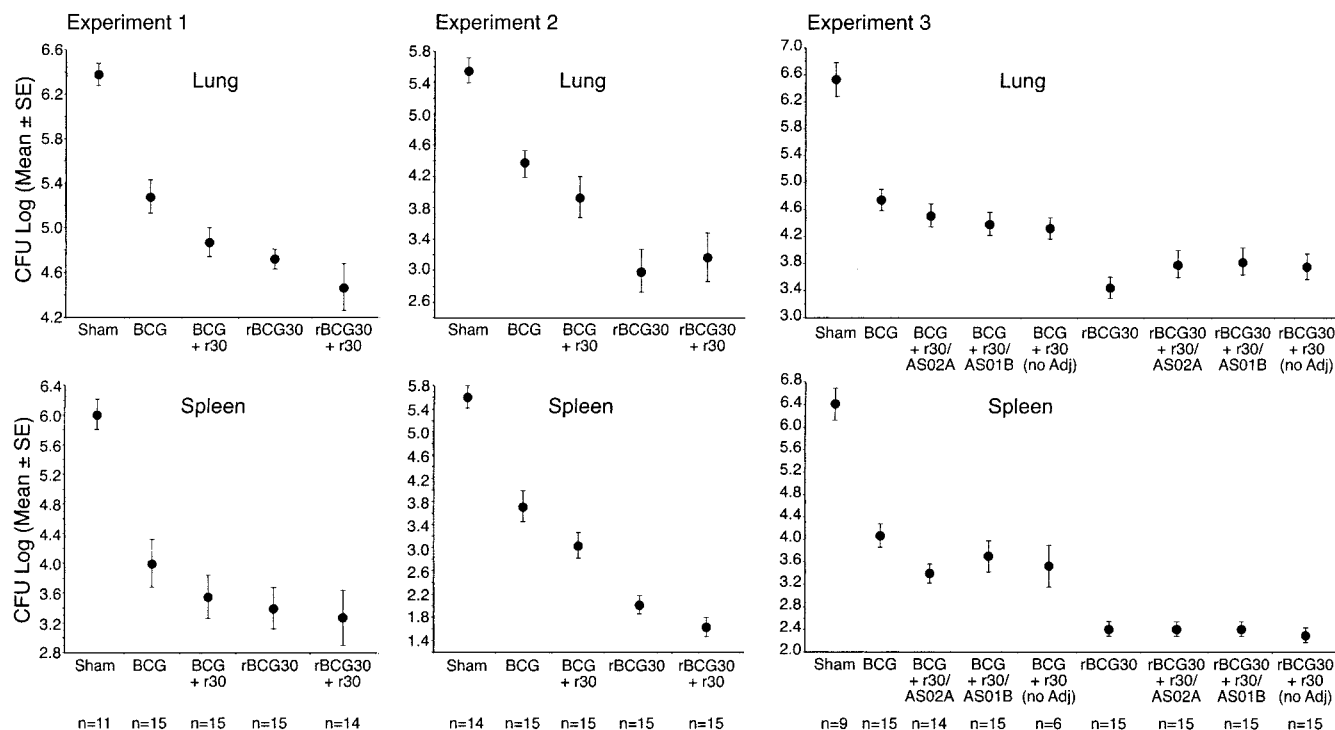


FIG. 4. Organ burden of bacteria after *M. tuberculosis* challenge. At the end of the 10-week observation period, the challenged animals analyzed in Fig. 3 were euthanized, and numbers of CFU of *M. tuberculosis* in the lung and spleen were determined. Data are the mean  $\pm$  SE for all animals in a group. The lower limit of detection was 1.0 log/organ in experiments 1 and 2 and 2.0 logs/organ in experiment 3.

CFU with r30 boosting was statistically significant in the lung or spleen in each of the three experiments ( $P \leq 0.05$  in the lung in experiment 1 and  $P \leq 0.03$  in the spleen in experiments 2 and 3 [AS02A adjuvant] by ANOVA or K-W [Table 2]). Analyzing all three experiments together, boosting BCG-immunized animals with r30 reduced CFU by an average of  $0.4 \pm 0.1$  logs in the lung ( $P = 0.03$ ) and  $0.6 \pm 0.1$  logs in the spleen ( $P = 0.002$ ).

Animals immunized with only rBCG30 had lower organ burdens of *M. tuberculosis* than animals immunized with only BCG. Compared with animals immunized with only BCG, animals immunized with only rBCG30 had an average of  $1.1 \pm 0.3$  logs fewer CFU in the lung and  $1.3 \pm 0.4$  logs fewer CFU in the spleen, differences which were highly significant in the lungs in all three experiments ( $P \leq 0.006$  in each experiment by ANOVA or K-W) and in the spleen in two of three experiments ( $P \leq 0.0001$  by ANOVA or K-W) (Table 2). Remarkably, animals immunized with only rBCG30 also had fewer CFU in their organs than animals immunized with BCG and boosted with r30. Animals immunized with only rBCG30 had an average of  $0.7 \pm 0.3$  logs fewer CFU in the lung and  $0.7 \pm 0.3$  logs fewer CFU in the spleen than animals immunized with BCG and boosted with r30, differences which were highly significant for both the lung and spleen in experiments 2 and 3 ( $P \leq 0.02$  in the lung and  $P \leq 0.004$  in the spleen by ANOVA or K-W [Table 2]).

Boosting animals immunized with rBCG30 had little impact on organ burden. The reduction in CFU was statistically significant only in the spleen in experiment 2 ( $P = 0.05$  by K-W).

Analyzing all three experiments together, for the lungs, there was no difference in numbers of CFU between boosted and nonboosted animals immunized with rBCG30. For the spleen, boosted animals averaged  $0.2 \pm 0.1$  logs fewer CFU than nonboosted animals.

Several animals, especially those immunized with rBCG30 but also two immunized with BCG and then boosted with r30, had no CFU detectable in the lung and/or spleen. In the lung, 1 animal in experiment 2 (rBCG30 group) and 1 animal in experiment 3 (rBCG30 + r30/AS02A group), and in the spleen, 1 animal in experiment 1 (rBCG30 + r30 group), three animals in experiment 2 (1 in rBCG30 group and 2 in rBCG30 + r30 group), and 32 animals in experiment 3 (2 in BCG + r30/AS01B group, 7 in rBCG30 group, 7 in rBCG30 + r30/AS02A group, 8 in rBCG30 + r30/AS01B group, and 8 in rBCG30 + r30 no-adjuvant group) had 0 CFU and were scored at the limit of detection (1.0 log/organ in experiments 1 and 2 and 2.0 logs/organ in experiment 3) for statistical purposes.

## DISCUSSION

Our study demonstrates that boosting BCG-immunized guinea pigs with r30 significantly enhances the cell-mediated and humoral immune response to r30 and protective immunity against aerosol challenge with highly virulent *M. tuberculosis*. In three consecutive experiments, the BCG-immunized animals boosted with r30 had significantly greater cutaneous DTH responses to r30, significantly greater antibody titers, and after

TABLE 2. Key statistical analyses of CFU in lung and spleen

| Expt | Comparison                     | Lung            |               | Spleen          |               |
|------|--------------------------------|-----------------|---------------|-----------------|---------------|
|      |                                | P value (ANOVA) | P value (K-W) | P value (ANOVA) | P value (K-W) |
| 1    | Sham vs. BCG                   | <0.0001         | 0.0001        | <0.0001         | 0.0002        |
|      | BCG vs. BCG + r30              | 0.04            | 0.05          | NS              | NS            |
|      | BCG vs. rBCG30                 | 0.006           | 0.004         | NS              | NS            |
|      | BCG + r30 vs. rBCG30           | NS <sup>a</sup> | NS            | NS              | NS            |
|      | rBCG30 vs. rBCG30 + r30        | NS              | NS            | NS              | NS            |
| 2    | Sham vs. BCG                   | 0.001           | 0.0002        | <0.0001         | 0.00007       |
|      | BCG vs. BCG + r30              | NS              | NS            | 0.02            | 0.03          |
|      | BCG vs. rBCG30                 | 0.0001          | 0.0007        | <0.0001         | 0.0001        |
|      | BCG + r30 vs. rBCG30           | 0.007           | 0.02          | 0.0008          | 0.004         |
|      | rBCG30 vs. rBCG30 + r30        | NS              | NS            | NS              | 0.05          |
| 3    | Sham vs. BCG                   | <0.0001         | 0.00006       | <0.0001         | 0.00007       |
|      | BCG vs. BCG + r30/AS02A        | NS              | NS            | 0.006           | 0.03          |
|      | BCG vs. BCG + r30/AS01B        | NS              | NS            | NS              | NS            |
|      | BCG vs. BCG + r30 No adj       | NS              | NS            | NS              | NS            |
|      | BCG vs. rBCG30                 | <0.0001         | 0.00004       | <0.0001         | 0.00001       |
|      | BCG + r30/AS02A vs. rBCG30     | <0.0001         | 0.0002        | <0.0001         | 0.0002        |
|      | BCG + r30/AS01B vs. rBCG30     | <0.0001         | 0.0002        | <0.0001         | 0.0004        |
|      | BCG + r30 No adj vs. rBCG30    | 0.009           | 0.006         | 0.0003          | 0.003         |
|      | rBCG30 vs. rBCG30 + r30/AS02A  | NS              | NS            | NS              | NS            |
|      | rBCG30 vs. rBCG30 + r30/AS01B  | NS              | NS            | NS              | NS            |
|      | rBCG30 vs. rBCG30 + r30 No adj | NS              | NS            | NS              | NS            |

<sup>a</sup> NS, not significant.

*M. tuberculosis* challenge, a significantly lower burden of *M. tuberculosis* in the lung or spleen.

A number of studies have explored prime-boost vaccination strategies against *M. tuberculosis*—all in the inbred mouse model—in which a DNA vaccine or a recombinant nonmycobacterium was utilized as the prime and a heterologous vaccine was utilized as the boost (9, 22–24, 31). However, we are aware of only one study that has explored heterologous boosting of BCG administered intracutaneously (4), the nearly universal method of administration in humans. In that study, in a single small experiment, mice primed with BCG and boosted with the *M. tuberculosis* 32-kDa major secretory protein (antigen 85A or FbpA) showed a trend toward improved immunoprotection compared with mice administered BCG and boosted with saline. The interpretation of these results was confounded by the fact that animals boosted with the protein, but not the other animals, received interleukin 2 with the boost, so it was not certain to what extent interleukin 2 may have influenced the results. In another study, mice primed by mucosal (intranasal) administration of BCG and boosted mucosally with the *M. tuberculosis* 32-kDa major secretory protein in a modified vaccinia Ankara construct showed enhanced immunoprotection compared with mice mucosally primed with BCG but not boosted (11). However, mucosal administration of BCG does not reflect the route by which BCG has been administered to the more than 4 billion people who have received it worldwide. Prior to our study, no studies have demonstrated significant efficacy of a prime-boost strategy or of heterologous boosting of BCG in guinea pigs, the most relevant small-animal model of tuberculosis. While our study and these studies utilized different animal models and/or routes of administration, all support the concept that extracellular pro-

teins of *M. tuberculosis* and other pathogens are key immunoprotective determinants (2, 13, 14, 25).

Boosting rBCG30 with r30 did not consistently enhance immunoprotection, perhaps because the level of protection induced by rBCG30 alone was so great. However, in these studies, guinea pigs were challenged relatively soon after immunization, when the level of immunoprotection was highest. Boosting with r30 might show greater benefit under conditions in which the immunization challenge interval is much longer. Indeed, humans may be naturally “challenged” with *M. tuberculosis* years or decades after BCG immunization. Our results with guinea pigs suggest the possibility that boosting rBCG30 with r30 may be of benefit to humans. Along this line, boosting with r30 may also reduce the occurrence of reactivation tuberculosis, the most common manifestation of tuberculosis in adults.

In our study, boosting with purified protein in adjuvant was not more efficacious than boosting without adjuvant. Again, whether this would be true under more demanding conditions, especially a much longer immunization-challenge interval, remains to be determined. Successful boosting of humans many years after BCG immunization, long after the initial immune response has peaked and waned, is likely to require a strong adjuvant. Human studies will be necessary to resolve this issue.

The context in which the 30-kDa protein is first introduced to the immune system is evidently critical to its capacity to enhance protective immunity upon boosting. In a previous unpublished study, we found that when guinea pigs were initially immunized with BCG and r30 in adjuvant at the same time and subsequently boosted with r30 in adjuvant, the guinea pigs did not develop greater protective immunity to *M. tuberculosis* challenge than guinea pigs immunized with only BCG

and not boosted. Taken together with the results of the present study, this indicates that for r30 boosting to enhance protective immunity, it is critical that the primary immunization establish a favorable type of immune response, such as a strong TH1 type of immune response, thought to be important in host defense against intracellular pathogens. Priming with BCG alone evidently allows the establishment of just such a favorable immune response to the 30-kDa protein, which is expressed and secreted by BCG, although not as abundantly as by *M. tuberculosis* or rBCG30 (14).

The timing of the booster dose is also likely critical to the cell-mediated immune response. A study by Ahmed and colleagues on the differentiation of antigen-specific CD8 T cells after lymphocytic choriomeningitis virus infection found that the development of mature memory CD8 T cells requires several weeks, and an optimal response to secondary antigen stimulation requires approximately 6 weeks (16). Assuming similar kinetics of memory cell differentiation for CD4 T cells, which play a more central role in the protective immune response against *M. tuberculosis*, our boosting with r30 at 6 to 7 weeks after the primary immunization with live BCG may have allowed for a vigorous immune response to the boost antigen.

Our study justifies a human trial to determine the efficacy of boosting BCG-immunized individuals with r30. Such a trial could explore the efficacy of one or more boosts and measure the impact of r30 boosting on the development of cell-mediated immune responses when the boost is administered years or decades after BCG, something impossible to do in small-animal models because of their limited life span.

That boosting intradermally administered BCG with r30 enhances immunoprotection in the challenging guinea pig model bodes well for human studies of the efficacy of an r30 booster vaccine. The guinea pig develops tuberculosis after aerosol challenge that closely resembles the disease in humans clinically, immunologically, and pathologically. Like humans, but unlike the mouse or rat, the guinea pig is susceptible to low doses of aerosolized *M. tuberculosis*; exhibits a high sensitivity to tuberculin, with a cutaneous DTH response characterized by a dense mononuclear cell infiltrate; and displays Langhans giant cells and caseation necrosis in tuberculous lung lesions (19). Given the absence of known correlates of protection against tuberculosis in humans, efficacy studies with the highly susceptible outbred-guinea-pig model are probably the best predictor of success of new vaccines in humans. Indeed, at a recent World Health Organization meeting of scientists and regulators involved in tuberculosis vaccine development, the participants concluded that efficacy studies with the guinea pig should be a prerequisite for entry of new live mycobacterial vaccines into human trials.

As a booster vaccine for BCG-immunized people, r30 has important advantages. First, r30 can be manufactured relatively easily. Large amounts of the protein can be readily obtained from culture filtrates of rapidly growing recombinant *M. smegmatis* (12); such protein is readily purified (12, 13) and virtually free of endotoxin (unpublished data). Second, this nonlive, non-DNA vaccine is likely to be safe for humans, including human immunodeficiency virus (HIV)-positive individuals. Persons infected with HIV have a greatly increased incidence of tuberculosis and comprise a sizable proportion of new cases of tuberculosis worldwide (8). Most HIV-positive

people live in areas of the world where BCG is administered routinely in childhood; hence, most were "primed" with BCG well before being infected with HIV. If a booster vaccine administered to HIV-positive individuals before their immune system deteriorates is able to augment their immunoprotective capacity against tuberculosis, this may help protect these high-risk persons from one of the most common and devastating opportunistic infections in AIDS.

As noted above, most cases of tuberculosis occur in people previously vaccinated with BCG. BCG is fairly effective at preventing meningitis and disseminated tuberculosis, but it offers relatively poor protection against adult pulmonary tuberculosis, the most common form (6, 28). One meta-analysis estimates the efficacy of BCG against adult pulmonary tuberculosis at 50% (6). If the immunoprotective capacity of BCG-immunized individuals can be augmented by even a modest amount with a booster vaccine, this would have a tremendous impact on the incidence of tuberculosis and the number of deaths from this disease, potentially saving hundreds of thousands of lives annually.

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