Mice Fed Lipid-Encapsulated *Mycobacterium bovis* BCG Are Protected against Aerosol Challenge with *Mycobacterium tuberculosis*

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Mice that consumed a single dose of 10⁷ lipid-encapsulated *Mycobacterium bovis* BCG bacilli showed significant pulmonary and systemic protection against aerosol challenge with *M. tuberculosis* H37Rv. As an extension of previous challenge studies with virulent strains of *M. bovis*, this report describes a reduction in *M. tuberculosis* infection in mice vaccinated orally with lipid-encapsulated BCG comparable to that observed in mice vaccinated subcutaneously with BCG. These results are consistent with the induction of tuberculin-specific cell-mediated immune responses.

Despite antibiotic treatment and a widely used vaccine, tuberculosis (TB) remains the leading cause of death by a single disease-causing organism (5). Intradermal inoculation of live attenuated *Mycobacterium bovis* BCG remains the most effective method of vaccinating humans against pulmonary TB. However, the efficacy of this method is highly variable, due in no small part to the requirement of delivering live bacilli in order to stimulate a protective cell-mediated immune (CMI) response (12). Accordingly, improved methods for the preparation and delivery of BCG, necessary to ensure an effective dose of viable, replicating, immunogenic microorganisms, are much sought after as a means of improving vaccine efficacy.

The BCG vaccine was administered orally to children between 1921 and 1976, although this route has since been largely abandoned as a public health measure due to concerns over vaccine efficacy and safety (6). Nevertheless, oral vaccination remains an attractive proposition due to its noninvasive nature, and recent research has seen renewed interest in the development of an oral live BCG vaccine (7–9, 11). We previously described a lipid coating method that maintains a high viability of encapsulated BCG bacilli for up to 4 weeks at room temperature (1, 2). This formulation is immunogenic when fed to BALB/c mice, promoting strong tuberculin-specific lymphoproliferative responses and gamma interferon (IFN-γ) secretion. Moreover, mice immunized with a single oral dose of the formulated vaccine show a significant degree of protection against aerosol challenge with a virulent strain of the homologous species (*M. bovis* strain 83/6235), with 1- to 2-log-unit fold reductions in pathogen burdens typically being observed in lung and spleen tissue samples. However, the protective efficacy of this method of vaccination against virulent human tubercle bacilli (*M. tuberculosis*) remains to be determined; this is an important issue, since comparative studies have suggested that BCG vaccination may provide less protection against *M. tuberculosis* than against virulent strains of *M. bovis* (16). In this report, we describe protection afforded to C57BL/6 mice against *M. tuberculosis* H37Rv after a single immunizing dose of lipid-encapsulated live BCG.

For oral vaccine preparation, mid-log-phase *M. bovis* BCG bacilli (Pasteur 1173P2 grown in Middlebrook 7H9 broth) were encapsulated into lipid microdroplets at 37°C as described previously (1). Two alternative formulations were used, each comprising >95% triglycerides with the following fatty acid (FA) profiles: lipid C (predominated by the monoenoic FA oleic acid at 50% and containing palmitic, stearic, linoleic, and myristic acids at 25, 15, 6, and 1%, respectively) and lipid K (predominated by the saturated FA lauric acid at 50% and containing myristic, palmitic, oleic, stearic, and linoleic acids at 18, 8, 6, 2, and 1%, respectively). Lipid-encapsulated BCG preparations were flavored with chocolate powder (1) and allowed to solidify at room temperature before being cut into standardized pellets containing approximately 10⁷ bacilli/pellet.

Specific-pathogen-free 6- to 8-week-old female C57BL/6 mice were kept in barrier conditions and maintained on standard mouse chow with water supplied ad libitum. For oral vaccination, mice were separated into individual cages, taken off food for 12 h, and then offered a single BCG-containing pellet in either the lipid C or the lipid K formulation; consumption was ensured by close monitoring. For subcutaneous immunization, positive control mice were vaccinated at the base of the tail with 4 × 10⁶ CFU of nonencapsulated BCG in 7H9 broth, while negative control animals received phosphate-buffered saline (PBS) alone.

Vaccination and immune response studies were conducted in laboratories at the University of Otago. At 15 weeks after vaccination, eight mice per group were euthanized. Single-spleen-cell suspensions (1 ml) containing 2.5 × 10⁶ splenocytes were stimulated in the presence of 60 μg of bovine purified protein derivative (PPD) (CSL Ltd., Melbourne, Victoria, Australia)/ml or PBS as a control as described elsewhere (1). After 72 h, cell-free supernatants were collected. Cytokines were assessed by using commercial IFN-γ and interleukin 2 (IL-2) capture enzyme-linked immunosorbent assays (Duoset kits; R&D Systems Inc., Minneapolis, Minn.). Significant levels...
of IFN-γ were detected in PPD-stimulated splenocyte culture supernatants for all three groups of vaccinated mice (Fig. 1), although antigen-stimulated production of IL-2 was detected only in subcutaneously vaccinated mice. IFN-γ production was highest among subcutaneously vaccinated mice; there was no apparent difference in IFN-γ production in mice vaccinated orally with BCG in the lipid C formulation and with BCG in the lipid K formulation.

Vaccination and challenge infection studies were conducted in a level III biohazard facility at Colorado State University. The challenge inoculum was obtained from a batch-lot frozen culture of M. tuberculosis H37Rv (1-ml aliquots, stored at −70°C) which had been grown from low-passage seed lots in Proskauer-Beck liquid medium containing 0.05% Tween 80 to 70°C which had been grown from low-passage seed lots in Proskauer-Beck liquid medium containing 0.05% Tween 80 to early mid-log phase (15). At 15 weeks after vaccination, eight mice per group were infected with M. tuberculosis H37Rv via respiratory challenge with an aerosol generation device (Glass-Col, Terre Haute, Ind.), which was calibrated to deliver approximately 100 bacilli/animal. Mice were sacrificed 4 weeks after M. tuberculosis infection. Spleen and lung homogenates were prepared in PBS–0.05% Tween 80 and plated in double serial fivefold dilutions on Middlebrook 7H11 Bacto agar for the enumeration of mycobacteria (with or without the addition of 2 μg of 2-thiophene-carboxylic acid hydrazide/ml to selectively inhibit the growth of any residual BCG bacilli in the test organs). Compared to the results obtained for control (nonvaccinated) animals, significant reductions in the geometric mean bacterial burdens were recorded for mice from all three vaccine groups for both organs (Table 1). Notably, there was a >1-log-unit reduction in bacterial burdens in the lungs and spleens of mice vaccinated orally with BCG in the lipid C formulation; this level of protection was comparable to or greater than the corresponding level of protection seen in subcutaneously vaccinated mice. There were no significant differences in protection among the various vaccination groups.

Despite recent developments in the protective efficacy of subunit protein and DNA vaccines (4, 10, 14), live BCG remains an effective vaccination agent for the establishment of protective immunity against TB, especially childhood disease. Parenterally administered live BCG is a standard method for inducing a protective CMI response against pulmonary infection, although recent progress has been made toward delivering BCG via the mucosal route to establish protection (3, 9). In order to invoke a protective response with BCG, live replicating bacilli must reach sites of immune induction. For that reason, our research has focused on developing an improved means of maintaining viable BCG bacilli in a lipid-encapsulated form as an oral delivery vehicle (1, 2).

In the present study, two different forms of lipid-encapsulated BCG were shown to invoke system-level lymphocyte sensitization in mice, as evidenced by in vitro tuberculin-specific IFN-γ production in splenocyte cultures. Stimulated splenocytes from lipid-encapsulated BCG-fed C57BL/6 mice assessed 15 weeks after vaccination produced 1,300 to 1,600 pg of IFN-γ/ml; these levels compare favorably to IFN-γ levels that we observed previously in lipid-encapsulated BCG-fed BALB/c mice assessed 8 weeks after vaccination (1). These results indicate that a strong PPD-specific CMI response can be sustained for over 3 months after a single oral vaccination. However, noteworthy from the present study was the fact that orally vaccinated mice produced lower levels of IFN-γ than mice vaccinated via a standard subcutaneous route and, moreover, only mice vaccinated via the parenteral route produced statistically significant levels of IL-2 in response to PPD stimulation. Together, these data suggest that although oral vaccination of

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**TABLE 1. Effects of subcutaneously delivered BCG or orally delivered lipid-encapsulated BCG vaccination on tissue bacterial burdens after aerosol challenge with M. tuberculosis**

<table>
<thead>
<tr>
<th>Group</th>
<th>Lungs</th>
<th>Spleens</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Log₁₀ CFU/organᵃ</td>
<td>Log₁₀ resistanceᵇ</td>
</tr>
<tr>
<td>Nonvaccinated (control)</td>
<td>6.24 ± 0.05</td>
<td>NA</td>
</tr>
<tr>
<td>Subcutaneous BCG</td>
<td>5.46 ± 0.05ᵃ</td>
<td>0.78</td>
</tr>
<tr>
<td>Oral BCG in lipid C formulation</td>
<td>5.22 ± 0.08ᵃ</td>
<td>1.03</td>
</tr>
<tr>
<td>Oral BCG in lipid K formulation</td>
<td>5.80 ± 0.15ᵃ</td>
<td>0.44</td>
</tr>
</tbody>
</table>

ᵃ Data are geometric means ± standard error of the means for duplicate analysis of eight mice in each group. The efficacies of different vaccinations were compared by a one-way analysis of variance of the log₁₀ CFU, followed by Dunnett’s post-hoc test. *P value of <0.05 was considered significant.

ᵇ Protective efficacy, expressed as log₁₀ reductions in bacterial loads compared to nonvaccinated control animals (which received a sham injection of PBS). NA: Not applicable.
mice with lipid-encapsulated BCG is able to invoke a strong
and sustained IFN-γ response, the magnitude of the CMI
response, at the systemic level at least, is not as great as that
observed after parenteral vaccination.

As anticipated (4, 13), subcutaneous vaccination of C57BL/6
mice with BCG was able to confer protection against aerosol
challenge with *M. tuberculosis* H37Rv, with significant reduc-
tions in bacterial burdens being observed in lung and spleen
tissue samples. In addition, both test preparations of orally
administered BCG (BCG in lipid C and lipid K formulations)
were shown to confer significant protection against *M. tuber-
culosis*, with BCG in the lipid C formulation in particular
reducing bacterial burdens to levels comparable to (splenic
tissue) or lower than (lung tissue) those observed in subcuta-
neously vaccinated mice. These results correlate with our pre-
vious observation that orally delivered lipid-encapsulated BCG
can protect against aerosol challenge with a virulent strain of
*M. bovis* (1) and are particularly relevant to interpretation of
the protective capacity of orally delivered BCG against virulent
mycobacteria that ordinarily infect via the respiratory route.
Other recent studies reported the outcome of systemic myco-
bacterial challenge in orally vaccinated mice, whereby oral
delivery of a high dose (2 × 10^9 CFU [9]) or a low dose (1 ×
10^7 CFU [11]) of BCG to mice was shown to confer protection
against intravenous challenge with a high dose of virulent
*M. tuberculosis* (1 × 10^5 CFU). In contrast, the present report
highlights protection against mycobacterial challenge via the
natural pulmonary route in a low-dose aerosol model.

Orally delivered preparations of BCG represent a poten-
tially valuable vaccine tool for the control of TB (7), and the
present study provides evidence that lipid encapsulation of
BCG microorganisms may further extend the utility of the oral
BCG vaccine. Our previous studies indicated that lipid encap-
sulation can increase the protective efficacy of orally delivered
BCG beyond that observed with bacilli in a conventional for-
mation (1, 2). Further studies are required to optimize this oral
delivery system (e.g., by identifying the particular immune in-
duction sites at which lipid-encapsulated BCG is active) as well
as to define patterns of lymphocyte trafficking and effector site
responsiveness that correspond to protection.

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challenged with *M. tuberculosis* and the Department of Animal Labo-
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