Enhanced immunoprotective potential of *Mycobacterium tuberculosis* Ag85 complex protein based vaccine against airway *Mycobacterium tuberculosis* challenge following intranasal administration

Pramod K. Giri, Indu Verma & Gopal K. Khuller

Department of Biochemistry, Postgraduate Institute of Medical Education & Research, Chandigarh, India

Correspondence: Gopal K. Khuller, Department of Biochemistry, PGIMER, Chandigarh 160 012, India. Tel.: + 91 (0)172 2755175; fax: + 91 (0)172 2744401; e-mail: gkkhuller@yahoo.co.in

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Abstract

This study examined the role of intranasal vaccination with *Mycobacterium tuberculosis* antigen85 complex proteins formulated in dimethyldioctadecylammonium bromide against airway *Mycobacterium tuberculosis* challenge in mice. Intranasal vaccination with antigen85A and antigen85B induced a significantly higher level of interferon-γ, interleukin-12 and interleukin-4 in cervical lymph nodes together with IgA and IgG, predominantly IgG2a isotype in nasal secretion over subcutaneous vaccination. Further, intranasal vaccination with antigen85A and antigen85B imparted protection comparable with that obtained from intranasal or subcutaneous *Mycobacterium bovis* bacillus Calmette-Guerin immunization. These results suggest that mucosal vaccination via the intranasal route is of importance in the development of vaccine for tuberculosis.

Introduction

Vaccination is a method of choice for the eradication of infectious diseases, including tuberculosis. Unfortunately, despite tremendous efforts using the sophisticated methods of modern science, no clear protection against this disease has emerged from traditional approaches of vaccination via parenteral routes. Because *Mycobacterium tuberculosis*, the causative agent of tuberculosis, gains entrance to the body via the respiratory mucosal surface, the importance of generating a ‘first line of defense’ by establishing pathogen-specific immunity at the site of entry has been well recognized. Recently, the intranasal route has received considerable attention for its potential for vaccine delivery against several mucosal infectious pathogens (Gallichan & Rosenthal, 1996; Belyakov et al., 1999). To develop safer and better vaccines, much effort has been devoted to defining and producing protective antigens from the appropriate pathogen. Secreted proteins are major antigens recognized by the protective immune response against tuberculosis. We have recently reported that intranasal vaccination with total culture filtrate proteins of *Mycobacterium tuberculosis* with dimethyldioctadecylammonium bromide imparts superior immunoprotection over subcutaneous vaccination (Giri et al., 2005). Immunization with whole culture filtrate, which contains many molecules, may induce irrelevant immune responses. Thus, a protein based vaccine, consisting of a few key protective molecules capable of inducing protective immunity, could have substantial advantages over whole culture filtrate proteins based vaccine. In recent years, of several mycobacterial proteins, Ag85A and Ag85B, which are members of the mycolyl transferase family of *Mycobacterium tuberculosis* (also called Ag85 complex), have been reported as the most promising tuberculosis vaccine candidates (Kaufmann, 2000). Systemic vaccination of mice with naked plasmid DNA encoding Ag85A and Ag85B has been shown to stimulate strong cell-mediated and humoral immune responses and confer significant protection against aerosol or intravenous challenge with *Mycobacterium tuberculosis* H37Rv (Huygen et al., 1996; Kamath et al., 1999). However, there is inadequate information in the literature about the protective efficacy of mycobacterial immunodominant antigens when administered by the intranasal route. We have investigated the protective efficacies of *Mycobacterium tuberculosis* Ag85 complex proteins in dimethyldioctadecylammonium bromide when administered...
via the intranasal route against airway challenge with *Mycobacterium tuberculosis* in a murine model.

**Materials and methods**

**Animals**

Mice (BALB/c, 6–8 weeks old) were housed in negative pressure animal isolators and fed on standard pellet diet (Hindustan Lever Ltd, Mumbai, India) and water *ad libitum*.

**Bacterial cultures**

*Mycobacterium tuberculosis* H$_{37}$Rv was originally obtained from the National Collection of Type Cultures (NCTC), London. *Mycobacterium bovis* bacillus Calmette-Guerin (BCG) (Danish – 1331 strain) was obtained from BCG Vaccine Laboratory, Chennai, India. Both strains used in the study were maintained on Lowenstein Jensen’s medium in our laboratory.

**Immunization with Ag85 complex proteins and BCG**

The purification and characterization of purified Ag85A and Ag85B proteins have been previously described (Sable SB *et al.*, 2005). For intranasal delivery of Ag85A (10 µg), Ag85B (10 µg) or Ag85A plus Ag85B in combination (Ag85AB; 20 µg, 10 µg of each antigen), a group of four or five animals was lightly anesthetized with ketamine (150 mg kg$^{-1}$ body weight). A 30-µL volume containing the above concentrations of antigens together with 250 µg dimethyldioctadecylammonium bromide in phosphate-buffered saline (PBS) was administered dropwise to the external nares of the mice (15 µL per nostril) with a fine pipette tip. For subcutaneous delivery of Ag85A, Ag85B or Ag85AB, animals received a 100 µL volume of the above concentrations of antigens together with 250 µg dimethyldioctadecylammonium bromide in PBS, injected subcutaneously. Control groups received only dimethyldioctadecylammonium bromide in PBS. This was prepared as previously described (Brandt *et al.* 2000) with minor modifications. Briefly, dimethyldioctadecylammonium bromide (Sigma, St Louis, MO) was suspended in distilled water (10 mg mL$^{-1}$ for intranasal and 2.5 mg mL$^{-1}$ for subcutaneous immunization), and a homogeneous dispersion of the powder was obtained by heating the suspension to 80°C for 5–10 min with continuous mixing. After cooling at room temperature, the suspension was mixed with antigens or PBS. Mice were immunized with a total of three doses at 2-week intervals. *Mycobacterium bovis* BCG was administered via the intranasal or subcutaneous route as a single dose of 5 × 10$^5$ CFU per 30 or 100 µL of PBS, respectively.

**Lymphocyte isolation and *in vitro* antigen stimulation**

Three weeks postvaccination, spleens and cervical lymph nodes were aseptically isolated and pooled for each group. Single cell suspensions were obtained, and erythrocytes were lysed by hypotonic shock using 0.84% NH$_4$Cl and cultured as previously described (Andersen *et al.*, 1991). Approximately 2 × 10$^5$ cells per well were seeded in 96-well tissue culture plates (Greiner, Bio-one, Essen, Germany) in 200 µL of RPMI-1640 medium containing 10% heat inactivated fetal calf serum, 25 mM HEPES, 2 mM l-glutamine, 1 mM sodium pyruvate, 100 IU mL$^{-1}$ penicillin and 100 µg mL$^{-1}$ streptomycin in the presence or absence of Ag85A (2 µg mL$^{-1}$), Ag85B (2 µg mL$^{-1}$) or Ag85AB (4 µg mL$^{-1}$, 2 µg of each antigen). Con-A (5 µg mL$^{-1}$) was used as positive control for this assay. The cells were incubated for 72 h at 37°C in 5% CO$_2$ and 100% humidity. Cell-free culture supernatants were harvested for cytokine measurements. Cells were then pulsed with 0.30 µCi of $[^3]$H-thymidine and incubated for 18–22 h. Cells were harvested on glass fiber filters with Nunc cell harvester (Intermed, Kamstrup, Denmark) and allowed to dry overnight at room temperature; radioactivity was then measured using a $\beta$-scintillation counter. Stimulation indices were calculated by dividing mean counts per minute in antigen-stimulated cells by mean counts per minute in unstimulated cells.

**Measurement of cytokines in cell culture supernatant**

The levels of interferon (IFN)-γ, interleukin (IL)-12 (p-40) and IL-4 were measured in cell culture supernatants using mouse specific ELISA kit (Opt EIA™ set, BD Pharmingen, Franklin Lakes, NJ). The sensitivity of detection for IFN-γ, IL-12, and IL-4 assays was 31.3, 15.4 and 7.8 pg mL$^{-1}$, respectively.

**Assessment of humoral immune responses**

Sera and nasal lavage were obtained 3 weeks postimmunization as described previously (Bowe *et al.*, 2004). Briefly, mice were sacrificed, and blood and nasal washes collected. Nasal lavage was obtained by flushing the nares three times with 500 µL PBS containing protease inhibitor (phenylmethylsulphonylfluoride, 0.1 mM). Serum and nasal lavage samples were stored at −20°C until use. Enzyme-linked immunosorbent assay (ELISA) was used to quantitate levels of anti-Ag85A, anti-Ag85B or anti-Ag85AB antibodies in the pooled sera and nasal lavage of immunized and unimmunized mice. Flat bottom, 96-well microtitre plates (Nunc-Maxisorp, Roskilde, Denmark) were coated overnight at 4°C with Ag85A (2 µg mL$^{-1}$), Ag85B (2 µg mL$^{-1}$) or Ag85AB (4 µg mL$^{-1}$, 2 µg of each antigen) in coating buffer (0.05 M...
Carbonate buffer, pH 9.5). Plates were washed three times with PBS-0.05% Tween 20 (PBS-T) prior to blocking with 250 μL of PBS containing 3% BSA at 37 °C for 1 h. Diluted (1:10) sera and nasal lavage samples were added to the plates (100 μL per well) in quadruplicate and incubated for 2 h at 37 °C. Unbound primary antibody was removed, and the wash step repeated. Bound IgG, IgG1, IgG2a and IgA were detected by using goat antimouse IgG, and IgA (Sigma). Following incubation for 2 h at 37 °C, plates were washed as above before incubation for 1 h with horseradish peroxidase (HRP)-conjugated antigoat IgG. Plates were washed as above and ortho-phenylenediamine dihydrochloride substrate (Sigma) was added. After 15 min, reaction was stopped by adding 1 M H2SO4 and the absorbance was measured at 492 nm. Antigen-specific antibody titre was expressed as described previously (Shibata et al., 2001).

**Pulmonary *Mycobacterium tuberculosis* challenge and bacterial enumeration**

Mice were lightly anaesthetized with ketamine (150 mg kg⁻¹ body weight) and challenged by intranasal inoculation of live *M. tuberculosis* H37Rv (10⁵ CFU in 30 μL PBS) 30 days after the final vaccination. Mice were sacrificed 5 weeks postinfection. Lungs and spleen were aseptically removed and homogenized in saline under sterile conditions. Appropriate serial dilutions of homogenates were plated on Middlebrook 7H11 agar supplemented with 10% oleic acid-albumin-dextrose-catalase (Becton Dickinson, Sparks, MD) and 0.5% glycerol. Organ homogenates from BCG-vaccinated animals were plated on enriched Middlebrook 7H11 agar supplemented with 2-thiophene carboxylic acid hydrazide (2 μg mL⁻¹) to selectively inhibit the growth of the residual BCG bacilli. Colonies were counted after 3–4 weeks of incubation at 37 °C and results were expressed as log₁₀ CFU.

**Data analysis**

Levels of statistical significance were evaluated by using an unpaired, two-tailed Student’s t-test. The difference was considered statistically significant when *P* ≤ 0.05.

**Results**

**Lymphocyte proliferation and cytokine responses**

To examine the effectiveness of intranasal vaccination for the induction of lymphocyte proliferative responses, splenocytes and cervical lymph node cells were isolated from vaccinated and unvaccinated groups 3 weeks after final immunization and stimulated in vitro with Ag85 complex proteins. Vaccination with adjuvanted Ag85AB via both intranasal and subcutaneous routes induced significantly higher (*P < 0.05*) lymphocyte proliferative responses by cervical lymph nodes cells and splenocytes than vaccination with adjuvanted Ag85A- or Ag85B-vaccinated animals (Fig. 1). Further, intranasal vaccination with Ag85A, Ag85B or Ag85AB induced significantly higher (*P < 0.05*) lymphocyte proliferation than subcutaneous vaccination in cervical lymph nodes (Fig. 1a). Both intranasal and subcutaneous vaccination induced comparable levels (*P > 0.05*) of lymphocyte proliferative responses at the systemic level (Fig. 1b).

To evaluate the capability of intranasal vaccination to induce cytokine profiles, culture supernatants of Ag85A, Ag85B or Ag85AB stimulated and unstimulated splenocytes and cervical lymph nodes cells were analyzed for different cytokines. Vaccination with adjuvanted Ag85AB via both...
the intranasal and the subcutaneous routes induced a significantly higher (P < 0.05) level of IFN-γ and IL-12 produced by cervical lymph node cells and splenocytes than animals vaccinated with adjuvanted Ag85A or Ag85B (Fig. 2). Further, intranasal vaccination with Ag85A, Ag85B or Ag85AB induced significantly higher (P < 0.05) levels of IFN-γ, IL-12 and IL-4 than subcutaneous vaccination at a local level (Figs 2a–c). Intranasal and subcutaneous vaccination induced comparable levels (P > 0.05) of IFN-γ, IL-12 and IL-4 at a systemic level (Figs 2d–f). These results demonstrate that intranasal vaccination elicited greater lymphocyte proliferation and Th1-dominant immune responses at both local (cervical lymph nodes) and systemic (spleen) levels.

Fig. 2. Cytokine responses in cervical lymph nodes (CLN) (a, b, c), and spleen (d, e, f) induced by intranasal and subcutaneous immunization with Ag85A-, Ag85B- or Ag85AB-dimethyldioctadecylammonium bromide (DDA). Three weeks postimmunization, cervical lymph nodes cells and splenocytes were isolated and pooled (n = 3–4 mice per group) from immunized or unimmunized (PBS-dimethyldioctadecylammonium bromide) mice and cultured in vitro with or without Ag85 complex proteins. Results are presented as means ± SD from triplicate determinations. The experiment was repeated two times with similar results. Significant difference determined by Student's t-test. *P < 0.05, **P < 0.01, ***P < 0.001, Ag85 AB compared to Ag85 A or Ag85B group. †P < 0.05, ††P < 0.01, †††P < 0.001, intranasal compared to subcutaneous groups. i.n., intranasal; s.c., subcutaneous.
Antibody responses in serum and nasal lavage

To assess the effect of the intranasal route of vaccination on the induction of specific antibodies, mice were vaccinated with Ag85 complex proteins formulated in dimethyldiota-cylammonium bromide via the intranasal or subcuta-neous route. Intranasal vaccination with adjuvanted Ag85AB induced significantly higher \((P < 0.05)\) levels of IgA, IgG and IgG2a in respiratory tract and IgG in serum than vaccination with adjuvanted Ag85A or Ag85B (Fig. 3). Intranasal vaccination with adjuvanted Ag85AB also induced significantly higher \((P < 0.05)\) levels of IgA, IgG and IgG2a in nasal secretions as compared to subcutaneous vaccination. Subcutaneous or intranasal vaccination with adjuvanted Ag85AB induced significantly higher \((P < 0.05)\) levels of IgG and IgG1 in serum than vaccination with adjuvanted Ag85A or Ag85B. Moreover, intranasal or subcutaneous vaccination with the Ag85 complex proteins induced comparable levels of IgG, IgG1 and IgG2a in serum. These results demonstrate that intranasal vaccination with Ag85AB induced the secretion of antigen-specific IgA, IgG, IgG1 and IgG2a in nasal lavage and IgG, IgG1 and IgG2a in serum.

Protection against airway Mycobacterium tuberculosis challenge

Figure 4 depicts the protective efficacy against experimental tuberculosis of adjuvanted Ag85 complex protein based vaccine formulations administered via the intranasal or subcutaneous route. Vaccination with adjuvanted Ag85AB via both routes induced significantly higher \((P < 0.05)\) protection in lung and spleen than vaccination with adjuvanted Ag85A or Ag85B (Fig. 4). Intranasal vaccination with adjuvanted Ag85 complex proteins induced significantly higher \((P < 0.05)\) protection in lung than subcutaneous vaccination (Fig. 4a). Further, intranasal vaccination with adjuvanted Ag85AB induced significantly higher \((P < 0.05)\) protection in lung than vaccination with adjuvanted Ag85A or Ag85B. Both intranasal and subcutaneous vaccination induced comparable levels \((P > 0.05)\) of protection at a systemic level (Fig. 4b).

Intranasal vaccination with BCG elicited significantly higher \((P < 0.001)\) immunoprotection in lung compared with subcutaneous BCG vaccination (Fig. 4c). Comparison of protective efficacy induced by intranasal Ag85AB vaccination with intranasal or subcutaneous BCG immunization indicated that intranasal vaccination with adjuvanted Ag85AB imparts immunoprotection comparable \((P > 0.05)\) to intranasal or subcutaneous BCG vaccination (Fig. 4c) in lung and spleen.

Discussion

The vast majority of pathogens, including M. tuberculosis, enter through the respiratory mucosa and strong immunity at this mucosal site is considered important for optimal protection against these pathogens. In the present study, we have investigated the effect of respiratory mucosal vaccination with Ag85 complex proteins emulsified in dimethyl-dioctadecylammonium bromide, on the induction of immunoprotection against Mycobacterium tuberculosis challenge. Increasing evidence suggests that mucosal vaccination via the respiratory tract is superior to vaccination at other sites in eliciting protection from mucosal infectious diseases (McGhee et al., 1999). However, direct administration of protein antigens to mucosal surfaces without adjuvant results in little or no response and can even lead to tolerance (Chen et al., 1995). Thus, mucosal adjuvants are essential for mediating mucosal as well as systemic responses to protei-naceous vaccine antigens. In the past, several adjuvants have been tested in animal models, but few are acceptable for use in humans, dimethyl-dioctadecylammonium bromide being one of them (Stanfield et al., 1973; Hilgers & Snippe, 1992). Recently, it has been reported that dimethyl-dioctadecylam-monium bromide induces both mucosal and systemic immune responses without toxicity when coadministered with some bacterial and viral proteins (Klinguer et al., 2001). Recently, we have also shown that intranasal immunization with total culture filtrate proteins of Mycobacterium tuberculosis with dimethyl-dioctadecylammonium bromide provides greater protection than subcutaneous vaccination (Giri et al., 2005).

In the last decade, systemic administration of several protein based subunit vaccines have been shown to impart partial protection against Mycobacterium tuberculosis challenge (Andersen, 1994; Horwitz et al., 1995; Roberts et al., 1995). Of the several mycobacterial proteins, Ag85A and Ag85B, members of Ag85 complex, have been reported as the most promising target for development of tuberculosis vaccine (Kaufmann SHE, 2000). In this report, intranasal vaccination with Ag85AB-dimethyl-dioctadecylammonium bromide has been observed to confer protection comparable to intranasal or subcutaneous BCG vaccination in lungs and spleen (Fig. 4c). Moreover, intranasal vaccination with Ag85AB-dimethyl-dioctadecylammonium bromide confers improved protection over Ag85A or Ag85B alone (Figs 4a and b). These results are consistent with a previous report (D’Souza et al., 2003) in which component-specific immu-nodominant epitopes on Ag85A and Ag85B were identified in the BALB/c mice model despite pronounced sequence homology between these antigens. This explains the varied levels of immunoprotection seen in groups vaccinated with adjuvanted Ag85AB, Ag85A or Ag85B. Further, comparison of the routes of vaccination with adjuvanted Ag85A+Ag85B indicated that intranasal vaccination induced significantly superior \((P < 0.01)\) immunoprotection in lungs as compared to subcutaneously vaccinated animals, reducing the bacterial burden by \(\sim 1.35 \log\) for
Fig. 3. Antigen-specific antibodies responses in nasal lavage (a, b, c, d) and serum (e, f, g, h) induced by intranasal and subcutaneous immunization with Ag85A-, Ag85B- or Ag85AB-dimethyldioctadecylammonium bromide (DDA). Three weeks postimmunization, serum and nasal lavage were collected and pooled (n = 3–4 mice per group) from intranasal or subcutaneous immunized or unimmunized (PBS-dimethyldioctadecylammonium bromide) mice. Results are presented as mean ± SD from quadruplicate determinations. The experiment was repeated three times with similar results. Significant difference determined by Student’s t test. *P < 0.05, **P < 0.01, ***P < 0.001, Ag85 AB as compared to Ag85 A or Ag85B group. **P < 0.05, ***P < 0.01, ****P < 0.001 intranasal compared to subcutaneous groups. †P < 0.05, ††P < 0.01, †††P < 0.001 immunized compared to control group. i.n., intranasal; s.c., subcutaneous.
in intranasal and \( \sim 0.84 \) log for subcutaneous vaccination (Fig. 4a). Intranasal vaccination with Ag85AB-dimethyldioctadecylammonium bromide could achieve a level of protection even better than intranasal culture filtrate proteins-dimethyldioctadecylammonium bromide vaccination (\( \sim 0.9 \) log and \( \sim 0.5 \) log in lungs and spleen, respectively) (Giri et al., 2005), reducing the bacterial burden by \( \sim 1.4 \) log and 0.8 log in lungs and spleen, respectively. This explains the potential of intranasal vaccination with adju-
vanted immunodominant antigens to activate antigen-specific immunity at both local and systemic levels. To the best of our knowledge, this is the first demonstration of comparable immunoprotection between intranasal Ag85AB-dimethyldioctadecylammonium bromide immunization and intranasal or subcutaneous BCG vaccination. After intranasal vaccination, T cells migrate and reside in the cervical lymph nodes; this has been correlated with the establishment of antigen-specific mucosal immune responses (Wu et al., 1997). Intranasal immunization with adjuvanted Ag85AB resulted in an elevated level of type 1 (IFN-\( \gamma \) and IL-12) cytokines in the cervical lymph nodes and spleen as compared to subcutaneous vaccination (Fig. 2). In addition, intranasal BCG vaccination induced high levels of IFN-\( \gamma \) (2500 and 1600 pg mL\(^{-1}\) for intranasal and subcutaneous vaccination, respectively) and comparable levels of IL-4 (35 and 28 pg mL\(^{-1}\) for intranasal and subcutaneous vaccination, respectively) production in cervical lymph nodes as compared to subcutaneous BCG vaccination (data not shown). Intranasal vaccination with Ag85AB induced Th1-dominant immune responses but it also induced significantly elevated levels of type 2 (IL-4) cytokine in cervical lymph nodes as compared to subcutaneous vaccination (Fig. 2c). This can be explained by the fact that the local production of type1 and type2 cytokines is induced only after intensive stimulation of lymphoid tissues associated with the respiratory tract by intranasal delivery of the antigens. The induction of type 1 responses dominated by IFN-\( \gamma \) and IL-12 following subunit vaccine formulations is considered essential for protection against tuberculosis (Cooper et al., 1993; Collins et al., 2003). The involvement of Th2 cytokines in controlling mycobacterial infection is debatable but it has been shown that IL-4 knockout mice are more susceptible to mycobacterial infection (Sugawara et al., 2000). Thus, IL-4 might play a crucial role in host protection against Mycobacterium tuberculosis infection. This contention is also supported by this study, in which intranasal administration of adjuvanted Ag85AB potentially induced significant increased levels of type 1 and type 2 cytokines at a mucosal level. These balanced immune responses have been correlated with the establishment of protection against pulmonary Mycobacterium tuberculosis infection. A more plausible explanation for the superior immunoprotection achieved by intranasal administration may be that the memory T and B cells generated by mucosal vaccination acquire mucosa-homing receptors and preferentially accumulate at the mucosal site of induction (Gallichan & Rosenthal, 1996). Thus, it is likely that the T cells, preferentially retained in the lungs of intranasally immunized hosts, potently protect from pulmonary tuberculosis. Our study also shows that intranasal vaccination induced significantly elevated levels of IgA response together with IgG in respiratory mucosa and IgG at a systemic level (Fig. 2). The higher
levels of IgA in the nasal secretions following intranasal immunization are consistent with the idea that intranasal vaccination targets the mucosa associated lymphoid tissue to initiate local immune responses (Kiyono et al., 1992). It has also been reported that intranasal delivery of specific IgA provides passive protection against Mycobacterium tuberculosis challenge, and IgA−/− mice were more susceptible to mycobacterial infection than IgA+ +/+ mice, as revealed by the higher bacterial loads in the lungs (Williams et al., 2004; Rodriguez et al., 2005). In this study, we have also found increased levels of predominantly IgG2a in nasal lavage. These findings warrant further evaluation of the role of these antibodies in the protection against Mycobacterium tuberculosis infection at the respiratory mucosal level.

In brief, this study provides evidence that intranasal immunization with the combination of Ag85A and Ag85B formulated in dimethylidioctadecylammonium bromide confers improved protection over subcutaneous vaccination and comparable protection with intranasal mucosal or systemic BCG immunization.

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References


