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The phosphoenolpyruvate carboxykinase of *Mycobacterium tuberculosis* induces strong cell-mediated immune responses in mice

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Abstract

Phosphoenolpyruvate carboxykinase (PEPCK) catalyzes guanosine or adenosine mononucleotide-dependent reversible conversion of oxaloacetate (OAA) and phosphoenolpyruvate (PEP). *Mycobacterium (M) tuberculosis* possesses a putative GTP-dependent PEPCK. To analyze the immune responses caused by PEPCK, the effects of PEPCK on the induction of $CD4^+$ T cells and cytokines such as IFN-γ, IL-12 and TNF-α were evaluated in mice. It was found that the number of $CD4^+$ T cells was increased in the PEPCK immunized mice although the change of the number of $CD8^+$ T cells was not significant. The cytokines IFN-γ, IL-12 and TNF-α were increased significantly in the mice immunized with PEPCK than those of incomplete adjuvant. These characteristics were further demonstrated in the mice infected by *pckA* mutated BCG strain. The results indicate that PEPCK can effectively induce cell-mediated immune response by increasing activity of cytokines and PEPCK may be a promising new subunit vaccine candidate for tuberculosis. (Mol Cell Biochem **288:** 65–71, 2006)

Key words: *Mycobacterium tuberculosis*, phosphoenolpyruvate carboxykinase, cell immune response

Introduction

Phosphoenolpyruvate carboxykinase (PEPCK) catalyzes guanosine or adenosine mononucleotide-dependent reversible conversion of oxaloacetate (OAA) and phosphoenolpyruvate (PEP) [1]. In most cases, the PEPCK activity has been postulated to catalyze the first committed step in gluconeogenesis, the formation of PEP from OAA. In adipose tissue, GTP-PEPCK primes glycerogenesis, especially under fasting conditions, when pyruvate, lactate and amino acids serve as the precursors for PEP [2], this tissue does not synthesize glucose. In parasites and in certain bacteria, PEPCK fulfills an anaplerotic role by carboxylating PEP to OAA [3–6].

Mycobacterium (M) tuberculosis is the pathogen of human tuberculosis which is one of the most rampant infectious diseases and a leading cause of death worldwide [7]. An analysis of recently determined whole genome sequence of *M. tuberculosis* [8] shows that this bacterium possesses a putative GTP-dependent PEPCK (Rv0211). It has been reported that *M. smegmatis* possesses a PEPCK that kinetically prefers the PEP synthesis direction. Such a preference allows a combination of pyruvate carboxylase and PEPCK activities to constitute a beginning of the gluconeogenesis and/or glycerogenesis route [9]. Collins [10] reported that *M. bovis* deficient in PEPCK expression were avirulent in guinea pigs and failed to induce a protective immune response. We

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found that the *pckA* gene mutated *M. bovis* BCG strain resulted in the reduced survival ability of the bacteria in both macrophages and in mice [11]. In this report we describe the induction of T cells and cytokines as well as the immune response caused by phosphoenolpyruvate carboxykinase from *M. tuberculosis*.

Materials and methods

Bacterial strains and growth conditions

M. bovis BCG (Pastuer) and H37Rv obtained from Barry Bloom (Albert Einstein College of Medicine, New York) were maintained in Middlebrook (MB) 7H9 media (Difco laboratories, Detroit, MI) with 10% (v/v) OADC enrichment (10 X OADC: per liter, 20 g Glucose, 8.5 g NaCl, 0.6 ml Oleic acid, 50 g BSA) and 0.05% Tween-80 at 37° C. *Escherichia coli (E. coli)* HB_{101} was purchased from China Academy of Sciences. For *E. coli* culture, Luria-Bertani (LB) medium (Fisher Biotech) was used.

*Preparation of BCG*Δ*pckA strain*

As reported previously [11], the shuttle vector pPR23 was used for inactivation of *pckA* gene through the allelic exchange. The plasmid pPR/pck/Kan constructed by inserting kanamycin resistance gene in EcoRI site of *pckA* gene open reading frame of *M. tuberculosis* H37Rv was used to transform BCG wild-type via electrophoration. The mutants were identified by Southern blot with kanamycin gene, *sacB* gene and *pckA* gene probes.

Preparation of PEPCK recombinant protein and antibody

M. tuberculosis H37Rv chromosome DNA was amplified with the primers designed from *pckA* gene open reading frame of *M. tuberculosis*: 5′-CGCCATATGACCTCAGCGACCATCCC-3′ and 5′-CGCCTCGAGCTAACCTAGGCGCTCCT-3′. The PCR product was cloned into the plasmid of pET-based vector p6HisF-11d linearized by NdeI and XhoI. *E. coli* HB_{101} were transformed with plasmid pHF/*pck* and cultured in LB medium with 50 μg/ml ampicillin and 50 μg/ml kanamycin. The selected clone was cultured at 30° C to OD_{600} of 0.5, and then shifts to 42 ° C for 30 min, 37 °C 1.5 h. The cells were harvested by centrifugation at 4000 × g at 4° C and the pellet was disrupted for 3 min through Homogenizer (Biospec). After centrifugation at 15000 × g for 30 min at 4° C, the protein was purified with Superflow Ni2+-nitrilotriacetic acid-agarose (Qiagen, Inc.) following the instruction of the manufacture. The recombinant PEPCK (r-PEPCK) was used to produce polyclonal antibody.

Western blot

The cell-free extracts were prepared as follows: after centrifugation of the bacterial cells at 3000 × g for 15 min at 4° C, the pellet was suspended in 1 ml extraction solution (50 mM imidazole pH7.5, 5 mM $MnCl_2$, 1 mM 2-mercaptoethonal, 50 μg/ml lysostaphin, 10 μ/ml DNAse I) for 50 ml culture. After incubation at room temperature (RT) for 15 min, the lysate was homogenized with bead beater through Homogenizer (Biospec) for 3 min at high speed. The supernatant was collected after centrifugation at 15000 × g for 30 min at 4° C and separated by SDS-PAGE at about 60 μg of protein through a 10% acrylamide gel. Then the proteins were blotted onto a nitrocellulose membrane. The membrane was blocked with 5% non-fat milk in PBS and reacted with an affinity-purified anti-tuberculosis recombinant PEPCK serum (1:500). After washing with PBS-TT (PBS buffer containing 0.05% Tween-20 and 0.05% Triton X-114) 3 times at RT, donkey anti-rabbit antibody conjugated to horseradish peroxidase was added and incubated at RT for 1 h. After washing with PBS-TT, the blot was developed with diaminobenzidine reagent solution from ECL kit as described by manufacture.

Immunizations

Emulsions comprising equal volumes of PEPCK antigen (Ag) in PBS and incomplete Freund's adjuvant (Difco) were prepared and administered as subcutaneous injections (in total 10 μg per mouse). Mice were boosted 2 weeks later with subcutaneous doses administered 2 weeks apart. In addition, to further verify the ability of PEPCK to induce the cell-mediated immune response, mice were infected with 1×10^6 *BCG*Δ*pckA* strain or BCG wild type strain via lateral tail-vein. The difference of induction of T cells and cytokines between the two strains was compared.

Flow cytometry

At 6 weeks post immunization, mice were sacrificed and single-cell suspension was prepared as described [12]. Fourteen days postimmunization of third time, spleens were removed and cell suspensions (1×10^7 cells per ml) were resuspended in RPMI medium 1640 (GIBCO) supplemented with 2 mM L-glutamine (GIBCO), 100 units/100 μg/ml penicillin/streptomycin solution (GIBCO), and 10% heat-inactivated fetal calf serum. The single cell suspension was added to 96-well, flat-bottomed microtiter plates. A volume of 10 mg/ml of r-PEPCK was added and the supernatants were collected from cultures after 72 h and assayed by flow cytometry. The following MAbs (BD Pharmingen) were used for flow cytometry: anti-mouse $CD4^+$ (L3T4) H129.19

conjugated with fluorescence isothiocyanate (FITC). Anti-mouse $CD8^+$(Ly-2) 53-6.7 conjugated with R-phycoerythrin (R-PE). Analysis was done on a flow cytometer FC500 (Beckman coulter).

Cytokine assays

At 14 days after the third time infection with mycobacteria, the spleens were collected, homogenized and cultured as described above. The supernatant was collected for cytokine assays. Gamma interferon (IFN-γ) was detected with rat anti-mouse McAbs AN18 (BD Pharmingen). Tissue necrosis factor (TNF-α) was detected with rat anti-mouse McAbs TN3-19.12 and interleukin 12 (IL-12) (p70) was detected with rat anti-mouse McAbs 9A5. The cytokines were tested with ELISA. The second antibody was rabbit anti-rat antibody conjugated to alkaline phosphatase (Sigma).

Statistical analysis

Single factor analysis of variance (ANOVA) was applied for statistical evaluation of the data generated by wild-type BCG (BCG-WT) and *BCG*Δ*pckA* strain. A p-values of <0.05 was considered as a statistically significant observation.

Results

pckA gene expressed 72kDa protein

To investigate if *pckA* gene encodes a functional protein in BCG, the open reading frame of *M. tuberculosis pckA* gene is cloned. The polyclonal antibody against the recombinant PEPCK protein was produced. The crude proteins of *M. tuberculosis*, wild type BCG and the *BCG*Δ*pckA* strain were used in SDS-PAGE and reacted with the polyclonal antibody via Western blotting. It was found that a band of 72kDa was present in both wild-type BCG and *M. tuberculosis* strain, which was equal to the predicted molecule size. However, no band was found in *pckA* mutated BCG, indicating that the PEPCK antigen was actively expressed *in vivo* (Fig. 1).

pckA-induced pathogenicity

By the end of immunization, the spleens of PEPCK immunized mice were expanded greatly than those immunized with only incomplete adjuvant (Fig. 2A). Under the microscopy, no granuloma was observed in the *BCG*Δ*pckA* strain immunized mice except a few scanty and diffuse infiltrates

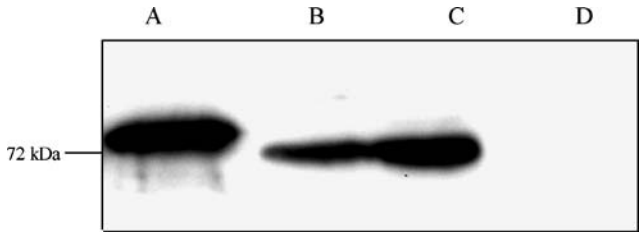

Fig. 1. Western blot. The crude proteins extracted from different mycobacterial strains were separated on 10% SDS-PAGE and analyzed with anti-PEPCK antibody. (A) The recombinant PEPCK fusion protein from *E. coli* HB_{101}. (B) The protein from *M. tuberculosis* H37Rv strain. (C) The protein from BCG wild-type strain. (D) The protein from *BCG*Δ*pckA* strain. Since a His-flag is presence in the vector, the band from the recombinant PEPCK is bigger than 72 kDa.

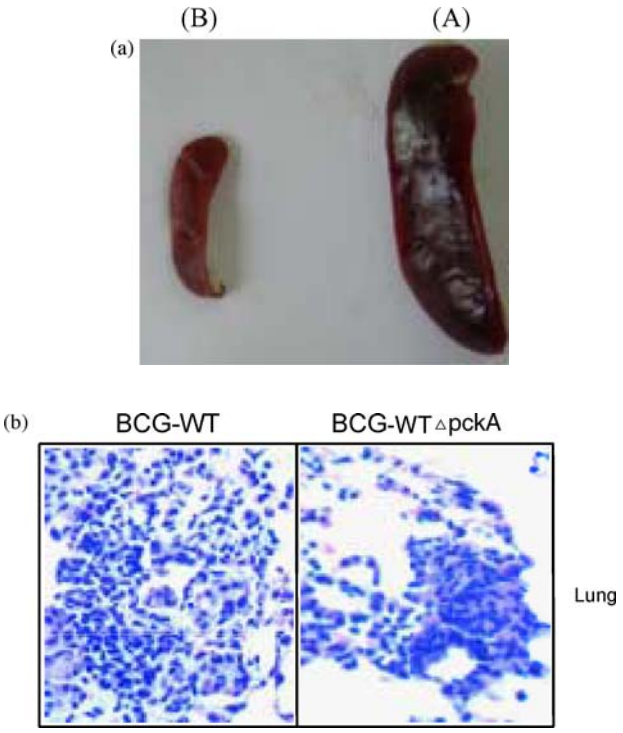

Fig. 2. (a) Organ change caused by the immunization of PEPCK. Mice were immunized with (A) the recombinant PEPCK plus incomplete Freund's adjuvant or (B) incomplete Freund's adjuvant only and spleens were harvested at 2 months of immunization. (b) The histological change caused by PEPCK. The sections of the lungs from the mice infected with BCG-WT or *BCG*Δ*pckA* were stained with Hematoxylin & Eosin and observed with 63 × oil immersion objective of Axioskop 2 plus inverted microscope. BCG-WT: mice infected with BCG wild type strain. *BCG*Δ*pckA*: mice infected with *pckA* mutant BCG strain.

containing mainly macrophages and T cells. In contrast, the lung of mice infected with BCG wild type contained granulomatous inflammation containing large macrophages and lymphocytes (Fig. 2B).

PEPCK induces strong CD4$^+$ T cell recruitment and activation

After immunization, the number of CD4$^+$ T cells from the PEPCK immunized group was significantly higher than that of adjuvant group immunized group ($p < 0.05$). There was no significant difference for CD8$^+$ T cells between the two groups (Fig. 3).

To further verify the ability of PEPCK to induce the cell-mediated immune response, mice were infected with *BCGΔpckA* strain or BCG wild type strain and the difference of induction of T cells and cytokines between the two strains was compared. It was found that CD4$^+$ T cells in wild-type BCG group were significantly higher than both of *BCGΔpckA* strain immunized group and PBS control group ($P < 0.001$). There was no significant difference for CD8$^+$ T cells among *pckA* mutant BCG strain, wild type strain and PBS immunized groups ($P > 0.05$) (Fig. 4). These results indicated that the mutated PEPCK was unable to induce T cell recruitment/activation.

The effect of PEPCK on the production of cytokines

Because IFN-γ, TNF-α and IL-12 cytokines play the important role in the protective immune response in mycobacteria, the secretion ability of these cytokines induced by PEPCK was studied. The result showed that the amount of TNF-α, IFN-γ and IL-12 in PEPCK immunized mice was higher than that of adjuvant group ($P < 0.05$) (Fig. 5). The amount of TNF-α from BCG-WT group was higher than that of BCG mutant group ($P < 0.001$). The IFN-γ from BCG-WT group was higher than that of BCG mutant group ($P < 0.001$). In addition, IL-12 from BCG-WT group was higher than that of BCG mutant group ($P < 0.001$) (Fig. 6). There was no significant difference for all the three cytokines between *BCGΔpckA* group and PBS control group. These results indicate that the mutation of *pckA* gene has inhibited the secretion of IFN-γ, TNF-α and IL-12 cytokines.

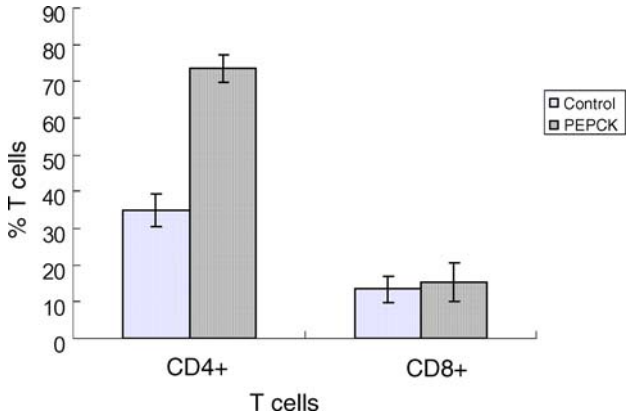

Fig. 3. FACS analysis of spleen T cells isolated from BALB/C mice immunized with PEPCK. Mice were immunized with the recombinant PEPCK plus incomplete Freund's adjuvant or incomplete Freund's adjuvant only. Spleen cells were obtained at 14 days after the third time infection and were analyzed by FACS. The data were representative of experiments performed on pools of cells from four mice.

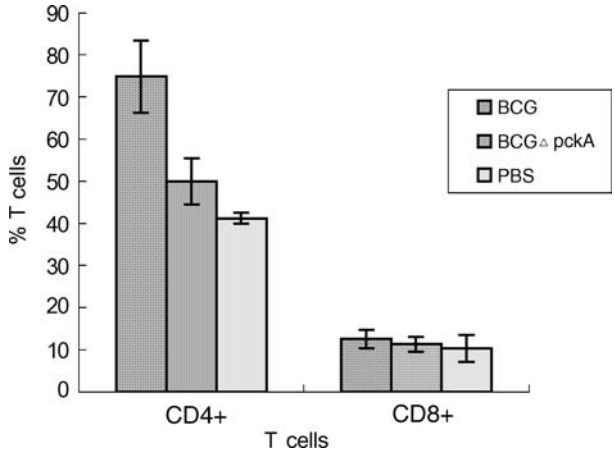

Fig. 4. FACS analysis of spleen T cells isolated from BALB/C infected with *BCGΔpckA*. The spleens of mice infected with 1×10^6 BCG-WT or *BCGΔpckA* were collected at 14 days after the third time infection. The supernatant of the cultured spleen cells was analyzed by flow cytometry. Data were analyzed with single factor analysis of variance and were representative the two independent experiments performed on pools of cells from four mice.

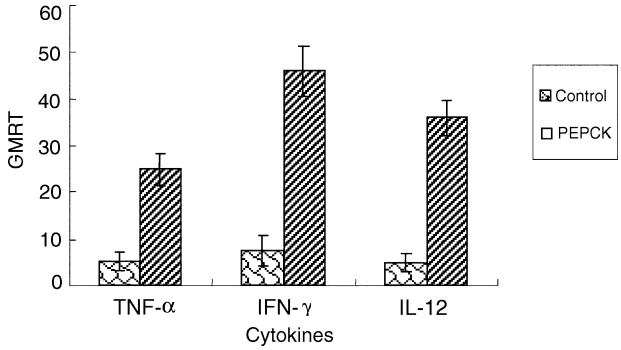

Fig. 5. Secretion of cytokines induced by PEPCK. The spleens of mice immunized with PEPCK were collected at 14 days after the third time infection. The supernatant from the cultured spleen cells of mice was analyzed with specific antibodies against cytokines by ELISA. The reaction was evaluated by the Geometric Mean Reverse Titer (GMRT). The number was representative of the experiments performed on pools of cells from four mice.

Discussion

Human tuberculosis is one of the most rampant infectious diseases and a leading cause of death worldwide [7], which

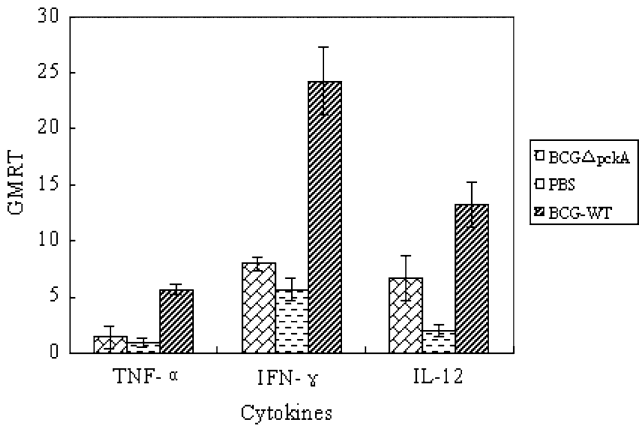

Fig. 6. Secretion of cytokines induced by *BCGΔpckA*. The spleens of mice infected with 1×10^6 BCG-WT or *BCGΔpckA* were collected at 14 days after the third time infection. The supernatant of the cultured spleen cells was analyzed with specific antibodies against cytokines by ELISA. The reaction was evaluated by Geometric Mean Reverse Titer (GMRT). The number was representative of the experiments performed on pools of cells from four mice.

is likely to become even more critical in coming years because of the high incidence of human immunodeficiency virus disease in regions where infection with the intracellular pathogen *M. tuberculosis* is endemic. Many factors contribute to this global burden of tuberculosis, but one cause is undoubtedly the lack of a more effective vaccine. The current vaccine, Bacille Calmette–Guérin (BCG), is an attenuated strain of *M. bovis* that was developed 80 years ago by the simple technique of subjecting a virulent *M. bovis* strain to serial culture *in vitro* for 13 years [13]. While BCG prevents childhood forms of the disease, it provides only limited protection against the vast majority of disease and in the most recent large trial it provided no protection at all [13–15]. Therefore, more effective antituberculosis vaccines are required to combat the tuberculosis epidemic. For developing vaccines, one of the criterions is to select the mycobacterial antigens which may induce a strong immune response. It is known that *M. tuberculosis* is an obligate intracellular organism and mainly causes cell-mediated immune response. The current study shows that PEPCK can effectively induce cell-mediated immune response by increasing CD4 T cells and cytokines such as IFN-γ, IL-12 and TNF-α indicating that PEPCK may be a promising new subunit vaccine candidate for tuberculosis.

M. tuberculosis resides primarily in a vacuole within the macrophage, and thus, major histocompatibility complex (MHC) class II presentation of mycobacterial antigens to $CD4^+$ T cells is an obvious outcome of infection. $CD4^+$ T cells are considered to be the major T cell subset responsible for immunity against *M. avium* [16] and *M. tuberculosis* [17]. The spatial distribution and kinetics of recruitment of $CD4^+$ T lymphocytes to the lung following infection of mice with *M. tuberculosis* have been well characterized [18, 19]. Murine studies with antibody depletion of $CD4^+$ T cells [20], adoptive transfer [21], or the use of gene-disrupted mice [22] have shown that the $CD4^+$ T cell subset is required for control of infection. In humans, the pathogenesis of HIV infection has demonstrated that the loss of $CD4^+$ T cells greatly increases susceptibility to both acute and reactivation tuberculosis [23]. In this study, the number of $CD4^+$ T cells from the PEPCK immunized group is significantly higher than that of adjuvant immunized group indicates that PEPCK may be an important immune protective antigen.

The importance of PEPCK in immune response against *M. tuberculosis* is demonstrated further by the induction of cytokines. In this study, it is found that IFN-γ and IL-12 are increased significantly in the PEPCK immunized group than those of control group. As reported that strong Ag-specific Th1 responses, characterized by IFN-γ secretion, are crucial for protection against mycobacterial infection [24]. Interferon-γ can enhance expression of MHC class II, and costimulatory molecules involved in antigen presentation, and modulate the expression of other molecules involved in antigen processing, such as immunoproteasome subunits and transporter associated with antigen processing, thus promoting optimum activation of $CD4^+$ T cells. It is documented that IL-12 is a crucial cytokine in controlling *M. tuberculosis* infection [25]. In the early phase of the immune response IL-12 drives the production of INF-γ from natural killer (NK) cells [26–29]. During the ensuing immune response, IL-12 plays a key part in driving the activation, differentiation, and expansion of antigen-specific T-helper type 1 cells.

The important role of PEPCK played in the immune response is also reflected by the histological change of mice. In this study there is no well-defined granuloma in the tissues of the mice infected with the *pckA* gene mutated BCG strain although the typical granuloma can be found in the BCG wild-type infected mice, indicating PEPCK plays an important role in the formation of granuloma. The role of PEPCK in the granulomatous formation was reported in schistosomiasis previously. The granulomatous formation in schistosomiasis, a disease with main immunopathological damage of granulomatous inflammation, was strictly dependent on $CD4^+$ T helper cells specific for schistosomal egg antigen phosphoenolpyruvate carboxykinase *(Sm-PEPCK)*. PEPCK strongly stimulated $CD4^+$ Th cells from BALB/c mice, and elicited significant gamma interferon production [17, 30]. In addition, TNF-α is important for walling off infection and preventing dissemination. Convincing data on the importance of this cytokine in granuloma formation in tuberculosis and other mycobacterial diseases has been reported [31, 32]. TNF-α affects cell migration and localization within tissues in *M. tuberculosis* infection. TNF-α influences expression of adhesion molecules as well as chemokines and chemokine

receptors, and this is certain to affect the formation of functional granuloma in infected tissues. TNF-α has also been implicated in immunopathologic response and is often a major factor in host-mediated destruction of lung tissue [33]. In our studies, the reduced formation of granuloma in the *pckA* gene mutated BCG strain and the increased level of TNF-α in the PEPCK immunized mice showed that PEPCK played an important role in the compartmentalized immune response.

Acknowledgements

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