Effect of Maternal Immune Status on Responsiveness of Bacillus Calmette-Guérin Vaccination in Mouse Neonates

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Abstract
Objectives: Bacillus Calmette-Guérin (BCG) vaccination has proven to be efficient in immunologically naive infants; however, it has not been investigated that maternal natural exposure to Mycobacterium and/or BCG vaccine could influence the characteristics of immune responses to BCG in newborns. In this study, we analyzed whether the maternal immune status to M tuberculosis (Mt) can affect neonatal immunity to BCG using a mouse model.

Methods: Neonates were obtained from mice that were previously exposed to live BCG, to live M avium, or to heat-killed Mt H37Rv, and from naive control mothers. One week after birth, the neonates were divided into two subgroups: one group immunized with live BCG via the subcutaneous route and the other group of neonates sham-treated. Interferon-gamma (IFNγ) secretion in response to in vitro stimulation with heat-killed BCG or purified protein derivative (PPD) was examined. Protection against Mt infection was evaluated by challenging mice nasally with live Mt H37Rv followed by counting colonies from spleen and lung homogenates.

Results: BCG-immunized neonates showed increased IFNγ secretion in response to heat-killed BCG or PPD. All mice in BCG-immunized neonates subgroups showed reduced bacterial burden (colony forming unit) in the lungs when compared with control naive neonate mice. However, no statistically significant difference was observed when comparing BCG-immunized mice born from mothers previously exposed to M avium or immunized with either heat-killed H37Rv or live BCG and mice born from naive mothers.

Conclusion: The maternal immune status to Mt does not appear to impact on the immunogenicity of BCG vaccine in their progeny in our experimental conditions.

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1. Introduction

*Mycobacterium tuberculosis* (*Mt b*) is an intracellular pathogen that causes severe infectious disease, tuberculosis (TB), which is responsible for high level of mortality and morbidity [1–3]. About 30% of the population is worldwide infected with this infectious agent [1]. Most of them are considered to have latent tuberculosis infection (LTBI) and only 10% of the infection [1]. Most of them are considered to have latent tuberculosis infection (LTBI) and only 10% of the infection will develop to serious pulmonary TB [2,4]. Nevertheless, new cases of this bacterial infection are still occurring mostly in developing countries, and immunologically immature infants in these regions can be easily exposed to infections with mycobacteria [5].

To prevent from TB infection, Bacillus of Calmette and Guérin (BCG) obtained from a live attenuated strain of *M bovis* (*M bovis*) has been used as an only authorized vaccine since 1921 [6]. Previous study showed outstanding effectiveness of BCG vaccination at birth in control of neonatal miliary TB and meningitis TB as well [7]. However, the vaccination with BCG shows inconsistent responsiveness and relatively low protection efficacy against predominant pulmonary TB in adults [8]. In animal and human studies, neonatal immunization with BCG showed relatively higher protection efficacy than that in adults, which is mediated by Th1-type immune responses [9,10].

Guirado and colleagues [11] reported that antibodies play an essential protective role against mycobacterial infections by passive immunization using sera obtained from mice treated with detoxified *Mt b* extracts in B cell-deficient mice, while it is generally accepted that cell-mediated immune response is important in controlling *Mt b* infections. While current BCG vaccine has proven to be efficient in immunologically naïve infants, a maternal history of natural exposure to mycobacteria or vaccination against TB has not been studied yet. Therefore, we hypothesized that maternal immune status to mycobacteria may play a critical role in the uptake and subsequent protective immunogenicity of BCG in newborns. In the present study, we analyzed whether the maternal immune status to *Mt b* can affect the neonatal immunity to BCG in mouse neonates by assessing in vitro IFNγ secretion by stimulated spleen cells [11–13] and by evaluating the protective efficacy after challenge experiments. We found that neonatal BCG immunization can reduce the bacterial load in pulmonary tissues. In addition, we showed that the maternal immune history mediated by either vaccination or pre-exposure to *Mt b* was insufficient to elicit the specific immune responses to BCG vaccine in their progeny. This may provide basic insights into importance of immunogenicity through neonatal vaccination and/or maternal vaccination, and it can be considered for further TB vaccine researches.

2. Materials and Methods

2.1. Animals

Six-weeks old female Balb/c mice (OrientBio or Daehan Biolink, Korea) were maintained under specific pathogen-free conditions in the animal facility, International Vaccine Institute (Seoul, Korea), where they were fed with sterilized food and water *ad libitum*. Breeding cages were checked daily for new births, and the pups were kept with the mother until weaning at 3 weeks of age. Animal infections with *Mt b* were performed at the Biosafety Level 3 facility of the institute. All experiments described were approved by appropriate institutional animal care and use committees.

2.2. Bacteria

*M bovis* BCG Pasteur (BCG) [14], *M tuberculosis* H37Rv (*Mt b* H37Rv) [15] and *M avium subsp. Hominsuis* (*M avium*)[16] were cultured in suspension in 7H9 Middlebrook media (BD Bioscience, San Diego, CA, USA) supplemented with 10% OADC (v/v, BD, Franklin Lakes, NJ, USA) and 0.05% Tween80.

2.3. Vaccination of mice

One week before mating, mice were immunized with live BCG [subcutaneously, 5 × 10⁵ colony forming units (CFU)], live *M avium* (intranasally, 1 × 10⁶ CFU), or heat-killed *Mt b* H37Rv (intranasally, 5 × 10⁷ CFU), respectively. Neonates obtained from individual mothers were divided into two subgroups and vaccinated 1 week after birth: one group immunized with live BCG (5 × 10⁵ CFU) via subcutaneous route and the other group of neonates sham-treated.

2.4. Antibody determination

Four weeks after BCG vaccination, immunoglobulin G (IgG) level in serum obtained from individual mice were analyzed by enzyme-linked immunosorbent assay (ELISA). Briefly, ELISA plates were coated with heat-inactivated BCG (5 × 10⁵ CFU/mL) in phosphate buffered saline (PBS) overnight at 4 °C. Next day, plates were washed four times with PBS-T (v/v, 0.05% Tween20, Sigma-Aldrich, St. Louis, MO, USA) and blocked with 1% bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, MO, USA) for 1 hour at room temperature. After washings, the samples were applied in serial dilutions and subsequently incubated for 2 hours at room temperature. Following extensive washings with PBS-T, horseradish peroxidase-conjugated goat anti-mouse IgG (Southern Biotech, Birmingham, AL, USA) was diluted in 1% BSA and applied. After 1-hour incubation at room temperature, the plates were washed four times, and the 3,3′, 5,5′-tetramethylbenzidine (TMB) peroxidase substrate (Moss, Inc., Pasadena, MD, USA) was subsequently added to develop the enzymatic color reaction. Optical density was detected.
by using a microplate reader (molecular device, Sunnyvale, CA, USA) at 450 nm, and concentrations were given in inverse log2 at OD of 0.2.

2.5. Mt b challenge of mice

Infection with Mt b H37Rv was performed 4 weeks after BCG immunization via intranasal route with an inoculum of 200 CFU/20 μl per head. The mice were sacrificed four weeks after challenge infection, and bacterial loads in lung and spleen were determined by incubating serial dilutions of lung and spleen homogenates from individual mice on 7H11 Middlebrook agar plates. The 7H11 Middlebrook agar plates were freshly prepared with 100 μg/mL of ampicillin (Sigma-Aldrich, St. Louis, MO, USA) and 10 μg/mL of fungizone (Gibco, Grand Island, NY, USA). The plates were incubated for 3–4 weeks at 37 °C with 5% CO2, and the numbers of bacterial colonies were counted.

2.6. Measurement of in vitro IFN γ production

Specific IFNγ response against heat-inactivated BCG (10 minutes at 95 °C) or purified protein derivative (PPD) (kindly provided by Institut Pasteur Korea) was measured by ELISA. It was detected after in vitro stimulation of splenocytes and lung cells obtained from individual mice at 3 weeks or 4 weeks after neonatal vaccination with BCG, respectively. Lung (5 × 10⁵ cells/well) and/or spleen cells (2 × 10⁵ cells/well) were obtained from individual mice and stimulated with either heat-killed BCG (5 × 10⁵ CFU/mL) or PPD (5 μg/mL) for 72 hours at 37 °C with 5% CO2. Following incubation, supernatants were harvested and stored at −80 °C until use. IFNγ level was determined by using mouse BD OptEIA IFNγ ELISA kit (BD Bioscience, San Diego, CA, USA) according to manufacturer’s instructions.

2.7. Statistical analysis

All statistical analyses using the unpaired Student t-test were performed in GraphPad Prism 5 (GraphPad Software Inc., La Jolla, IL, USA). A p < 0.05 value was considered statistically significant.

3. Results

3.1. Determination of optimal subcutaneous BCG inoculum

Although T cell-mediated immunity plays critical roles in protection against Mt b infection, antigen-specific antibodies induced by successful vaccination are thought to be also involved in modifying the course of Mt b infection. We first assessed humoral immune response in response to BCG vaccination. Different doses of live BCG, 1 × 10⁴, 5 × 10⁴, 1 × 10⁵, and 5 × 10⁵ CFU were subcutaneously administered into 1-week-old neonate mice and specific anti-BCG IgG titer in serum was measured after 3 weeks of vaccination. Anti-BCG IgG production was detected solely in mice vaccinated with 5 × 10⁵ CFU of live BCG; this dose was therefore used as a standard for further immunizations in this study (Figure 1).

3.2. Impact of maternal immune status on in vitro IFNγ production in neonates after BCG immunization

We analyzed cellular immune responses of neonates following BCG vaccination by measuring in vitro IFNγ secretion in response to BCG or PPD, since it has been described that protective immune responses to Mt b infection is mainly mediated by T-cell immunity.

First, to compare different maternal immune backgrounds, 6-week-old female mice were administered with either live BCG subcutaneously, heat-inactivated Mt b H37Rv intranasally, live M avium intranasally, or sham-treated, respectively. Mating occurred 1 week later. Neonate mice born from mothers previously exposed to above described mycobacteria were divided into two subgroups; one group consisting of neonates vaccinated with BCG at the age of 7 days and the other group of neonates sham-treated (Figure 2). After 3 weeks, spleen and lung cells from naïve and BCG immunized neonates born from either naïve or BCG immunized mothers were analyzed for IFNγ production.

Figure 1. IgG level following vaccination with BCG. To determine the optimum dose of BCG vaccine, we first quantified the IgG level in serum in response to BCG vaccine by enzyme-linked immunosorbent assay. One week after birth, neonates (n = 4 to 6 heads per each group) were subcutaneously primed with different doses of live BCG. Three weeks after the BCG-priming, specific anti-BCG IgG level was measured. Anti-BCG IgG production was detected solely in mice vaccinated with 5 × 10⁵ CFU of live BCG. Representative data are shown as reciprocal log2 Immunoglobulin G titer. Data are mean ± SD. BCG = Bacillus Calmette-Guérin; CFU = colony forming unit; IgG = immunoglobulin G; SC = subcutaneous; SD = standard deviation.
secretion in response to BCG. No statistically significant
difference was observed when comparing subgroups of
BCG-immunized mice born from mothers previously
immunized with BCG or from naive mothers in both
tissues (Figure 3A and B).

Next, we examined splenic IFN-γ secretion in
response to PPD (Figure 3C). Statistically significant
difference was detected only between BCG immunized
progeny born from naïve mothers and from BCG
immunized mothers (Figure 3). These results suggest
that neonatal BCG vaccination can elicit cellular
immune response regardless of maternal pre-exposure to
mycobacteria; however, maternal pre-exposure to BCG
can increase PPD-specific IFN-γ production in their
progeny.

3.3. Influence of maternal immunity on
protective efficacy of neonatal BCG
vaccination

We thereby performed Mtb challenge experiment in
order to see whether different maternal immune status
can affect BCG vaccine efficacy in their progeny. Intranasal Mtb H37Rv challenge was performed after 4
weeks of BCG vaccination, and bacterial numbers were
determined from lung and spleen of individual mice
after 4 weeks of challenge (Figure 2).

When compared with nonvaccinated neonates, neonates mice administered with BCG exhibit a significant
decrease of the bacterial loads in the lung following Mtb infection; however, there was no statistical significance
between BCG-immunized mice born from female
mice previously exposed to either heat-killed Mtb H37Rv,
live M avium, or live BCG, or naïve mothers. These results
suggest that maternal exposure to different Mtb-related antigens does not have impacts on neonatal immunogenicity induced by BCG vaccination. No statistically
significant reduction of bacterial burden in the spleen
was observed in any of the groups. These results suggest
that the impact of maternal immunity on the protective
immunity induced by neonatal vaccination with BCG is
minimum.

4. Discussion

In this study, the effect of maternal pre-exposure to
mycobacteria has been investigated on BCG vaccine
efficacy in neonates using a mouse model. We found
that maternal immune status to mycobacteria does not
impact on the protective immunogenicity of BCG
vaccine in their progeny under our experimental
conditions.

The main component of immune protection trans-
ferred from mother to child is antibody. Maternal anti-
body is transferred to the fetus and newborns across
the placenta using the neonatal Fc receptor and via
breast milk, respectively [17]. Unlike pigs, horses and
ruminants, which rely mainly on colostral transfer, mice
allow placental transfer of maternal immunoglobulin.
Therefore, neonatal mice can be applied as an alterna-
tive to human neonatal vaccination [17].

To generate enough quantity of antibodies, we first
determined the optimal subcutaneous BCG inoculums in
neonate and used the 5 x 10⁵ CFU as a standard dose for
immunization (Figure 1). BCG-immunized neonates
showed increased IFN-γ secretion in response to BCG or
PPD. In vitro IFN-γ secretion in response to PPD from
splenocytes of BCG-immunized neonates born to BCG
vaccinated mother is higher than BCG-immunized
neonates born to naïve mothers. This statistically signif-
cant difference might indicate that maternal pre-
exposure to BCG influences the neonates’
responsiveness to PPD by an immune mechanism still unknown. No statistical difference in protective efficacy against \( \text{Mt} \text{b} \) infections was observed suggesting that the increased IFN\( \gamma \) production by PPD-stimulated cells was not sufficient to prevent the infection.

Protection against \( \text{Mt} \text{b} \) infection was evaluated after intranasal challenge with \( \text{Mt} \text{b} \) H37Rv. BCG-immunized mice were protected when compared to naive mice. However, no statistically significant difference was observed between BCG-immunized mice born from mothers previously exposed/immunized with heat-killed \( \text{Mt} \text{b} \) H37Rv, BCG or \( \text{M} \text{avium} \) and mice born from naive mothers (Figure 4).

In humans, multiple factors, for instance, environmental and genetic background, nutritional and socio-economic status, may affect the BCG efficacy [18,19]. Successful neonatal immunity achieved by vaccination can effectively protect the high-risk population from \( \text{Mt} \text{b} \) infections. This study showed that the efficacy of

**Figure 3.** Impact of maternal immune status on neonatal IFN\( \gamma \) production in response to BCG. Specific IFN\( \gamma \) responses against heat-inactivated BCG (A and B) or PPD (C) were detected by in vitro stimulation using splenocytes and lung cells obtained from individual mice (\( n = 8 \) to 16 heads per each group) either at 3 weeks or 4 weeks post neonatal vaccination with BCG, respectively. There was no statistically significant difference between groups. Data are mean \( \pm \) SD. \(*p < 0.05 \) (Student’s \( t \)-test). B = Bacillus Calmette-Guérin (BCG)-vaccinated; hk \( \text{Mt} \text{b} \) = heat-inactivated \( \text{Mt} \text{b} \) H37Rv-administered; \( \text{M avium} \) = live \( \text{M avium} \)-administered; N = naive; SD = standard deviation.

**Figure 4.** Bacterial burden in lung and spleen. Bacterial numbers (CFUs) were determined 4 weeks after challenge with virulent \( \text{Mt} \text{b} \) H37Rv in lung and spleen (\( n = 3 \) to 11 heads per each group) of mice (Balb/c) primed with BCG vaccine. Data are shown as log\(_{10}\) CFU. Data are mean \( \pm \) SD. \(*p < 0.05 \) (Student’s \( t \)-test) compared with the naive and BCG-vaccinated neonates of each subgroup. \(* *p < 0.01. \) B = Bacillus Calmette-Guérin (BCG)-vaccinated; CFU = colony forming unit; hk \( \text{Mt} \text{b} \) = heat-inactivated \( \text{Mt} \text{b} \) H37Rv-administered; \( \text{M avium} \) = live \( \text{M avium} \)-administered; N = naive; SD = standard deviation.
neonatal vaccination with BCG or with recently developed new BCG-based live vaccine against tuberculosis might not be affected by maternal immunity transferred to newborns in regions endemic for \( M. \) \( t \) \( b \) or \( M. \) \( a \) \( v \) \( i \) \( u \) \( m \).

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