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Commonly administered BCG strains including an evolutionarily early strain and evolutionarily late strains of disparate genealogy induce comparable protective immunity against tuberculosis

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Abstract

BCG has been administered to over 4 billion persons worldwide, but its efficacy in preventing tuberculosis in adults has been highly variable. One hypothesis for its variability is that different strains of BCG vary in protective efficacy, and moreover, that evolutionarily early strains are more efficacious than the more attenuated evolutionarily late strains, which lack region of deletion 2. To examine this hypothesis, we tested six widely used BCG strains – the evolutionarily early strain BCG Japanese, two evolutionarily late strains in DU2 Group III (BCG Danish and Glaxo), and three evolutionarily late strains in DU2 Group IV (BCG Connaught, Pasteur, and Tice) – in the guinea pig model of pulmonary tuberculosis. With the exception of BCG Glaxo, which had relatively poor efficacy, we found no substantial differences in efficacy between the early strain and the late strains, and only small differences in efficacy among late strains. BCG Tice was the most efficacious BCG vaccine, with significantly fewer *M. tuberculosis* in the lung and spleen than BCG Danish and BCG Japanese, although absolute differences in the organ burden of *M. tuberculosis* among these three vaccines were small (≤ 0.2 log). BCG Tice and Pasteur were not significantly different. rBCG30, a recombinant BCG Tice vaccine overexpressing the *M. tuberculosis* 30-kDa major secretory protein (Antigen 85B), was more potent than any BCG vaccine ($P < 0.0001$ for differences in organ burden). Our study shows that late strains are not less potent than an early strain and argues against strain differences as a major factor in the variability of outcomes in BCG vaccine trials.

Keywords

BCG; Vaccine; Protective Immunity; Tuberculosis

1. Introduction

BCG is the most widely used vaccine in history, having been administered to ~ 4 billion persons. BCG immunization provides significant protection against childhood tuberculosis and disseminated forms of tuberculosis such as miliary tuberculosis and meningitis [1]. However, efficacy against adult pulmonary tuberculosis is inconsistent, ranging from –35% to +80%

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[2]; moreover, the efficacy of BCG appears dependent upon geography, with relatively good efficacy seen in temperate regions and poor efficacy seen in tropical regions of the globe [3]. A number of theories have been proposed as explanations for the variable efficacy of BCG including a) prior exposure to environmental mycobacteria that mask or interfere with the protective immune response [4]; b) exposure to parasites that skew the immune response toward a Th2 type of response rather than a Th1 type of response [5,6], the type believed most important for immunoprotection; and c) the use of different strains of BCG with theoretically different efficacy. With respect to the last hypothesis, after BCG was developed by Calmette and Guérin in the early years of the 20th century by serial passage on artificial medium, the vaccine was distributed widely throughout the world and subsequently maintained and passaged independently and under different conditions, with the result that the strains differentiated from each other. Comparative genomics studies have revealed differences among the strains including both deletions and insertions of genetic material and tandem duplications that occurred with passage [7,8]. On the basis of comparative genomics studies, strains were divided into early and late strains, the major difference being that the latter strains have lost deletion region 2 [7,9]. The early strain BCG Japanese and the late strains BCG Pasteur, Danish, Glaxo, Connaught, and Tice have been used extensively for vaccination and/or treatment of bladder cancer. In part on the basis of a study [10] showing that percutaneous, and to a lesser extent intradermal, administration of the evolutionarily early strain BCG Japanese induced higher levels of the Th1 cytokines IFN γ and TNF and a lower level of the Th2 cytokine IL-4 and greater CD4⁺ and CD8⁺ T cell proliferation than the evolutionarily late strain BCG Danish, Brosch et al. postulated that early BCG vaccines may confer better protection against tuberculosis than the later strains that are in more widespread use [8].

To investigate if different strains of BCG differ in their protective efficacy in a relevant animal model, we tested six widely used BCG vaccine strains in the outbred guinea pig model of pulmonary tuberculosis. This model, in which the animals are challenged by aerosol with virulent *M. tuberculosis*, is the gold standard for testing vaccines against tuberculosis because the disease in guinea pigs closely resembles the disease in humans. We shall show that, with the exception of BCG Glaxo, the early strain and the late strains are comparably efficacious.

2. Materials and methods

2.1. Strains

M. bovis strains were obtained from the following sources: BCG Connaught from Connaught Laboratories, now Sanofi Pasteur; BCG Danish (a.k.a. BCG Danish 1331; BCG Denmark) from the Statens Serum Institut; BCG Glaxo (a.k.a. BCG British) from ATCC (#35741); BCG Japanese (a.k.a. BCG Japan or BCG Tokyo) from ATCC (#35737); BCG Pasteur (a.k.a. BCG Pasteur 1173P) from ATCC (# 35734); and BCG Tice from Organon, now Schering-Plough. rBCG30 Tice has been described [11]. All BCG strains were cultured at the same time to midlog phase in 7H9 medium plus 2% glucose, pH 6.7 (Difco) at 37°C in 5% CO₂/95% air as unshaken cultures, sonicated to separate clumps and washed three times so as to obtain a single cell suspension, resuspended in phosphate buffered saline (PBS), and counted in a Petroff-Hauser counting chamber. *M. tuberculosis* Erdman strain was obtained from ATCC (#35801), passaged through guinea pigs to maintain virulence, and maintained as described [11].

2.2. Protective immunity

Specific-pathogen free 250–300 g outbred male Hartley strain guinea pigs from Charles River Breeding Laboratories were injected intradermally with an estimated 10³ CFU of each BCG strain (n=15/group) or sham-immunized with buffer (PBS) only (n=9/group); based upon culture of the suspension administered, the actual CFU injected for each vaccine was as follows: BCG Connaught, 752; BCG Danish, 718; BCG Glaxo, 738; BCG Japanese, 710; BCG Pasteur,

726; BCG Tice, 712; rBCG30, 692. Ten weeks later, the guinea pigs were challenged by aerosol with *M. tuberculosis* Erdman with a dose that results in ~10 primary lung lesions, as described [11]. Afterwards, the animals 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 weighed weekly. Ten weeks after challenge, the guinea pigs were euthanized, their lungs and spleens homogenized, and CFU in each organ determined as described [11]. CFU in the lung and spleen were the primary endpoints. Secondary endpoints were a) spleen weight and b) number of surface liver lesions based upon the count of a single observer.

2.3. Statistical analysis

Both parametric analysis of variance (ANOVA) methods and non-parametric Kruskal-Wallis (K-W) methods were used to compare the mean and median log CFU and spleen weight across immunization groups. Non-parametric K-W methods were used to compare the number of liver lesions across immunization groups. For the ANOVA method, post hoc comparisons were judged statistically significant using the Fisher-Tukey LSD criterion.

3. Results and Discussion

We cultured six BCG strains (Connaught, Danish, Glaxo, Japanese, Pasteur, and Tice) under identical conditions in 7H9 medium, harvested the vaccines at mid-logarithmic phase, and administered 10^3 CFU intradermally to six-week old guinea pigs in groups of 15. In addition, as controls, we sham-immunized nine animals with PBS, and we immunized 15 animals with 10^3 CFU of rBCG30 (Tice), a recombinant BCG overexpressing the *M. tuberculosis* 30-kDa major secretory protein (a.k.a. Antigen 85B) and previously demonstrated significantly more potent than parental BCG Tice [11]. The dose of 10^3 bacteria for each vaccine was chosen as sufficiently high to eliminate vaccine dose as a major factor; although BCG is effective over a wide dose range – e.g. in a study utilizing BCG Tice, the vaccine was shown to be equally effective over a dose range of 10^2 to 10^6 CFU [12] – some vaccines may show dose-dependency at very low levels [12,13]. Ten weeks after immunization, all guinea pigs were challenged by aerosol with the virulent *M. tuberculosis* Erdman strain.

With the exception of BCG Glaxo and BCG Connaught, all groups of vaccinated guinea pigs gained weight normally after challenge (Fig. 1). As is typically the case, sham-immunized animals lost weight between weeks 3 and 5, corresponding to a period when *M. tuberculosis* disseminates from the primary site of infection in the lung to other organs in the body; subsequently gained weight for several weeks, corresponding to a period when the immune system is able to counter the infection with some success; and then again lost weight during the final two weeks of the experiment, corresponding to a period when lung capacity is severely compromised by the pathological response to the infection and the appetite is diminished. Animals immunized with BCG Glaxo showed a pattern of weight gain and loss similar to that of sham-immunized animals. Differences in weight gain at 10 weeks between BCG Glaxo and all other BCG strains were statistically significant [$P \leq 0.0004$ by both analysis of variance (ANOVA) and Kruskal-Wallis (K-W) non-parametric analysis]. BCG Connaught-immunized animals showed a pattern of continuous weight gain over the 10 week period similar to that of animals immunized with the other BCG strains excepting BCG Glaxo, but weight gain in BCG Connaught immunized animals significantly lagged that of the animals immunized with these other BCG strains ($P \leq 0.002$ by ANOVA and K-W analysis).

Ten weeks after challenge, the guinea pigs were euthanized and the burden of *M. tuberculosis* in the lungs and spleens of the animals determined. Again with the exception of BCG Glaxo, the various BCG strains gave similar levels of protection (Fig. 2). Compared with sham-immunized animals, BCG-immunized animals had ~2½ logs fewer CFU in the lung and

spleen ($P < 0.0001$ by ANOVA and K-W). With the exception of BCG Glaxo, differences among BCG strains were small (≤ 0.2 logs). BCG Tice had the lowest organ burden of *M. tuberculosis* in both the lung and spleen -0.1 logs fewer CFU in the lung than runner-up BCG Connaught and 0.1 log fewer CFU in the spleen than runner-up BCG Pasteur, differences that were not significant. The evolutionarily early strain, BCG Japanese, was not more efficacious than the evolutionarily later strains. In fact, the late strain BCG Tice was significantly more efficacious than BCG Japanese ($P < 0.02$ in the lung and $P < 0.002$ in the spleen by K-W analysis), although absolute differences in CFU in the lungs and spleens in animals immunized with these vaccines were small (≤ 0.2 logs). BCG Tice was also significantly more efficacious than BCG Danish ($P < 0.006$ in the lung and $P < 0.02$ in the spleen by K-W analysis), but again, the absolute differences in CFU in the lungs and spleens in animals immunized with these vaccines were small (≤ 0.2 logs).

BCG Glaxo was significantly less potent than the other strains; CFU in both the lung and spleen in BCG Glaxo-immunized animals were significantly greater than in animals immunized with each of the other BCG strains ($P < 0.0001$ in both organs by ANOVA and K-W analysis). Interestingly, in a previous study in which bank voles were immunized intraperitoneally with BCG strains and subsequently challenged intravenously with *M. bovis*, BCG Glaxo was similarly found to have lower protective efficacy than other BCG strains including BCG Danish [13].

Consistent with the previous 16 consecutive experiments, rBCG30-immunized animals had significantly fewer CFU in the lung and spleen than animals immunized with the parental BCG Tice strain; similarly rBCG30-immunized animals had significantly fewer CFU in these organs than animals immunized with any other BCG vaccine. rBCG30-immunized animals had 0.9 log fewer CFU in the lung and 1.3 log fewer CFU in the spleen than animals immunized with parental BCG Tice ($P < 0.0001$ by ANOVA and K-W analysis) and on average 1.0 log fewer CFU in the lung and 1.4 log fewer CFU in the spleen than animals immunized with BCG Connaught, Danish, Japanese and Pasteur ($P < 0.0001$ by ANOVA and K-W analysis).

At necropsy, for each animal, the spleen was weighed, and the number of tubercles on the liver counted. These results generally paralleled the CFU results (Fig. 3). That is, the sham and BCG Glaxo-immunized animals had significantly greater mean spleen weight and a significantly greater number of liver lesions than the animals immunized with the other vaccines. Differences among the animals immunized with BCG Connaught, Danish, Japanese, Pasteur, Tice, and rBCG30 Tice were relatively small. Interestingly, BCG Connaught-immunized animals had a significantly greater mean spleen weight ($P < 0.03$ by K-W analysis) and mean number of liver lesions ($P < 0.006$ by K-W analysis) than animals immunized with each of the other vaccines (excepting BCG Glaxo), in line with the somewhat lesser weight gain observed for these animals (Fig. 1). The evolutionarily early strain BCG Japanese was not superior to the evolutionarily late strains. In fact, animals immunized with the late strain BCG Tice, as well as rBCG30 Tice, had at least five-fold fewer liver lesions than animals immunized with each of the other vaccines, including BCG Japanese.

Of the evolutionarily early strains, we studied only BCG Japanese, which has demonstrated strong Th1 immunogenicity in infants, as noted above[10]. We can not exclude the possibility that other evolutionarily early strains may be more potent. In a study in which BCG-immunized mice were challenged intravenously with BCG Pasteur [14], and in another study in which BCG-immunized bank voles were challenged intravenously with *Mycobacterium bovis* [13], BCG Russian was more potent than BCG Japanese.

Although we studied only one evolutionarily early BCG strain, our study fails to support the hypothesis that evolutionarily early strains of BCG are more efficacious vaccines against

tuberculosis than evolutionarily late strains, or that differences in the efficacy of BCG vaccination in various human trials emanates from differences in the potency of BCG vaccine strains. Our result is also consistent with a meta-analysis showing no difference in efficacy among BCG strains (all evolutionarily late strains) in human vaccine trials [3]. Further evidence against a major effect of vaccine strain on the outcome of vaccine trials comes from a human trial showing no difference in efficacy between BCG, attenuated from *Mycobacterium bovis*, and an attenuated *Mycobacterium microti* strain [15].

A WHO panel has recommended that different laboratories settle upon a single BCG vaccine in their animal studies to eliminate the potential effect of vaccine strain on the outcome of pre-clinical efficacy studies and to facilitate comparisons of the results among different laboratories [16]. While this recommendation may yet have merit, our study shows that strain differences are unlikely to account for major differences in efficacy among different laboratories.

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Fig. 1.

Vaccine-induced protection against weight loss after aerosol challenge with *M. tuberculosis*. Guinea pigs were immunized with 10^3 CFU of the various vaccines, and ten weeks later, the animals were challenged by aerosol with *M. tuberculosis*. The animals were weighed weekly for 10 weeks. Data are the mean net weight gain or loss $\pm$ SE for each group of animals compared with their weight immediately before challenge. Differences in weight gain at week 10 between either sham-immunized animals ($P < 0.0001$ by ANOVA and K-W analysis) or animals immunized with BCG Glaxo ($P \leq 0.0004$ by ANOVA and K-W analysis) and the animals immunized with the other vaccines were statistically significant. Differences in weight gain at week 10 between animals immunized with BCG Connaught and animals immunized with the other BCG vaccines (excepting Glaxo) were also statistically significant ($P = 0.0002$ by ANOVA and $P = 0.002$ by K-W analysis).

Fig. 2.

Vaccine-induced protection against *M. tuberculosis* growth in the lung and spleen after aerosol challenge. Ten weeks after challenge with *M. tuberculosis*, guinea pigs were euthanized and CFU were determined in the right lung and spleen of each animal. Differences in CFU in the lung and spleen between sham-immunized animals and animals immunized with BCG Glaxo were statistically significant ($P < 0.0001$ in the lung and $P < 0.0002$ in the spleen by ANOVA and K-W analysis). Differences in CFU in the lung and spleen between animals immunized with BCG Glaxo and each of the other BCG vaccines were statistically significant ($P < 0.0001$ in the lung and spleen by ANOVA and K-W analysis). Differences in CFU in the lung and spleen between animals immunized with any of the BCG vaccines and rBCG30 were statistically significant ($P < 0.0001$ in the lung and spleen by ANOVA and K-W analysis). BCG Tice had significantly fewer CFU in the lung and spleen than BCG Danish ($P < 0.006$ in the lung and $P < 0.02$ in the spleen) and BCG Japanese ($P < 0.02$ in the lung and $P < 0.002$ in the spleen) by K-W analysis.

Fig. 3.

Spleen weight and number of liver lesions in vaccinated animals challenged with *M. tuberculosis*. At necropsy of each animal, the spleen was weighed and the number of tubercles on the liver counted. Sham or BCG Glaxo-immunized animals had significantly greater mean spleen weights than animals immunized with each of the other BCG strains ($P < 0.003$ vs. BCG Connaught and $P < 0.0001$ vs. each of the other BCG strains by ANOVA and K-W analysis). BCG Connaught-immunized animals had significantly greater mean spleen weight than animals immunized with BCG Danish ($P = 0.002$), BCG Japanese ($P = 0.03$), BCG Pasteur ($P = 0.001$), and both BCG Tice and rBCG30 Tice ($P = 0.01$) by K-W analysis. Sham and BCG Glaxo-immunized animals had a significantly greater number of liver lesions than animals immunized with each of the other BCG strains ($P < 0.0001$ by K-W analysis) and BCG Connaught-immunized animals had a significantly greater number of liver lesions than animals immunized with each of the other BCG strains excepting BCG Glaxo ($P < 0.006$ vs. BCG Danish, BCG Japan, or BCG Pasteur and $P < 0.0005$ for BCG Tice or rBCG30 Tice by K-W analysis). BCG Tice and rBCG30 Tice each had significantly fewer liver lesions than BCG Danish ($P = 0.04$ by K-W analysis).