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Mycobacterium bovis BCG Immunization Induces Protective Immunity against Nine Different *Mycobacterium tuberculosis* Strains in Mice▽†

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Recent preclinical and epidemiologic studies have suggested that certain *Mycobacterium tuberculosis* genotypes (in particular, Beijing lineage strains) may be resistant to *Mycobacterium bovis* BCG vaccine-induced antituberculosis protective immunity. To investigate the strain specificity of BCG-induced protective responses in a murine model of pulmonary tuberculosis, C57BL/6 mice were vaccinated with BCG vaccine and then challenged 2 months later with one of nine *M. tuberculosis* isolates. Four of these strains were from the W-Beijing lineage (HN878, N4, NHN5, and ChS) while four were non-Beijing-type isolates (C913, CDC1551, NY669, and NY920). As a control, the WHO standard *M. tuberculosis* Erdman strain was evaluated in these vaccination/challenge experiments. To assess the protective responses evoked by BCG immunization, organ bacterial burdens and lung pathology were assessed in vaccinated and naïve mice at 4, 12, and 20 weeks postchallenge as well as during the day of infection. At 4 weeks after the aerosol challenge with each of these strains, significantly reduced bacterial growth in the lungs and spleens and significantly improved lung pathology were seen in all vaccinated animals compared to naïve controls. After 12 weeks, reduced organ bacterial burdens were detected in vaccinated animals infected with six of nine challenge strains. Although lung CFU values were lower in vaccinated mice for only three of nine groups at 20 weeks postchallenge, significantly decreased lung inflammation was seen in all immunized animals relative to controls at 20 weeks postchallenge. Taken together, these data demonstrate that BCG vaccination protects against infection with diverse *M. tuberculosis* strains in the mouse model of pulmonary tuberculosis and suggest that strain-specific resistance to BCG-induced protective immunity may be uncommon.

As one of the world's most devastating infectious diseases, tuberculosis (TB) is responsible for approximately 2 million deaths per year (41). This high rate of mortality has persisted despite the availability of an attenuated vaccine (using *Mycobacterium bovis* BCG) for more than 50 years. The reasons for the overall ineffectiveness of the BCG vaccine in controlling global TB are complex and certainly not well understood (4, 5, 11). Potential factors which may contribute to the varying effectiveness of BCG vaccination in controlling TB include the limited durability of BCG-induced protective immunity, genetic variations among vaccinated individuals, and interference with vaccine activity by host presensitization with environmental mycobacterial strains.

Recently, it has been hypothesized that the anti-TB protective immunity induced by BCG may be *Mycobacterium tuberculosis* strain specific and that this strain specificity may also contribute to the limited effectiveness of this vaccine (1). The proposed strain specificity is consistent with the increasing awareness in recent years of the genetic heterogeneity and phenotypic differences among *M. tuberculosis* strains (13, 17, 24). Epidemiologic studies have suggested that the differences in virulence may be associated with the various genetic backgrounds of clinical *M. tuberculosis* isolates (20). Preclinical experiments have clearly shown that *M. tuberculosis* isolates exhibit different levels of virulence and induce various immune responses in animal models (9, 10, 19, 21, 28, 29, 35). Differential host-pathogen interactions caused by phenotypic differences among *M. tuberculosis* isolates could alter BCG-induced immunity, resulting in reduced anti-TB protective responses to specific pathogenic strains (25). Interestingly, previous studies have shown that genetic variability among *Plasmodium* and *Streptococcus pneumoniae* strains can reduce the effectiveness of vaccines against these pathogens in specific geographic regions (17).

Among the most prominent of the *M. tuberculosis* genotypes are the W-Beijing lineage strains which have been associated with worldwide TB outbreaks for more than a decade (2, 3, 12, 14). Recent epidemiological studies have suggested that mass vaccination with BCG may have been a selective force for the emergence of the W-Beijing genotype (15, 38). In these studies, W-Beijing family strains were isolated more frequently from BCG-vaccinated TB patients than from nonimmunized patients. Based on these data, Abebe and Bjune have suggested that BCG immunization may actually increase the risk of developing tuberculous disease in areas with a high prevalence of W-Beijing strain infections (1). The overall conclusion from these studies is that W-Beijing strains may be resistant to BCG-induced protective immunity and that BCG vaccination

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may actually promote the spread of these isolates. Previous results of preclinical studies have supported the strain-specific BCG resistance hypothesis. Lopez et al. have reported that BCG vaccination of mice provided less protection against infection with an *M. tuberculosis* W-Beijing isolate than against challenge with the H37Rv *M. tuberculosis* laboratory strain (19). Moreover, Tsenova and colleagues have shown in rabbits that BCG vaccination confers poor protection against central nervous disease caused by infection with the *M. tuberculosis* HN878 W-Beijing strain (36). It should be noted, however, that only a limited number of comparative preclinical studies have been designed to examine postvaccination protection induced against virulent W-Beijing isolates. Additionally, other epidemiological studies which directly compared isolates from BCG-vaccinated and nonimmunized patients have found minimal association between BCG vaccination and the prevalence of W-Beijing strains (2). Consequently, the linkage between the emergence of W-Beijing strains and the overall efficacy of BCG immunization is still uncertain and remains to be proven conclusively (20).

Clearly, the importance of the phenotypic variations among *M. tuberculosis* isolates on the design and development of new TB vaccines has not been adequately assessed. Here, we describe initial investigations aimed at examining the effect on TB vaccine efficacy of using different *M. tuberculosis* challenge strains in a mouse model of pulmonary TB. In these studies, mice were vaccinated with the licensed BCG vaccine and then were challenged with either the standard laboratory *M. tuberculosis* Erdman strain, Beijing lineage *M. tuberculosis* clinical isolates, or non-Beijing *M. tuberculosis* strains. Five of these strains (C913, CDC1551, HN878, N4, and NHN5) have been associated with outbreaks of TB (24, 26, 34, 37). In this report, we show by evaluating mycobacterial growth in relevant organs and comparing lung pathology in vaccinated and naïve mice that BCG immunization induces protective immune responses that limit the proliferation of aerosol infections by each of the nine *M. tuberculosis* strains tested.

MATERIALS AND METHODS

Bacterial strains. The *M. tuberculosis* Erdman strain was prepared as a preclinical standard for TB vaccine testing under a collaborative agreement between the World Health Organization, the Food and Drug Administration's Center for Biologics Evaluation and Research, and the Aeras Global TB Vaccine Foundation. The C913 and N4 *M. tuberculosis* strains were obtained from the strain collections at the Public Health Research Institute in Newark, NJ, while the HN878 and the NHN5 strains were kindly provided by Clifton Barry of the National Institutes of Health in Rockville, MD. The CDC1551, ChS, NY669, and NY920 strains had been previously characterized at the Food and Drug Administration's Center for Biologics Evaluation and Research. Each strain was grown in Middlebrook's 7H9 medium (Difco, Detroit, MI) supplemented with 10% of Middlebrook's OADC (oleic acid, albumin, dextrose, and catalase) enrichment medium (BBL, Sparks, MD) until late log phase. The cells were frozen at a concentration of 2×10^8 CFU/ml.

Genetic analyses of *M. tuberculosis* isolates. For DNA isolation, *M. tuberculosis* isolates were inoculated in 10 ml of Middlebrook 7H9 medium and incubated at 37°C with constant shaking until the mid-log phase was reached. The bacterial cells were collected by centrifugation at 10,000 × *g* and resuspended with distilled water. Resuspended bacterial cells were autoclaved at 121°C for 15 min, and the supernatant was used directly for PCR.

The W-Beijing and non-W-Beijing-type strains were differentiated using unique primer sets as described earlier (39). To identify a strain belonging to the Beijing evolutionary lineage, the following primers were used: ACCGAGCTG ATCAAACCCG (forward) and ATGGCACGGCCGACCTGAATGAACC (reverse). Using these primers, a 239-bp region of an IS*6110* element (which is unique for Beijing lineage isolates) was amplified (39). Non-Beijing isolates were identified with a primer set complementary to the Rv2819c gene: GGTGCGA GATTGAGGTTCCC (forward) and TCTACCTGCAGTCGCTTGTGC (reverse). The principal genetic groups and the cluster number were determined as described previously (13, 34). For further genetic analysis based on differences in variable-number tandem repeats (VNTR), three primer sets (VNTR0580, VNTR1955, and QUB1895) were selected to discriminate the isolates of *M. tuberculosis* (32, 33). The specific primer sequences used were the following: VNTR 0580, CTGCGGTCAAACAGGTCA (forward) and CATACATCGGT ACCCGAC (reverse); VNTR1955, AGACGTCAGATCCCAGTT (forward) and ACCCGACAACAAGCCCA (reverse); and QUB1895, GGTGCACGGCC TCGGCTCC (forward) and AAGCCCCGCCGCCAATCAA (reverse). Each PCR mixture contained 10 mM Tris-HCl, pH 9.0, 50 mM KCl, 1.5 mM $MgCl_2$, a 200 μM concentration of each deoxynucleoside triphosphate, a 0.5 μM concentration of each primer, and 2.5 U of *Taq* DNA polymerase (Perkin Elmer, Emeryville, CA). Two microliters of template DNA was added to each reaction mixture. For negative controls, 2 μl of sterile distilled water was added to the PCR mixtures. After an initial denaturation at 94°C for 5 min, a touch-down PCR was undertaken with 8 cycles consisting of a denaturation step at 94°C for 30s, an annealing step from 65°C to 57°C (each cycle at each temperature) for 1 min, and an elongation step at 72°C for 2 min, followed by 30 cycles of 94°C for 30 s, 52°C for 1 min, and 72°C for 2 min. A final elongation step at 72°C for 10 min terminated the program. The amplified products were analyzed by agarose gel electrophoresis.

Evaluation of BCG-induced protective immunity using a murine aerogenic infection model. The vaccination/challenge studies were performed as described earlier (7). Pathogen-free C57BL/6 mice were obtained from the Jackson Laboratories (Bar Harbor, ME). For the mycobacterial growth experiments, five mice were evaluated for each group. Initially, mice were vaccinated once subcutaneously with BCG Pasteur (10^6 bacteria in 0.1 ml of phosphate-buffered saline [PBS]). Eight to 10 weeks after the immunization, mice were aerogenically challenged with the *M. tuberculosis* isolates suspended in PBS at a concentration known to deliver 200 CFU in the lungs over a 30-min exposure time in a Middlebrook chamber (GlasCol, Terre Haute, IN). To assess the level of pulmonary exposure during the aerosol challenge, the number of CFU in the lung were measured at 4 h after the tuberculous infection. To determine the extent of bacterial growth in the lungs and spleens, the mice were sacrificed at 4, 12, and 20 weeks postchallenge. The lungs and spleens were then removed aseptically and homogenized separately in PBS using a Seward Stomacher 80 blender (Tekmar, Cincinnati, OH). The lung and spleen homogenates were diluted serially in 0.4% PBS–Tween 80, and 50-μl aliquots were placed on Middlebrook 7H11 agar (Difco) plates supplemented with 10% OADC enrichment (Becton Dickinson, Sparks, MD) medium, 2 μg/ml 2-thiophenecarboxylic acid hydrazide (TCH) (Sigma), 10 μg/ml ampicillin, and 50 μg/ml cycloheximide (Sigma). The addition of TCH to the agar plates inhibits the growth of BCG but not *M. tuberculosis*. All plates were incubated at 37°C for 14 to 17 days in sealed plastic bags, and the colonies were counted to determine the organ bacterial burdens.

Assessment of lung inflammation. To evaluate the level of inflammation in the lungs of mice infected with *M. tuberculosis*, lung sections stained with hematoxylin and eosin were photographed using a Nikon Optiphot 2 microscope fitted with a camera which was connected to a computer. The photos were taken at a magnification of ×5 or ×10. Spot Advanced software was used to save the computer images. The Image Pro Plus program (Media Cybernetics, Silver Spring, MD) was utilized to objectively assess the level of inflammation present in each image. In these images, the inflamed areas stained a more intense purple than the noninflamed areas. For these analyses, colors were assigned as follows: red to represent the inflamed areas, green to represent noninflamed areas, and yellow to represent the background. After the color assignments were established, the computer software identified inflamed and noninflamed sections on each slide. The percentage of the lung sections staining red, green, or yellow was then determined by the computer software. To quantitate the percent area inflamed, we determined the mean percent red area from three to five lung sections of each of the different groups.

Statistical analyses. The protection results and the lung inflammation data were analyzed using the GraphPad Prism, version 4, program.

RESULTS

***M. tuberculosis* clinical isolates.** To assess the effectiveness of BCG vaccination against an aerogenic challenge by different *M. tuberculosis* clinical isolates, we selected nine *M. tuberculosis* strains (described in Table S1 in the supplemental material).

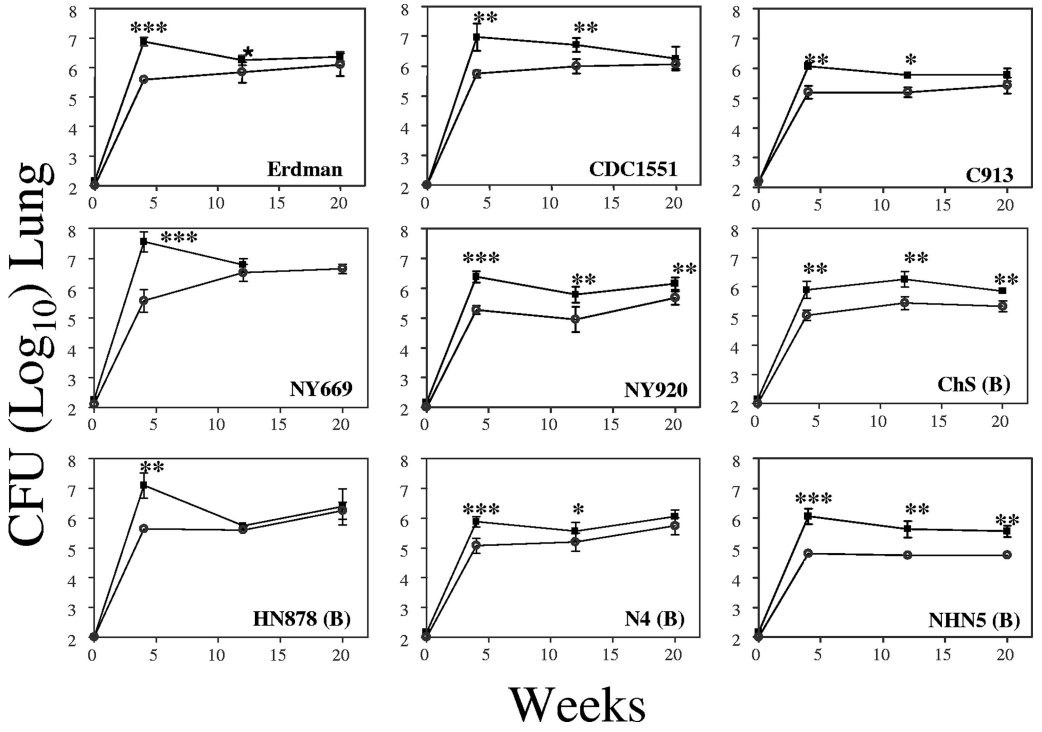

FIG. 1. Growth of nine different *M. tuberculosis* strains in the lungs of BCG-vaccinated and naïve mice. Mice were challenged with about 200 CFU of each of these *M. tuberculosis* isolates and then were sacrificed 4, 12, and 20 weeks postchallenge to determine pulmonary bacterial burdens. To determine the aerosol infection dose, groups of mice were also sacrificed at 4 h postchallenge. The results are representative of two to three experiments. Significant differences between the numbers of lung CFU for vaccinated (open circles) and control mice (closed squares) are shown with asterisks. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$. In these studies, the ChS, HN878, N4, and NHN5 strains are Beijing (B) lineage strains.

The Erdman strain is a common laboratory strain which has been designated by the World Health Organization as a standard infection strain for the preclinical evaluation of new TB vaccines. The other eight strains are drug-susceptible clinical isolates; four of these isolates were previously shown to be Beijing family strains (24, 26, 34, 37). To verify their genotypes, the strains were evaluated using a PCR assay developed to detect strains from the Beijing evolutionary lineage (39). The results of this assay confirmed that the HN878, N4, NHN5, and ChS isolates were Beijing family strains while the CDC1551, C913, NY669, and NY920 isolates did not have the W-Beijing genotype. To further characterize these strains, the strains were assigned to a major genetic cluster based on its single nucleotide polymorphism profile and to a principal genetic group as defined by polymorphisms at the *katG* codon 463 and the *gyrA* codon 95 (13, 24, 34). Finally, molecular typing was done using VNTR PCR primers designed to detect tandem repeats of interdispersed repetitive units within mycobacteria. Using this methodology, novel PCR profiles were detected for each strain. These analyses confirmed that each of these nine strains was unique.

Mycobacterial growth in naïve mice after aerosol infections. To evaluate whether BCG vaccination restricted the growth of the nine *M. tuberculosis* strains after a low-dose challenge, mice were initially vaccinated with 10^6 CFU of BCG Pasteur. Two months later, the relative mycobacterial growth rates in relevant organs of naïve and BCG-vaccinated mice were determined by infecting C57BL/6 mice via the aerosol route and then sacrificing the infected animals at 4, 12, and 20 weeks postchallenge. The 200-CFU infection dose was verified by sacrificing mice and plating lung homogenates at 4 h after an aerogenic challenge with *M. tuberculosis* bacilli. Representative mycobacterial growth data from two to three experiments with each of these strains are shown in Fig. 1. Direct comparisons of the bacterial growth of these strains are shown in Fig. S1 in the supplemental material. Interestingly, the growth profiles of the different *M. tuberculosis* strains in the lungs of naïve mice were generally similar for the first 3 months postchallenge. During the first month, rapid growth of the TB organisms was seen for all strains, with a 4 to 5 $\log_{10}$ increase in the lung infection being observed in naïve mice. After the lung bacterial burdens peaked at 4 weeks, the pulmonary concentrations of *M. tuberculosis* declined between 4 and 12 weeks postchallenge, with significant 0.4 to 1.2 $\log_{10}$ decreases in the number of mycobacterial CFU (relative to 4-week CFU values) detected for seven of nine test strains. Surprisingly, the pulmonary bacterial burdens for seven of the clinical isolates and the standard Erdman strain were not different at 20 weeks following the aerogenic challenges. At this time point, mice infected by aerosol with these eight strains had chronic infections with lung CFU values of approximately 6.0 $\log_{10}$. In contrast, by 20 weeks postchallenge, all of the NY669-infected naïve mice had died, and their deaths were associated with elevated pulmonary bacterial burdens.

Mycobacterial growth is limited in the lungs of BCG-vaccinated mice infected by the aerosol route. Substantial growth of

TABLE 1. Protective responses in the lungs and spleens of BCG-vaccinated mice at 4 and 12 weeks postchallenge with different *M. tuberculosis* strains

Infection strain[a]	Mean (± SEM) protective response in tissue for the indicated time postchallenge (CFU)[b]			
	Lung		Spleen	
	4 wk	12 wk	4 wk	12 wk
Erdman	1.26 ± 0.13	0.49 ± 0.03	1.27 ± 0.40	0.71 ± 0.17
C913	0.87 ± 0.20	0.57 ± 0.10	0.81 ± 0.10	0.89 ± 0.20
CDC1551	1.02 + 0.21	0.62 ± 0.15	1.32 ± 0.08	0.74 ± 0.20
ChS	0.75 ± 0.27	0.82 ± 0.02[d]	0.55 ± 0.04	0.58 ± 0.25
HN878	1.25 ± 0.11	0.40 ± 0.11[c]	0.84 ± 0.26	0.35 ± 0.02[e]
N4	0.92 ± 0.12	0.37 ± 0.05[c]	0.77 ± 0.25	0.14 ± 0.05[e]
NHN5	1.11 ± 0.13	0.95 ± 0.10[d]	0.48 ± 0.10	0.33 ± 0.12[e]
NY669	1.15 + 0.42	0.34 ± 0.25[c]	0.93 ± 0.02	0.63 ± 0.14
NY920	1.11 ± 0.22	0.80 ± 0.19	0.75 ± 0.02	0.68 ± 0.20

[a] In this study, the ChS, HN878, N4, and NHN5 isolates are Beijing-lineage strains.

[b] Mean protection is the average protective response (number of CFU in naïve mice − number of CFU in vaccinated mice) for two to three experiments.

[c] All of the BCG-induced protective responses in the lung at 4 and 12 weeks postchallenge were significant relative to the naïve controls except for the 12-week protection against the HN878, N4, and NY669 infections.

[d] Immunization with BCG induced significantly better protection against the ChS and NHN5 infections than against the Erdman challenge at 12 weeks post-challenge.

[e] All of the BCG-induced protective responses in the spleen at 4 and 12 weeks post-challenge were significant relative to the naïve controls except for the 12 week protection against the HN878, N4 and NHN5 infections.

these *M. tuberculosis* strains (3 to 3.5 $\log_{10}$) in the lungs was also seen in the BCG-vaccinated mice during the first 4 weeks (Fig. 1). However, the extent of mycobacterial growth was limited relative to naïve mice. Significantly decreased pulmonary concentrations of TB organisms were seen at this time point in immunized mice challenged with each *M. tuberculosis* isolate. Table 1 shows the mean protective values ($\log_{10}$ CFU for naïve mice minus $\log_{10}$ CFU for immunized mice) at 4 and 12 weeks postchallenge for two to three experiments. As seen in Table 1, BCG vaccination induced significant protective responses in the lungs against each *M. tuberculosis* challenge strain at 4 weeks after the tuberculous infection. Average levels of protection between 0.75 and 1.26 $\log_{10}$ CFU were detected in BCG-immunized mice at this early time after the infection. The levels of protection at 4 weeks postchallenge were not significantly different among these isolates (including the standard Erdman strain).

Although the lung bacterial burdens in naïve mice decreased substantially after 4 weeks postchallenge, the lung CFU levels in vaccinated mice generally increased during these later time periods. However, the rates of growth of the bacilli in the lungs of BCG-vaccinated mice slowed considerably between the 4- and 20-week time points, with increases of less than 0.5 $\log_{10}$ CFU generally being detected. Significantly elevated rates of growth were only detected for the NY669 strain, where a 1 $\log_{10}$ increase was seen in the 4- to 12-week time interval and the HN878, N4, and NY920 strains which increased by at least 0.5 $\log_{10}$ CFU in the lungs during the 12- to 20-week time period ($P < 0.05$). Interestingly, significant levels of protection in the lungs of BCG-vaccinated mice (relative to naïve mice) were detected at 12 weeks after the challenge in mice infected with all of the test strains except for the NY669, HN878, and N4 isolates (Fig. 1 and Table 1). However, significant differences in the lung bacterial CFU values of BCG-vaccinated and control mice were only observed in the NHN5, ChS, and NY920 groups at the 20-week time point (Fig. 1). It should be emphasized that significantly increased BCG-induced protective responses, compared to the standard Erdman strain, were only seen at 12 weeks postinfection for the ChS and NHN5 Beijing lineage isolates ($P < 0.05$).

Postinfection dissemination to the spleen in naïve and BCG-vaccinated mice. After a low-dose aerosol infection of C57BL/6 mice with *M. tuberculosis*, dissemination of the organisms to the spleen is detectable about 2 to 3 weeks postchallenge, and by 4 weeks the concentrations of splenic mycobacteria generally reach a chronic steady-state level for naïve mice (31). For most of the test strains, similar overall splenic growth profiles were seen in naïve mice with no substantial changes in mycobacterial concentrations detected between 4 and 20 weeks (Fig. 2; see also Fig. S1 in the supplemental material). Significant increases in splenic TB levels between 4 and 20 weeks postchallenge were only detected for naïve mice infected with the NY669, NY920, and HN878 isolates. Importantly, relative to the naïve controls, BCG vaccination delayed or reduced dissemination to the spleen of all of the different *M. tuberculosis* challenge strains. As seen in Table 1, significant decreases of about 60 to 95% in splenocyte CFU values (0.48 to 1.32 $\log_{10}$) were seen in immunized animals at 4 weeks following infection with all of the different *M. tuberculosis* isolates. At 12 weeks, significant levels of protection in the spleen (relative to naïve mice) were detected in BCG-vaccinated mice aerogenically challenged with the Erdman, CDC1551, C913, ChS, NY669, and NY920 isolates. By 20 weeks postchallenge, spleen CFU values were not statistically different between vaccinated animals and naïve controls after infection with all of the nine *M. tuberculosis* test strains.

Improved lung pathology in BCG-vaccinated mice compared to naïve controls after aerogenic *M. tuberculosis* infections. The relative postinfection lung pathology in control and immunized mice is a critical parameter in the evaluation of the effectiveness of new TB vaccines. To assess lung pathology after challenge, lung sections were removed after sacrifice, and these sections were stained with the hematoxylin and eosin reagent. Representative lung sections from mice infected with the Erdman and HN878 strains are shown in Fig. S2 to S5 in the supplemental material. At 4 weeks after the challenge with the different TB strains, three types of lung pathology patterns were observed in naïve mice. For animals infected with the C913, ChS, N4, NHN5, and NY920 strains, modestly sized, nonconsolidated lesions containing moderate lymphocyte infiltration were seen in lung sections. At this time point, interstitial and peribronchial swelling were common. For the Erdman, HN878, and NY669 strains, substantially more inflammation was apparent, and large coalescing inflammatory lesions were detected in the lungs at 28 days postchallenge. Interestingly, the CDC1551 aerogenic infections induced exaggerated early pathological responses. Substantial consolidation with lymphocyte aggregates was apparent, and considerable phagocytic infiltrate was seen in CDC1551 infected lungs at 4 weeks postchallenge. This CDC1551 strain has been previously associated with early vigorous inflammatory responses in mice and elevated rates of tuberculin conversion in humans (22, 37).

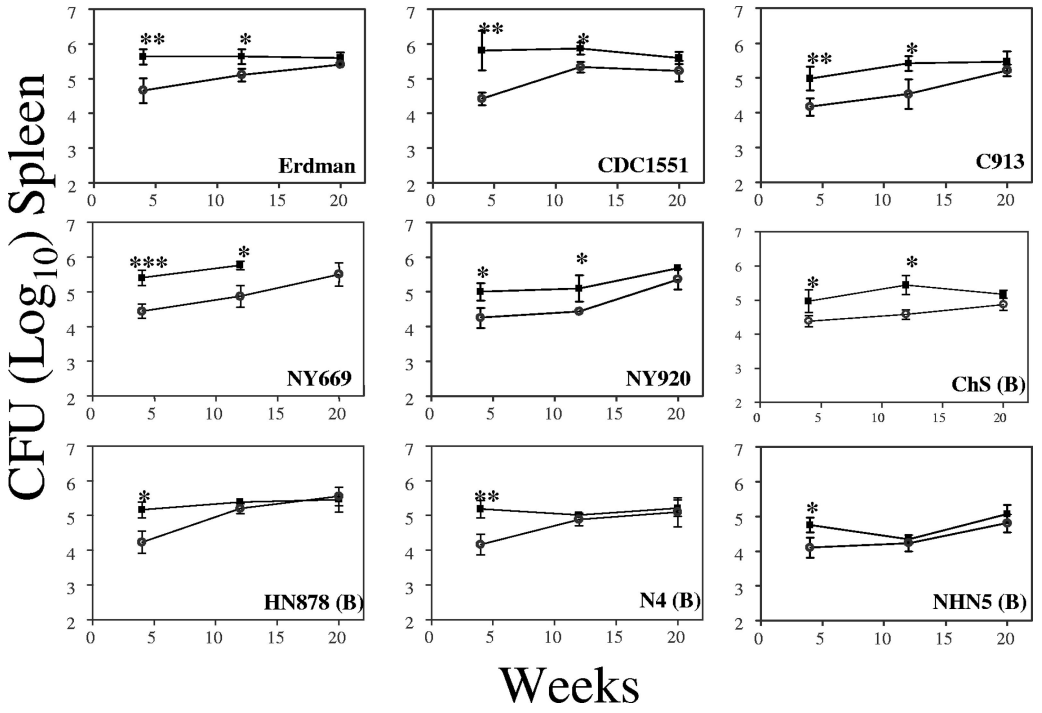

FIG. 2. Growth of nine different *M. tuberculosis* strains in the spleens of BCG-vaccinated and naïve mice. Mice were sacrificed at 4, 12, and 20 weeks following a 200-CFU aerogenic challenge with each of these *M. tuberculosis* isolates. To determine the aerosol infection dose, groups of mice were also sacrificed at 4 h postchallenge. The results are representative of two to three experiments. Significant differences between the numbers of lung CFU for vaccinated (open circles) and control (closed squares) mice are shown with asterisks. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$. In these studies, the ChS, HN878, N4, and NHN5 strains are Beijing (B) lineage strains.

By 20 weeks postchallenge, the inflammatory responses had increased but remained moderate in naïve mice infected with the C913, NHN5, and NY920 strains. Also, the inflammatory responses had subsided at this time in the CDC1551-infected animals. For these mice, large lesions containing lymphocyte aggregates were present in lung sections, but areas of relatively noninflamed lung tissue were also seen. In contrast, substantial disease progression was observed in lung sections of mice infected with the Erdman, HN878, NY669, ChS, and N4 strains at the 20-week time point. For these isolates, nearly complete consolidation of some lung regions was observed. Also, macrophage and neutrophil influx and occasional necrosis were seen in specific lesions.

In contrast, at 4 weeks postchallenge, substantially less inflammation was observed in the lungs of BCG-vaccinated animals. The granulomatous-type structures were more condensed, mature, and lymphocyte rich in the lungs of BCG-vaccinated mice than the larger, immature granulomas seen in naïve mice at this early time point. For the BCG-vaccinated animals at 20 weeks after the aerosol infections, smaller lesions and overall less inflammation were also observed (relative to naïve mice). The central pathological features within the lungs of BCG-vaccinated mice at later time points were the large areas of aggregated lymphocytes often surrounded by macrophages within the inflammatory lesions.

Reduced lung inflammation values in BCG-vaccinated mice following aerogenic *M. tuberculosis* infections. To quantitatively compare the pathological immune responses postinfection, the lung sections were evaluated using the Image Pro Plus analysis system (see Fig. S2 to S5 in the supplemental material). With this imaging system, the proportion of the lung section that is inflamed can be quantitatively defined. Previous analyses to validate the system had shown that the inflammation value for lung sections of moribund $CD4^{-/-}$ mice was 80% at 28 days postinfection while the degree of inflammation was 30% for BCG-vaccinated $CD4^{-/-}$ mice (S. Derrick, unpublished results). These inflammation values correlated with the mean survival times postinfection since the BCG-vaccinated $CD4^{-/-}$ mice (156 ± 22 days) survived fivefold longer than naïve $CD4^{-/-}$ mice (33 ± 6 days) in these earlier vaccination/challenge studies (8). The mean inflammation values for the lung sections of mice infected 4 or 20 weeks earlier with the different isolates of *M. tuberculosis* are listed in Table 2. These computer-generated inflammation values were generally consistent with the lung pathology observations described above. The levels of inflammation in the lungs of naïve mice at 4 weeks postchallenge with the different strains varied between 34 to 72%. Similar to our the lung pathology observations, the highest computer-generated inflammation values (72%) were seen after infection with the CDC1551 isolate at 4 weeks after the aerosol infection. Consistent with the significantly lower mycobacterial burdens and improved lung pathology detected in BCG-vaccinated mice at 4 weeks, the pulmonary inflammation values (13 to 35%) of immunized animals were significantly decreased, relative to naïve controls, for all nine strains tested ($P < 0.05$).

At 16 or 20 weeks postchallenge, significantly elevated lung inflammation values (relative to 4 weeks) were seen for five

TABLE 2. Lung inflammation in naïve and BCG-vaccinated mice at 4 and 20 weeks postchallenge with nine different *M. tuberculosis* strains

Infection strain	Mean inflammation value (% of lung section inflamed) for the group at:[a]			
	4 wk postchallenge		20 wk postchallenge	
	Naïve mice	BCG-vaccinated mice	Naïve mice	BCG-vaccinated mice
Erdman	48.8 ± 3.0	13.1 ± 2.1**	38.7 ± 1.5	25.5 ± 1.1**
C913	36.4 ± 4.2	24.8 ± 2.4*	34.0 ± 0.8	18.0 ± 1.7**
CDC1551	72.3 ± 2.5	35.9 ± 1.8**	37.8 ± 1.7	23.6 ± 2.5**
ChS	34.4 ± 2.5	11.7 + 4.3**	53.9 ± 7.7	24.4 ± 1.3**
HN878	49.3 ± 4.0	16.5 ± 1.4**	43.6 ± 5.2	23.2 ± 2.7*
N4	39.6 ± 3.4	15.4 ± 3.8*	47.3 ± 9.0	15.2 ± 2.5*
NHN5	35.3 ± 3.7	24.7 ± 2.8*	42.7 ± 2.9	16.8 ± 1.4**
NY669	47.5 ± 7.0	16.0 ± 0.6**	56.1 ± 0.2[b]	29.9 ± 3.5*
NY920	38.9 ± 5.8	19.4 ± 1.2**	36.3 ± 6.2	20.1 ± 4.4*

[a] Mean percentage of the area of inflammation ± standard error of the mean. Significantly reduced lung inflammation in the BCG-vaccinated mice relative to naïve controls is indicated as follows: *, $P < 0.05$; **, $P < 0.01$.

[b] Since naïve mice infected with NY669 died prior to the 20-week time point, these mice were sacrificed at 16 weeks postchallenge.

strains (Erdman, ChS, HN878, N4, and NY669). Considerable disease progression had been noted for all of these strains in visual pathological assessments. Importantly, our evaluation of the relative lung pathology at 20 weeks postinfection showed that the decreased inflammation in the BCG-vaccinated animals persisted for each strain tested. At this time point, the inflammation values were generally two- to threefold lower for vaccinated animals than values in the naïve controls. For the highly virulent NY669 strain, lung pathology assessments were done at 16 weeks because of the rapid mortality rate of naïve mice infected with this strain. Although the naïve animals challenged with NY669 were highly inflamed at 16 weeks (56.1%), a significant reduction in the inflammation value for BCG-vaccinated mice (29.9%) was detected.

DISCUSSION

The genetic and phenotypic differences that have been identified among *M. tuberculosis* isolates during the past decade have raised concerns that the protective responses elicited by new TB vaccines may be strain specific and, therefore, that these novel TB vaccine preparations may not protect against all *M. tuberculosis* strains. In fact, it has been speculated that the geographic variability in the efficacy of BCG vaccination may be due to BCG's inability to protect against the various types of *M. tuberculosis* strains that are endemic in specific regions of the world (1). Consistent with this hypothesis, intriguing studies in mice and rabbits have suggested that BCG vaccination is not effective at controlling infections by *M. tuberculosis* W-Beijing strains (19, 36). Selected epidemiologic data have also predicted that the W-Beijing strains may be resistant to BCG-induced protective immunity (1). However, in contrast to these findings, the results of our experiments did not support the hypothesis that specific *M. tuberculosis* strains are resistant to the anti-TB immunity evoked by BCG. In our studies, mice immunized with BCG were protected following aerosol infections with a classic laboratory strain, four Beijing clinical isolates, and four non-Beijing strains. After aerosol infections with all nine of these strains, statistical differences in the lung bacillary burdens and the lung inflammation values were detected between vaccinated and naïve mice at the 4-week time point. Relative growth of the infecting organisms was also statistically lower in BCG-vaccinated animals, relative to naïve mice, for six of the strains (including the Erdman strain and three of the W-Beijing isolates) at 12 weeks postchallenge. Interestingly, BCG immunization induced better protective responses against two of the Beijing lineage strains than against the standard Erdman strain at the 12-week time point. Although pulmonary bacterial burdens were reduced in vaccinated mice only when they were challenged with three of these strains at 20 weeks postchallenge, significant differences in the lung inflammation values and lung pathology between vaccinated and naïve animals were detected at the end of the study for all of the test strains. Importantly, the BCG-induced protection against two of these strains, as measured by decreases in pulmonary mycobacterial growth and reductions in lung pathology, correlated with extended survival periods for the vaccinated animals reported in earlier studies. Mice immunized with BCG and then aerogenically challenged with either the virulent *M. tuberculosis* HN878 or Erdman strains survived significantly longer than naïve controls infected with the same virulent strains (6, 16). Additionally, in this study, while all naïve mice infected with the NY669 strain died by 20 weeks postchallenge, all of their BCG-immunized counterparts survived until the 20-week time point.

The factors that have contributed to the different BCG immunization protection results seen in our study compared to earlier published reports remain uncertain but may include differences in animal models, *M. tuberculosis* challenge methods, lung pathology analyses, and strains used for infection and vaccination. Regarding the strains, various production methods can yield mycobacterial strain preparations with contrasting immunogenic activities because of different ratios of live and dead organisms, different bacterial concentrations, and altered surface compositions. For example, the failure to standardize production protocols can result in *M. tuberculosis* challenge strains with inconsistent levels of virulence. In a previous comparative study, the use of a subpotent *M. tuberculosis* Erdman preparation for murine infections led to improper initial assessments of the virulence of the CDC1551 strain (18). By contrast, the impact of immunizing with various live attenuated vaccine strains (including different BCG preparations) is unclear. Although different BCG strains have been shown to induce unique immune responses in animal models and humans, both preclinical study results and data from clinical trials have strongly suggested that different BCG preparations usually yield similar levels of protection when given at equivalent doses by the same route of administration (4, 40, 42). In fact, we have shown that the BCG Pasteur and SSI BCG strains induce statistically indistinguishable protective responses against the *M. tuberculosis* HN878 and *M. tuberculosis* Erdman strains (S. Derrick, unpublished data). To reduce this strain heterogeneity with the aim of improving the TB vaccine testing process, our laboratory has collaborated with the World Health Organization to provide standard BCG vaccine preparations and *M. tuberculosis* challenge strains to researchers throughout the world. Overall, the availability of these refer-

ence strains has facilitated and improved the comparative evaluation of new TB vaccines.

In recent reports, phenotypically different *M. tuberculosis* isolates have been shown to have various levels of virulence in animal models (9, 10, 21, 28, 30). In our experiments, the growth profiles for eight of nine *M. tuberculosis* strains were similar, and the pulmonary CFU values for most of these isolates at 20 weeks postchallenge were nearly equivalent. However, the importance of the number of culturable *M. tuberculosis* bacilli in the lungs at several months postinfection is unclear, and whether the lung CFU values correlate with virulence (as measured in survival studies) is uncertain. In an earlier report, North et al. concluded that the growth rate of mycobacteria in mice is not a reliable indicator of mycobacterial virulence (27). More recently, Palanisamy et al. showed that organ pathology is a better correlate of virulence than the number of viable organisms in animal tissues (30). At 16 or 20 weeks postinfection in our study, five of the test strains had elevated lung inflammation values including three strains—HN878, NY669, and Erdman strains—that have been shown to be virulent in mice in this and earlier studies (6, 23). Moreover, the modestly virulent CDC1551 strain was among the isolates with low inflammation values at the later time points (18, 30). Therefore, our data suggest that virulence may correlate with the extent of postinfection lung pathology but is not necessarily directly related to pulmonary CFU levels. Obviously, survival studies involving all of the strains in this study are needed to further support the association between virulence and lung inflammation.

In sum, we have evaluated nine different *M. tuberculosis* strains in a mouse model of pulmonary TB and have shown that while organ mycobacterial growth profiles were generally similar for eight of nine strains, various lung pathological responses were induced after the infection. Most importantly, we showed that BCG vaccination induced significant protective immune responses against all of these strains including four W-Beijing strains. The levels of BCG-induced protective immunity against the eight clinical isolates and the standard Erdman strain were generally similar, especially at the 4-week time point. Overall, we could not demonstrate the strain-specific resistance to BCG-induced protective immunity in our mouse model that has been suggested by other studies. It should be noted, however, that the specific protective immune responses induced by live attenuated vaccines such as BCG vaccine may differ from the protective immunity induced by protein-based, viral vectored, or DNA vaccines against TB. Further studies are needed to assess whether the anti-TB immune responses induced by nonliving TB vaccines also protect against various *M. tuberculosis* phenotypes.

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