Oral vaccination of guinea pigs with *Mycobacterium bovis* Bacille Calmette-Guérin (BCG) vaccine in a lipid matrix protects against aerosol infection with virulent *M. bovis*

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Increased incidence of bovine tuberculosis (TB) in the United Kingdom caused by infection with *Mycobacterium bovis* is a cause of considerable economic loss to farmers and Government. The Eurasian badger (*Meles meles*) represents a wildlife source of recurrent *M. bovis* infection to cattle in the UK and their vaccination against TB with *M. bovis* Bacille Calmette-Guérin (BCG) is an attractive disease control option. Delivery of BCG in oral bait holds the best prospect for vaccinating badgers over a wide geographical area. Using a guinea pig pulmonary challenge model, we evaluated the protective efficacy of candidate badger oral vaccines, based on broth-grown or ball-milled BCG, delivered either as aqueous suspensions or formulated in two lipids with differing fatty acid profiles; one being animal-derived and the other vegetable-derived. Protection was expressed in terms of increasing body weight after aerosol challenge with virulent *M. bovis*, reduced dissemination of *M. bovis* to the spleen, and in the case of one oral formulation, restricted growth of *M. bovis* in the lungs. Only oral BCG formulated in lipid gave significant protection. These data point to the potential of the BCG-lipid formulation for further development as a tool for controlling tuberculosis in badgers.
There was an estimated 225% increase in the number of bovine tuberculosis (TB) incidents in Great Britain between the years 1996 and 2006 (7). This adversely affects animal health and welfare, and is a cause of considerable economic loss to farmers and Government. Although transmission of Mycobacterium bovis between cattle is an important factor in spread of the disease (31), the Eurasian badger (Meles meles) represents an additional wildlife source of recurrent M. bovis infection to cattle in both the UK and Ireland (25, 26, 32). As well as badgers being afforded protection under law in the UK, their culling as a means to reducing bovine TB is both controversial (19) and demonstrated to have both positive and negative effects on the incidence of bovine TB (24, 25).

In this context, the vaccination of badgers against TB is an attractive option as a possible means to reduce and control bovine TB. It has long been recognised that delivery of vaccine in oral bait holds the best prospect for vaccinating badgers over a wide geographical area (8) and is well-attested for mass vaccination of other wildlife species (21). In the short to medium term, M. bovis Bacille Calmette-Guérin (BCG) represents the best available option for vaccination of wildlife against TB (8, 20). BCG has the advantage of a long history of safety and efficacy in a variety of animal species (8) but has the limitation of little to no efficacy if delivered in a non-viable state (9, 41). This is exacerbated in the case of oral delivery by inactivation in the low pH environment of the stomach (5, 11, 41).

Therefore, the success of an oral BCG vaccine for wildlife will depend in large part on the ability to formulate BCG so it maintains a viable state for prolonged periods in bait and is delivered to the gastrointestinal tract (GIT) in such a way as to establish bacterial replication in the lymphoid tissues sufficient to maintain protective immunity (41). These
issues have recently been addressed through the incorporation of BCG vaccine into a lipid-based matrix (1-4, 6, 12, 46). Use of the lipid-matrix allowed BCG to be retained in a viable, but static state for several weeks at ambient temperature (6). In rodent models and brushtail possums, oral delivery of lipid formulations containing live BCG was shown to establish populations of viable, replicating BCG in the alimentary tract lymphatic system (1, 46), which in mice persisted for at least seven months post-vaccination (3). Voluntary uptake of the vaccine (which can be readily induced following flavouring of the lipid matrix) was shown to confer protection against virulent *M. bovis* or *M. tuberculosis* aerosol challenge in mice (2, 3, 6), and in possums and cattle against challenge to the pulmonary tract with virulent *M. bovis* (4, 12). The duration of protection after oral vaccination was shown to be maintained for at least seven months in mice and 12 months in possums (3, 10).

For a commercial vaccine to comply with EU regulatory requirements, it is necessary for the raw materials to be of consistent quality and defined specifications. The production methodology also has to be validated and consistent. In the case of a licensed oral BCG-lipid vaccine for badgers, this must include sourcing BCG from a GMP (Good Manufacturing Practice) compliant supplier, and the BCG-lipid formulation being manufactured in a GMP facility. Experimental vaccines used to generate data for regulatory submission should be shown to be representative of the proposed final product. With a view to facilitating EU regulatory requirements, we have evaluated a commercial source of BCG (Danish Strain 1331, Statens Serum Institute, Denmark), as well as a pharmaceutical-grade version of Lipid K, which has been previously reported as a lipid delivery matrix for oral BCG vaccination of brushtail possums (4).
BCG crosses the intestinal barrier through the M cells of Peyer’s patches (PP) (28) from where it can stimulate lymphocyte responses in the spleen, MLN, and PP themselves (1, 28, 34, 38). Therefore, one limitation on efficient uptake of BCG by the gut-associated lymphoid tissue (GALT) is likely to be particle size. From a variety of different experimental systems in rodents it emerges that uptake into the PP and passage to the liver and spleen via the MLN, generally decreases with increasing particle size (23, 33, 37). In this context, previous work with BCG-lipid formulations has utilised broth-grown cultures of BCG; both for their high viability and their dispersion into single cells. Whilst, use of a GMP preparation of BCG Danish 1331 would facilitate the eventual licensing of an oral vaccine, the commercially available BCG vaccine from the Statens Serum Institute is a ball-milled preparation that contains considerably more aggregates of BCG than its broth-grown counterpart (data not shown). We therefore evaluated both forms of BCG Danish 1331 in order to establish whether the form of BCG would impact its efficacy when delivered orally.

Proof of concept for the oral BCG vaccine was evaluated in a guinea pig vaccination-challenge model, chosen because of its susceptibility to M. bovis (17, 47) and the relevance of the challenge route for badger TB pathogenesis (18). Lipid-encapsulated BCG delivered to guinea pigs orally was immunogenic and live bacilli were recovered from the mesenteric and cervical lymph nodes. Protection against aerosol challenge with virulent M. bovis was demonstrated for BCG only when delivered in lipid and there was little difference between the ball-milled and broth-grown forms of the BCG. These data point to the potential of the BCG-lipid formulation for the further development of a licensed oral TB vaccine for badgers.
MATERIALS AND METHODS

Bacteria and media. Three preparations of BCG Danish strain (1331) were used: lyophilised; ball-milled in sodium glutamate solution; and broth-grown. Both the lyophilised and ball-milled preparations were obtained from the Statens Serum Institute, Copenhagen, Denmark. Broth-grown BCG was prepared from the lyophilised BCG by culture in Middlebrook 7H9 medium + Albumin-Dextrose-Catalase (ADC) + 0.01% (v/v) Tween 80 to an optical density at 600nm of approximately 0.2 (mid-log-phase growth). Virulent Mycobacterium bovis isolate AF2122/97 (29) was propagated in Middlebrook 7H9 medium and stored as frozen aliquots prior to aerosol infection of guinea pigs. For routine bacterial enumeration, BCG was plated onto modified Middlebrook 7H11 + oleic acid-albumin-dextrose-catalase (OADC) + glycerol, and M. bovis was plated onto modified Middlebrook 7H11 + OADC + pyruvate.

Lipid formulation of M. bovis BCG. Two lipids with differing fatty acid profiles were used to encapsulate BCG for oral vaccination of guinea pigs. Lipid C consisted of an animal-derived fractionated lipid complex as described previously (4). Lipid PK consisted of a pharmaceutical grade, non-hydrogenated derivative of Lipid K - a vegetable-derived lipid containing a mixture of triglycerides of fatty acids as previously described (4). Broth-grown or ball-milled BCG bacilli were encapsulated into Lipid C or Lipid PK as previously described (4).

Post-vaccination BCG quantitative bacteriology and immune assessment. In order to confirm that orally-administered lipid formulations were effectively delivering
BCG bacilli to guinea pigs, bacteriological assessment was undertaken on lymphoid tissues, excised from animals in the absence of *M. bovis* challenge. Guinea pigs (eight per group) were orally vaccinated with 2.6x10⁷ colony-forming units (CFU) broth-grown BCG in Lipid C or 3.0x10⁷ CFU broth-grown BCG in Lipid PK. Eight weeks later, the guinea pigs were euthanased by CO₂ anaesthesia. Separate tissue homogenates were prepared in DMEM base medium from each animal for mesenteric lymph nodes (MLN), cervical-region lymph nodes (CLN), PP and the spleen, as described previously for processing murine tissues (1). Homogenates were centrifuged, resuspended in Middlebrook 7H9 broth and plated in duplicate onto Middlebrook 7H11 agar; BCG were enumerated after 20 days bacterial growth. Additionally, sub-samples of spleen homogenates were washed in DMEM, and resuspended to a concentration of 5x10⁶ viable mononuclear leukocytes/mL in DMEM supplemented with 10% fetal calf serum, 100U/ml penicillin/100µg/ml streptomycin, and 5.5x10⁻⁵ M 2-mercaptoethanol. One hundred microlitre aliquots of cell suspensions were plated, in triplicate, into 96 well tissue culture plates (Nunc, Denmark) and cultured in the presence or absence of 50 µg/mL *M. bovis* purified protein derivative (PPD-B; Prionics Inc, Switzerland) for a total period of four days. Lymphocyte proliferation, due to antigen-stimulation, was assessed by tritiated thymidine incorporation over the final 18h of culture, as described previously for murine cells (22). Data were expressed as a stimulation index as described previously (4, 6, 22), and responses among vaccinees were compared against a control group (N=4) comprising non-vaccinated guinea pigs.
Vaccination of guinea pigs for evaluation of vaccine efficacy. Eight separate groups of vaccinated guinea pigs were used to evaluate vaccine efficacy. Out-bred female Dunkin Hartley guinea pigs (weighing between 250-300g) free from inter-current infection were obtained from Harlan, UK and were vaccinated in groups of eight animals. Two groups received broth-grown or ball-milled BCG without formulation in lipid. Oral vaccines were pre-loaded in 1 ml syringes and delivered at the back of the mouth slowly to ensure the full dose was swallowed. The efficacy of each oral vaccine was evaluated against lyophilised BCG vaccine as the positive control. A single vial of lyophilised BCG vaccine was reconstituted with 1 ml Sauton diluent supplied by the Statens Serum Institute with the vaccine and delivered subcutaneously in a volume of 0.1 ml in the nape of the neck (s.c. BCG). The delivered dose of BCG was determined by plating an aliquot of the vaccine preparation. The delivered doses were: 2-8x10^5 CFU for s.c. BCG; 1.7x10^8 CFU for broth-grown BCG (or 9.2x10^7 CFU when in lipid); and 7.9x10^7 CFU for ball-milled BCG.

Aerosol challenge with M. bovis. Twelve weeks after vaccination all animals were challenged via the aerosol route with M. bovis strain AF2122/97 using a fully contained nose-only exposure Henderson apparatus as previously described (16, 47). A fine particle aerosol with a mean diameter range of 2 µm (diameter range, 0.5-7 µm) (36) was generated with a saline suspension containing 1x10^6 CFU/ml in order to obtain an estimated retained inhaled dose of approximately 10-20 CFU delivered to the lungs of each animal. The Henderson apparatus allows a controlled delivery of aerosols to the animals and the reproducibility of the system and relationship between numbers of lesions and concentration of bacilli in the Collison nebuliser have been described previously (17, 47).
Monitoring of animals post-challenge and necropsy. Following aerosol challenge, the guinea pigs were housed at ACDP containment level 3 and weighed regularly as a marker of disease progression. The animals were euthanased by peritoneal overdoses of sodium pentobarbitone five weeks after challenge or when an individual weighed 20% less than its maximal body weight (humane endpoint). The spleen and lungs were aseptically removed and the lungs weighed after removal of the trachea and pulmonary associated lymphoid tissues. Spleen and lungs were placed into separate sterile tubes for storage at -20°C until processed for bacteriology. Frozen tissues were thawed and homogenised in sterile deionised water using a rotating blade macerator system. Viable counts were performed by preparing decimal dilutions in sterile deionised water and plating 100 µl aliquots onto Middlebrook 7H11 agar + OADC + pyruvate. Plates were incubated at 37°C for 5 weeks before counting the number of *M. bovis* colonies (CFU).

**RESULTS**

**BCG delivery and vaccine immunogenicity using lipid formulations.** Given that this was the first time the lipid-BCG formulations had been evaluated in the guinea pig, we first conducted a preliminary experiment to establish the delivery and immunogenicity of lipid-BCG to guinea pigs. Eight weeks post-vaccination, all guinea pigs which had been orally-vaccinated with broth-grown BCG in lipid were culture-positive for BCG in the alimentary tract lymphatic system (Fig. 1); no viable BCG bacilli were isolated from splenic tissues of any animals. Average total BCG loads per animal were 1006 and 479 CFU, for Lipid C and Lipid PK vaccinees, respectively. Distribution profiles indicated that
BCG had successfully colonised the CLN tissues in all guinea pigs receiving BCG in Lipid PK (Fig. 1A), whereas all guinea pigs receiving BCG in Lipid C had BCG persistent in their MLN tissues (Fig. 1B). However, there was no statistically significant difference between the levels of colonisation of the MLN and CLN for either lipid (Fisher’s Exact test). BCG was only recovered in low numbers from the PP of one animal that received BCG in Lipid PK and from three that received BCG in Lipid C. Broth-grown BCG delivered in either lipid matrix invoked a proliferative response among guinea pig splenic lymphocytes, cultured in the presence of PPD-B antigen (Fig. 2). There was no statistically significant difference between the two vaccinated groups.

**BCG-in-lipid formulations protect against weight loss following challenge with* M. bovis.** Having established that broth-grown BCG in lipid was delivered successfully to the alimentary tract lymphatics of guinea pigs resulting in a systemic immune response, separate groups of guinea pigs were vaccinated orally with BCG alone or formulated with lipid in order to evaluate vaccine efficacy against aerosol challenge with virulent *M. bovis*. Additional groups received either Lipid PK orally or BCG s.c. as negative and positive controls, respectively. Two different preparations (ball-milled or broth-grown) of BCG Danish were used to see if this had a bearing on protective efficacy.

Following challenge, all guinea pigs survived to the end of the experiment (five weeks post-challenge) but there were differences in disease progression between groups. Groups of animals that received BCG in lipid showed an increase in body weight over the period of study. In contrast, the two groups that received BCG orally without lipid showed little overall change in body weight. Animals in the group that received lipid alone lost on average 11% of their body weight over the four week period (data not shown).
All BCG-in-lipid formulations confer equivalent protection to the spleen following challenge with *M. bovis*. Figure 3A shows the mean concentration of *M. bovis* in the spleens from each group, five weeks after aerosol challenge with a low dose of *M. bovis*. All BCG-in-lipid formulations conferred significant protection to the spleen compared with the unvaccinated control (Lipid PK alone). BCG (whether broth-grown or ball-milled) failed to give significant protection when given orally in the absence of lipid, although the mean bacterial load was lowered approximately ten-fold.

A single BCG-in-lipid formulation confers protection to the lung following challenge with *M. bovis*. Compared to the spleen, protection conferred to the lung by vaccination was considerably lower. Figure 3B shows the mean concentration of *M. bovis* in the lungs from each group. Parenterally administered BCG (s.c. BCG) conferred 1.57 log$_{10}$ of protection to the lungs; approximately 40% of that achieved in the spleen. This was statistically significant (P = 0.01). For the oral vaccines, neither BCG without lipid nor BCG administered in Lipid C gave statistically significant protection to the lung (P = 0.576 and 0.132, respectively), whereas ball-milled BCG in Lipid PK conferred significant protection (1.07 log$_{10}$ reduction, P = 0.045). Broth-grown BCG in Lipid PK was very similar (0.94 log$_{10}$ reduction), only just failing to achieve statistical significance (P = 0.078).

**DISCUSSION**

The present study demonstrates that oral vaccination of guinea pigs with BCG in a lipid matrix can give significant protection against aerosol infection with *M. bovis*. As the
respiratory route is considered the principal route of exposure to natural *M. bovis* infection in the badger (18), and guinea pigs are particularly susceptible to aerosolised *M. bovis* (17, 47), this was considered a relevant and stringent animal model in which to evaluate the efficacy of oral vaccines for protection against *M. bovis* colonisation of the lungs and subsequent dissemination to the spleen. This is the first report of the evaluation of the BCG-lipid vaccine in the guinea pig challenge model. Our study extends the growing body of literature on the utility of the lipid matrix for the oral delivery of BCG (1-4, 6, 12, 46).

Protection was only seen for oral BCG when formulated in lipid, and was expressed in terms of increasing body weight after challenge with virulent *M. bovis*, reduced dissemination of *M. bovis* to the spleen, and in the case of ball-milled BCG in Lipid PK, restricted growth of *M. bovis* in the lungs.

Several hundred-fold more BCG were delivered orally than administered subcutaneously and in all cases but one, they still failed to confer statistically significant protection to the lungs. Even with the gastric protection provided to BCG by the lipid, it is evident that a higher titre of BCG will need to be administered orally than parenterally to achieve comparable levels of protection. Whether this proves to be a serious constraint on the application of oral BCG for use in wildlife remains to be seen, and will depend on studies in the target species to define the minimal oral dose required for protection, together with studies *in vitro* to define the formulated dose of BCG required to achieve this in the field.

Recent work demonstrated the association between the establishment and persistence of BCG in the GALT of mice following oral delivery in lipid, and the persistence of effector immunity (22), and by inference protection from challenge with
virulent mycobacteria (3). In the present study, BCG administered orally in lipid resulted in colonisation of the upper (CLN) and lower (MLN) alimentary tract lymphatic tissues, and to a lesser extent the PP also. Although we did not determine whether BCG were still present in the GALT at the time of challenge with *M. bovis*, BCG has been reported to persist in the murine GALT for at least seven months after oral delivery in lipid (3).

In the non *M. bovis*-challenged guinea pigs, BCG was recovered from the alimentary tract lymphatic tissues of all animals which had received broth-grown BCG-in-lipid formulation, indicating regular lymphatic colonisation. Among these animals, BCG was isolated variably from the different lymphoid sites, although MLN and CLN tissues were the main sites of BCG replication. It was interesting to note that viable bacilli were recovered from 100% of the MLN tissues in animals vaccinated with BCG-Lipid C, as has been reported previously in murine studies (1, 3). In contrast 100% colonisation of CLN tissues occurred in animals which had received BCG-Lipid PK. The same associations have been observed in equivalent studies in mice (F. E. Aldwell, unpublished observations): voluntary consumption of BCG in Lipid PK most frequently results in replicating bacterial populations in upper alimentary tract lymph nodes (i.e. those draining the oropharyngeal region), in contrast to consumption of BCG in Lipid C, where replicating BCG is most frequently recovered from the lower alimentary tract lymph nodes (i.e. those draining the small intestinal region). Thus, although the two lipid matrices appear to favour BCG tropism at different lymphatic sites, results from the present study suggest that the magnitude of the ensuing CMI response and the subsequent degree of protection against *M. bovis* infection is equivalent between the two matrices. This is in accordance with a study in which a high dose of BCG given to mice either orally (so that BCG colonised the CLN) or
intragastrically (thereby resulting in uptake exclusively by the MLN and PP), resulted in equivalent splenic T-cell responses and comparable levels of protection against intravenous challenge with *M. tuberculosis* (34).

Although the precise mechanism by which the lipid matrix enhances the efficacy of oral BCG is unknown, it is clear from the present study that the lipid plays a significant role in protecting BCG during transit through the GIT and/or augmenting delivery to the GALT.

In regard to the latter mechanism, lipid delivery systems have been studied for some time for their ability to enhance the oral bioavailability of poorly absorbed compounds (14); recently reviewed in (40), although there are likely to be significant differences in the way drugs are handled by the GIT compared with live BCG. Nonetheless, it is interesting that the mixing of polystyrene nanoparticles (0.5 μm) with 6% lecithin (a mixture of glycolipids, triglycerides, and phospholipids) (43) was shown to significantly enhance the uptake of the nanoparticles into rat MLN, compared with oral delivery of the microparticles in saline or 6% oleic acid (45).

Previous work with BCG-lipid formulations has utilised broth-grown cultures of BCG both for their high viability and their dispersion into single cells. With a view to facilitating eventual licensing, we evaluated a ball-milled preparation of BCG Danish 1331 obtained from the Statens Serum Institute, Denmark. The bacteria are first grown as a surface pellicle and then ball-milled in a solution of sodium glutamate to aid dispersion. Even so, the ball-milled preparation contains considerably more aggregates of BCG than its broth-grown counterpart (data not shown). The fact that we found lipid-formulated ball-milled BCG to be as protective as broth-grown BCG, suggests that the ball-milled BCG
preparation contains sufficient singlet bacilli for effective delivery to the GALT.

Interestingly, other studies have shown that although a greater quantity of smaller (2 µM) particles may be taken up by the GIT, larger (6 µM) particles appear more efficiently translocated to lymph nodes (15) and can even reach the lung (35). In a detailed study in mice, ovalbumin (OVA) was delivered orally using microspheres over a 0.6 – 26.0 µm size range. The quantity of microspheres taken up into PP and subsequently translocated to the spleen was determined in relation to particle size, and the influence this had on systemic and mucosal antibody responses to OVA evaluated. It was concluded that the body distribution pattern of microspheres following PP uptake was a key factor in determining the induction of systemic or mucosal immune responses (44). As well as particle size, other factors affecting uptake of microparticles in the GIT include surface charge and hydrophobicity (27). Especially in the case of lipid-based delivery systems, the surface charge (30) or other characteristics of the lipid (39) may be more significant than the size of the particle droplet.

In conclusion, we have demonstrated for the first time in guinea pigs that oral vaccination with BCG in lipid is both immunogenic and able to confer a level of protection against aerosol challenge with virulent M. bovis. This supports previous observations in other animal hosts (1-4, 6, 12, 46), and points to the potential of the BCG-lipid formulation for the further development of an oral vaccine for use in badgers in the UK. A previous study using a parenteral route of BCG vaccination in badgers demonstrated enhanced cell-mediated immunity, prolonged survival following intradermal M. bovis challenge, and delayed excretion of the organism (42). The reduction in M. bovis load in the lungs, and in
bacterial dissemination to the spleen in the present study using oral BCG is encouraging in terms of the effect of BCG vaccination required for the badger, where a reduction in excretion of \textit{M. bovis} may be sufficient to break on-going transmission to badgers and cattle (13). Since BCG is stable and the lipid is solid at temperatures most likely to be encountered in the environment (4), it also has the potential to serve as the bait-delivery system itself; as has proven successful in delivery to captive possums in New Zealand (4, 10, 46), although further work is required to evaluate the palatability and uptake of a lipid bait to wild badgers.

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FIG 1. BCG distribution profiles in alimentary tract lymphatic tissues, following oral vaccination of guinea pigs with BCG formulated in lipid PK (A) or lipid C (B). Data represent BCG CFU counts per total organ homogenate for mesenteric lymph nodes (MLN), cervical-region lymph nodes (CLN) and Peyer’s patches (PP). Closed symbols represent viable BCG cultured from the tissue; open symbols represent culture-negative tissue.

FIG. 2. Splenic lymphocyte proliferative responses to PPD-B stimulation, among guinea pigs vaccinated orally with lipid-formulated BCG. PPD-B-induced stimulation indices (SI) for individual animals are shown with the group mean as a bar. The raw SI data were non-normally distributed (Anderson-Darling normality test), subsequently normalised by log$_{10}$ transformation prior to statistical analysis (one-way ANOVA, Dunnett’s post-hoc test) and graphical presentation. Asterisks refer to mean SI responses significantly higher in vaccinated guinea pigs compared to non-vaccinated (control) animals (⋆ ⋄ P = 0.018, ⋆ ⋆ ⋄ P = 0.003).

FIG. 3. Influence of vaccination on bacterial load of *M. bovis* in the spleens (A) and lungs (B) of guinea pigs infected by the aerosol route five weeks earlier. The mean +/- SEM are shown for each group. Significant differences compared with the Lipid PK group are represented by asterisks (⋆ ⋄ P < 0.05, ⋆ ⋆ ⋄ P < 0.01, ⋆ ⋆ ⋆ ⋄ P < 0.001) and were derived using Dunn’s Multiple Comparisons test (for the spleen data) and Dunnett Multiple Comparisons test (for the lung data) following non-parametric and parametric ANOVA’s,
respectively. NS = not significant. The log_{10} difference in group means compared with the Lipid PK group are shown numerically. BG = broth-grown; BM = ball-milled.
Figure 1
Lymphocyte stimulation index (log scale)

- Non-vaccinated controls
- Oral lipid-formulated BCG
- Lipid-PK
- Lipid-C

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Figure 3