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Oral immunogenicity of a plant-made, subunit, tuberculosis vaccine

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Abstract

Transgenic plants are a novel way to produce and deliver oral vaccines. *Arabidopsis thaliana* material shown previously to express the tuberculosis (TB) antigen ESAT-6 fused to the B subunit of *Escherichia coli* heat-labile enterotoxin (LTB) was fed to mice and the resulting immune response investigated. The plant-made LTB-ESAT-6 fusion protein induced antigen-specific responses from CD4+ cells and increased IFN-γ production, indicating a Th1 response. In addition, a Th2 response was induced in the Peyer's patch. This is the first report of an orally delivered, subunit, tuberculosis vaccine priming an antigen-specific, Th1 response.

Keywords: Tuberculosis vaccine; Mucosal delivery; Transgenic plants

1. Introduction

Tuberculosis (TB) is the leading infectious disease in the developing world. In humans, TB is primarily caused by *Mycobacterium tuberculosis*. Since the portal of entry and primary site of infection of *M. tuberculosis* is the respiratory track, mucosal vaccination should have inherent advantage to inducing a protective immune response. Unfortunately, parenteral immunization, the traditional means of vaccine delivery, is a poor inducer of mucosal immunity. Mucosal delivery is the best way to induce a mucosal immune response. It has the added advantage of not needing needle administration, thereby cutting cost and increasing ease of vaccination. It has been suggested that transgenic plants expressing therapeutic proteins are ideal vehicles to produce and orally deliver protective antigens. It has been speculated that the plant cell wall delays digestion of plant-made and delivered antigen; thus, more antigen is taken up and presented to the gut associated lyphoid tissue [1]. The purpose of this study was to investigate the ability of transgenic plants to serve as a novel delivery system for an oral tuberculosis vaccine. We have previously produced transgenic *Arabidopsis thaliana* plants expressing the immuno-dominant TB antigen ESAT-6 (early secretory antigenic target, 6 kDa) fused to the B subunit of the *Escherichia coli* heat-labile enterotoxin (LTB) [2]. In the present study, we tested the oral immunogenicity of the plant-made, subunit vaccine in mouse feed trials.

2. Materials and methods

2.1. Animals

Specific pathogen free female, C57BL/6 mice were purchased from Charles River Laboratories (Wilmington, MA) at 8–10 weeks of age and kept under SPF conditions at the Laboratory Animal Resources Facility and Bioenvironmental Hazard Research Building at Colorado State University. The mice were housed in isolator cages (Thoren Caging Systems Inc., PA) to which air is supplied directly and then exhausted. Four mice were housed per cage. Upon arrival, mice were rested 1 week before the start of

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Table 1
Summary of treatments performed in the mice challenge trials

Group	Treatment	Animal number
1	Unvaccinated mice	5
2	Fed, wildtype plant	5
3	Fed wildtype plant plus saponin	5
4	Fed, transgenic plant	5
5	Fed transgenic plant plus saponin	5
6	Oral BCG	5

the experiment. When not being fed test treatments, the mice had free access to sterile water and standard mouse chow. Mice were monitored on a daily basis for signs of weight loss and condition. Each treatment contained five mice.

2.2. Vaccination

The treatments are summarized in Table 1. For feed immunizations, test animals were transferred to individual cages and fasted for 8 h before introduction of the test diet. The mice had continuous supply of water. The test diet remained with the animals for 16 h and consisted of powdered, freeze-dried plant cells mixed with apple cider in a ratio of 1:3, respectively (w/v). In treatments using saponin, 10 mg of food grade Quillaja extract powder (Garuda) were added per gram of plant material. Test animals were provided with 3 g of the mix (92.6 μg of plant-made LTB-ESAT-6). Feed treatments were given on days 0, 7, 14 and 28. The Bacillus Calmette-Guérin (BCG) vaccine was used as a positive control. Mice were orally vaccinated by initially receiving 100 μl of 3% (w/v) sodium bicarbonate prepared in sterile, pyrogen-free water (Abbott Labs, IL) before 100 μl BCG at 1×10^8 colony forming units (CFU) per milliliter. The BCG suspension was administered via oral gavage on days 0, 14, 28 and 42.

2.3. Mouse challenge model

Mice were challenged as per the NIH standard TB challenge short protocol. *M. tuberculosis* strain H37Rv (Trudeau Mycobacterial Culture Collection-TMCC #102) was grown to mid-log phase in Proskauer–Beck medium containing 0.01% Tween-80 and stored at −80 °C. Three weeks following the last immunization, mice were infected via the aerosol route using a Glas-Col aerosol generator (Glas-Col, Terre Haute, IN), such that ∼100 viable bacteria were deposited in the lungs of each animal. After 50 days, animals were sacrificed by carbon dioxide asphyxiation, as approved by the CSU Animal Care and Use Committee. The number of viable bacteria in the lungs and spleen was determined at specific time points. Serial dilutions of individual partial organ homogenates were plated on nutrient Middlebrook 7H11 agar, and counting bacterial colonies after 21 days of incubation at 37 °C.

2.4. Isolation and characterization of pulmonary, lymph node and spleen cells

Lungs from each mouse were perfused through the heart with 10 ml cold PBS containing 3 U/ml heparin (Sigma–Aldrich, St. Louis, MO). The lungs were removed from the pulmonary cavity and placed in cold Dulbecco's minimal essential medium (DMEM; Cellgro, Herndon, VA). The tissue was then disrupted using sterile razor blades and incubated for 30 min at 37 °C in a final volume of 2 ml DMEM containing 0.7 mg/ml collagenase D (Roche Applied Science, Indianapolis, IN) and 30 μg/ml deoxyribonuclease (Sigma–Aldrich). Following incubation, the digested lung tissue was gently passed through a sterile 70 μm nylon screen. The cells were rinsed using 8 ml cold DMEM supplemented with 10% heat-inactivated endotoxin low fetal calf serum (Atlas Biologicals, Fort Collins, CO), 10mM HEPES buffer (Sigma–Aldrich), 2 mM L-glutamine (Sigma–Aldrich), 2% MEM-non-essential amino acids (100×; Sigma–Aldrich), 50 μM 2-mercaptoethanol (Sigma–Aldrich) and centrifuged at 200 × *g*. Spleens and lymph nodes were also passed through a wire screen to prepare single-cell suspensions. Red blood cells were lysed using Gey's solution (0.15 M NH_4Cl, 10 mM $KHCO_3$) and the samples were centrifuged and resuspended in DMEM plus supplements. A cell aliquot from each mouse was counted using a hemacytometer and aliquots were taken for flow cytometric analysis. Viable cells were enumerated by trypan blue exclusion.

2.5. Preparation of leukocytes for flow cytometric analysis

Counted cells were centrifuged for 5 min, 1200 rpm, at 4 °C, resuspended in 200 μl PBS with 0.05% sodium azide and incubated on ice for 15–30 min. Cells were pelleted by centrifugation and resuspended. Twenty-five microliters of fluorochrome labeled antibodies against appropriate surface markers were added to appropriate wells, and the plates incubated on ice. All antibodies were purchased from BD PharMingen (San Diego, CA). Cells were incubated in the dark for 20 min at 4 °C with specific antibody (directly conjugated to fluorescein isothiocyanate [FITC], phycoerythrin [PE], peridinin chlorophyll *a* protein [PerCP], or allophycocyanin [APC] at 3 μg/ml), followed by two washes using PBS with sodium azide. For T cell analysis, cells were washed twice and incubated separately with an equal volume of cRPMI containing Monensin (10 ml cRPMI, 30 μl Monensin, [40 μl anti-CD28, 4 μl anti-CD3]) at 37 °C, 90% humidity, 5% CO_2 for 4 h. Cells were washed twice, centrifuged and divided into two wells on 96-well plates before being washed and centrifuged. Surface staining antibodies were added and the plate incubated at 4 °C in the dark for 30 min. The cells were washed twice then incubated with Cytofix™ Buffer (BD Biosciences, Cat. No. 2014KZ) at 4 °C for 30 min. The cells were washed twice with 200 μl 1 × Perm/Wash™ Buffer (BD Biosciences, Cat. No.

2091KZ) then resuspended in 200 μl Perm/Wash™ Buffer before centrifugation and addition of intracellular staining antibodies. The plates were incubated at 4 °C in the dark for 30 min before washing twice. Two hundred microliters of Ab-stained cells were analyzed in a FACSCaliber (BD Biosciences).

2.6. Flow cytometric analysis

Cell-surface markers were analyzed with the following specific antibodies: FITC-CD44 (clone IM7), CD3ε (clone 145-2C11), CD62L (clone MEL-14), PerCP-CD8α (clone 53-6.7), CD4 (clone RM4-5), APC-CD3 (clone 145-2C11), CD127 (clone A7R34). For intracellular antibody staining, a single-cell suspension was prepared as described above and the cells were resuspended in DMEM with supplements and stimulated with 0.1 μg/ml anti-CD3ε (clone 145-2C11), 1 μg/ml anti-CD28 (clone 37.51), in the presence of 3 μM Monensin for 4 h at 37 °C, 5% CO_2. Cells were stained with antibodies for cell-surface molecules as described above, before permeabilization with BD Cytoperm/Cytofix (BD PharMingen, San Diego, CA) according to the manufacturer's instructions. Cells were incubated with FITC anti-IFN-γ (clone XMG1.2) for 20 min at 4 °C, washed twice, and resuspended in PBS with sodium azide before analysis. Appropriate isotype controls were prepared simultaneously and were included in each analysis.

The FACSCaliber is calibrated weekly through analysis of single-stained beads (FITC, PE, PerCP, APC and unstained) in conjunction with the FACSComp program to determine the sensitivity of the PMTs and to set the time delay between the 488 and 635 lasers. In addition, unstained and single-stained cells were prepared and run with each experimental time-point to determine the correct compensation values to eliminate spectral overlap.

3. Results

The amount of test diet eaten by each mouse varied with the individual; however, the majority of mice consumed above 75% of the diet. After pulsing with ESAT-6, cells from mice inoculated with the plant-made LTB-ESAT-6 fusion had significantly (ANOVA, $\alpha = 0.05$) more CD4+ lymphocyte proliferation in the mesenteric lymph nodes than other treatments (Fig. 1b). No significant difference could be determined between the treatments with CD4+ cells in the Peyer's patches (Fig. 1a) or CD8+ cells in the Peyer's patches (Fig. 1c) or mesenteric lymph nodes (Fig. 1d). There was, however, a trend for the transgenic plant material treatments and BCG treatment in the Peyer's patches to have higher CD8+ cell proliferation (Fig. 1c). Mice inoculated with the plant-made LTB-ESAT-6 fusion with adjuvant and oral BCG had significantly more CD4+ cells producing IL-10 in the Peyer's patches (ANOVA, $\alpha = 0.05$) (Fig. 2a) while mice inoculated with the plant-made LTB-ESAT-6 fusion without adjuvant had significantly more CD4+ cells producing IFN-γ than any other treatment (Fig. 2b). There was no significant differ-

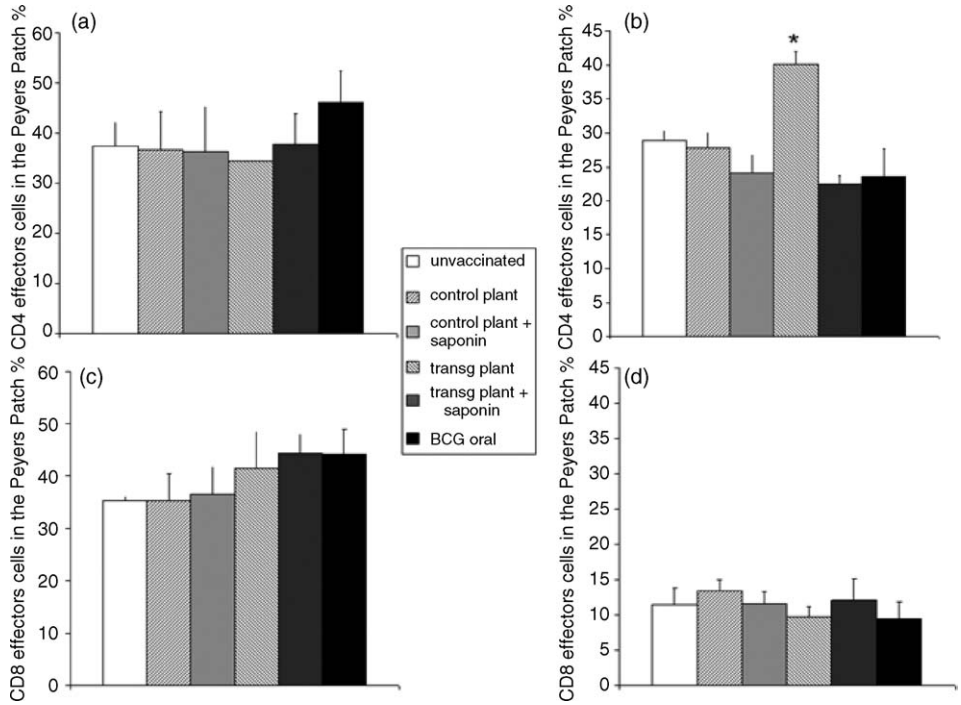

Fig. 1. Proliferation of lymphocytes in Peyer's patches (a) and (c) and mesenteric lymph nodes (b) and (d) taken from animals prior challenge. Blc/6 mice were vaccinated on days 0, 7, 14 and 28. Where control plants were wildtype, plant feed treatments, and trans plant represents transgenic plants feed treatments. Symbols indicate a significantly different result ($\alpha = 0.05$). The bars represent the average of five samples and the error bars the standard deviation of the means.

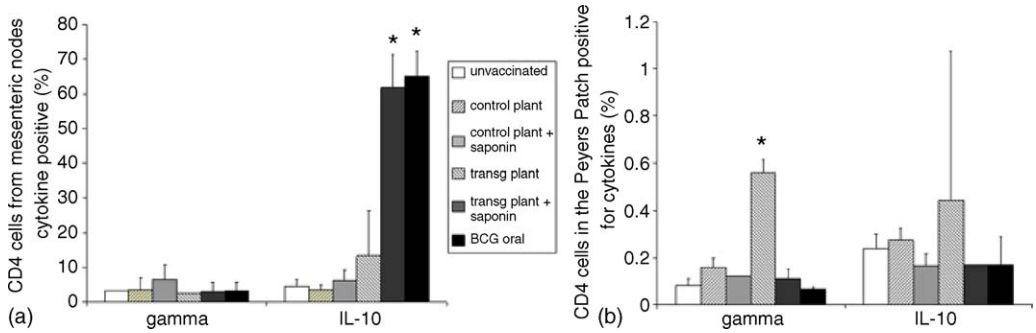

Fig. 2. Percentage of lymphocytes producing cytokines in the Peyer's patches (a) and mesenteric lymph nodes (b) taken from animals prior challenge. Blc/6 mice were vaccinated on days 0, 7, 14 and 28. Where control plants were wildtype, plant feed treatments; trans plant represents transgenic plants feed treatments. IFN-γ: interferon gamma; IL-10: interleukin 10. Symbols indicate a significantly different result ($\alpha = 0.05$). Bars with the same symbols are not significantly different. The bars represent the average of five samples and the error bars the standard deviation of the means.

ence between IL-10 producing CD4+ cells in the transgenic plant plus adjuvant and oral BCG treatments in the Peyer's patches (Fig. 2a); any treatment with regards to IFN-γ in the Peyer's patches (Fig. 2a); or IL-10 production in the mesenteric lymph nodes (Fig. 2b). No difference was seen between treatments concerning cytokine production by CD8+ cells (data not shown). We observed significant induction of IFN-γ by the transgenic plant treatment in the mesenteric lymph nodes and evidence of ESAT-6 specific CD8+ cell proliferation in the Peyer's patches. However, despite oral BCG treated mice having a one log drop in colony forming units in the lung and spleen, no protection was given by the transgenic plant treatments (with or without saponin) when mice were challenged with *M. tuberculosis* (H37Rv strain) (data not shown).

4. Discussion

An effective response against *M. tuberculosis* involves a complex interaction of a range of immune components. Since *M. tuberculosis* is an intracellular pathogen, a strong cell-mediated immune (CMI) response assisted by a humoral response is needed to control infection. The CMI response involves CD4+ and CD8+ T cells, macrophages and cytokines including IFN-γ, IL-12, TNF-α and IL-6 [3]. Many preliminary TB vaccine studies look for induction of subsets of these components, particularly IFN-γ; however, the best known way to find resistance afforded by a new TB vaccine is to challenge the vaccinated animals with virulent Mtb, preferably by inhaling a low dose of bacteria [4].

Oral delivery of a plant-made vaccine based on the model tuberculosis antigen ESAT-6 stimulated CD4+ cells to produce IFN-γ and therefore induced a Th1 response in the mesenteric lymph nodes. This is the first report of a plant-made vaccine inducing an immune response to a tuberculosis antigen and of an orally delivered, subunit, tuberculosis vaccine priming a Th1 response. Crude saponin was used as an adjuvant since it has been found to stimulate mixed immune responses skewed toward a Th1 response [5,6]. In this study, the plant-made, LTB-ESAT-6 fusion adjuvanted with saponins stimulated CD4+ cells to produce IL-10 in the Peyer's patches. Production of IL-10 could be indicative of a Th2 response or oral tolerance [7]; unfortunately, the studies required to determine if oral tolerance was induced, for example analysis of TGF-β production, was not performed. ESAT-6 is a major target molecule for IFN-γ producing Th1 cells in the murine model of TB infection [5]; but fusion to the LTB carrier protein may have changed the way in which ESAT-6 was taken up and detected by the mucosal immune system [8]. There is considerable evidence that the ganglioside binding by LTB plays an important role in the signal transduction pathways inducing cellular immunomodulation, proliferation, differentiation, transformation and suppression. Crude saponin has been suggested to amplify the immune response and not modulate it [9]. Since the LTB used in this experiment primarily induces a Th2 response, we believe CD4+ cell production of IL-10 results from saponins amplifying an ESAT-6 specific Th2 response in the Peyer's patches because of different signal transduction pathways triggered by fusion to LTB. Hence, in the current fusion format, the delivery of the test diet with saponin held no advantage. It merely amplified and undesired type of immune response.

Since the efferent lymphatics of the Peyer's patches' drain to the mesenteric lymph nodes [10], it was interesting to see a Th2 response by CD4+ cells in the inductor site (Peyer's patches), but a Th1 response by CD4+ cells in the secondary site of the mesenteric lymph nodes. We believe the LTB-ESAT-6 fusion induced CD4+ cells to help produce a local, antigen-specific immune response in the enriched source of IgA committed plasma precursor cells found in the Peyer's patches [11]; however, upon entering the different microenvironment of the mesenteric lymph nodes, an antigen-specific Th1 immune response was induced.

Oral delivery of a plant-made LTB-ESAT-6 fusion protein induced promising responses (antigen-specific responses from CD4+ cells; IFN-γ production; equivalent CD8+ proliferation to BCG). However, as previously found, production of IFN-γ did not correlate with ability to reduce bacterial load [12] and the mice were not protected against challenge with

M. tuberculosis. Given the encouraging data from our initial attempts to produce a plant-made vaccine against tuberculosis, we are currently making new constructs using a different targeting system. The long-term goal of this novel approach for vaccination is to provide a flexible, tuberculosis vaccine that is safe and effective for naïve or BCG immunized children, adults and animals.

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