Tuberculosis (TB) remains the leading cause of death by a single disease-causing organism, with an estimated 2 million deaths each year (18, 37). In addition, the disease is a threat to the agricultural industry due to persistence of TB in farmed animals (16). Human TB is predominantly caused by *Mycobacterium tuberculosis*, while TB in animals is caused by *Mycobacterium bovis*. Both *M. tuberculosis* and *M. bovis* are members of the TB complex and are very closely related genetically. While effective chemotherapy for human TB has been available for many years, treatment is lengthy and relatively expensive, making widespread control of the disease difficult to achieve in developing countries. Prophylactic vaccination may be a better option for controlling TB.

*M. bovis* bacille Calmette-Guérin (BCG) is the world’s most widely used vaccine, with an estimated 3 billion doses having been administered since its discovery and initial testing in the 1920s (20, 33). *M. bovis* BCG consists of a live attenuated strain of *M. bovis* and was originally derived from a cow with tuberculosis mastitis. Despite its widespread use, the efficacy of *M. bovis* BCG remains highly variable, with success rates in human and animal trials ranging between 0 and 80% protection (14, 15, 20). In a recent meta-analysis of human *M. bovis* BCG vaccination trials, *M. bovis* BCG was shown to reduce the risk of pulmonary TB by 50% and the number of deaths due to TB by 71% (5). There are a number of reasons proposed for the variation in efficacy; however, it is clear that killed *M. bovis* BCG is poorly protective and that acquired immunity against TB depends on survival and persistence of live *M. bovis* BCG, or its secreted products, in the host (17, 21, 36). These reports suggest that under the appropriate conditions, *M. bovis* BCG can be effective against TB. Furthermore, to date no TB vaccine has been shown to provide greater protection than *M. bovis* BCG against experimental infection with TB. Taken together, these data lend weight to arguments favoring modification of *M. bovis* BCG to improve its efficacy.

Oral administration of *M. bovis* BCG was used with children between 1921 and 1976, but this route was abandoned due to low rates of delayed-type hypersensitivity conversion and occasional development of cervical lymphadenitis (21). Since these trials, there have been substantial improvements in oral delivery systems and the use of immune modulators for oral vaccines (4, 41). Oral administration of a vaccine against TB has a number of advantages, including ease of administration, low cost, and avoidance of needles and the associated reduced risk of disease transfer. Furthermore, oral immunization more effectively targets the mucosal immune response. Control of bovine TB is difficult in countries which have wildlife reservoirs of *M. bovis* infection, such as New Zealand and the United Kingdom. Vaccination of wildlife against bovine TB is currently being considered, and an oral bait containing *M. bovis* BCG would be a promising option if the *M. bovis* BCG could be protected from degradation in the stomach (9).

Oral immunization of guinea pigs and mice with *M. bovis* BCG has been shown to induce immune responses in spleen and lymph node cell populations as well as purified protein derivative (PPD)-specific delayed-type hypersensitivity and antibody responses (28, 29, 36). Mice immunized orally with high doses of *M. bovis* BCG showed similar levels of protective immunity to mice immunized via the subcutaneous route and induced protection against intravenous challenge with *M. tuberculosis* (29). These reports suggest that mucosal immunization with *M. bovis* BCG can be an effective means of inducing protective systemic immune responses.

Current commercially available human *M. bovis* BCG vaccines (e.g., Glaxo) are derived from freeze-dried *M. bovis* BCG preparations. Although in animal trials, fresh *M. bovis* BCG is frequently used (2, 8, 22, 30, 35), the freeze-drying procedure results in reduced viability of bacteria, with reports of between 30 and 50% loss of viability and impaired recovery of remain-
ing live bacteria (21). A method of preparing M. bovis BCG that retains optimal viability of the bacteria prior to vaccination would contribute greatly to the success of this vaccine.

Lipids are only partially degraded in the stomach, as the majority of lipolytic enzymes are present in the large intestine (11, 13, 24, 32). Lipids may therefore provide some protection for the M. bovis BCG vaccine against degradation in the stomach. Furthermore, ingestion of lipids decreases gastrointestinal motility, and the presence of lipid digestive products within the upper small intestine induces the secretion of biliary and pancreatic fluids that dramatically alter the luminal environment (13). These conditions may assist in the transfer of live M. bovis BCG through the intestinal epithelium. Based on these observations, we hypothesized that protection of M. bovis BCG against gastric secretions with lipids would enhance viability and improve uptake of viable bacilli across the intestinal wall. We report here the development and testing of a novel lipid-based M. bovis BCG formulation for oral vaccination of mice.

MATERIALS AND METHODS

Bacteria. M. bovis BCG Pasteur 1173P2 was used as the vaccine strain. The virulent M. bovis strain used for macrophage infection studies and for aerosol challenge of mice was M. bovis 83/6235, which has been described in previous studies (1, 6). For M. bovis BCG formulation and macrophage infection, bacteria were grown to mid-log phase in 175-ml flasks (Falcon) containing Middlebrook 7H9 medium (Difco, Detroit, Mich.) supplemented with albumin-dextrose-catalase (8). Bacilli were harvested by centrifugation and washed twice in phosphate-buffered saline (PBS) prior to storage at −70°C. For aerosol challenge of mice, M. bovis was grown to mid-log phase in Tween albumin broth containing a Dubos broth base (Difco) supplemented with 0.006% (vol/vol) alkaline oleic acid, 0.5% (wt/vol) albumin fraction V, and 0.25% (wt/vol) glucose. The number of CFU of M. bovis BCG or virulent M. bovis was determined retrospectively by plating on 7H11 agar plates as described previously (7).

Lipid composition. A fractionated lipid product was selected for formulation of M. bovis BCG on the basis of melting temperature (32°C) and the ability to maintain bacilli in a viable and uniform suspension as assessed by counting CFU (data not shown). The lipid consisted of triglycerides of fatty acids comprising 1% myristic acid, 25% palmitic acid, 15% stearic acid, 50% oleic acid, and 6% linoleic acid.

Formulation of M. bovis BCG. Glucose (10 mg/ml), monosodium glutamate (1 mg/ml; Sigma), and 10% vol/vol albumin-dextrose-catalase were added to the lipid (lipid formulation). These additives were dispersed with the lipid in the liquid phase (40°C) and were previously shown not to affect viability of M. bovis BCG. Pelleted M. bovis BCG was resuspended in liquefied formulation medium which had been warmed to 40°C. M. bovis BCG formulations were transferred to 15-ml tubes (Falcon) and allowed to solidify with gentle mixing at 4°C. Formulations were removed from the tubes and aseptically cut into 1-g pellets as required for viability testing and vaccination studies.

Vaccination of mice. Female BALB/c mice (6 to 8 weeks old) were obtained from the University of Otago Department of Animal Laboratory Sciences, Dunedin, New Zealand. Mouse experiments were conducted under ethics approval from the University of Otago Animal Ethics Committee (approval no. 51/2000). For time course experiments, mice were vaccinated subcutaneously with 107 CFU of BCG. For oral vaccination, mice were separated into individual cages housed in a clean room and were given 107 CFU of BCG. For oral vaccination, mice were separated into individual cages housed in a clean room and were given 107 CFU of BCG. For aerosol challenge experiments, mice were given aerosolized suspensions of 107 CFU/ml (final 106 CFU/ml) in a phosphate-buffered saline (PBS) vehicle (pH 7.2) containing 1% vol/vol Tween 80. Mice were infected via the respiratory route with virulent M. bovis. Aerosol challenge of M. bovis was performed using a method described in previous studies (26, 31, 39, 43). Peritoneal exudate cells were obtained by lavage from female BALB/c mice. Cells were collected in PBS supplemented with 1% bovine serum albumin and 20 U of heparin/ml washed once and resuspended in DMEM containing 10% fetal calf serum and 1,000 U of penicillin/ml (supplemented DMEM) at 2 × 106 cells/ml. One hundred micro-liter of cell suspension was dispensed into a 96-flat-well plate (Nunc). After incubation for 2 h in 5% CO2 at 37°C, the nonadherent cells were removed, washed, and resuspended at a density of 5 × 105/ml in supplemented DMEM. Nonadherent cells were selectively depleted of the remaining adherent population by incubation in 25-mI flasks (Falcon). Nonadherent peritoneal exudate cells (NPEC) were determined to comprise >90% lymphocytes following May-Grünwald/Giemsa staining. Warm supplemented DMEM was added to the adherent monolayer, which was estimated to contain 5 × 105 cells/well. This population was found to be 98% positive with a nonspecific esterase staining kit (catalog no. 181-B; Sigma, St. Louis, Mo.) and is henceforth referred to as macrophages. Macrophages were infected with M. bovis at a multiplicity of infection of two bacilli per macrophage as described previously (3). Nonphagocytosed bacteria were removed by washing. Bacteria were cultured on 7H11 agar plates as described previously (7).

Spleen cell proliferation assay. Spleen cell suspensions were prepared by filtering cells through a cell strainer (70-μm-pore-size mesh; Becton Dickinson). Cells were washed twice in PBS and resuspended to a concentration of 109/ml in Dulbecco’s modified Eagle medium (DMEM) containing 10% fetal calf serum, 20 mM HEPES penicillin (100 U/ml), streptomycin (100 μg/ml), 5.5 × 10−5 M 2-mercaptoethanol (all from Gibco-BRL). Cells were resuspended to a concentration of 5–107 per ml in RPMI 1640 medium with 10% fetal calf serum (5 × 105 per well) were plated out in triplicate wells in 96-well plates (Nunc). Cells were cultured for 5 days at 37°C, and media were changed at 48 and 72 h. One hundred microliters of culture supernatant was collected and frozen at −70°C for cytokine analysis. Interleukin-2 (IL-2) and gamma interferon (IFN-γ) capture enzyme-linked immunosorbent assays (ELISAs) were performed according to the manufacturer’s instructions using a commercial kit (Duoset; R&D Systems, Minneapolis, Minn.). Cytokine levels in culture supernatants were quantified by extrapolation from standard curves. The minimum sensitivities of the two ELISAs were determined to be 50 pg/ml for IFN-γ and 35 pg/ml for IL-2.

M. bovis inhibition assay. Peritoneum-derived macrophages were tested for inhibition of intracellular growth of M. bovis following coculture with or without autologous lymphocytes. Experiments were performed according to a modification of previously described protocols (26, 31, 39, 43). Peritoneal exudate cells were obtained by lavage from female BALB/c mice. Cells were collected in PBS supplemented with 1% bovine serum albumin and 20 U of heparin/ml washed once and resuspended in DMEM containing 10% fetal calf serum and 1.00 U of penicillin/ml (supplemented DMEM) at 2 × 106 cells/ml. One hundred microliters of cell suspension was dispensed into a 96-flat-well plate (Nunc). After incubation for 2 h in 5% CO2 at 37°C, the nonadherent cells were removed, washed, and resuspended at a density of 5 × 105/ml in supplemented DMEM. Nonadherent cells were selectively depleted of the remaining adherent population by incubation in 25-mI flasks (Falcon). Nonadherent peritoneal exudate cells (NPEC) were determined to comprise >90% lymphocytes following May-Grünwald/Giemsa staining. Warm supplemented DMEM was added to the adherent monolayer, which was estimated to contain 5 × 105 cells/well. This population was found to be 98% positive with a nonspecific esterase staining kit (catalog no. 181-B; Sigma, St. Louis, Mo.) and is henceforth referred to as macrophages. Macrophages were infected with M. bovis at a multiplicity of infection of two bacilli per macrophage as described previously (3). Nonphagocytosed bacteria were removed by washing. Bacteria were cultured on 7H11 agar plates as described previously (7).

Isolation of M. bovis. Mice were euthanized between 37 and 40 days after aerosol challenge. The lungs and spleen from each mouse were processed indi-
vidually for mycobacterial isolation. The organs were homogenized in a Ten-Broeck grinder, and samples were centrifuged at 3,500 × g for 20 min. The deposits were resuspended in 1 ml of distilled water. Appropriate dilutions were made in Tween albumin broth, and a 0.1-ml volume of a diluted or undiluted sample was inoculated onto a modified mycobacterium 7H11 agar containing sodium pyruvate (7). In the absence of glycerol, sodium pyruvate is selective for viable mycobacterium 7H11 agar containing sodium pyruvate (7). In the absence of glycerol, sodium pyruvate is selective for viable bacteria in lung and spleen homogenates was 10 CFU/lung or spleen. Two replicates were prepared for each dilution. Culture conditions and methods for identification of isolates were carried out as previously described (7).

Analysis of data. Statistically analyses of differences in the mean cytokine levels and log_{10} transformed spleen cell proliferation responses for the vaccine groups were determined using the Student t test. The bacterial counts from the lung and spleen were log_{10} transformed and analyzed using analysis of variance. For statistical purposes, when no bacteria were cultured from tissues, half the lowest detectable count (percent CFU/organ) was used.

RESULTS

A lipid product has been identified as being compatible with M. bovis BCG. Chemical analysis of the lipid showed that it consists of a mixture of medium and long-chain fatty acids. The lipid is liquid at 37°C but forms a solid at 30 to 33°C. Formulation of M. bovis BCG in the lipid matrix has no immediate detrimental effect on bacterial viability. M. bovis BCG is maintained and stored in a solid matrix which is suitable for oral delivery.

M. bovis BCG viability is retained following formulation. Retrospective testing of M. bovis BCG viability following formulation with lipid showed that the number of CFU recovered immediately following formulation did not significantly decrease. Over time however, the number of CFU decreased in relation to increased storage temperature. Figure 1 shows the number of CFU of M. bovis BCG which could be recovered following storage in lipid formulation. Formulated M. bovis BCG could be stored for at least 8 weeks at 4°C with minimal loss of viability. Further tests at 16 weeks after storage at 4°C showed similar levels of M. bovis BCG viability (data not shown). However, with storage at room temperature, viability of M. bovis BCG began to decrease after 32 days. By 8 weeks of storage at this temperature, few viable M. bovis BCG could be detected. These results show that the shelf life of formulated M. bovis BCG is maintained at a low temperature but shortens considerably at higher temperatures.

Oral delivery of formulated M. bovis BCG induces immune responses in mice. To determine a suitable method of measuring systemic immune responses following oral delivery of M. bovis BCG, we compared bovine PPD-induced splenocyte proliferation (LTA) and splenic IL-2 and IFN-γ responses with subcutaneous BCG vaccination at 8 weeks. Mice were fed 5 × 10^7 CFU of lipid-formulated M. bovis BCG or M. bovis BCG in jam (nonformulated M. bovis BCG) or were subcutaneously vaccinated with 10^6 CFU of M. bovis BCG. Table 1 shows that while both the LTA and IFN-γ assays showed significant differences between the formulated and nonformulated oral M. bovis BCG groups, the differences for the IL-2 assay were not significant. The IFN-γ assay was used in further experiments to monitor systemic immune responses due to the importance of IFN-γ for protection against TB.

To determine the effect of dose of M. bovis BCG following oral delivery, we compared splenic IFN-γ responses to bovine PPD in mice vaccinated with various doses of formulated or nonformulated M. bovis BCG at 8 weeks postvaccination. Figure 2 shows that a low level of IFN-γ (<200 pg/ml) was detected in the formulated group following oral immunization with 10^6 CFU of M. bovis BCG, but there were no significant differences between the vaccine groups. When the dose was increased to 10^7 CFU, IFN-γ responses in the nonformulated group remained low whereas responses to formulated M. bovis BCG increased significantly (P < 0.05). Similar differences were seen with 10^8 CFU of M. bovis BCG. When the vaccine dose was increased to 10^9 CFU of BCG, an increase in the levels of IFN-γ was seen in the nonformulated group while levels in the formulated group remained high. At doses of M.
bovis BCG ranging from $10^7$ to $10^9$ CFU, IFN-$\gamma$ responses in the formulated M. bovis BCG group were significantly greater than those of nonformulated M. bovis BCG. The increase in IFN-$\gamma$ responses seen at the high dose in the nonformulated group shows that considerably higher doses of oral M. bovis BCG are required for induction of immune responses than are required with formulated M. bovis BCG. To determine the time course of immune responses to oral M. bovis BCG, we compared splenic IFN-$\gamma$ responses at 2-week intervals following oral or subcutaneous vaccination with M. bovis BCG. Figure 3 shows that IFN-$\gamma$ responses following subcutaneous vaccination peaked at 4 weeks and gradually declined at weeks 6 and 8. By comparison, IFN-$\gamma$ responses following oral vaccination with formulated M. bovis BCG first increased at 6 weeks and remained high at 8 weeks postvaccination. IFN-$\gamma$ responses to nonformulated M. bovis BCG or formulation material alone remained low between 2 and 8 weeks. These results show that immune responses following oral vaccination with formulated M. bovis BCG are delayed compared to results with subcutaneous vaccination but appear to persist at least to 8 weeks.

Peritoneum-derived lymphocytes from mice orally vaccinated with formulated M. bovis inhibit growth of M. bovis in autologous macrophages. The addition of NPEC to M. bovis-infected macrophages from mice vaccinated with oral M. bovis BCG formulations was performed in order to determine whether lymphocyte-mediated effector mechanisms could inhibit intracellular growth of M. bovis. Growth of M. bovis in macrophages was determined by $[^3H]$uracil uptake. The growth of M. bovis within macrophages alone or when cocultured with NPEC from orally vaccinated mice is illustrated in Fig. 4. Macrophages prepared from mice orally vaccinated with formulated or nonformulated M. bovis BCG or mice given formulation material alone showed no differences in their ability to control M. bovis growth. When NPEC from mice vaccinated with formulated M. bovis BCG were cocultured with autologous M. bovis-infected macrophages, the $[^3H]$uracil counts were significantly reduced from those for coculture of NPEC from mice vaccinated with nonformulated M. bovis BCG or formulation material alone ($P < 0.05$). These results demonstrate that lymphocytes from mice orally vaccinated with formulated M. bovis BCG activate macrophages to inhibit

**TABLE 1.** Bovine PPD-stimulated spleen cell responses in mice 8 weeks after vaccination

<table>
<thead>
<tr>
<th>Immunization</th>
<th>IL-2 (pg/ml)</th>
<th>IFN-$\gamma$ (pg/ml)</th>
<th>LTA (SI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Formulation only</td>
<td>110.62 (±14.06)</td>
<td>51.44 (±14.38)</td>
<td>1.67 (±0.49)</td>
</tr>
<tr>
<td>Nonformulated oral BCG</td>
<td>153.51 (±25.22)</td>
<td>65.10 (±20.05)</td>
<td>1.83 (±0.51)</td>
</tr>
<tr>
<td>Formulated oral BCG</td>
<td>230.23 (±54.13)</td>
<td>778.95* (±76.80)</td>
<td>6.26* (±0.33)</td>
</tr>
<tr>
<td>Subcutaneous BCG</td>
<td>330.56 (±61.12)</td>
<td>536.88* (±86.70)</td>
<td>8.22* (±0.73)</td>
</tr>
</tbody>
</table>

* Asterisk indicates a mean which is significantly different from the mean of the formulation-only control group. $P$ value, $<0.05$ (Student $t$ test). Standard errors are given in parentheses. SI, stimulation index.

**FIG. 2.** Bovine PPD induced IFN-$\gamma$ responses following oral vaccination with various doses of formulated M. bovis BCG. Mice were sacrificed at 8 weeks after oral immunization with formulated M. bovis BCG (circles), nonformulated M. bovis BCG (triangles), or formulation material only (diamonds). Splenocytes were incubated with bovine PPD for 72 h. Supernatants were then collected and analyzed using a sandwich ELISA. Each treatment group contained six mice. Splenograms were individually processed. Results are expressed in picograms/milliliter and are presented as means of triplicate determinations. Bars indicate standard errors.
in intracellular growth of *M. bovis*. Control of intracellular growth of *M. bovis* in vitro may reflect growth inhibition in vivo, leading to reduced dissemination of *M. bovis* in the host.

**Oral vaccination with formulated *M. bovis* BCG protects against aerosol challenge with virulent *M. bovis***. In order to determine the protective efficacy of formulated oral *M. bovis* BCG, mice were orally vaccinated with 5 × 10^7 CFU of formulated *M. bovis* BCG or subcutaneously vaccinated with 10^8 CFU of *M. bovis* BCG. Nonvaccinated mice served as controls. Mice were challenged with virulent *M. bovis* by the aerosol route 8 weeks after vaccination and euthanatized 37 to 40 days after challenge. Table 2 shows that subcutaneous *M. bovis*
BCG vaccination reduced the bacterial count in the lungs by approximately 2.34 logs and reduced the bacterial count in the spleen by 1.90 logs. By comparison, formulated oral M. bovis BCG reduced the lung bacterial count by approximately 1.0 log and the spleen bacterial count by 1.48 logs. The results in Table 2 showed that oral formulated M. bovis BCG and subcutaneous M. bovis BCG induced significant protection against aerosol challenge with virulent M. bovis, although the protective efficacy of subcutaneous M. bovis BCG in the lung was greater than that for the oral formulated M. bovis BCG group.

**DISCUSSION**

This study demonstrates that encapsulation of M. bovis BCG in a lipid matrix enhances vaccine efficacy following oral delivery. Consumption of lipid-formulated M. bovis BCG by mice induced strong protective immune responses compared to results with nonformulated M. bovis BCG. This finding is important for developing live vaccines which can be administered through the oral route. Oral delivery of vaccines is also likely to target mucosal immune responses at the barriers where most pathogens enter the host.

Our study extends earlier studies with young children as well as those with mice and guinea pigs which used high doses of oral M. bovis BCG to vaccinate against TB (10, 29). In these studies the oral route of delivery was shown to be less effective than subcutaneous or intradermal administration for preventing aerosol or intravenous infection with TB. High doses of oral M. bovis BCG sometimes resulted in lymphadenitis.

Oral vaccination of BALB/c mice with formulated M. bovis BCG stimulated protective immune responses and provided protection against aerosol challenge with M. bovis. Oral delivery of formulated M. bovis BCG to mice induced strong immune responses—as indicated by tuberculosis-induced lymphocyte proliferation responses, IFN-γ levels in the spleen, and macrophage inhibition—compared to nonformulated M. bovis. The results from the time course study showed that the mean IFN-γ response of the oral formulated M. bovis BCG group remained high at 8 weeks after vaccination, while the response for the subcutaneous M. bovis BCG group declined between 4 and 8 weeks after vaccination. Oral vaccination of mice with formulated M. bovis BCG also reduced the number of M. bovis organisms in the spleen and lungs compared to results with mice vaccinated with nonformulated M. bovis BCG, although the level of protection was greater for the subcutaneous M. bovis BCG group at this time point. The immunological data suggested that a more sustained immune response may have been induced in the oral formulated M. bovis BCG group, and further studies are in progress to determine if the level of protection was greater for the oral formulated M. bovis BCG group when the vaccination-challenge interval is increased.

The present results demonstrate that oral formulation of M. bovis BCG enhanced delivery of M. bovis BCG to the immune system. The capacity of formulated M. bovis BCG but not nonformulated M. bovis BCG to stimulate strong systemic immune responses suggests that the formulation procedure protects M. bovis 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. Others have shown that high numbers of M. bovis BCG (2 × 10⁹ to 5 × 10⁹ CFU) administered either orally or intragastrically can reduce the bacterial burden in the spleen, liver, and lungs following intravenous challenge with M. tuberculosis (30, 29). Our data show that similar levels of protection can be achieved against aerosol challenge using considerably fewer M. bovis BCG organisms which are protected in a lipid formulation.

Maintenance of BCG viability prior to vaccination is an important first step for oral vaccine formulation since killed M. bovis BCG has been shown to induce weaker protection against TB than live M. bovis BCG (17, 21). The second step is preventing loss of M. bovis BCG viability in the stomach. Finally, successful oral vaccination against TB depends on uptake of viable M. bovis BCG through the gastrointestinal tract and replication of bacilli in the host. In the present study, lipid formulation of M. bovis BCG resulted in minimal loss of viability after storage for up to 16 weeks at 4°C. This suggests that certain lipid preparations are suitable for long-term storage of M. bovis BCG. Further work is required to determine the conditions under which the viability of lipid-formulated M. bovis BCG can be maintained at temperatures above 4°C.

Since formulated oral M. bovis BCG elicited stronger immune responses than nonformulated BCG, we suggest that the formulated M. bovis BCG was protected from degradation in the stomach by the lipid formulation. The majority of enzymes involved in lipolysis are secreted in the lower intestine (13, 25, 44). This may have allowed the lipid formulation to protect M. bovis BCG from the harsh acidic and enzymatic environment of the stomach. In humans, the process of fat digestion begins in the stomach, with a fraction of the triacylglycerols being hydrolyzed by preduodenal lipases (12, 23, 38). The dietary lipids are also emulsified in the stomach by the action of peristalsis (11). However, lipid digestion in the stomach is incomplete, and dietary fats are delivered to the intestinal lumen as crude lipid emulsion particles in association with the undigested triacylglycerols and phospholipids (25, 44). The mech-

<table>
<thead>
<tr>
<th>Vaccine group</th>
<th>Lung bacterial countb</th>
<th>Log₁₀ resistancec</th>
<th>Spleen bacterial countd</th>
<th>Log₁₀ resistancee</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonvaccinated</td>
<td>5.837 (±0.362) (A)</td>
<td>NA</td>
<td>4.565 (±0.189) (A)</td>
<td>NA</td>
</tr>
<tr>
<td>Formulated oral BCG</td>
<td>4.774 (±0.270) (B)</td>
<td>1.06</td>
<td>3.084 (±0.176) (B)</td>
<td>1.48</td>
</tr>
<tr>
<td>Subcutaneous BCG</td>
<td>3.498 (±0.237) (C)</td>
<td>2.34</td>
<td>2.660 (±0.181) (B)</td>
<td>1.90</td>
</tr>
</tbody>
</table>

a Figures in columns with the same parenthetical letter are not significantly different (P > 0.05).

b Values are log₁₀ numbers of CFU (± standard error) of M. bovis from the lungs or spleens of six animals per group 37 to 40 days postchallenge.

c Data are expressed as levels of log₁₀ resistance calculated by subtracting the log₁₀ mean number of bacilli in the organs of vaccinated animals from the log₁₀ mean number of bacilli in the organs of nonvaccinated animals. NA, not applicable.
anism of enhanced oral delivery of lipid-formulated BCG remains to be determined, and it is possible that lipids may also act as mucosal adjuvants in the small intestine. Although this study did not examine the in vivo dissemination of viable _M. bovis_ BCG across the gut wall, the observation that immune responses were detected in the spleens of mice suggests that _M. bovis_ BCG was able to attach to and cross the gastrointestinal barrier. Live mycobacteria are known to use the complement receptor and the mannose receptor to bind to and invade epithelial cells (19, 27). The onset of immune responses following oral immunization supports the hypothesis that _M. bovis_ BCG was able to translocate across the gut and replicate in the host. Our data support the hypothesis that _M. bovis_ BCG multiplys following translocation across the gut and that it is the replication of live bacilli in the host that leads to the generation of immune responses and to subsequent protection against TB.

We have previously demonstrated that direct administration of _M. bovis_ BCG into the duodenum induced enhanced immune responses and protection against bovine TB compared to responses following intragastric administration of _M. bovis_ BCG (6). These results showed that administration of _M. bovis_ BCG into the duodenum was an effective means of stimulating mucosal immune responses in the lungs and suggested that if _M. bovis_ BCG vaccine is protected from degradation in the stomach its efficacy should improve.

Lipid-formulated BCG has several properties which distinguish it from the currently available BCG vaccine. The lipid matrix is hydrophobic and is hence able to be stored under humid or moist conditions without gross contamination. Additives can be included in the lipid matrix as required—for example, to improve palatability or survival of the bacteria in vitro or following ingestion. Lastly, the lipid matrix maintains high levels of _M. bovis_ BCG viability and hence vaccine efficacy.

In conclusion, a method of formulating and protecting _M. bovis_ BCG in a viable state for oral delivery has been developed. Oral delivery of formulated _M. bovis_ BCG to mice induced strong immune responses, as indicated by splenic IFN-γ levels and by macrophage inhibition of virulent _M. bovis_, compared to nonformulated _M. bovis_ BCG. Oral vaccination induced significant protection in mice against aerosol challenge with virulent _M. bovis_. Our data suggest that formulated oral _M. bovis_ BCG is an effective means of inducing protective immune responses against TB. Lipid-based orally delivered mycobacterial vaccines may be a safe and effective means of controlling TB in humans and animals.

**ACKNOWLEDGMENTS**

This work was supported by a grant from the New Zealand Animal Health Board.

We are grateful for the assistance of Barry Wards for aerosol challenge and Gary Yates and Maree Joyce for the bacteriological examinations. We thank Clare Fitzpatrick for assistance with cytokine ELISAs and BCG survival. We acknowledge the assistance of the staff of the Wallaceville Small Animal Unit and the University of Otago Department of Animal Laboratory Sciences in caring for the mice.

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