

Immune Response Following Oral Immunization with BCG Encapsulated in Alginate Microspheres

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ABSTRACT

Background: Different methods have been used for BCG vaccination. Alginate microspheres are useful in delivery of vaccines to the gastrointestinal tract by oral route. **Objective:** To compare the immune response following oral microencapsulated and subcutaneous (SC) route administration of BCG vaccine in BALB/c mice. **Methods:** Alginate microspheres were produced by an internal emulsification method within olive oil. Four groups of mice were studied, including two groups receiving oral gavages of microencapsulated and free BCG, one receiving SC injection of BCG, and a control group. T cell proliferation, specific anti-BCG total IgG, and IgG subclasses (IgG1 and IgG2a) were compared between groups 5 and 12 weeks after vaccination. **Results:** The best result was achieved using oral microencapsulated form in comparison with oral BCG alone. **Conclusion:** Delivery of oral BCG with alginate microspheres is an effective way to induce immune response in BALB/c mice.

Keywords: Alginate, BCG, Emulsification, IgG, Microsphere

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INTRODUCTION

Most of the vaccines available today are injected parenterally and not effective in inducing immunity at mucosal surfaces. However, majority of pathogens invade the body through mucosal surfaces. There is a compelling evidence indicating that mucosal immunity is effectively induced when vaccines are delivered to the mucosa-associated lymphoid tissue (MALT) located at mucosal surfaces (1-3). Thus, there is a need for effective mucosal vaccine delivery to have better disease protection against mucosal pathogens.

Oral vaccination is the preferred route of immunization to induce mucosal immunity in the gastrointestinal tract (GIT), having several advantages over other mucosal routes. Effectively delivered oral vaccines can induce mucosal, as well as systemic immunity (4). Additionally, oral immunization uses a physiologic delivery process (ingestion) that is non-invasive and eliminates needle injections which are frequently associated with tissue reaction. However, successful oral vaccination faces significant challenges due to the anatomic and physiologic barriers presented by the GIT (5). Oral administration of non-replicating vaccine antigens is usually associated with poor immune responses (6). This is in part due to vaccines being degraded in the GIT before reaching the gut-associated lymphoid tissue (GALT), the inductive site for mucosal immune responses. Consequently, to achieve effective oral immunization, a "protective" vehicle is required to deliver vaccine antigens to the GALT. Many such antigen delivery systems have been evaluated. Biodegradable microspheres have been proven to be one of the most promising approaches (7,8). "M" (microfold) cells, located within the follicle-associated epithelium (FAE) overlying the Peyer's patches, sample particulate antigens from the intestinal lumen (9). This uptake of particulate material appears to be size dependent. Microparticles with a diameter around 10 μm are taken up by Peyer's patch M-cells (10,11).

There are relatively few reports of using microspheres made from sodium alginate, a naturally occurring gelling polysaccharide, extensively used in the industry as a stabilizer and thickening agent. Sodium alginate is water soluble and can polymerize into a solid matrix upon contact with divalent cations (12). Procedures for formulating alginate microspheres are compatible with the use of a variety of antigens. Various drugs (13), vaccines (14,15), peptides (16,17), cells (18-20), and genes (21) have been formulated successfully in alginate microparticles. Additionally, alginate microspheres are stable at low pH, and can be formulated into a variety of particle sizes that can pass through the GIT (22).

Bacillus Calmette Guerin (BCG) is the world's most widely used vaccine. It consists of a live attenuated strain of *Mycobacterium bovis* (*M. bovis*). It is cleared that killed BCG is poorly protective and acquired immunity depends on survival and persistence of live bacilli, or its secreted products, in the host (23). Microencapsulation of BCG in alginate microspheres has been used by some investigators for delivery of the bacilli to the lungs following intravenous injection (24).

In this experiment, we hypothesized that protection of *M. bovis* BCG against gastric secretion with calcium alginate microspheres would enhance viability and improve uptake of viable bacilli across the intestinal wall. Therefore, we report the development of a novel alginate-based BCG formulation for vaccination of mice.

MATERIALS AND METHODS

BCG Pasteur strain 1173-P2 (60 mg/mL, 17.00×10^6 CFU/mg) and 6-8 weeks old BALB/c mice were kindly provided by Pasteur Institute of Iran. Sodium alginate was kindly donated by FMC biopolymer (Drammen, Norway). $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ was obtained from Merck (Darmstadt, Germany) and 96 well ELISA plate from Griener (GmbH, Germany). Olive oil (highly refined with low acidity), bovine serum albumin, goat anti-mouse IgG, rabbit anti-goat IgG, and TMB substrate were obtained from Sigma (GmbH, Germany). PPD (purified protein derivative) was purchased from Razi Institute of Iran. ^3H -thymidine was prepared from Amersham (Buckinghamshire, England).

Encapsulation of Vaccine in Alginate Microspheres. In this study, alginate-BCG microspheres were prepared with modification of a previously described emulsification method by Sone et al. in 2002 (21). Briefly, sodium alginate 2% W/V concentration was dissolved in distilled water with low heat and constant stirring. The same volume of BCG (2×10^9 CFU/mL) was added to alginate and mixed thoroughly to form a final mixture of 1% W/V sodium alginate and 10^9 CFU/mL BCG. 20mL of alginate-BCG mixture was added to approximately 30mL of olive oil. A W/O emulsion of the alginate-BCG in oil was prepared by mixing at 7000 rpm using a high speed stirrer for 5 min. 0.5g of CaCl_2 powder was added to this emulsion and stirred for 20 min to form jelling microspheres. This suspension was further centrifuged and the microspheres were separated. The microsphere size analysis was performed by laser diffraction (Malvern Mastersizer X, Malvern, UK), with a mean diameter of around 11 μm .

Immunization of Mice. 6-8 weeks old female BALB/c mice were divided into 4 groups: group A was immunized with 1mL/mouse gavage of 10^8 CFU of BCG encapsulated in alginate microspheres. Group B was given 1mL/mouse of 10^8 CFU of BCG alone. Group C was injected subcutaneously (SC) with 100 μL /mouse of 10^7 CFU of BCG at the base of tail and group D was given no vaccine, defined as control group. There were 7 mice in each group and the experiment was repeated 3 times.

Cell Proliferation Assay. Cell proliferation assay was performed to determine immunization level of mice. Briefly, at five and twelve weeks post vaccination, spleen cells of each group were isolated aseptically, pooled, and cultured with PPD (10 μg /mL) and concavaline A in micro culture plates. Cultures were incubated for 4 days at 37 $^\circ\text{C}$ under 5% CO_2 and pulsed with 0.5 μCi of ^3H -thymidine per well for the last 18 hours of incubation. Cell stimulation index (SI) was obtained by dividing the count per minute (cpm) of stimulated cultures by the cpm of unstimulated cultures.

Antibody Assay. Sera of mice immunized with BCG vaccine were used to titrate specific antibodies against whole BCG bacilli by enzyme-linked immunosorbent assay (ELISA). Briefly, at 5 weeks post vaccination blood samples were collected, allowed clotting, and sera were separated and pooled in each group of mice. ELISA plates were coated overnight with 10 μg /mL of sonicated BCG then blocked with PBS + 1% bovine serum albumin. Serial dilutions of serum samples were added and plates were incubated for 2h at 37 $^\circ\text{C}$. For detection of total specific IgG, peroxidase-conjugated goat anti-mouse antibody was added to each well and the plate was incubated for 1h at 37 $^\circ\text{C}$ before the addition of TMB substrate. For IgG subclasses, goat anti-mouse IgG1 and IgG2a antibodies were added and plates were incubated for 1h at 37 $^\circ\text{C}$. Peroxidase-conjugated rabbit anti-goat antibody was added to each well and

plates were incubated for 1h at 37 °C, before addition of TMB substrate. Reactions were stopped by addition of 1N H₂SO₄ and optical density was detected at 450 nm.

Statistical Analysis. ANOVA was used and a *P*-value of < 0.05 was considered significant. Data are expressed as mean ±SD, unless otherwise stated.

RESULTS

Spleen Cell Proliferation Response. Comparison of SIs 5 weeks post vaccination shows that SI of group A (7.06 ±0.6) is significantly greater than group B (3.24 ±0.2) (*P* < 0.001). The difference between groups A and C (6.85 ±0.8) was not statistically significant after 5 weeks (*P*>0.05), however, the mean SIs of groups A and C after 12 weeks (15.47 ±0.7, 12.40 ±0.4, respectively) were significantly higher than those at 5 weeks. Furthermore, after 12 weeks the mean SI of group A was significantly higher than the other groups (*P*<0.005). The mean SIs of group B and control were 3.14 ±0.2 and 2.04 ±0.6, respectively. (Figure 1)

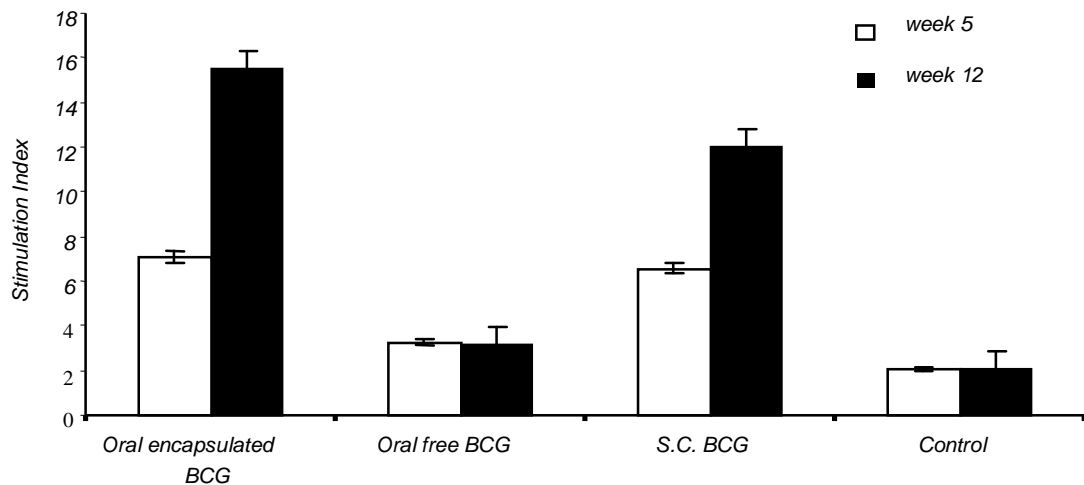


Figure 1. Diagram of spleen cell stimulation index at 5 and 12 weeks post BCG vaccination of mice (error bars represent ± S.D.).

Antibody Response. Specific serum IgG, IgG1 and IgG2a were assayed by ELISA (Table 1). All vaccinated groups showed antibody titers higher than control group (*P*<0.001). The IgG level in group vaccinated with encapsulated BCG was significantly higher than the group received free BCG in oral form (*P*<0.001). There was no significant difference between the groups received oral encapsulated and SC BCG. In all vaccinated groups antibody response was predominantly of IgG2a isotype. IgG2a levels were significantly higher in group A compared with groups B and C (*P* <0.05). Although the differences in IgG1 levels were not significant between groups A and C, titers were significantly higher in group A than B (*P* < 0.05). As IgG2a and IgG1 are Th1 and Th2 related antibodies, respectively, IgG2a/ IgG1 ratio is a parameter indicating whether Th1 or Th2 response dominates in vaccine-induced immunity (25). Table 1 also shows that serum levels of IgG2a are dominant

over IgG1 in all groups of BCG vaccinated mice and IgG2a/IgG1 antibody ratio was significantly higher in group A ($P < 0.05$).

Table 1. Specific serum IgG, IgG1, and IgG2a subclasses and IgG2a/IgG1 ratio

Group	total IgG	IgG1	IgG2a	IgG2a/IgG1
Oral encapsulated BCG (A)	1.580 ± 0.1	0.325 ± 0.02	1.035 ± 0.06	3.18
Oral free BCG (B)	0.673 ± 0.2	0.074 ± 0.02	0.100 ± 0.01	1.35
SC BCG (C)	1.333 ± 0.3	0.263 ± 0.01	0.550 ± 0.05	2.09
Control (D)	0.304 ± 0.1	N.D.	N.D.	---

N.D. = Not Detectable

DISCUSSION

Oral administration of free BCG induces a low cell mediated immune response mainly due to reduced viability of bacteria in acidic medium of stomach (23). To protect BCG against gastric secretions, the bacilli were formulated in alginate microspheres. Alginates are extensively used in industry as stabilizer and thickening agents. Calcium alginate microspheres shrink at acidic pH and erode at alkaline pH (26); thus, they can effectively deliver vaccines and peptides to the intestine, which has an alkaline pH. Moreover, alginate is mucoadhesive and is likely to stick to intestinal mucosa for a prolonged period of time (27,28). In the present study we compared the immune response of BALB/c mice induced by oral administration of BCG vaccine encapsulated in alginate microspheres with that induced by free BCG administered orally and subcutaneously.

The proliferative response to PPD antigens was compared between different groups. Spleen lymphocytes from the group vaccinated with oral encapsulated BCG showed stronger proliferation response than groups vaccinated with free BCG by oral and SC routes. This finding is in agreement with the findings of Aldwell et al, who reported the development of a lipid-based BCG formulation for oral vaccination of mice. Their oral administration of formulated BCG induced a strong T cell response in comparison with nonformulated BCG (23). Furthermore, the mean proliferative response of groups vaccinated orally with encapsulated BCG and subcutaneously with BCG was increased from 5 to 12 weeks post vaccination. Higher proliferative response after 2 months also has been reported by Aldwell et al (23).

Higher specific IgG levels in mice vaccinated with encapsulated BCG in comparison with the mice receiving free BCG by oral route confirms stronger capacity of formulated BCG to induce immune response. Th1 type immune response plays an essential role in protection against intracellular infections (29). It is shown that IgG1 and IgG2a subclasses are associated with Th2 and Th1, respectively (25). To assess the type of immune response induced by encapsulated BCG, IgG1 and IgG2a titers were determined. Our results showed a more prominent rise of IgG2a and IgG2a/IgG1 ratio in mice vaccinated with encapsulated BCG, suggesting stronger Th1 response. Strong Th1 immune response after oral immunization of cattle with lipid-formulated BCG is also reported by Buddle et al (30). Petricevich et al (31) have reported a higher ratio of IgG1/IgG2a after intravenous (IV) administration of BCG to BALB/c mice, meaning a greater Th2 immune response. After mucosal immunization of mice, a mixed Th1/Th2 response with a predominant Th1 response was observed, whereas

after SC or IV immunization, Th2 response is more remarkable. However, the preferential Th1 response induced by mucosal vaccination of mice with BCG gave more efficient immunity than the mixed Th1/Th2 response induced by SC vaccination (32). The capacity of encapsulated BCG to stimulate strong systemic immune responses suggests that the encapsulation procedure protects BCG against degradation in the stomach or enables live bacilli to be more efficiently taken up through the gastrointestinal mucosa for processing and presentation to the immune system (23). To conclude, this study demonstrates that oral administration of encapsulated BCG is an effective mechanism for inducing Th1 immune response, which is the effective response against tuberculosis. This data also suggest that alginate is a suitable polymer for utilization in oral BCG vaccination.

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