Isocitrate lyase encoding plasmids in BCG cause increased survival in

ApoB100-only LDLR^{-/-} mice

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Agnes Mira Szabo[™], Valeria Endresz, Ferenc Somogyvari, Andras Miczak, Ildiko Faludi Department of Medical Microbiology and Immunobiology, University of Szeged, Szeged, Hungary 6720 Dóm tér 10. Email: szabo.agnes.mira@gmail.com **Abstract** ApoB100-only LDLR^{-/-} (B6;129S-ApoB^{tm2Sgy}Ldlr^{tm1Her}/J) mice were inoculated with *Mycobacterium bovis* BCG harbouring plasmids carrying the gene for isocitrate lyase. The presence of ~29 times more copies of this gene resulted in a higher bacterial yield in the spleens and lungs of the infected mice. The spleen was 3-4 times heavier, and in the spleen the bacteria survived over 10 days longer than did the bacteria with the control plasmid. Propionate was less toxic for bacteria carrying *icl* plasmids *in vitro*. This recombinant BCG can be a possible vaccine candidate.

Keywords BCG immunization isocitrate lyase M. tuberculosis

Introduction

More than one-third of the world's population is infected with tuberculosis (TB) bacilli. *Mycobacterium tuberculosis* is a very successful pathogen. There were an estimated 9.4 million new cases of TB in 2009 and an estimated 1.7 million deaths (including 380 000 people with HIV), making this disease one of the world's most significant infectious killers. Millennium Development Goal 6 is that the incidence of TB should be falling by 2015 [1].

Although *Mycobacterium bovis* BCG is the only approved vaccine used for the prevention of TB in humans, the protective efficacy of the BCG vaccine varies widely in different parts of the world, reaching a maximum of 78% [2]. Most current efforts to improve the level of protective immunity provided by BCG include the development of recombinant BCG vaccines expressing different antigens [3].

The survival of *M. tuberculosis* in infected macrophages requires the activity of isocitrate lyase (Icl), a key enzyme in the glyoxylate cycle. The glyoxylate shunt permits the effective utilization of two-carbon compounds, such as acetate (from the β -oxidation of fatty acids), to satisfy carbon requirements [4]. The glyoxylate cycle is required for the virulence of other bacteria and fungi [5, 6]. In mycobacteria, Icl has a dual role in the metabolism of fatty acids. It participates in the methylcitrate cycle too [7]. β -oxidation of odd-chain or branched-chain fatty acids results in the production of propionyl-CoA which is toxic for bacteria and fungi. [8,9]. Propionyl-CoA is metabolised in the methylcitrate cycle. The other way to use up proponyl-CoA is the methylmalonyl pathway. It requires a vitamin-B₁₂-derived cofactor [10].

A key to the intracellular survival of *M. tuberculosis* is its ability to prevent the fusion of phagosomes containing the internalized bacterium with the lysosomal system of the host cell. The addition of certain exogenous fatty acids modulates phagosome maturation, resulting in the killing of pathogenic bacteria, whereas other fatty acids stimulate pathogen growth [11]. Thus the role of lipids is multifaceted.

We used ApoB100-only LDLR^{-/-} mice to determine the survival of *M. bovis* BCG carrying plasmids coding for Icl. This double-knock-out (KO) mouse strain produces only apolipoprotein-B-100 and is deficient in low-density lipoprotein (LDL) receptors. This mouse model has a lipid profile similar to that in most humans with atherosclerosis. Extended BCG survival is observed if the bacteria carry plasmids with the gene coding for Icl. We assume that this increased survival can result in more effective protection.

Materials and methods

Bacterial strains and growth conditions

M. bovis BCG was grown in Middlebrook 7H9 broth or 7H10 agar (Difco Laboratories, Detroit, MI, USA) enriched with 10% Middlebrook OADC (oleic acid/albumin/dextrose/catalase) (Difco) and 0.05% Tween 80 (Sigma–Aldrich Chemie GmbH, Steinheim, Germany) with the appropriate antibiotic. *Escherichia coli* DH5α was used for plasmid preparation.

Plasmid construction

We constructed pMV262(icl), which carries *icl* after the mycobacterial *icl* promoter (GenBank accession no. CAE55284.1) in pMV262 [12]. II 5'-ACT ATC TAG ATC CGC AGG ACG TCG A-3 and I2 5'-GTA AGC TTC AGA CTA GTG GAA CTG G-3' primers were used to synthetize *icl* with *M. tuberculosis* H37Rv chromosomal DNA as template. Primers were planned by using the sequence of H37Rv [13]. The PCR amplification conditions were as recommended by the manufacturer, with a GeneAmp II (Applied Biosystems, Foster City, CA, USA) thermocycler with Advantage GC cDNA polymerase (BD Biosciences Clontech, Mountain View, CA, USA). The amplified DNA was cut with XbaI and HindIII and inserted into pMV262 opened with the same enzymes.

Gene copy number determination

The High Pure PCR Template Preparation Kit (Roche Diagnostic GmbH, Mannheim, Germany; Cat. No.: 1796828) was used for DNA extraction. Each DNA sample was analysed in triplicate. The reaction volume was 15 µl, containing 3 µl of DNA, 1 µmol/l of each of the primers (2µl), 7.5 µl of reaction buffer (IQ[™] 2X Supermix, Bio-Rad Laboratories, Hercules, CA, USA) 0.6 µl of EVAGreen (20x EVAGreen[™] Biotium Inc., Hayward, CA, USA) and 1.9 µl of distilled water. We used the forward primer 5'-AGC GCA TAT GTC TGT CGT CGG-3' and reverse primer 5'-GTA AGC TTC AGA CTA GTG GAA CTG G-3' for *icl*. The reference gene (GenBank accession no. O53899) primer set was 5' TGG CAT ATG AAC CGG CAA CCT ATC 3' and 5' GAG GAT CCT CAT TCA TAG GAC GTG 3'. The primer sets have very similar efficiencies. A BIO-RAD CFX 96 instrument (Bio-Rad, Hercules, CA, USA) was used for quantitation. The PCR conditions were initial denaturation at 95 °C for 10 min, followed by 35 cycles of denaturation (94 °C for 20 s), annealing (55 °C for 30 s) and extension (68 °C for 45 s). The emitted fluorescence was measured after the extension step.

Quantitation was performed by online monitoring for identification of the exact time point at which the logarithmic linear phase could be distinguished from the background (crossing point). The gene copy number was calculated by the Δ Ct method with Bio-Rad CFX Manager 1.1 Gene Expression software.

Electroporation of M. bovis BCG

Competent *M. bovis* BCG prepared in 10% glycerol was transformed with pMV262 or pMV262(icl) by electroporation with a Gene Pulsar (Bio-Rad, München, Germany) set at 2.5 kV and 25 μ F, and with the pulse controller resistance set at 1,000 Ω [14, 15]. Transformed BCG was selected on Middlebrook 7H10 agar plates supplemented with 30 μ g/ml kanamycin.

Mouse experiments

We used ApoB100-only LDLR^{-/-} (B6;129S-ApoB^{tm2Sgy}Ldlr^{tm1Her}/J; 16 females per group and their weight ranged from 25-30 g) with the genetic background of a mixture of C57BL/6 and a particular 129 strain from Jackson Laboratories (Bar Harbor, Maine, USA). These mice express full-length ApoB-100 in their LDL particles and have 3-fold higher plasma levels of ApoB100 than those in LDLR^{-/-} mice. The mice were maintained under standard husbandry conditions and were supplied with food and water *ad libitum*. Intraperitoneal infection of the KO mice was started at 12-14 weeks of age and consisted of 100 µl of PBS containing 10⁶ colony-forming units (CFU) of either BCGpMV262 or BCGpMV262(icl). Four mice from each group were sacrificed on days 10, 14, 21 and 35 post-infection. The mice were euthanized and their spleens and lungs were excised.

All experiments complied with the University of Szeged Guidelines for the Use of Laboratory Animals.

Culturing of BCG from the lungs and the spleen

The dissected spleens were homogenized by pressing them through nylon mesh into PBS (1 ml) containing 0.05% Tween 20. The lungs were removed and homogenized mechanically in 1 ml of PBS containing 0.05% Tween 20. 100 μ l of tissue suspension was used. Ten fold dilutions of these suspensions were plated on Middlebrook 7H10 agar for cultivation of the bacteria. CFUs were determined after 21 days of incubation at 37°C.

Western blot

Cell lysates of *E. coli* over-expressing Icl and purified control (chlamydial LcrH) [16] protein were heated to 95°C for 5 min in sample buffer and separated by SDS-10% PAGE. The separated proteins were blotted onto a polyvinylidene difluoride membrane (SERVA Heidelberg, Germany). The membranes were blocked with PBS containing 5% skim milk and 0.05% Tween 20 overnight at 4°C. Membranes were probed with BCGpMV262(icl) immunized and control (BCG pMV262) mouse homogenized lungs (1:10 dilution in 5% skim milk and 0.05% Tween 20 containing PBS). After washings, the filter was incubated with HRP-conjugated anti-mouse IgG for 2 hours, and the colour was developed using diaminobenzidine tetrahydrochloride (DAB, Sigma) with hydrogen peroxide in 10 mM Tris pH 7.5 according to the manufacturer's instructions.

Statistical analysis

Statistical analysis of the data was carried out with GraphPad Prism 5 software, using the Student t test. Differences were considered significant at P < 0.005.

Results

The copy number of the pMV262(icl) in the BCG strain used for infection was 29. Figure 1 shows the spleen weights of mice infected with BCG carrying Icl plasmids and the control. Twenty one days after infection, the spleens from mice that were infected with bacteria that carried the additional *icl* genes were three to four times larger than those of the controls (Fig. 2).

Bacteria with one copy of *icl* (the chromosomal gene) appeared in the spleen earlier and their clearance was faster than that from the lungs (Fig. 3). In the spleen, very few bacteria were found 21 days after infection and they had disappeared by the end of the experiment (35 days), whereas some bacteria were still detected in the lungs at this time. Bacteria containing the plasmid with the *icl* gene survived much longer and their yield was 8-250 times higher (Fig. 3). Lungs from the BCG pMV262(icl) -immunized mice had increased antibody level against Icl as shown in Figure 4.

We determined the toxic effect of propionate produced from the β -oxidation of odd-chain or branched-chain fatty acids. Figure 5 shows that propionate was less toxic for bacteria carrying *icl* plasmids *in vitro*. Although vitamin-B₁₂ increased the growth rate of both strains BCG pMV262(icl) multiplied much faster.

Discussion

Several vaccines against tuberculosis are currently undergoing clinical trials [17]. Some of them are subunit vaccines. Another approach is the use of recombinant BCG expressing antigens from *M. tuberculosis*. A recombinant *M. smegmatis* was recently shown to induce potent bactericidal immunity against *M. tuberculosis* [18].

The Rv0467 (CAE55284.1) gene codes for Icl in *M. tuberculosis* H37Rv. In *M. bovis* BCG, the *icl* gene (GenBank accession no. CAL70492) differs in only one base and the amino acid composition is identical. The avirulent strain (*M. tuberculosis* H37Ra) has the same protein (GenBank accession no. ABQ72194). Sequence search was done using NCBI. Although we cloned *icl* from H37Rv, because of the identity the extended survival we observed should be due only to the increased copy number.

The tricarboxylic acid cycle and the glyoxylate shunt together supply bacteria with both energy and precursors for carbohydrate synthesis from fatty acids. Icl has an important role in the methylcitrate cycle too where the processing of the toxic propionate occurs [7]. Thanks to the suggestion of an anonymous reviewer we determined the growth rate of the bacteria in the presence of propionate *in vitro*. Bacteria with increased *icl* copy multiplied much faster wich can contribute to the increased number and survival in mice. The "lipid lunch" [19] results in long term survival. Being an enzyme expressed in the later stages of infection by *M. tuberculosis*, Icl may induce long-term protection if overexpressed from multicopy plasmids during BCG vaccination. In addition, the extended survival of this recombinant BCG itself may increase the efficacy of the vaccine. Western blot shows increased antibody response. It would be interesting to know the protective effect of this recombinant BCG. Because of the lack of biosafety level 3 facilities, we cannot perform these types of experiments.

The mycobacterial cell wall is very rich in lipids: there are around 250 distinct enzymes involved in fatty acid metabolism, five times more than in *E. coli* [13]. We used ApoB100-only LDLR^{-/-} mice to gain an insight into the role of a high-fat diet on the course of infection, but no significant differences were observed with the different diets (unpublished data).

Conflict of interest The authors declare no competing interests.

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Fig. 1 Spleen masses (g) after 10 (\square), 14 (\square), 21 (\square) and 35 (\square) days of infection.

Fig. 2 Differences in spleen size. Spleens were removed from mice 21 days after infection with BCGpMV262 (lower) or BCGpMV262(icl) (upper).

Fig. 3 Colony-forming units in 100 μ l of tissue suspension from the spleen and lungs of infected animals after 10 (\boxdot), 14 (\blacksquare), 21 (\boxdot) and 35 (\boxminus) days of infection. The CFUs were significantly higher in the samples from BCGpMV262(icl) than in those from BCGpMV262-infected mice (*P*<0.005).

Fig. 4 BCGpMV262(icl) immunized and control mouse homogenized lungs were tested in Western blot assay. Purified control protein (chlamydial LcrH) (lanes 1, 3) and cell lysates from Icl expressing *E. coli* (lanes 2, 4) were probed with BCGpMV262(icl) immunized (lanes 1, 2) and with BCGpMV262 immunized mouse homogenized lungs in 1:10 dilution (lanes 3, 4).

Fig. 5 Growth curves in the presence of sodium propionate (0.1%) and with vitamin- B_{12} addition (10µg/ml). Growth was monitored by measuring the optical density of cultures using at least two independent experiments.