Influence of Advanced Age on *Mycobacterium bovis* BCG Vaccination in Guinea Pigs Aerogenically Infected with *Mycobacterium tuberculosis*

Shihoko Komine-Aizawa,1,2* Toshio Yamazaki,3 Tsuyoshi Yamazaki,4 Shin-ichiro Hattori,1,5 Yuji Miyamoto,6 Naoki Yamamoto,1,7 Shinji Haga,8 Masahiko Sugitani,9 Mitsuo Honda,1,2 Satoshi Hayakawa,1,2 and Saburo Yamamoto10

AIDS Research Center,1 Division of Biosafety Control and Research,3 Department of Mycobacteriology, Leprosy Research Center,6 and Department of Bacteriology,5 National Institute of Infectious Diseases, Division of Microbiology2 and Division of Pathology,9 Department of Pathology and Microbiology, Nihon University School of Medicine, and Japan BCG Laboratory,10 Tokyo, School of High-Technology for Human Welfare, Tokai University, Kanagawa,4 and Division of Hematopoiesis, Center for AIDS Research, Kumamoto University, Kumamoto,7 Japan, and Department of Microbiology, Yong Loo Lin School of Medicine, Singapore7

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*Mycobacterium bovis* bacillus Calmette-Guérin (BCG) is the only tuberculosis (TB) vaccine currently available, but its efficacy against adult pulmonary TB remains controversial. BCG induces specific immune responses to mycobacterial antigens and may elicit protective immunity against TB. BCG remains a major public health problem, especially among the elderly, yet the efficacy of BCG in the elderly is unknown. We investigated the ability of BCG vaccination to prevent TB in young (6-week-old), middle-aged (18-month-old), and old (60-month-old) guinea pigs. BCG-Tokyo vaccination reduced the growth of *Mycobacterium tuberculosis* H37Rv in all three groups. By use of an enzyme-linked immunospot (ELISPOT) assay, antigen-specific gamma interferon (IFN-γ)-producing cells were detected in the 60-month-old guinea pigs after a booster vaccination with BCG-Tokyo. Our findings suggest that BCG-Tokyo has a protective effect against tuberculosis infection regardless of age.

Tuberculosis (TB) remains a major public health problem, especially among elderly people. Patients ≥60 years of age account for ≥50% of new cases in Japan (29). The increasing susceptibility of the elderly to *Mycobacterium tuberculosis* is generally thought to be associated with age-related changes in immune system function, especially losses or delays in antigen-specific CD4+ T-cell function (14). Compromised antigen-specific CD4+ T-cell responses may contribute to increased susceptibility to *M. tuberculosis* infection in mice (27).

*Mycobacterium bovis* bacillus Calmette-Guérin (BCG) is the only TB vaccine currently available. BCG has been used for more than 80 years (41), and vaccination with BCG is the standard for TB prevention in most countries. BCG induces specific immune responses to mycobacterial antigens and may elicit protective immunity against tuberculosis. BCG provides efficient protection against severe and disseminated TB, such as tuberculosis meningitis and miliary tuberculosis, in children (33, 34, 40). Although the long-term efficacy of BCG has been documented (3, 6), with several reports indicating efficient protection against disseminated TB in newborns and children, it appears to have less efficacy against adult pulmonary TB (2).

In fact, its efficacy against pulmonary TB in both adults and the elderly is controversial, as is the efficacy of revaccination (5).

In the present study, we examined the efficacy of BCG against TB at different ages in a common guinea pig model (15, 25, 30). We used three age-segregated groups—young (6 weeks old), middle-aged (18 months old), and old (60 months old)—and we measured the number of antigen-specific gamma interferon (IFN-γ)-producing cells as an indicator of the efficacy of the vaccine against TB.

**MATERIALS AND METHODS**

**Animals.** Female pathogen-free outbred Hartley guinea pigs were purchased from Japan SLC (Shizuoka, Japan). The guinea pigs were divided into the three groups described above and were housed in accordance with the guidelines for animal experimentation of the Japanese Association for Laboratory Animal Science (1987) and in full compliance with the Law for the Humane Treatment and Management of Animals (Japan). The guinea pigs were fed and maintained in accordance with the guidelines set forth by the Institutional Animal Care and Use Committee of the National Institute of Infectious Diseases (NIID), Japan. Once approved by an institutional committee for animal experiments, these studies were conducted at the Animal Facility of Toyama Campus, NIID, Japan, in accordance with the requirements specifically stated in the Laboratory Biosafety Manual of the World Health Organization.

**BCG vaccination.** The guinea pigs were vaccinated with 5 × 107 CFU of BCG (strain Tokyo 172) injected subcutaneously into the left or right inguinal region. The vaccination schedules were as follows. The old guinea pigs were vaccinated with BCG, maintained for 60 months, and then revaccinated with BCG 6 weeks before *M. tuberculosis* infection (group 1; n, 2). The middle-aged guinea pigs were vaccinated either 18 months or 6 weeks before the infection (groups 2 and

* Corresponding author. Mailing address: Division of Microbiology, Department of Pathology and Microbiology, Nihon University School of Medicine, 30-1 Oyaguchi-Kamicho, Itabash-ku, Tokyo, Japan. Phone: 81-3-3972-8111. Fax: 81-3-3972-9560. E-mail: ashiho@med.nihon-u.ac.jp.

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BCG vaccination was administered to guinea pigs aged 6 weeks before challenge with virulent Mycobacterium tuberculosis. The number of virulent bacteria was maintained at 2.5 ml of a 10^4 CFU/ml suspension. This number was determined by a trypan blue dye exclusion test. Single-cell suspensions were cultured with or without PPD (10 μg/ml) at 37°C in a humidified 5% CO₂ environment for 40 h. The supernatant by an immunoaffinity column, and its bioactivity was measured.

Microbial enumeration. The dissected lung samples from each guinea pig were fixed in 4% formalin for 40 h. The sections from these tissues were 4 μm thick and were stained with hematoxylin and eosin (H&E) or with Ziel-Helenes stain for acid-fast bacilli.

Preparation of cells. Mononuclear cells were isolated from the peripheral blood of the guinea pigs. Approximately 0.1 ml of blood was harvested from the animals by cardiac puncture at 0, 6, and 11 weeks after BCG vaccination. Before blood collection, the animals were anesthetized with ketamine (44 mg/kg). Peripheral blood mononuclear cells (PBMCs) were prepared with lymphoprep (JBL Co., Ltd., Gunma, Japan) and were then adjusted to 1 x 10^6/ml in complete medium (RPMI 1640 supplemented with 10% fetal bovine serum). Cell viability was determined by a trypan blue dye exclusion test. Single-cell suspensions were cultured with or without PPD (10 μg/ml) at 37°C for 18 months before infection (group 4), 6 months before revaccination with BCG 6 weeks before infection (group 4), or were not vaccinated (group 5).

Blood sample. Approximately 10 ml of blood was harvested from the peripheral blood of the guinea pigs. Approximately 10 ml of blood was harvested from the peripheral blood of the guinea pigs. Approximately 10 ml of blood was harvested from the peripheral blood of the guinea pigs. Approximately 10 ml of blood was harvested from the peripheral blood of the guinea pigs.

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Statistical analysis. The data were analyzed using the Tukey-Kramer test and Pearson’s correlation coefficient test using Statease 2 software. Differences between treatments were determined by the least-squares significant difference multiple-comparison method. A probability level of 5% (P < 0.05) was considered statistically significant.

RESULTS

DTH skin responses of guinea pigs to PPD. DTH was assessed on the skin of guinea pigs 6 or 11 weeks after BCG inoculation both before and after challenge with Mycobacterium tuberculosis. At week 6, significant DTH responses to PPD were detected in all of the guinea pigs vaccinated with BCG, while no response to PPD was detected in unvaccinated guinea pigs. The mean diameters of the indurations were as follows: 17.0 ± 4.2 mm (group 1), 20.0 ± 0.6 mm (group 2), 18.0 ± 1.7 mm (group 3), 15.3 ± 2.1 mm (group 4), and 5.5 ± 1.3 mm (group 5). No significant difference was observed among the groups vaccinated with BCG. DTH responses were detected in all groups at 5 weeks after the challenge with Mycobacterium tuberculosis (Fig. 2).

BGC-induced PPD-specific T-cell responses. We examined the stimulation by PPD of IFN-γ production by the PBMCs of...
the guinea pigs. To investigate T-cell functions specific for PPD, an IFN-γ ELISPOT assay was performed for groups 1, 4, and 5. The old guinea pigs of group 1 were inoculated with BCG 60 months before the infection and at week zero, and the young guinea pigs of group 4 were inoculated with BCG at week zero. Another group of young guinea pigs, group 5, was not inoculated. IFN-γ production by PBMCs was examined in each group at weeks zero, 6, and 11. At week 6 after the BCG vaccination, significant and specific IFN-γ responses to PPD were detected in groups 1 and 4 (Fig. 3). The mean numbers of SFCs among the PBMCs from the animals in groups 1 and 4 were 216.25 ± 24.50 and 108.75 ± 9.57, respectively. No significant IFN-γ production was detected in group 5. PPD-specific IFN-γ-secreting cells were more frequent among the PBMCs from group 1 than among those from group 4. Five weeks after the challenge with M. tuberculosis (at week 11 after the BCG vaccination), a significant increase in IFN-γ production by PBMCs following stimulation with PPD was observed in groups 1 and 4, although there was no difference between the groups in the mean frequency of cells responding specifically to PPD. The number of SFCs was also higher in group 5 after the challenge with M. tuberculosis. However, the number of SFCs was significantly lower than those in the BCG-vaccinated groups (P < 0.01). At week zero of BCG vaccination, no increase in IFN-γ production was detected by the ELISPOT assay in group 1 in spite of the early BCG vaccination; groups 4 and 5 also showed no increase.

**Effect of BCG vaccination on bacterial growth in young, middle-aged, and old guinea pigs challenged with M. tuberculosis H37Rv.** To determine the impact of BCG vaccination on bacterial growth in young, middle-aged, and old guinea pigs, bacterial replication in the lungs (Fig. 4A), tracheal lymph nodes (Fig. 4B), and spleen (Fig. 4C) was examined for each group. In all cases, those animals vaccinated with BCG showed less bacterial growth in the lungs and tracheal lymph nodes than unvaccinated animals. In the spleen, no bacterial replication was detected except in groups 2 and 5. In group 2, which received BCG vaccination 18 months before the M. tuberculosis challenge, the effect of BCG may have been attenuated. Group 1, which was revaccinated with BCG before the challenge, showed significantly less bacterial growth in the lungs than the unvaccinated guinea pigs (P < 0.05). In the tracheal lymph nodes, bacterial growth was also reduced, but the difference was not significant. We also found a significant negative correlation between the number of IFN-γ SFCs and the residual number of bacteria in the lungs (expressed in log10 CFU) 5 weeks after M. tuberculosis challenge (r = −0.6696; P = 0.04852) in groups 1, 4, and 5 (Fig. 5). This finding suggests that PPD-specific T-cell responses induced by BCG are crucial for the host defense against M. tuberculosis infection. Thus, BCG appears to have a protective effect in guinea pigs at all ages.

**Histopathology.** Figure 6 shows histopathological images of the lungs of young unvaccinated guinea pigs (Fig. 6a and b) and BCG-vaccinated guinea pigs (Fig. 6c and d) 5 weeks after M. tuberculosis challenge. Figure 6c shows a lung from a young BCG-vaccinated guinea pig (group 4), and Fig. 6d shows a lung from an old BCG-revaccinated guinea pig (group 1). In the lungs from unvaccinated guinea pigs, large granuloma nodules with central necrosis were predominant and consisted of epithelioid cells. Acid-fast bacilli were detected in the granulomas by Ziehl-Neelsen staining (Fig. 6b). Although granuloma nodules were also observed in the lungs of vaccinated guinea pigs...
Fig. 6c and d), vaccination with BCG reduced granuloma nodule formation in the lungs of both young and old guinea pigs, and no acid-fast bacilli were detected in the granulomas by Ziehl-Neelsen staining (data not shown).

Fig. 5. Correlation between IFN-γ production and bacterial growth in the lungs. There was a statistically significant negative correlation between the number of IFN-γ SFCs detected 5 weeks after *M. tuberculosis* challenge and bacterial growth in the lungs (r = −0.6696; *P* = 0.04852 as determined by Pearson’s correlation coefficient test). Circles, group 4 (young, vaccinated); squares, group 5 (young, unvaccinated); triangles: group 1 (old, revaccinated).

Fig. 4. Effects of BCG vaccination on bacterial growth in young, middle-aged, and old guinea pigs challenged with *M. tuberculosis* H37Rv. To determine the impact of BCG vaccination on bacterial growth, bacterial replication in lung (A), tracheal lymph node (B), and spleen (C) specimens from each guinea pig was examined. The minimum detectable level of bacilli in the tissue homogenate was 1.52 log_{10} CFU. Error bars represent standard deviations. Asterisks indicate that the mean numbers of *M. tuberculosis* CFU in an organ were significantly different. *, *P* < 0.05, and **, *P* < 0.01, as determined by analysis of variance (ANOVA) followed by a posthoc Tukey-Kramer test.

Fig. 6. Histopathology of lungs from guinea pigs infected with *M. tuberculosis* H37Rv. Shown are histopathological observations in the lungs of young unvaccinated (a and b), young BCG-vaccinated (c), and old BCG-revaccinated (d) guinea pigs 5 weeks after *M. tuberculosis* challenge. Bars, 1 mm (a, c, and d) and 50 μm (b).
DISCUSSION

BCG is the only TB vaccine currently available, and it has been used since 1921. It is inexpensive and safe, with few complications reported in infants. BCG provides efficient protection against severe and disseminated TB, such as tuberculosis meningitis and miliary TB, in children (33, 34, 40). However, its efficacy at preventing pulmonary TB in adults is controversial. Aronson et al. reported that BCG vaccination had long-term efficacy for American Indians and Alaskan natives (3), suggesting that a single dose offered protection for 50 to 60 years. The long-term efficacy estimates from clinical trials, observational case-control studies, and contact studies range from 0 to 80% (7), although the efficacy for the elderly is unknown. In the present study, we demonstrated that revaccination of elderly guinea pigs with BCG-Tokyo reduced bacterial replication in the lungs, alveolar lymph nodes, and spleen. In addition, in 60-month-old guinea pigs, PPD-specific IFN-γ responses were observed after the BCG-Tokyo booster vaccination. These findings suggest that BCG-Tokyo has a protective effect at all ages.

However, the efficacy of BCG revaccination is a matter of international debate (5). Several studies have shown that BCG revaccination had no protective efficacy against TB (19, 28, 32). Fjällbrant et al. reported that both primary vaccination and revaccination of tuberculin skin test-negative young adults caused a significant increase in the T-helper type 1 (Th1) immune response (12), a result consistent with the present findings in the old guinea pig model. This result suggests that BCG revaccination has a protective effect against TB. However, other factors that determine the efficacy of BCG revaccination, including age, duration of vaccination, and the influence of environmental mycobacteria, must be considered.

Cell-mediated immune responses play an essential role in the control of M. tuberculosis infection and TB. In particular, CD4+ and CD8+ T-cell subsets are considered to play important roles in the production of cytokines such as IFN-γ and tumor necrosis factor alpha (TNF-α). These cytokines are involved in inflammatory processes, including macrophage activation, control of M. tuberculosis replication, and granuloma formation (1, 10, 13). Using guinea pig models, Jeevan et al. suggested that BCG vaccination induces upregulation of IFN-γ and TNF-α after M. tuberculosis challenge (16). In the present study, we investigated IFN-γ responses by using an ELISPOT assay. To the best of our knowledge, this is the first study that used a guinea pig model together with an antigen-specific ELISPOT assay to show that BCG induces PPD-stimulated IFN-γ responses. The secretion of PPD-specific IFN-γ was observed in both young and old BCG-vaccinated guinea pigs. The number of PPD-specific IFN-γ-secreting cells was greater in 60-month-old vaccinated guinea pigs (group 1) than in young vaccinated guinea pigs (group 4). Because this was the second BCG vaccination for group 1, a booster effect may have occurred. Bacterial growth in the lungs, lymph nodes, and spleen was higher in unvaccinated guinea pigs (group 5) than in the vaccinated groups. The number of PPD-specific IFN-γ-producing PBMCs in the unvaccinated guinea pigs was significantly lower than that in the vaccinated guinea pigs. The results of our M. tuberculosis aerosol infection experiment suggest that the number of PPD-specific IFN-γ-producing PBMCs correlates with the level of protection against M. tuberculosis. While TNF-α is another important cytokine that protects against M. tuberculosis, BCG vaccination appears to modulate the potentially harmful effects of TNF-α and to reduce M. tuberculosis replication (26, 42). Recent studies have shown that general immune responses are important for resistance to M. tuberculosis. Interleukin-12 (IL-12) is required for dendritic cell migration (22), maintenance of pulmonary Th1 cells (11), and macrophage activation and subsequent production of IFN-γ. IL-27 has both proinflammatory and anti-inflammatory properties. IL-12 and/or M. tuberculosis-induced IL-27 gene expression in human macrophages may regulate macrophage function during M. tuberculosis infection (31). In addition, the importance of Th17 responses, including IL-17 and IL-23, in the pathophysiology of M. tuberculosis infection has been reported recently (4, 20, 21). M. tuberculosis-specific Th1 (IL-12 and IFN-γ) and Th17 responses play roles in the increased expression of cytotoxic T lymphocyte antigen 4 (CTLA-4) and programmed death-1 (PD-1), while IL-23 induces IFN-γ and supports the IL-17 response in the lungs. McMurray and colleagues, using a laser capture microdissection (LCM) technique, reported cytokine mRNA responses in situ in the pulmonary granulomas of nonvaccinated and BCG-vaccinated guinea pigs (23, 24). TNF-α mRNA was dominant in primary lesions microdissected from nonvaccinated guinea pigs at both 3 and 6 weeks postinfection, while the cytokine profiles of granulomas from BCG-vaccinated guinea pigs shifted from type 1 cytokine mRNA (IFN-γ and IL-12p40) at 3 weeks to a predominantly anti-inflammatory profile dominated by transforming growth factor-β (TGF-β) at 6 weeks (23, 24). These results suggest that BCG vaccination modulates cytokine responses in the lungs to promote antimycobacterial functions while controlling the potentially damaging inflammatory response.

A DTH skin test for PPD has been employed in the diagnosis of TB. While the test is highly sensitive for PPD, its specificity in the diagnosis of TB infection is controversial, because after BCG vaccination, a DTH response is detected. In the present study, DTH responses to PPD were detected in all of the guinea pigs vaccinated with BCG, and no significant difference was observed among the age groups. However, IFN-γ production by PBMCs was significantly different between the groups, and the number of PPD-specific IFN-γ-producing PBMCs correlated with the degree of protection against M. tuberculosis. These results suggest that ELISPOT assays that detect TB-specific immune responses may be the most accurate means of monitoring immunity against TB.

In Japan, individuals 65 years old or older represented 22.1% of the population in 2008, and this age group is expected to grow to one-third of the population by 2035 (8). This trend is seen in other countries as well. Currently, in Japan, more than 50% of the new cases of TB occur in patients ≥60 years old (29). The increasing susceptibility of the elderly to M. tuberculosis is generally thought to be associated with immune senescence, the most significant change being the loss of delayed production of antigen-specific CD4+ T cells (14). In mice, an inadequate antigen-specific CD4+ T-cell response is thought to contribute to increased susceptibility to M. tuberculosis infection (27). However, the mouse model has revealed that old mice express early resistance to pulmonary tuberculo-
sis infection (9, 39). CD8+ T cells contribute to TB resistance via IL-12p70-dependent production of IFN-γ (35–38). However, this innate immune response is antigen independent, and the early resistance cannot be sustained. The bacterial load in the lungs of old mice increases about 90 days after infection (9), and the lungs of old mice are eventually more susceptible to bacterial growth (39). In the present study, antigen-specific IFN-γ production was observed after BCG revaccination of 60-month-old guinea pigs. In humans, the elderly have more preexisting diseases, such as diabetes mellitus (DM) and hypertension, some of which may be associated with an increased risk of TB (17). Clearly, further evaluation of BCG in the elderly is necessary.

In conclusion, we found that vaccination of elderly guinea pigs with BCG-Tokyo reduces bacterial replication in the lungs, alveolar lymph nodes, and spleens of infected animals. In addition, PPD-specific IFN-γ responses were observed after the second BCG-Tokyo vaccination. These findings suggest that BCG-Tokyo has a protective effect at all ages.

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