

# Construction of Recombinant BCGs Overexpressing Antigen 85 Complex and Their Protective Efficacy against *Mycobacterium tuberculosis* Infection in a Mouse Model

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## 항원 85 복합체를 과발현하는 재조합 BCG의 개발 및 마우스 모델에 있어서의 결핵균 감염에 대한 방어 효능

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Tuberculosis (TB) remains an enormous global health problem, and a new vaccine against TB more potent than the current inadequate BCG vaccine is urgently needed. We constructed three recombinant *Mycobacterium bovis* BCG (rBCG) strains over-expressing antigen (Ag) 85A, Ag85B, or both of *M. tuberculosis* using their own promoter and secretory sequence, or hsp60 promoter. SDS-PAGE analysis of rBCG proteins showed over-expression of Ag85A and Ag85B proteins in higher level than of those in their parental strain of BCG. In addition, rBCG(rBCG/B.FA) over-expressing Ag85A and Ag85B induced strong IFN- $\gamma$  production in splenocytes. However, there was no significant difference in protective efficacy between rBCG and their parental BCG strain. In this study, therefore, rBCG over-expressing Ag85A, Ag85B, or both failed to show enhanced protection against *M. tuberculosis* infection in a mouse model. (*Tuberc Respir Dis* 2004; 57:125-131)

**Key words** : Tuberculosis, Vaccine, Recombinant BCG.

### Introduction

Tuberculosis (TB) has been one of the leading infectious diseases in the world, and the World Health Organization (WHO) estimated 80 million new cases of tuberculosis in this decade<sup>1</sup>. In addition, the emergence of drug-resistant strains of *M. tuberculosis* have emphasized once again that drug therapy has its intrinsic limitations.

The only currently available TB vaccine is an attenuated strain of *Mycobacterium bovis*, bacillus Calmette-Guérin(BCG). BCG continues to be widely administered to children, yet its efficacy has demonstrated variable protective efficacies ranging from 0 to 85% in different field trials<sup>2</sup>. Thus, an improved vaccine is urgently needed to replace BCG and to prevent TB effectively. Experimental approaches to develop an improved vaccine against TB have included the use of live attenuated mycobacteria<sup>3</sup>, subunit vaccine<sup>4-6</sup>, recombinant BCG, and DNA vaccine<sup>7,8</sup>.

The protective antigens for tuberculosis are still not precisely defined, but the prevailing hypothesis that they predominantly reside within the secreted or exported proteins of the bacillus has been recently supported by observations in animal model<sup>9</sup>. The three components of the antigen (Ag) 85 complex, a

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30- to 32- kilodalton family of proteins (Ag85A, Ag85B, and Ag85C), constitute a major fraction (20-30%) of the secreted proteins in the culture filtrate (CF)<sup>10</sup>. Ag85 homologues are found in all mycobacterial species. The Ag85 complex induces strong T-cell proliferation, interferon- $\gamma$  (IFN- $\gamma$ ) production and cytotoxic T lymphocyte (CTL) activity in most healthy individuals infected with *M. tuberculosis* or *M. leprae*<sup>11</sup>. Sensitization with Ag85 complex occurs in the first phase of mycobacterial infection<sup>12</sup>. Hence, recognition of these antigens may be an effective strategy of the host to control initial bacterial replication, consequently making the proteins of the Ag85 complex reasonable vaccine candidates.

In the present study, we have constructed three rBCG strains over-expressing only Ag85A, Ag85B, or both and evaluated their immunogenicity and protective efficacy against *M. tuberculosis* infection in a mouse model. However the enhanced *in vitro* immunological response of the recombinant vaccine, rBCG/B.FA, afford no greater protection against *M. tuberculosis* infection.

## Materials and Methods

### 1. Bacteria

*M. tuberculosis* H37Rv and *M. bovis* BCG Pasteur 1173P<sub>2</sub> that had been maintained at the Korean Institute of Tuberculosis were used in this study. *M. tuberculosis* Erdman strain was provided by P.J. Brennan (Colorado State University, Fort Collins, Co., U.S.A.). All mycobacteria were maintained in Ogawa media. *E.coli* JM109 was used for cloning.

### 2. Preparation of culture filtrate protein(CFP)

Culture filtrate protein (CFP) from *M. tuberculosis* was provided by Department of Microbiology, Yonsei University College of Medicine.

Culture filtrate protein was purified from *M. tuber-*

*culosis. M. tuberculosis* bacteria ( $2 \times 10^6$  CFU/ml) were grown in modified Sauton medium without Tween80 on an orbital shaker for 7 days. The culture supernatants were sterile filtered and concentrated by ultrafiltration, over an Amicon ultrafiltration stirred cell (Amicon, Danvers, MA, USA) fitted with a PM10 membrane (Millipore, Bedford, MA, USA). The total protein concentration was estimated by the bicinchoninic acid protein assay (Pierce, Rockford, IL, USA).

### 3. Construction of the plasmids

The plasmid pEM that was provided by Hye-Young Lee (Yonsei University, Wonju, Korea)<sup>13</sup>, an *Escherichia coli*-*Mycobacterium* shuttle expression plasmid, was employed for over-expression of *M. tuberculosis* genes in BCG. The plasmid pEM was engineered to express recombinant Ag85A of *M. bovis* BCG and Ag85B of *M. tuberculosis* from its own promoter and *hsp60* promoter. Genes encoding Ag85A from *M. bovis* BCG and Ag85B from *M. tuberculosis* were amplified by PCR using primers containing *Bam*HI or *Spe*I. After cloning into pT7Blue T-vector (Novagen, Madison, WI, USA), amplified DNAs were digested with *Bam*HI or *Spe*I and ligated to *Bam*HI or *Spe*I-digested pEM. The orientation of Ag85A and Ag85B gene inserts were then confirmed by restriction enzyme digestion. The recombinant plasmids were introduced by electroporation into BCG Pasteur 1173P<sub>2</sub>. Recombinant strains were maintained in Middlebrook 7H11 (M7H11) agar (Difco, Sparks, MD, USA) medium containing kanamycin at concentration of 25  $\mu$ g/ml. The expression and export of recombinant proteins were verified by SDS-PAGE.

### 4. Preparation of immunization inocula

Wild type BCG and rBCGs were cultured on Sauton medium at 37°C as unshaken cultures. Kanamycin 25  $\mu$ g/ml was added to rBCG cultures. The surface pellicles were collected and disrupted with 6 mm glass

beads by gentle vortexing. Viable organisms were counted by plating serial dilutions on M7H11 agar (Difco, Sparks, MD, USA).

### 5. Immunization of animals

Specific pathogen-free female C57BL/6 mice were purchased from Japan SLC, Inc. Shizuoka, Japan. Groups of eight mice each injected subcutaneously with a single dose of BCG and rBCGs [ $2 \times 10^5$  colony forming unit (CFU)/mouse]. Negative controls were injected subcutaneously with 200  $\mu$ l of sterile, pyrogen-free saline. All mice used were 6- to 7- weeks of age and were housed in cages contained within a BL-3 biohazard animal room at Yonsei University Medical Research Center. Animals were allowed free access to water and standard mouse chow.

### 6. Measurement of IFN- $\gamma$ production

Four weeks after immunization, immunized or control mice were sacrificed, and their spleens were removed aseptically. The spleens from three mice were pooled in each group. Spleen cells ( $2 \times 10^5$  cells/well) were cultured in 96-well cell culture plates (Nunc, Roskilde, Denmark) in RPMI1640 supplemented with 50  $\mu$ M 2-mercaptoethanol, 1% penicillin-

streptomycin, 1 mM glutamate and 10% (v/v) fetal calf serum. The culture filtrate protein (CFP) were added to the wells at a final concentration of 10  $\mu$ g/ml. Supernatant were harvested after 6 days incubation at 37°C in a humidified CO<sub>2</sub>. Measurement of IFN- $\gamma$  was performed with a mouse IFN- $\gamma$  OptEIA™ Set (Pharmingen, San Diego, CA, USA).

### 7. Challenge infection

Six weeks after immunization, five mice in each group were challenged with an aerosol generated from a 10 ml, single-cell suspension containing a total of  $5 \times 10^5$  CFU of *M. tuberculosis* Erdman strain. These aerosol doses delivered approximately 200 live bacilli to the lungs. Mice were sacrificed 4 weeks after challenge. The spleens and lungs were removed aseptically and cultured for CFU of *M. tuberculosis*. Differences in CFU between groups were analyzed statistically by the Student's *t*-test.

## Results

### 1. Expression of Ag85A and Ag85B from rBCGs

Three recombinant BCGs expressing and secreting

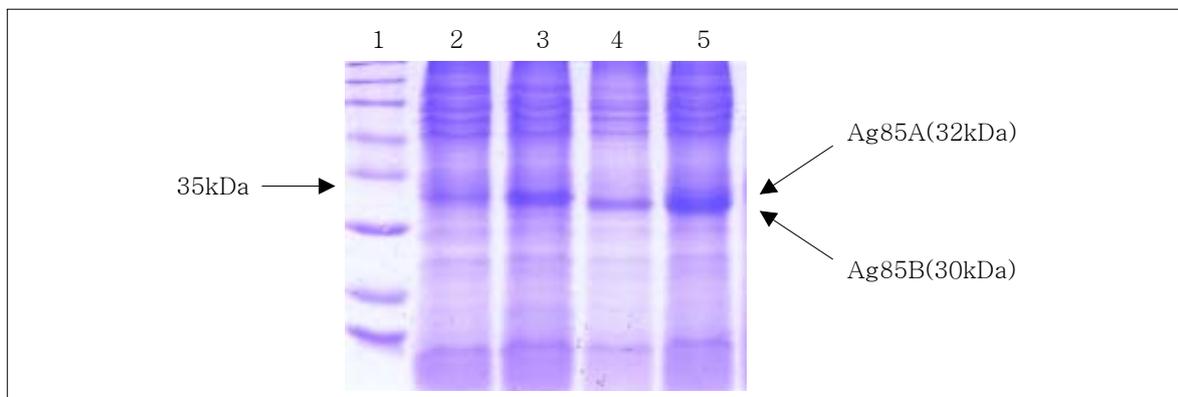


Figure 1. SDS-PAGE analysis of culture filtrate protein of recombinant BCGs. Lane 1, molecular weight standard; lane 2, BCG; lane 3, rBCG/FA; lane 4, rBCG/FB; lane 5, rBCG/B.FA. Protein bands were visualized by Coomassie blue staining.

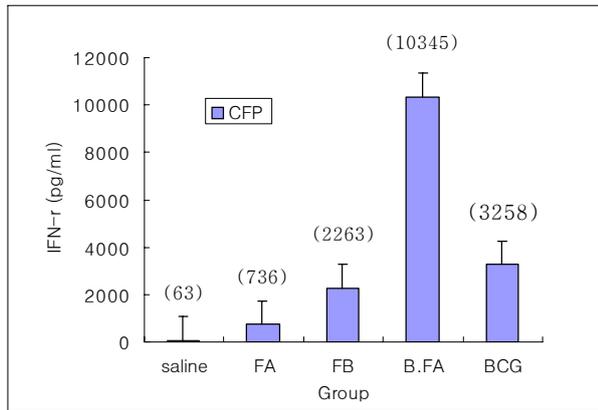


Figure 2. IFN- $\gamma$  production by splenocytes of immunized mice. C57BL/6 mice were immunized subcutaneously with 200  $\mu$ l of saline or  $2 \times 10^5$  CFU of FA (rBCG/FA), FB (rBCG/FB), B.FA (rBCG/B.FA), or BCG. Three mice per group were sacrificed after 4 weeks. Splenocytes were cultured with culture filtrate protein (10  $\mu$ g/ml) of *M. tuberculosis* H37Rv. Supernatants were harvested after 6 days of incubation for IFN- $\gamma$  assay. Data represent the mean and standard error of three mice per group. ( ) : IFN- $\gamma$  concentration(pg/ml)

Ag85A (rBCG/FA), Ag85B (rBCG/FB), or both (rBCG/B.FA) were prepared by transformation of wild-type BCG Pasteur 1173P<sub>2</sub> with recombinant plasmids containing genes encoding the antigens with their own promoters or *hsp60* promoter. SDS-PAGE was used to analyze and staining of SDS-PAGE gel with Coomassie brilliant blue revealed all rBCGs expressed and secreted a relatively high level of recombinant Ag85A, Ag85B, or both proteins (Fig. 1).

## 2. IFN- $\gamma$ production of splenocytes

IFN- $\gamma$  release was evaluated after *in vitro* stimu-

lation with culture filtrate protein(10  $\mu$ g/ml). The results of IFN- $\gamma$  ELISA showed that splenocytes from mice immunized only with rBCG/B.FA could produce higher IFN- $\gamma$  level than those from the parental BCG group (Fig. 2).

## 3. Protective efficacy of the rBCG vaccines in the mouse model

BCG and rBCGs-immunized mice were challenged by aerosol with *M. tuberculosis* Erdman. Lungs and spleens were removed 4 weeks after challenge. Animals immunized with rBCGs or BCG had a fewer organisms in the lungs and spleens (except the rBCG/FA group) than the saline control mice ( $P < 0.05$ , Student *t*-test). There was, however, no significant difference in protective efficacy between rBCGs and BCG (Table 1).

## Discussion

Most of the candidate vaccines that have been developed against TB have performed either inferior to BCG or at the most at par with BCG. This has given a lot of impetus to the proposal that BCG itself should be developed into a more potent vaccine. For development of an improved BCG vaccine, several cytokine genes have been expressed in recombinant BCG(rBCG) system, in an attempt to boost the Th1 inducing ability of BCG<sup>14</sup>. It is also been suggested

Table 1. rBCG vaccine-induced protective efficacy in the mouse model

Vaccine group <sup>a</sup>	Mean log <sub>10</sub> CFU of <i>M. tuberculosis</i> ± SEM (n=5) <sup>b</sup>			
	Lung	<i>p</i> -value compared to BCG	Spleen	<i>p</i> -value compared to BCG
Saline (Control)	5.10 ± 0.21		3.69 ± 0.48	
rBCG/FA	4.55 ± 0.18*	0.70	3.36 ± 0.22	0.27
rBCG/FB	4.54 ± 0.04**	0.72	2.96 ± 0.22*	0.31
rBCG/B.FA	4.40 ± 0.18**	0.67	3.13 ± 0.21*	0.91
BCG	4.48 ± 0.36*		3.15 ± 0.33	

<sup>a</sup>C57BL/6 mice were injected once subcutaneously with saline(200  $\mu$ l), BCG, or rBCGs ( $2 \times 10^5$  CFU).

<sup>b</sup>Number of bacteria isolated from the spleen and lung 4 weeks after aerosol challenge. \*,  $P < 0.05$ ; \*\*,  $P < 0.001$  compared to controls (Student *t*-test).

that overexpression of immunodominant antigens in BCG can also lead to improved induction of Th1 response.

Both Ag85A and Ag85B antigens of *M. tuberculosis* have been widely used as new vaccine candidates against TB. The recent finding reported by Horwitz and coworkers on the better protective efficacy of a recombinant BCG expressing an epitope (Ag85B) of *M. tuberculosis* compared to the parent strain have strengthened the justification for using such a strategy. The authors showed that guinea pigs immunized with the recombinant BCG and challenged with virulent *M. tuberculosis* via aerosols, had fewer bacilli in their lungs and spleen and smaller and fewer lesions in these organs as compared to those immunized with the parent BCG strains. To date, this recombinant BCG vaccine is the first vaccine reported to induce greater protective immunity against TB than the standard BCG vaccine in an animal model<sup>15</sup>. Also, rBCG which produced large amounts of Ag85A enhanced the potential to protect the host<sup>16</sup>. Ohara *et al.*<sup>17</sup> reported that rBCG overproducing Ag85A, Ag85B, and MPB51(rBCG/BA51) reduced the multiplication of *M. leprae* in the foot pads than that by parental BCG. It is expected that the combination of several important protective antigens would induce stronger protective immunity in broad human population.

This study describes three rBCGs that overexpressed Ag85A, Ag85B, or both. To prepare rBCGs, we used the hsp60 promoter and their own promoters for expressing Ag85A, Ag85B, or both. The recombinant BCGs successfully produced large amounts of Ag85A and Ag85B.

The ability to produce IFN- $\gamma$ , the hallmark of a TH1 type of T-cell response, is widely accepted as a strong correlate of protective immunity to TB<sup>18</sup>. We focused on this cytokine response in the vaccinated animals. IFN- $\gamma$  levels produced in response to

culture filtrate protein of *M. tuberculosis* were higher in mice immunized with rBCG/B.FA than in BCG-immunized mice, suggesting that rBCG/B.FA enhance the protective immunity of the host more potently than BCG. In the protective efficacy experiments, immunization with rBCG/FB or with rBCG/B.FA gave a 0.5 - log reduction in CFU in the lungs and spleens compared with control. However, although the rBCG/B.FA elicited higher IFN- $\gamma$  level than their parental BCG strain, it didn't show greater protective efficacy greater than that of parental BCG against *M. tuberculosis* infection. The results showed the difference between immune responses to antigens *in vitro* and protective immunity *in vivo*. The reasons are to be explored.

IFN- $\gamma$  is a central cytokine in control of *M. tuberculosis* infection. This cytokine is produced by both CD4 and CD8 T cells in tuberculosis<sup>19-21</sup> and is important in macrophage activation and perhaps other functions. Although IFN- $\gamma$  production alone is insufficient to control *M. tuberculosis* infection, it is required for the protective response to this pathogen. IFN- $\gamma$  is produced by T cells from healthy PPD+ subjects as well as those with active tuberculosis. Although some studies suggest that IFN- $\gamma$  levels are depressed in patients with active tuberculosis<sup>22</sup>, this cytokine may not be ideal as an immune correlate of protection. The recent report that *M. tuberculosis* has developed mechanisms to limit the activation of macrophages by IFN- $\gamma$  suggests that the amount of IFN- $\gamma$  produced by T cells may be less predictive of outcome than the ability of the cells to respond to this cytokine<sup>23</sup>.

Several approaches may be considered for further enhancement of the immunogenicity and protective efficacy of rBCG vaccine: (i) using shuttle vectors with high-efficiency promoters to increase expression of antigens; (ii) adding more kinds of protective antigens, such as ESAT-6<sup>24</sup>, CFP10 and MPT64; (iii)

coexpressing genes of cytokines, such as IL-2, IFN- $\gamma$ , and IL-12(14); and (iv) utilizing other methods of administration, such as intranasal and oral vaccination<sup>25</sup>.

Some scholars have previously proposed that genetic engineering BCG is a candidate vaccine against TB. Despite the unsatisfactory protective efficacy yielded in our research, the increased immunogenicity compared with BCG and the advantages of BCG itself(e.g., it is safe to use, stable to store, available for immunization at birth, and cost-effective) over other types of new vaccines make rBCG attractive in TB vaccine development.

### SUMMARY

결핵균 감염에 대한 주요 방어 항원 물질로서 항원 85 복합체(Ag85A, B, C)가 주목되고 있다. 우리는 이들 항원들을 과발현하는 재조합 BCG를 클로닝하였고, 마우스 모델을 이용하여 결핵균 감염에 대한 재조합 BCG의 방어 효능을 알아보려고 하였다. 항원 85A를 과발현하는 재조합 BCG를 rBCG/FA, 항원 85B를 과발현하는 재조합 BCG를 rBCG/FB, 그리고, 이들 두 항원을 과발현하는 재조합 BCG를 rBCG/B.FA라고 명명하였고, 이들 항원들의 과발현 여부를 SDS-PAGE상에서 확인한 결과, 재조합 BCG에서 항원 85A와 B 단백질이 BCG에 비해 과발현된 것을 알 수 있었다. 면역주사한 마우스에서 분리한 비장세포를 *M. tuberculosis* H37Rv의 culture filtrate protein(CFP)으로 자극하여 분비된 IFN- $\gamma$  농도를 측정된 결과에서는 rBCG/B.FA만이 BCG에 비해 높은 IFN- $\gamma$  농도를 나타내었으나, 마우스 모델을 이용한 결핵균 감염에 대한 재조합 BCG의 방어 효능 실험에서는 BCG와 뚜렷한 차이를 나타내지 못하였다.

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