Mycobacterium bovis BCG Substrains Confer Different Levels of Protection against *Mycobacterium tuberculosis* Infection in a BALB/c Model of Progressive Pulmonary Tuberculosis

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Mycobacterium bovis BCG is the only available vaccine against tuberculosis. Reasons for why diverse BCG substrains induce different levels of protection in clinical trials remain unclear. The aim of this study was to compare the effectiveness of 10 BCG substrains in a mouse model of pulmonary tuberculosis. BALB/c mice were subcutaneously vaccinated and 2 months later were challenged with *Mycobacterium tuberculosis* H37Rv by intratracheal injection. Two and 4 months after challenge, delayed-type hypersensitivity (DTH) response, lung tissue affected by pneumonia, CFU, T-cell counts, and cytokine expression (interleukin-2 [IL-2], IL-4, IL-10, and gamma interferon) were determined. A differential protective effect of the diverse BCG substrains was found. BCG Phipps led to the largest and most persistent reduction of CFU counts and of the area of pneumonia at 2 and 4 months after challenge. This protection was accompanied by reduced IL-10-producing T cells. Contemporary BCG substrains induce a wide range of protection in this animal model. These data can help in the selection of the best vaccine for human immunization and for the development of novel recombinant BCG-based vaccine.

Tuberculosis (TB) remains one of the world's leading causes of morbidity and mortality by a single infectious agent (4, 13), and more than 90% of new cases of tuberculosis occur in developing countries, where the BCG vaccination is not highly effective; thus, the search for a novel, more effective vaccine is paramount (4, 15). The attenuated *Mycobacterium bovis* strain bacille Calmette-Guérin (BCG) is, since 1921, the only vaccine currently available against TB (30, 33, 36). Nevertheless, its effectiveness against tuberculosis has been highly variable, showing an average risk reduction of pulmonary tuberculosis of 50% (8, 11).

The most accepted hypotheses for explanations of discrepancies in BCG effectiveness comprise progressive loss of BCG capacity to stimulate a durable immune response and, on the other hand, the high prevalence of and continuous exposure to environmental mycobacteria commonly known as nontuberculous mycobacteria, which could block or mask BCG vaccination-induced immune responses. However, neither of these has strong clinical and research support (5, 7, 14).

After the distribution of BCG to several countries for worldwide application, this vaccine was preserved by subculture until adoption of the seed-lot system. During this period different BCG strains, now known as BCG daughter strains, have been

described; these strains derive from the original strain attenuated by Calmette-Guérin or from an ancestor derived from them. During BCG diversification, some daughter strains have lost genetic regions (Table 1) which affect their antigenic content and maybe their protective efficacy (3, 5, 6, 9, 27). To obtain a successful vaccine that can be eventually substituted for the currently employed BCG vaccine, it is necessary to understand how different BCG vaccine strains drive the immune response to Th2 profile and why, in some cases, BCG vaccine has failed before we can rationally develop the next generation of tuberculosis vaccines. To decrease the wide variation in vaccine protection seen in clinical trials, the World Health Organization has designed certain requirements to reduce the variability among BCG strains by encouraging each manufacturer to correlate laboratory test results with clinical effectiveness data (3, 30, 34).

In terms of efficacy, no BCG strain has been definitely shown to be better than another, and there is no global consensus as to which strain of BCG is optimal for general use. Although there is considerable heterogeneity among strains of BCG vaccine in use at present, several studies have failed to demonstrate significant differences in effectiveness among these strains (5, 25, 30, 37). To understand such differences, it is necessary to compare currently available BCG substrains in an animal model that mimics the natural human disease. In this regard, several animal models have been used in TB research, but specific characteristics of the models have strongly influenced outcomes (1, 17, 25, 29, 32, 35, 37).

This study was designed to assess the protectiveness of 10 different BCG substrains in immunized BALB/c mice intratracheally infected with *M. tuberculosis* H37Rv. To measure pro-

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TABLE 1. *M. bovis* BCG substrains used in this study

Strain (synonym)	Deletion(s) (ORFs) ^a	Genealogy ^b
Pasteur	RD1, RD2, RD14 (28)	
Phipps (Philadelphia)	RD1, RD2 (20)	
Frappier (Montreal)	RD1, RD2, RD8, RDFrappier (27)	
Connaught (Toronto)	RD1, RD2, RD8 (24)	
Tice (Chicago)	RD1, RD2 (20)	
Denmark (Danish 1331)	RD1, RD2, RDDenmark (22)	
Mexico ^c	RD1, RD2, RDDenmark (22)	
Birkhaug	RD1 (9)	
Sweden (Gothenburg)	RD1 (9)	
Moreau (Brazil)	RD1, RD16 (15)	

^a ORF, open reading frame; RD, region deleted.

^b Modified from the genealogy published in reference 5a, with permission of the publisher.

^c Mexico substrain is a vaccine produced by the Health Council of Mexico from 1970 to 1997 from the Danish 1331 strain.

tection, we evaluated survival curves, loads of CFU of *M. tuberculosis* in lungs, delayed-type hypersensitivity (DTH) response, tissue damage (percentage of lung surface affected by pneumonia), and cytokine profiles.

MATERIALS AND METHODS

Bacterial strains. BCG substrains (listed in Table 1), including Moreau, Frappier, Tice, Phipps, Connaught, Birkhaug, and Sweden, were kindly provided by Marcel A. Behr from McGill General Hospital, Montreal, Canada. BCG-Pasteur (Pasteur Institute) was provided by Raúl Mancilla at the Universidad Nacional Autónoma de México Institute of Biomedical Research. BCG Mexico was obtained from the Mexican Health Council. BCG-Danish strain was kindly provided by Mahavir Singh from Lionex Diagnostics and Therapeutics, GmbH, Braunschweig, Germany, and a reference strain of *M. tuberculosis* H37Rv (ATCC 27294) was grown to early mid-log phase in Middlebrook 7H9 medium (Difco, Detroit, MI) supplemented with albumin-dextrose-catalase (BBL, Cockeysville, MD) and 0.05% Tween 80 (Sigma Chemical Co., St. Louis, MO). Cultures were incubated at 37°C with 5% CO₂ and shaken continuously. Bacteria were harvested by centrifugation at 5,000 × g, washed in aliquots containing 10⁶ bacteria/ml, and stored at -70°C until use. Aliquots were thawed before bacterial counts, and viability was determined by fluorescence microscopy using fluorescent diacetate in phosphate-buffered saline (PBS), employing a Neubauer camera and an always-certified absence of bacterial clumping. Bacterial concentration was determined by serial dilution plating of each substrain on Middlebrook 7H10 agar plates supplemented with oleic acid-albumin-dextrose-catalase. After 21 days, total CFU were determined.

Growth cultures of the 10 different *M. bovis* BCG substrains were carried out under uniform conditions in Middlebrook 7H9 supplemented with albumin-dextrose-catalase broth and incubated at 37°C until cultures reached a growth density comparable to McFarland's tube 3 (~10⁸ CFU/ml). Growth rates varied between BCG substrains, ranging from 5.63 days for BCG Frappier to 7.37 days for the Mexico substrain.

Mice vaccination and infection. Groups of eight male BALB/c mice 8 weeks of age were vaccinated subcutaneously (s.c.) at the base of the tail with 10⁴ CFU of each *M. bovis* BCG substrain diluted in 100 µl of PBS; one group of animals was not vaccinated and is referred to in this text as the control group. Eight weeks after vaccination, the mice were anesthetized intraperitoneally (i.p.) with pentothal (56 mg/kg of body weight), the trachea was exposed via a mid-incision, and 2.5 × 10⁵ CFU of viable *M. tuberculosis* H37Rv (standardized doses to evaluate pulmonary TB in 4 months) was injected. The incision was then sutured with sterile silk, and mice were maintained in groups of four in cages fitted with

microisolators connected to negative pressure. All procedures were performed in a laminar flow cabinet at a biosafety level III facility. Twenty animals per group were left untouched, and deaths during the experiment were recorded to construct survival curves. Mice were sacrificed at days 60 and 120 after *M. tuberculosis* challenge. Animal work was performed in accordance with the Institutional Ethics Committee and Mexican national regulations on animal care and experimentation.

Measurement of cutaneous DTH. Culture filtrate was harvested by filtration from *M. tuberculosis* H37Rv grown in Proskauer- and Beck-modified Youmans media for 5 to 6 weeks. Culture filtrate antigens were precipitated with 45% (wt/vol) ammonium sulfate and washed and dissolved in PBS. For delayed-type hypersensitivity (DTH) measurement, each mouse received an injection of 20 µg of antigen in 40 µl PBS into the hind footpad. The footpad was measured with a precision micrometer before and 24 h after the antigen injection as previously described (16). Each data point represents the means of eight mice, four from each time point and from experiments comparing the different groups.

Preparation of lung tissue for histology and automated morphometry. Groups of four mice per group vaccinated with a BCG substrain in two different experiments were killed by exsanguinations at 60 and 120 days after intratracheal infection. Blood was collected, and serum was obtained by centrifugation and stored frozen at -70°C until use. For histologic study, four lungs, right or left, were perfused with absolute ethanol via the trachea, immersed for 24 h in the same fixative, and embedded in paraffin. Five-micrometer-thick sections taken through the hilum were stained with hematoxylin and eosin. In these slides, the percentage of lung surface affected by pneumonia was determined using an automated image analyzer (QWin Leica; Milton Keynes).

Bacterial loads in the lungs. To assess CFU of *M. tuberculosis* in lungs of BCG substrain-vaccinated mice, four lungs per group 2 and 4 months postchallenge were homogenized with Polytron (Brinkmann Instruments, Rexleid, Canada) in 3 ml of isotonic salt solution containing 0.05% Tween 80 (Sigma). CFU were counted by plating 10-fold serial dilutions of the homogenates onto Middlebrook 7H10 nutrient agar and incubating at 37°C. To distinguish bacterial clumps from single cells, colonies were counted twice under a stereoscopic microscope after 10 and 21 days of incubation. The resulting data are representative of two independent evaluations and are expressed as the 25th, 50th, and 75th percentile of the distribution of log₁₀-transformed CFU values in each group of mice.

Antibodies for flow cytometry. To assess T-cell subpopulations in lungs, cells were stained with the following surface marker rat anti-mouse monoclonal antibodies from Pharmingen (BD Bioscience, Piscataway, NJ): CD8a (Ly-2) fluorescein isothiocyanate (FITC)-conjugated clone 56-6.7, CD4 (L3T4) R-phycoerythrin (R-PE) clone RM4-5, and hamster anti-mouse CD69 FITC clone H1-F3. The following clones were used for intracellular cytokine staining:

TABLE 2. DTH response at 2 and 4 months after *M. tuberculosis* challenge in mice immunized with 10 different BCG substrains

BCG substrain	Footpad swelling (mm) at:			
	2 Months		4 Months	
	Median	IQR ^a	Median	IQR
Control	0.73	0.24–1.15	1.29	0.97–1.38
Tice	2.47 ^b	2.11–2.89	1.82	1.40–2.31
Mexico	1.94 ^b	1.65–2.64	1.90	1.62–2.25
Frappier	1.41 ^c	0.94–1.53	1.90 ^c	1.74–2.37
Moreau	1.53	1.17–1.84	2.06	1.52–2.49
Phipps	1.75 ^b	1.46–1.83	2.23	2.19–2.27
Birkhaug	1.21	0.97–1.54	2.27	1.94–3.24
Sweden	1.77 ^b	1.55–2.54	2.35	2.15–2.79
Connaught	2.26 ^b	1.97–2.51	2.67	2.27–3.08
Danish	2.19	1.94–2.27	2.75	2.35–3.24
Pasteur	2.03	1.86–2.43	2.84	2.43–3.08

^a IQR, interquartile range.^b 0.05 > *P* > 0.01 compared with the control group.^c Comparison between 2 and 4 months. *P* = 0.029.

gamma interferon (IFN- γ)-FITC clone XMG1.2, interleukin-4 (IL-4) R-PE clone BVD4-1D11, IL-2-R-PE clone, and IL-10 allophycocyanin clone JES5-16E3.

Flow cytometry analysis. Following surface or intracellular labeling, cells and cytokine-producing cells were discriminated according to specificity of excitation or wavelength emissions of fluorophores used.

(i) **Cell surface markers.** Lung cells were taken from infected mice 4 months after challenge, and single-cell suspensions were produced. Briefly, frozen lungs were thawed in a water bath at 37°C infused with PBS containing brefeldin A at 5 μ g/ml, collagenase type IV at 0.5 U/ml (Sigma Aldrich, St. Louis, MO), and 0.025% DNase I. Lungs were cut into small pieces (<3 mm) and placed in the previously mentioned solution for 1 h at 37°C under vigorous shaking. Tissue fragments and debris were removed and separated on a Ficoll-Hypaque (Histoprep; Sigma) gradient, washed twice in PBS, and adjusted to 1×10^7 cells/ml in PBS. Mononuclear cells were stained for cell surface markers using antibodies against CD8-FITC, CD4-R-PE, and CD69-FITC in PBS for 30 min at 4°C according to manufacturer recommendations, fixed with 4% paraformaldehyde for 16 h, and evaluated by fluorescence-activated cell sorter analysis using Expo Altra v.2 software (Beckman Coulter System). Cells were gated on lymphocyte population by size and forward side scatter.

(ii) **Intracellular staining.** A single-cell suspension of lungs was prepared as described previously. Cells were diluted in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum and 1% antibiotic anti-mycotic solution (Sigma Aldrich) containing 3 μ g/ml of brefeldin A (Sigma Aldrich), incubated for 16 h, and then treated with Citofix/Citoperm (BD Bioscience) solution to fix and permeabilize lymphocytes. Finally, cells were stained with monoclonal antibodies against IL-2-R-PE, IL-4-R-PE, IL-10-allophycocyanin, and IFN- γ -FITC. After intracellular staining, cells were fixed with 4% paraformaldehyde. Preserved cells were stored at 4°C in the dark for 16 h and tested by flow cytometry.

Statistical analysis. Statistical analysis was performed with the SPSS 11.0 software program. Medians and interquartile ranges were calculated as descriptive statistics. Differences between groups of immunized mice were assessed with the Mann-Whitney U test. Association between variables was assessed with Spearman correlation coefficients (*R*). A significant *P* value was set as <5%. Analysis of concordance of CFU values in a set of animals under the same experimental conditions was performed through the estimate of the intraclass correlation coefficient and its 95% confidence interval.

RESULTS

Delayed-type hypersensitivity (DTH) responses against total mycobacterial antigens in mice vaccinated with different BCG substrains after 2 and 4 months of intratracheal challenge with H37Rv. Table 2 shows the DTH response (median footpad swelling size) in mice, at 2 and 4 months after challenge, according to the different BCG substrains. Mice vac-

TABLE 3. Area of pneumonia at 2 and 4 months after *M. tuberculosis* challenge in mice immunized with different BCG substrains

BCG substrain	% Area of pneumonia at:			
	2 Months		4 Months	
	Median	IQR ^a	Median	IQR
Control	12.3	9.4–14.7	57.0	43.4–66.3
Danish	15.0 ^b	8.3–23.5	43.6 ^d	36.2–48.0
Pasteur	18.0 ^b	14.5–21.5	39.9 ^d	26.2–49.2
Tice	13.7 ^b	12.7–18.1	34.0 ^d	26.5–48.2
Frappier	10.1 ^c	5.6–13.5	27.5 ^e	18.0–51.5
Birkhaug	8.0 ^c	7.4–8.9	25.3 ^e	21.4–32.8
Mexico	3.0 ^c	2.2–3.9	22.0 ^e	13.0–27.0
Moreau	6.9 ^c	4.6–8.1	21.5 ^e	16.0–24.4
Sweden	5.0 ^c	33.3–6.1	18.0 ^e	12.7–29.0
Connaught	2.3 ^c	1.7–3.4	17.6 ^e	13.0–37.4
Phipps	5.0 ^c	4.0–5.6	13.4 ^e	7.6–16

^a IQR, interquartile range.^b Pooled data from these mice; median = 16%.^c Pooled data from these mice; median = 5%. Difference between these two groups at 2 months; *P* < 0.001.^d Pooled data from these mice; median = 37.5%.^e Pooled data from these mice; median = 18.8%. Difference between these two groups at 4 months; *P* < 0.001.

nated with the Tice, Mexico, Phipps, Sweden, and Connaught strains showed a DTH response, at 2 months, significantly higher than that in nonvaccinated (control) mice. There was a more than 100% increase (compared to nonimmunized animals) in footpad swelling, at 4 months, in mice immunized with substrains Connaught, Danish, and Pasteur; however, these differences did not reach statistical significance. A significant rise in the DTH response at 4 months (compared to that at 2 months) after challenge was observed in mice immunized with the Frappier (*P* = 0.029) substrain. Mice immunized with the remaining substrains had a statistically nonsignificant change in DTH at 4 months compared to that at 2 months.

Percentage of pneumonia-affected lung area in mice vaccinated with different BCG substrains and challenged with H37Rv via the intratracheal route. Areas with pneumonia were characterized by abundant intra-alveolar vacuolated macrophages with some multinucleated giant cells and numerous alveolar and interstitial lymphocytes surrounding perivascular and peribronchial areas. The percentage of pneumonia-affected lung surface, as an indicator of tissue damage by the mycobacterial infection, was compared between control animals and mice vaccinated with the different BCG substrains. Control animals showed 12% of lung surface affected by pneumonia after 2 months of H37Rv intratracheal challenge, while 2 months later there was nearly a fivefold increase (Table 3).

Mice vaccinated with Phipps, Connaught, Sweden, Moreau, Mexico, Birkhaug, and Frappier BCG substrains had a significantly (*P* < 0.001) smaller area of pneumonia both at 2 (median percent area of pneumonia, 5%) and 4 months (median percent area of pneumonia, 18.8%) after challenge compared with animals immunized with the remaining three substrains, Danish, Pasteur, and Tice (16 and 37.5% at 2 and 4 months, respectively).

Lung live bacilli loads in mice vaccinated with the different BCG substrains after 2- and 4-month postintratracheal challenge with H37Rv. According to the calculated intraclass cor-

TABLE 4. CFUs in lung tissue at 2 and 4 months after *M. tuberculosis* challenge in mice immunized with 10 different BCG substrains^a

BCG substrain	Log ₁₀ of CFUs at:			
	2 Months		4 Months	
	Median	IQR ^a	Median	IQR
Control	6.94	6.89–6.97	7.21	7.00–7.31
Tice	5.71 ^b	5.70–5.73	6.27 ^d	6.10–6.32
Moreau	5.71 ^b	5.71–5.72	6.25 ^d	6.20–6.27
Danish	5.38 ^b	5.25–5.55	6.06 ^d	6.01–6.18
Sweden	5.58 ^b	5.55–5.60	5.95 ^d	5.70–6.17
Frappier	5.92 ^b	5.87–5.96	5.89 ^d	5.80–5.97
Mexico	6.03 ^b	5.97–6.06	5.75 ^d	5.41–6.11
Connaught	5.28 ^c	4.95–5.46	5.81 ^d	5.76–5.87
Birkhaug	5.32 ^c	5.03–5.52	5.72 ^d	5.54–5.83
Pasteur	5.52 ^c	5.21–5.78	5.70 ^d	5.53–5.79
Phipps	5.32 ^c	5.27–5.44	5.68 ^d	5.46–5.85

^a IQR, interquartile range; CFUs, colony forming units.

^b Pooled data from these mice; median = 5.71.

^c Pooled data from these mice; median = 5.33.

^d Pooled data from these mice; median = 6.08.

^e Pooled data from these mice; median = 5.78. Difference between groups at 2 and 4 months; $P = 0.001$.

relation coefficient of the log₁₀-transformed CFU, there was a good concordance of these values among mice immunized in two independent experiments with the same BCG substrain: intraclass correlation coefficient = 0.69 (95% confidence interval = 0.52 to 0.85). Nonvaccinated (control) mice had a median of 6.94 log₁₀ of CFU after 2 months of intratracheal infection; this number of live bacilli increased to 7.21 log₁₀ of CFU at 4 months postinfection. All vaccinated mice showed significantly lower CFU counts compared to those of the control group at 2 months after challenge. At 4 months, no group of immunized mice had a statistically significant difference in CFU compared to the control animals (Table 4). Worth noting is the finding that mice immunized with Birkhaug and Phipps substrains had the greatest decrease in CFU (≥ 1.5 log₁₀ compared with the count in nonimmunized animals) at both times. Median log₁₀ of CFU in mice immunized with Connaught, Birkhaug, Pasteur, and Phipps substrains at 2 months (5.33) and 4 months (5.78) were significantly ($P = 0.001$) lower than those in animals immunized with the remaining substrains (5.71 and 6.08, respectively).

In summary, immunization with substrains Connaught and

Phipps led to the greatest and most persistent reduction of the area of pneumonia and a significant drop in CFU at months 2 and 4. Interestingly, the DTH response magnitude was independent of the level of reduction in both bacterial load and pneumonic surface (R for size of footpad swelling and log₁₀ of CFU, -0.12 [$P > 0.05$]; R for the size of footpad swelling and percent area of pneumonia, 0.31 [$P > 0.05$]). Phipps BCG substrain-immunized animals had the lowest CFU count and the least extended pneumonia at 4 months, despite the fact that their DTH response at 2 and 4 months was relatively low. In contrast, the greater the area of pneumonia the higher the mycobacterial load in the lung (R for the percent of area of pneumonia and the log₁₀ of CFU, 0.51 [$P = 0.016$]).

Lungs infiltrate T cells 4 months after challenge. After 4 months of intratracheal challenge, percentages of CD4⁺ and CD8⁺ lymphocytes were very similar in control mice and in animals vaccinated with the different BCG substrains. Results revealed that mice vaccinated with BCG Sweden, which induced good protection, presented the highest CD4⁺ T-cell counts of all vaccinated groups, followed by Connaught and Mexico substrains, which also induce good protection. Interestingly, Connaught and Frappier substrains induced a higher percentage of CD8⁺ lymphocytes than CD4⁺ T lymphocytes. Tice substrain-vaccinated mice showed only 21% lymphocyte infiltration of lungs 4 months postinfection (Fig. 1). In terms of quantifying activated lymphocytes, mice vaccinated with Tice and Pasteur substrains induced the lowest percentages (4.1 and 6.8%, respectively), different from all other BCG substrains tested. The ratio between T-cell activation level and CD4⁺/CD8⁺ T-cell presence was lowest in nonvaccinated mice, with a ratio of 1.7:1. In vaccinated groups, the ratio ranged from 2.1:1 to 4.2:1, with the exception of mice vaccinated with the Mexico substrain, which demonstrated a ratio of 7.5:1.

Lung cytokine production of T cells. Cells isolated from lungs of vaccinated and nonvaccinated mice were analyzed by flow cytometry to determine cytokine profile. A characteristic response to BCG vaccination is release of cytokines that attract or activate other leukocyte populations. Therefore, expression of these cytokines is an indicator of the type of immune response developed in infected tissue. IL-2 and IFN- γ were considered representative of a Th1 response, and IL-4 and IL-10 were considered Th2 markers.

In all mice, $<7\%$ of lymphocytes expressed IFN- γ , as seen in Fig. 1. The percentage of IL-2-expressing cells ranged from

FIG. 1. CD4⁺, CD8⁺ lymphocyte subset found in the lungs of mice immunized with 10 different BCG daughter strains 4 months after infection with *Mycobacterium tuberculosis* H37Rv. Analysis was performed on suspensions of pooled cells from four mice per group. Each bar represents the percentage of CD4⁺ and CD8⁺ T cells.

FIG. 2. Local immune response in the lungs of mice immunized using BCG substrains and infected with *Mycobacterium tuberculosis* H37Rv. Nonvaccinated (control) and BCG-vaccinated groups were sacrificed 4 months after infection. Analysis was performed on suspensions of pooled cells from four mice per group. Data represent the percentage of cytokine-producing cells.

6.3% in Birkhaug-vaccinated mice to 16% in Mexico substrain-vaccinated mice. In terms of IL-10 expression, the antagonistic cytokine for IFN- γ activity, Phipps BCG-vaccinated mice demonstrated the lowest production level (2%). In the Sweden substrain-vaccinated group, we observed a mixed Th1/Th2 response; 15% of cells produced IL-4 and 15.3% produced IL-2. The percentages of IL-4-producing cells in all groups except mice vaccinated with Sweden were higher than the percentages of IL-10-producing cells (Fig. 2). Differences observed among groups demonstrated particular profiles for each BCG substrain, indicating a differing level of inducement for Th1 and Th2 responses, which suggested a difference in immunogenic antigen expression.

Among all mice, no significant association between magnitude of DTH response and lymphocyte subsets or cytokine expression was found. There was a significant association between CD4 $^{+}$ and CD69 $^{+}$ cell counts ($R = 0.64$, $P = 0.034$) and between IL-2 and IFN- γ levels ($R = 0.93$, $P < 0.001$).

DISCUSSION

Since 1993, global efforts to combat tuberculosis have been focused primarily on the development of new drugs for reducing treatment time and new, more efficient vaccines with greater stability for maintaining protection levels of >85% in the general population. Current research attempts to develop a novel and more effective vaccine by exploring the use of subunit, auxotrophic strains of *M. tuberculosis* or BCG, naked DNA, phylogenetically closely related mycobacteria, and recombinant vaccines that overexpress immunodominant antigens of *M. tuberculosis*. However, with the exception of recombinant BCG vaccines, all these alternatives confer less protection than that offered by the current BCG vaccine (10, 12, 21, 29, 31). In this regard, it is necessary to evaluate the effectiveness of currently available BCG substrains to better select the strain used for recombination.

In the 1950s, macroscopic observations of colony morphology were the first differences observed among BCG vaccines used worldwide. Some showed an indistinguishable growth compared with *M. tuberculosis* strains, which appeared as irregular aggregates or which lay in parallel strands on individual cells, while others showed that nonpathogenic strain morphology lacked direction (3, 34). Phenotypical observations led to

BCG substrains being classified into two groups according to production of HSP64 and MPB70 antigens, the loci of which were lost in the second deletion that occurred in 1931 (2, 6, 26, 28). In this study, however, the level of protection offered by each BCG substrain did not correlate with loss of these immunodominant antigens.

The current study analyzed the effectiveness of 10 different *M. bovis* BCG substrains in a mouse model of progressive pulmonary tuberculosis with intratracheal infection which attempted to mimic the natural route of infection as closely as possible. In a previous study, Lagrandier et al. showed variable protection induced by vaccination with BCG substrains Prague, Japanese, Glaxo, Pasteur, and Russian, given that the first two were unable to confer any protection. Nevertheless, their findings are based on a model of mice vaccinated i.v. and challenged with recombinant BCG substrains that are not pathogenic for mice (25). Our results showed that all BCG substrains protect against disease progression but that none prevent infection, as demonstrated by the presence of lung live bacilli and tissue lesions, suggesting that all mice actually suffered slow progressive disease.

We considered lung live bacilli load determinations as the principal parameter to define the effectiveness conferred by the different BCG substrains, and according to this determination, we classified the effectiveness of the diverse BCG substrains as poor or good vaccines. Poor protective vaccines showed six to eight times fewer CFU than control mice. Animals vaccinated with Birkhaug, Tice, Danish, and Moreau BCG demonstrated extended pneumonia and high DTH responses. Interestingly, Danish substrain-vaccinated mice showed high tissue damage and DTH response, suggesting that this strain induces a high proinflammatory response postchallenge. Sweden, Mexico, and Frappier substrains exhibited 20 times fewer lung CFU and 60% less pneumonia with moderate increase of DTH than the nonvaccinated control group. Finally, Birkhaug and Phipps substrains were the most efficient BCG substrains, showing close to 1.5 log $_{10}$ fewer CFU than control mice, the Phipps substrain-vaccinated animals exhibiting less lung surface affected by pneumonia and moderate DTH responses. In general, lung CFU determination correlated with tissue damage; animals vaccinated with some strains such as the Danish substrain induced percentages of pneumonia-affected lung surface similar to that of control animals,

probably because this strain induces a higher proinflammatory response. Our results also demonstrated that vaccination with poorly protective BCG substrains, associated with more lung inflammation, corresponded to the highest DTH responses, while good protective substrains induced low DTH responses. Thus, it is possible that more activated lymphocytes are recruited by mycobacterial antigens deposited in footpad s.c. tissue in mice with higher bacilli loads and pulmonary inflammation in mice vaccinated with poorly protective BCG substrains.

Interesting information from CFU determinations was gleaned from the comparison between the 2- and 4-month postchallenge data. In general, lung bacilli loads at 2 months postintratracheal inoculation were increased significantly at 4 months after challenge in vaccinated and control animals; however, mice vaccinated with Birkhaug and Phipps substrains showed significantly lower CFU compared with the remaining substrains. This result could indicate that these strains produced more prolonged activation of the immune system, which permits better control of bacilli proliferation at late infection. This observation using an experimental animal model is in agreement with clinical human trials that permitted comparison of five BCG substrains that have been in use worldwide since 1921. Only the Frappier substrain showed a consistent 80% protection, as reported previously (5). The Danish, Tice, and Pasteur BCG substrains showed clear reduction in vaccine protection (from ~80 to 0%), while the Phipps substrain, which was the most effective but apparently does not induce lung protection, demonstrated variations from 83 to 40% (5, 8, 11).

Other studies have demonstrated that smaller doses of *M. bovis* BCG induced a Th1 response confirmed by the presence of IL-2 and IFN- γ , whereas high doses favored development of Th2 immune response indicated by the presence of IL-4 and IL-10 (16, 18, 22–24, 35). A BCG immunization dose of 10^4 CFU selected for the current study was based on findings of mortality rates postchallenge in preliminary experiments in which we evaluated two different vaccination doses (data not shown).

A second finding in this study was the magnitude of DTH response presented by *M. bovis* BCG-vaccinated mice compared with the control group. The Sweden substrain BCG-vaccinated group demonstrated the greatest footpad swelling in response to mycobacterial antigens, although DTH response did not correlate with levels of protection induced by each BCG substrain; in fact, there was no correlation between DTH response and the percentage of pneumonia observed. Histological findings varied at 2 months postchallenge in vaccinated mice. Phipps BCG-vaccinated mice showed a lower percentage of pneumonia with very few CFU. In contrast, by the fourth month mice vaccinated with Pasteur, Danish, Frappier, and Tice BCG substrains showed greater pneumonia areas, between 33 and 42%, with larger areas of infiltrated inflammation and granuloma; their reduction in efficacy suggests substrain overattenuation. In the current study, the Frappier BCG substrain showed significant reduction in the percentage of pneumonia, but it was unable to reduce *M. tuberculosis* load in lungs.

Histologic damage and CFU revealed that the protective effectiveness of different BCG substrains was variable under

the same conditions. Results of this study suggest that the Phipps BCG substrain would be the preferred choice of vaccine because it induced the highest protection level, with statistically significant reduction in pneumonia and higher control of *M. tuberculosis* in the lungs. It is important to mention that this study does not demonstrate prevention of *M. tuberculosis* H37Rv infection but does show that BCG vaccination diminishes disease severity.

The study results support the idea of overattenuation of the parental *M. bovis* BCG vaccine during strain propagation by production in laboratories throughout the world between 1929 and 1956 (2, 3, 6, 33). However, sensitization with saprophyte mycobacteria, which could be blocking, masking, or altering the efficacy of the BCG vaccine, need to be tested in this mouse model in future BCG evaluation studies and during the development of new vaccines (19, 32).

Looking back to the development of the tuberculosis vaccine, the more effective alternative is the recombinant BCG vaccines. Some of these were developed according to the availability of BCG strains used in the region, and it remains uncertain whether these vaccines induced greater protection than all BCG substrains or whether the recombination of other BCG substrains with the same antigens improved protection against disease and increased killing bacilli, thus avoiding development of the latent disease (10, 20). Similarly, use of recombinant BCG vaccines that overproduce secreted antigens may be of particular significance with regard to induction of a protective immune response; nonetheless, they similarly may produce increased tissue damage. This study demonstrates the different protection levels induced by vaccination with diverse vaccine substrains, suggesting the use of Phipps BCG as the reference strain for evaluation of new vaccines in this mouse model of pulmonary tuberculosis. In conclusion, using a well-characterized model of progressive pulmonary tuberculosis in BALB/c mice, we demonstrated wide protection variability conferred by different BCG substrains, suggesting that differences in BCG genotypes used for human vaccination could be a significant factor in the well-demonstrated variability of BCG protection observed in human populations from diverse geographic regions of the world.

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