Intranasal vaccination of mice against infection with *Mycobacterium tuberculosis*

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**Abstract**

The intranasal (i.n.) route of immunisation, has recently been of active interest in endeavours to improve the efficacy of vaccination against a number of respiratory infections. Here, we examined the outcome of tuberculous infection in BALB/c mice. I.n. application of the BCG-Pasteur strain was found to be highly protective against challenge infection with the pathogenic H37Rv strain given after a 4-week interval, reflected by the 100-fold reduction of CFUs in both lungs and spleens. Vaccination with the recombinant PstS1 antigen and cholera toxin significantly protected against the challenge given 10 days later, but only marginally after 12 weeks. Histological examination showed, that i.n. vaccination abrogated the confluent infiltration of lungs with inflammatory cells, which surrounds the granulomas in H37Rv challenged control mice. In conclusion, the strong protection demonstrated by BCG suggests that the i.n. route of vaccine delivery deserves further attention toward improving vaccination against tuberculosis. © 2000 Elsevier Science Ltd. All rights reserved.

**Keywords:** Intranasal vaccination; Tuberculosis; Mucosal immunity

1. Introduction

Intranasal (i.n.) immunisation in man offers distinct advantages over the oral or parenteral route of vaccination against infections at both local and distant sites [1]. Following vaccination in experimental animal models, pronounced mucosal IgA, as well as systemic IgG, Th1 and Th2 responses accompany protection against aerogenic challenge with a number of bacterial pathogens of the upper and lower respiratory tract [2–7]. I.n. inoculation of recombinant *Mycobacterium bovis* BCG induced mucosal and systemic immune responses against heterologous antigens [8,9], but its effect in respect of tuberculous challenge has so far not been reported. A possible reason for this omission could be that the nasally induced Th2 and secretory IgA responses are perceived to be outside the prevailing paradigm, attributing a mandatory role to Th1 cell mediated protection against tuberculosis [10,11].

We decided to explore the mucosal vaccination against tuberculosis with the premise, that: (i) systemic BCG vaccination is not fully protective despite its strong Th1 bias [12]; (ii) mucosal immunity may overcome an organ specific failure of the lungs to benefit from otherwise potent systemic immunity [13]; and (iii) transcytosing secretory IgA antibodies may act against intracellular infectious pathogens [14]. In this paper, we examined the protective effect of i.n. application of...
BCG and of the PstS-1 protein against the challenge of mice with *M. tuberculosis* by the same i.n. route. The vaccinal efficacy was evaluated on the basis of viable bacterial counts in the lungs and spleen and by histopathology of the lungs.

2. Materials and methods

2.1. Bacteria and antigens

*M. tuberculosis*, strain H37Rv and *Mycobacterium bovis*–bacillus Calmette-Guerin (BCG) strain Pasteur were grown as suspension cultures in the Middelbrook 7H9 medium (Difco Laboratories, Detroit, MI, USA). The recombinant PstS-1 (38 kDa) protein antigen was produced and purified by previously reported methods [15] and supplied by Mr. M. Singh (GBF Braunschweig, Germany).

2.2. Vaccination

BALB/c female mice, 6–8 weeks old, (Harlan/Olac, Bicester, UK) were vaccinated either with BCG (10^6 CFU/mouse) or with 10^15 mg of PstS-1 mixed with 1 mg cholera toxin (CT Sigma). Mice under mild halothane anaesthesia were inoculated using a fine plastic tip onto the external nares with 30 μl volume in fractions spread over a period of 45–60 s, alternating between the nostrils. The mice were given two inoculations of BCG or three inoculations of PstS-1, separated by 2-week intervals. The repeated inoculations were also made with the intention to stimulate the mucosal IgA antibody response effectively.

2.3. Tuberculous challenge and colony forming unit (CFU) assay

The vaccinated and control mice were challenged by i.n. inoculation (see Section 2.2) of 1.25 × 10^6 H37Rv viable bacilli. Lungs and spleens harvested at different time intervals were homogenised in 5 ml distilled water using a stomacher (Seward London, UK). Homogenates in five-fold serial dilutions were plated on Petri dishes of Middlebrook 7H11 agar (Difco) and incubated at 37°C for 2–3 weeks, until visible colonies were formed. The limit of detection in this assay system is 100 CFUs per organ. A ‘protection index’ was calculated as a difference in mean CFUs between vaccinated and control mice. The significance of the protection index was calculated by the non-parametric U-test, and intra-group individual variations represented by standard errors (S.E.) from the mean CFU values. Furthermore, the number of protected mice was defined arbitrarily as the number of vaccinated mice with at least 10-fold reduced CFU counts.

2.4. Tissue sections

Lungs harvested 8 weeks after H37Rv challenge were fixed in 5 ml of 10% formalin and embedded in paraffin blocks. Sections of 4 μm thickness were cut at the widest organ diameter and stained with haematoxylin–eosin or by the Ziehl-Neelsen procedure for acid-fast bacilli.

2.5. ELISPOT technique

Using previously described methods [16], single cell suspensions were prepared from nasal associated lymphoid tissue (NALT), cervical lymph nodes, lungs and spleen in 5 ml of Dulbecco’s minimal essential medium containing 10% foetal calf serum (DMEM, Gibco BRL, Paisley, Scotland). Polystyrene 96-well microtitre plates (Nunc-Immuno MaxiSorp, Fisher Scientific, Loughborough, UK) were coated with the 38 kDa protein at 5 μg/μl in 100 μl PBS/well overnight at 4°C. Plates were washed three times with PBS containing 0.5% Tween 20 (PBS-T) and then blocked with 1% bovine serum albumin for 1 h at 37°C (200 μl/well). Cell suspensions in serial four-fold dilutions were added (100 μl) to wells and plates then incubated overnight at 37°C in 5% CO2. Cells were lysed using PBS-T and incubated for 2 h at 37°C with either 100 μl of 1:500 diluted goat anti-IgG (γ chain specific) or anti-IgA (α chain specific) unconjugated antiserum (Sigma). Plates washed three times were incubated for 2 h at 37°C with 1:1000 rabbit anti-goat, alkaline phosphatase conjugate and washed in PBS-T with a final wash.
in sterile water. ELISPOTs were developed with 5-bromo-4-chloro-3-indolyl phosphate disodium salt and individual spots were counted using a low power inverted light microscope.

3. Results

3.1. Protection against M. tuberculosis challenge infection following intranasal vaccination

In preliminary experiments, we determined the time course of H37Rv infection (Fig. 1). The results showed that CFU counts were fully restricted to the lungs for 7 days. Subsequently, the infection spread to the spleen, though 100 times lower CFU counts were sustained for at least 3 weeks. For the evaluation of the post-vaccine challenge, we chose the early harvest of lungs between 9 and 14 days and the late harvest of both lungs and spleens after 8 weeks.

The results obtained following the BCG vaccination (Fig. 2) showed a 10-fold difference in mean CFU counts between vaccinated and control mice at the 2 week harvest of lungs ($P = 0.016$, Wilcoxon–Mann–Whitney U-test). At the subsequent 8 weeks harvest, a 100-fold difference between vaccinated and control group mean CFUs was found in both lungs ($P = 0.004$) and spleens ($P = 0.029$). Furthermore, CFUs in the lungs increased between 2 and 8 weeks post infection in control mice, but decreased during the same time period in vaccinated mice. Thus, the protection imparted by i.n. BCG intensified within that time interval for the progression of the infection in the

![Graph](image)

Fig. 2. Protection following vaccination with M. bovis BCG. BALB/c mice were inoculated i.n. with $10^6$ BCG (strain Pasteur) and challenged 4 weeks later by the same route with $1.25 \times 10^6$ H37Rv organisms. Columns represent mean ($n = 4–6$) CFU counts; bars = S.E.

<table>
<thead>
<tr>
<th>Vaccine-challenge</th>
<th>Incubation with CTb</th>
<th>Organ</th>
<th>Mean log$_{10}$ CFU</th>
<th>S.E.</th>
<th>P</th>
<th>Protection index</th>
<th>Number of protected total number of mice</th>
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<tr>
<td>10 days</td>
<td>9 days</td>
<td>PstS-1</td>
<td>Lungs</td>
<td>3.49</td>
<td>0.62</td>
<td>0.048</td>
<td>1.81</td>
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<td></td>
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<td>5.29</td>
<td>0.46</td>
<td>&gt; 0.5</td>
<td>0.24</td>
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<td></td>
<td></td>
<td>PstS-1</td>
<td>Spleen</td>
<td>3.92</td>
<td>0.46</td>
<td>0.016</td>
<td>1.08</td>
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<td></td>
<td></td>
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<td></td>
<td>5.32</td>
<td>0.46</td>
<td>&lt; 0.01</td>
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<td></td>
<td>5.0</td>
<td>0.14</td>
<td>&gt; 0.5</td>
<td>0.24</td>
</tr>
</tbody>
</table>

Table 1 Protection following intranasal vaccination with the PstS-1 antigen

$^a$ Challenge: intranasal inoculation of $1.25 \times 10^6$ M. tuberculosis H37Rv viable bacilli.

$^b$ Intranasal inoculation of $10 \mu g$ PstS-1 protein mixed with $1 \mu g$ cholera toxin.

$^c$ Control-vaccinated CFU.

$^d$ Wilcoxon-Mann-Whitney U-test.

$^e$ CFU > 10 times below the median control CFUs.
Fig. 3. Histopathology of lungs 8 weeks after intranasal H37Rv infection of BALB/c mice. Lung sections were stained with haematoxylin-eosin (A, C, D) or by the Ziehl–Neelsen (B) staining method. A (×4) and B (×40) = non-vaccinated controls; C (×4) and D (×10) = BCG-vaccinated mice (see legend to Fig. 2).
lungs and also strongly interfered with the dissemination of infection to the spleen.

Vaccination was done with the PstS-1 protein as a representative antigenic subunit, mixed with CT as the proven mucosal adjuvant. The results showed a significant decrease in the H37Rv CFUs ($P = 0.048$, Wilcoxon–Mann–Whitney U-test, protection index 1.81) for the early challenge, 10 days after vaccination (Table 1). However, the degree of protection diminished for the 12 week challenge; the difference in lung CFUs had only low significance ($P = 0.151$, protection index 1.19), though it remained significant ($P = 0.016$, protection index 1.08) for the splenic CFUs.

3.2. The effect of i.n. vaccination on the histopathology of H37Rv infected lungs

Lung sections were examined in the light of recent studies, which indicated the importance of monitoring the host pathology as well as infection following vaccination [13,17]. Representative sections of H37Rv infected lungs (Fig. 3) demonstrate extensive (30–60% of section) confluent macrophage and lymphoid cell infiltration surrounding the lung granulomas (containing densely packed lymphocytes) in non-vaccinated mice (Lung section A). Acid fast staining bacilli were detected in mainly epithelioid type macrophages located outside, though often close to granulomas (Lung section B). In contrast to controls, the lungs of BCG vaccinated mice contained granulomas surrounded by healthy alveolar architecture (Lung section C), without demonstrable acid-fast-staining bacilli. The granulomatous areas have been of irregular shape and they often localised around blood vessels (Lung section D). Nevertheless, these granulomatous foci appeared to be of similar size in both vaccinated and control infected mice.

3.3. B cell response to intranasal vaccination

Repeated i.n. inoculations of Pst-S1 with CT favoured the induction of a mucosal antibody response and therefore it was of interest to monitor the antibody producing cells in different lymphoid tissues. (see Table 2) The results of the ELISPOT assay showed similar numbers of anti-PstS-1 IgM, IgG and IgA antibody forming B cells in the lungs and spleens of immunised mice. A predominantly IgA response was found in NALT cells, whereas the cervical LN cells produced predominantly IgG antibodies. These results are consistent with the interpretation that IgA antibodies produced in the lungs and NALT could have contributed toward the protective immunity.

4. Discussion

BCG vaccination in man was given orally when originally introduced [18], but that route was discontinued because of loss of viable BCG during gastric passage and frequent cervical adenopathy. In experimental animal species, aerosol or intra-tracheal delivery of BCG was without adverse effects [19], but varied in efficacy from superior protection than parenteral inoculation in primates [20], mice [21] and guinea pigs [12,22] to no apparent advantage over the subcutaneous route in other studies [23–25].

We have demonstrated in this pilot study, that i.n. application of BCG imparted a strong protection against subsequent challenge by the same route with the pathogenic H37Rv strain of *M. tuberculosis*. The observed profound protective effect is documented by the 100-fold reduction of both pulmonary and splenic CFU counts 8 weeks post challenge. Although this degree of CFU reduction exceeds that reported in most studies following parenteral BCG inoculation (usually about 10-fold), the relative efficacy of the intransal route needs to be confirmed in future experiments, comparing directly the different routes of vaccination.

The protective mechanisms could have involved several factors, such as the activation of T lymphocytes with preferential lung homing [26], upregulation of co-stimulatory molecules, e.g. B7.2 [27], activation of macrophages [28] or secretory IgA antibodies. Analysis of B cells by the ELISPOT

### Table 2

<table>
<thead>
<tr>
<th>Organ/tissue</th>
<th>Cell yield $\times 10^6$</th>
<th>Number of ELISPOTS $\times 10^{-3}$ per organ/tissue</th>
<th>IgA/IgG ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>IgM</td>
<td>IgG</td>
</tr>
<tr>
<td>Spleen</td>
<td>75</td>
<td>3.37</td>
<td>14.55</td>
</tr>
<tr>
<td>Lungs</td>
<td>4</td>
<td>2.95</td>
<td>15.98</td>
</tr>
<tr>
<td>LNC</td>
<td>5</td>
<td>0.35</td>
<td>17.76</td>
</tr>
<tr>
<td>NALT</td>
<td>3</td>
<td>0.37</td>
<td>1.99</td>
</tr>
</tbody>
</table>

*ELISPOT assay of cell suspensions (LNC = cervical lymph nodes, NALT = nasal lymphoid tissue) pooled from 4 mice/group, 7 days after three i.n. inoculations of PstS-1 with CT.*
assay demonstrated that the employed immunisation schedule with pstS-1 plus CT initiated a strong IgA response in the lungs, NALT and spleen. The observed highest IgA/IgG ratio in the NALT confirms previous observations [29]. The employment of two inoculation regimens of a relatively large BCG dose was also likely to stimulate a mucosal antibody response and shift the T cell response toward the Th2 phenotype [30].

The PstS-1 antigen was used previously for the vaccination of mice with a variable outcome of protection against parenteral challenge: the recombinant protein (the same as used here) entrapped in biodegradable microparticles given subcutaneously decreased CFUs only in two out of three experiments [31], while the intramuscular injection of DNA was found protective in one study [32] but not in the other [33]; the latter discrepancy could be due to the use of different plasmid constructs. In the present study, i.n. vaccination with the PstS-1 imparted protection of lesser degree and of shorter duration than BCG. These results corroborate with the findings for other antigen subunits following parenteral immunisation [34].

Histological evaluation of protection has been of interest in the light of a recent report, that antibody opsonisation of tubercle bacilli influenced tissue distribution, but not the extent of organ infection [17]. Interestingly, we found that vaccination with either BCG or PstS-1 abrogated the inflammatory infiltrate surrounding the granulomas, but without apparent reduction in the size or numbers of the granulomas per se. This outcome could have resulted from discrete changes in the maturation or activation of macrophage populations [35], T cell cytokine production [36] or an influence of antibodies [37]; these factors following mucosal immunisation need further characterisation.

In conclusion, the presented results suggest, that intranasal delivery could improve the efficacy of vaccination against tuberculosis. While our results demonstrated that i.n. BCG is strongly protective in BALB/c mice, its direct comparison with other routes of inoculation must await further studies. The superior protective effect of BCG to the Pst-s1 antigen could be due to the additive effect of multiple antigens and/or to the more suitable adjuvant properties of BCG. i.n. vaccination is known to engage both T and B cell mediated effector mechanisms in nasal and bronchus associated mucosal tissues, which differ from other mucosa-associated lymphoid tissues [38,39]. However, the mucosal immune reactions in the lungs are much less understood and their merits will need to be judged not only on the grounds of protective efficacy, but also for the possible risk to respiratory function from the infiltration of the lungs. Therefore, further characterisation of both cellular and humoral elements of bronchial and pulmonary mucosal immunity is needed for the design and development of more efficient vaccination against tuberculosis.

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References


