Veterinary Tuberculosis Vaccine Development

J. Frank T. Griffin

From the Division of Immunology, Department of Microbiology, University of Otago, Dunedin, New Zealand

Tuberculosis caused by Mycobacterium bovis in domestic livestock and wildlife is a significant problem in many countries worldwide. Wildlife reservoirs of tuberculosis confound programs for tuberculosis eradication from domestic livestock. Successful vaccination against tuberculosis in domestic animals or wildlife could contribute to tuberculosis eradication. Bacille Calmette-Guérin (BCG) has been used as the prototype vaccine for domestic livestock and wildlife. The majority of studies have been carried out with BCG-vaccinated animals challenged experimentally with M. bovis. Although protection against disease has been evident in all these studies, protection against infection has rarely occurred. Results obtained with BCG vaccination of cattle, deer, ferrets, opossums, and rabbits are presented here and highlight the need for appropriate animal models for vaccination and control of the variables that influence the efficacy of BCG vaccine. Refinement of the existing animal models is essential for the advancement of tuberculosis vaccine research of relevance to animals and humans.

Tuberculosis in Domestic Livestock and Wildlife

Tuberculosis presents with widely varying pathology in the diverse species of animals that are infected naturally. Marked variation in disease patterns may be seen even within a single species such as cattle, and farm management systems may affect patterns of transmission and the resultant pathology. Phenotypic differences among ruminants (buffalo, cattle, and deer), omnivores (pigs), mustelids (ferrets and badgers), and marsupials (opossums) produce disease with distinctly different specific patterns of pathology [1]. Difficulties associated with the isolation of virulent Mycobacterium bovis, the range of outcomes following infection, and the chronic, insidious nature of the disease present challenges for research in this area [2]. Nonetheless, considerable progress has been made in developing vaccine protocols for domestic livestock and wildlife that should allow tuberculosis prophylaxis to be applied in the field within the next 5 years. Data obtained from studies in this area should also provide valuable generic information on tuberculosis with regard to pathogenesis, diagnosis, and protective immunity, which will have direct application to human vaccination for tuberculosis.

Animal Models for Tuberculosis and Vaccines

The low prevalence of tuberculosis in naturally infected populations, combined with the chronic nature of the disease, makes it extremely difficult to conduct studies on tuberculosis vaccine efficacy under field conditions. This necessitates the availability of animal models for studying vaccines more cost-effectively [3].

To enable the study of vaccine responses, it is essential to develop animal models that fulfill minimal criteria to validate their contribution. Models for experimental infection should produce levels of infection and disease at a high prevalence, yet the pathogenesis of disease that occurs after artificial infection must mimic disease patterns that occur naturally. Definitive markers that correlate with protective immunity must be elucidated so that candidate vaccines and protocols for their use can be evaluated without expensive field studies.

All of the existing small-laboratory animal models for tuberculosis vaccination and experimental challenge have intrinsic limitations. Although guinea pigs and rabbits may be adequate to study the pathogenesis of tuberculosis [4], they are inordinately susceptible [5, 6] to progressively fatal disease after natural infection with low doses of virulent M. bovis or Mycobacterium tuberculosis. The progressive experimental disease is unrepresentative of disease patterns found in naturally infected animals or humans. Prolonged survival and attenuated pathology are the only indicators of vaccine-mediated protection that can be studied in these models [7, 8]. The early evaluations of BCG vaccine efficacy in uniquely susceptible guinea pigs [7] and rabbits [8] challenged with virulent M. bovis or M. tuberculosis may have distracted these researchers from the goal of achieving protection against infection. Although mice constitute an excellent model to monitor fundamental parameters of immunity, they are innately resistant to tuberculosis [9] and produce pathology [10] unlike that found in any naturally susceptible host. No consistent parameter of protection has been established in extensive studies that involve BCG vaccination of guinea pigs, mice, and rabbits [11].

Comparative studies of these animals fail to establish either a consistent level of protection with a given vaccine or a representative ranking of efficacy of different vaccines [12]. An ideal animal model for tuberculosis should produce susceptibility patterns and disease parameters similar to those found...
in naturally infected animals or humans. After choosing an animal model, it is necessary to establish parameters of infection that mimic disease etiology in the target host [6]. Critical variables that affect disease outcome include the species of mycobacterium (M. bovis or M. tuberculosis), the dose of virulent organisms used for infectious challenge, and the route by which they are administered [13]. The early studies in guinea pigs and rabbits [5,6] established that M. bovis was significantly more virulent than M. tuberculosis. A similar pattern has also been confirmed in mice [14]. Although this does not invalidate animal studies that use M. bovis as a model for human tuberculosis caused by M. tuberculosis, it serves to caution against the use of M. tuberculosis to study animal tuberculosis caused by M. bovis. The dose of virulent organisms used should be the minimum necessary to cause infection. Routes of infection could include subcutaneous, intragastric, intranasal, intratracheal, and intratracheal administration.

The necessity of having robust animal models for tuberculosis infection becomes evident upon reviewing the inconsistent outcomes of the extensive studies of BCG vaccination of humans over the past 50 years [15]. Many factors have confounded studies of vaccine efficacy in naturally infected populations. Most important among these is the serious limitation that there are no defining in vitro immune correlates for protection after vaccination.

Until recently, the only widely available marker for immunity was the delayed type hypersensitivity (DTH) skin test reaction, which is used as a putative marker for both infection and protective immunity in humans and for testing the potency of vaccines in experimental animals as a quality-assurance measure before their release for human use. Although DTH is a reasonably accurate marker for infection and disease, it correlates poorly with protective immunity [16]. Since skin testing has limited sensitivity and has been used exclusively to select subjects for vaccine trials, it has not been possible to exclude all infected subjects from vaccine efficacy trials [17]. The inclusion of infected individuals in vaccine trials may have compromised efficacy estimates, because vaccines can more readily prevent primary infection from becoming established than resolve existing infection or disease [18].

Other variables that confound field studies in humans and animals are phenotypic modifiers of host immunity, such as immunosuppressive stressors [19] or prior sensitization by saprophytic mycobacteria from the environment [20]. The strain of vaccine [21], storage conditions, and viability [15] may also influence efficacy, since different stocks of BCG vaccine have variable levels of immunogenicity. It is essential, therefore, to develop standardized vaccination and challenge protocols for use in animal models to elucidate protective efficacy of tuberculosis vaccines.

Vaccination of Cattle

The first studies [22] that used BCG vaccines in cattle were carried out soon after the development of BCG vaccine by Calmette and Guérin. However, since these involved massive doses (10^9 cfu) of vaccine and virulent M. bovis, they are not comparable with the more refined studies carried out over the past 30 years. Field studies of BCG vaccination carried out from 1966 through 1972 in Malawi [23] showed that a single inoculum of BCG vaccine (Glaxo Pharmaceuticals, Research Triangle Park, NC) given sc at a dose of 10^6 cfu provided significant protection against lesions in animals infected experimentally (orally or sc) with virulent M. bovis. Subsequent field data [24] showed that vaccination caused a significant reduction in condemnation after slaughter of vaccinated animals exposed to M. bovis naturally under field conditions. Later field studies suggested that protection noted in young cattle waned by adulthood (aged >5 years) among vaccinated calves [25].

More recent studies [26] have shown that subcutaneous vaccination of calves with 10^7 to 10^9 cfu of BCG (Pasteur 1173P2) caused a significant reduction in the prevalence and severity of lesions, 5 months after challenge with virulent M. bovis by the intratracheal route. Intratracheal vaccination with BCG caused a significant reduction in pulmonary lesions after challenge with virulent M. bovis [27]. No protection was seen after sc inoculation of killed Mycobacterium vaccae (10^9 cfu).

<table>
<thead>
<tr>
<th>Outcome of challenge</th>
<th>Primary or booster vaccination</th>
<th>Vaccine dose, cfu</th>
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<tr>
<td></td>
<td>L-BCG</td>
<td>K-BCG</td>
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<tr>
<td>Uninfected</td>
<td>5/10</td>
<td>8/10</td>
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<tr>
<td>Infected</td>
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<td>2/10</td>
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<tr>
<td>Diseased</td>
<td>1/10</td>
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NOTE. Data are no. of deer positive/no. tested.

* Live freshly cultured BCG, single dose (2 x 10^7 cfu).
* Live BCG, 2 doses (2 x 10^7 cfu).
* Killed BCG in oil adjuvant, 2 doses (5 x 10^7 cfu).
* Dexamethasone treatment before administration of BCG, single dose (2 x 10^7 cfu).
* Live BCG.
* Lyophilized BCG.
Vaccination of Opossums and Ferrets

Studies have been carried out to evaluate the protective efficacy of vaccines in opossums and ferrets vaccinated with BCG (Pasteur 1173P2) and challenged with virulent *M. bovis*. Significant protection against pulmonary disease after intratracheal challenge with virulent *M. bovis* was seen in opossums vaccinated sc or by aerosol [28]. No protection was seen in animals vaccinated intragastrically with BCG. Subsequent studies by the same group of investigators [29] showed that injection of BCG vaccine directly into the duodenum caused a significant reduction in pathology, weight loss, and clinical disease in opossums challenged with virulent *M. bovis*. Protection in the above studies was manifested by a reduction in the severity of disease in experimentally infected opossums and a concomitant reduction in the number of recoverable virulent organisms from the tissue of affected animals. There was no evidence of sterile immunity (i.e., protection of animals against the establishment of infection) in any of the cases.

Ferrets given $10^8$ cfu of BCG vaccine (Pasteur 1173P2) orally in bait vaccines [30] had a significant reduction in pathology after oral challenge with virulent *M. bovis*. No macroscopic lesions were found in the gut-associated tissues of any of the vaccinated animals after challenge with virulent *M. bovis* by the oral route.

Vaccination of Deer

Extensive studies have been carried out to evaluate the critical variables that influence the protective efficacy of BCG vaccine (Pasteur 1173P2) in experimentally infected deer. A sensitive infection model has been developed in which deer infected with $10^1$–$10^2$ cfu of virulent *M. bovis* develop infection and disease patterns indistinguishable from those seen in naturally infected animals [14]. During development of this model, different routes of experimental infection were studied to determine which produced typical disease. The intranasal route, though producing typical lung pathology, yielded highly unpredictable results, whereas intratracheal inoculation produced atypical lung abscesses. Intratonsilar inoculation produced highly reproducible results, with patterns of disease identical to those found in naturally infected animals. Challenge of deer with a 0.2-mL inoculum ($10^5$ cfu) of virulent *M. bovis*, introduced onto the mucosal surface of the crypt of the tonsil, resulted in infection levels in $>90\%$ of animals and disease in $>50\%$. This level of infection allows cost-effective vaccine efficacy studies to be carried out with small groups of animals (10–20) challenged with virulent *M. bovis* ($10^5$ cfu) after vaccination.

Natural spread of infection in deer occurs at such a low prevalence rate (5%–10%) that vaccine efficacy studies of naturally infected animals would require experimental groups of >200 animals. The foundation experimental rabbit studies of tuberculosis carried out by Lurie [6] arose from concern that guinea pigs do not contract tuberculosis by aerogenous spread, when housed in contact with infected animals. Aerogenous transmission of *M. bovis* occurs readily in rabbits exposed to low doses (1–10 cfu) of virulent organisms.

Apart from the unacceptably low levels of natural transmission of tuberculosis under field conditions, experimental infection has the additional advantage of excluding complications associated with prior tuberculosis infection, an intangible factor that confounds field studies of vaccine efficacy. A series of experiments have been carried out with use of the deer model to identify the critical variables (dose, route, boosting, and formulation live/dead) that influence the protective efficacy obtained with BCG vaccines [31]. A summary of the findings from these studies is given in table 1.

The initial study examined the influence of vaccine dose ($10^7$–$10^8$ cfu) on postvaccinal protection by use of 2 sc injections of vaccine given at 6-week intervals. Doses between $10^5$ and $10^7$ cfu provided significant levels of protection against both infection and disease in vaccinated animals challenged experimentally with virulent *M. bovis*. Although high-dose vaccine ($10^8$ cfu) protected against disease, it did not protect against infection.

A study of the effect of boosting showed that, although single-dose vaccines protected against disease, boosting was required to protect against infection. Vaccine administered onto the mucosal surface of the tonsilar crypt produced levels of protection equivalent to those obtained with a similar vaccine given sc. Lyophilized vaccine produced levels of protection against infection and disease equivalent to those obtained with freshly cultured BCG. Killed BCG vaccine in an oil adjuvant did not protect animals against either infection or disease, although it evoked an aggressive immune response after vaccination. The protective response to live BCG vaccine was completely ablated in animals given slow-release dexamethasone just before vaccination.

The most striking findings to emerge from these experiments were that low-dose BCG vaccine ($5 \times 10^4$ cfu) was highly efficacious and that booster vaccination was necessary to protect against the establishment of infection and disease. Less optimal vaccination produced protection against disease but did not prevent infection from becoming established.

Immune Parameters of Vaccination and Protection

Vaccine studies in cattle showed that animals vaccinated with live BCG produced significant but transient levels of IL-2 and IFN-γ [27]. There was no correlation between the levels of cytokines produced after vaccination and the protection against tuberculosis after experimental infection. Conversion to skin reactivity occurred routinely in animals given live BCG vaccine by sc injection but not in calves vaccinated via the intratracheal route. Studies of opossums vaccinated intraduodenally [29] with BCG showed that there was a correlation between postvaccin-
fection levels of lymphocyte transformation to PPD and subsequent levels of protection after challenge with virulent *M. bovis*.

Results of deer studies show that active disease produces patterns of immunity typified by specific lymphocyte transformation and ELISA (antibody) reactivity, combined with increased levels of inflammatory proteins (fibrinogen and haptoglobin) and plasma viscosity. These responses were most evident in the weeks immediately following intradermal skin testing. Similar immunologic responses that did not produce protection were seen in animals vaccinated with killed BCG in oil adjuvant [33]. By contrast, in animals that were vaccinated with booster doses of live BCG, lymphocyte transformation occurred in peripheral blood mononuclear cells, but there was no antibody or inflammatory activity.

Studies [34] of the cytokine profiles of animals given 1 or 2 doses of live BCG vaccine revealed that primary exposure to BCG vaccine results in immune activation of cells that produce cytokines (IL-4 and IFN-γ), the phenotype that is typically associated with immature T-helper (Th0) cells. Deer vaccinated with booster doses of BCG produce IFN-γ alone, a response that is associated with Th1 cells. Animals vaccinated with killed BCG in oil produce a Th2 response, with activation of IL-4-producing cells.

**Conclusions**

Animal species that contract tuberculosis after natural exposure to *M. bovis* or *M. tuberculosis* provide experimental model systems of immediate relevance to the study of human tuberculosis. Although large animals are costly to maintain under experimental conditions, they provide unique opportunities to carry out studies of vaccines and tuberculosis prophylaxis. The deer model provides high rates of infection and prototypical pathology after challenge with low numbers of virulent *M. bovis* organisms introduced into the tonsilar crypt. This model has allowed for protection against infection, measured by sterile clearance of the challenge dose of virulent *M. bovis* (infection) and reduced or altered pathology (disease), both measured independently in small numbers of vaccinated animals.

The deer model has for the first time shown protection against infection, a previously unattainable goal with regard to tuberculosis vaccination in animal models or human field trials. It has been established that unless booster vaccination with BCG is used, there is no significant protection against infection. It is important to note that booster doses were given 6–8 weeks after primary vaccination, rather than months or years after such vaccination. The deer studies have confirmed that primary vaccination protocols are less optimal and provide protection only against disease. This observation is compatible with data available from the use of BCG as a single-dose human vaccine.

The widely used single-dose BCG vaccination protocol for humans evokes protection against disease-related pathogenesis and bacteremic spread [15] but does not provide protection against infection or infectious spread. The efficacy of single-dose BCG vaccination in humans seems to be related to the prevalence of tuberculosis within the target population [35]. BCG vaccination had an efficacy of 80% in a group of North American Indian infants, where the tuberculosis prevalence among nonvaccinated controls was 1.56% [36]. By contrast, it produced no protection in infants in Georgia, where the tuberculosis prevalence was 0.011% [36]. Among children in Puerto Rico [37], for whom the prevalence of tuberculosis was intermediate (0.043%), 31% were protected by the BCG vaccine. These findings could be reassessed within the context that exposure of infants to virulent *M. tuberculosis* soon after primary vaccination with BCG serves as a natural booster to generate higher levels of protective immunity.

Systematic studies involving short-term, double-dose BCG vaccination in animals or humans are rare. Ironically, some of the earliest animal studies of BCG vaccination, in rabbits [8, 38], showed that high levels of protection could be obtained with booster BCG vaccination. It is surprising that these observations were not factored into human vaccine protocols, since there are no data available on the use of short-course booster BCG vaccination in humans.

Although revaccination is carried out in some countries in Eastern Europe, intervals between primary and booster vaccinations range from 1 to 12 years [39]. A large trial carried out in Malawi [40] showed that revaccination of children at varying intervals after primary vaccination did not confer additional protection against tuberculosis, although it appeared to enhance protection against leprosy.

The presence of postvaccinal scars on children’s arms was the only evidence used to designate prior vaccination. None of the human trials have carried out systematic short-course BCG vaccine boosting of designated individuals. At best, these human trials have evaluated a “recall” rather than a “booster” response in individuals vaccinated years earlier. Short-course “imprinting” [35, 41] of the immune system through boosting may be essential to achieve optimal protection. Indeterminate intervals between primary inoculation and revaccination may result in intermittent exposure to saprophytic mycobacteria and may override any benefit gained from primary vaccination, in populations in which the prevalence of tuberculosis is low [42].

The continuing worldwide debate concerning the variable efficacy of BCG as a human vaccine may relate more to the protocol for its usage than to any deficit in its immunogenicity or protective efficacy per se [43]. With the exception of BCG vaccine, no currently registered human or animal vaccine, even those involving live-attenuated microorganisms, is used as a single-dose vaccine. In light of these experiences, it may not be reasonable to expect that single-dose BCG vaccination could provide protection against infection. Single-dose BCG vaccination may provide suboptimal protection in clinical trials, but its use in this way highlights a concern that the efficacy of new candidate vaccines used in multiple-inoculation protocols is be-
ing compared with that of single-dose BCG vaccine. Although no new vaccine has been shown to be superior to BCG in primary vaccination, a more valid test must be to compare new vaccines with BCG vaccine used optimally in a booster protocol.

Although it is reasonable to be concerned about side effects associated with booster vaccination with BCG, the good protection that can be achieved with low-dose vaccines (10^4 cfu) preempts such problems. The fact that protection can be achieved in the absence of DTH reactivity postvaccination suggests that successful booster vaccination should not have any necrotizing effect and not produce more adverse side effects than primary vaccination.

References


