Vaccination of animals against *Mycobacterium bovis*

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**Summary**

Vaccination could potentially be used as a practical means of controlling bovine tuberculosis in countries in which a wildlife reservoir of the disease is present, and also in those countries which cannot afford conventional control strategies. An understanding of the processes involved in the protective immune response to tuberculosis is desirable for the rational development and testing of new vaccines for tuberculosis. The authors review current knowledge regarding the processes involved in protective immune responses to tuberculosis, much of which has been derived from studies in mice. This knowledge is discussed in relation to the problem of using vaccination to induce protective immunity in cattle, deer and wildlife. Challenge models have now been developed to test candidate vaccines in many domestic animals and wildlife species and these models are being used to evaluate tuberculosis vaccines. Most studies of the efficacy of tuberculosis vaccines in target animals have focused on the use of bacillus Calmette-Guérin (BCG), an attenuated strain of *Mycobacterium bovis*. Recent advances in immunology and the molecular biology of mycobacteria have greatly increased the options for candidate vaccines and future studies will test new types of vaccines including new attenuated strains of *M. bovis*, sub-unit protein vaccines and recombinant deoxyribonucleic acid vaccines. Several of these vaccines have shown promising results when tested in small animal models. Although progress has been made in the development of vaccine delivery systems for animals, the technical problems associated with vaccination of wildlife remain a challenge.

**Keywords**


**Introduction**

Bovine tuberculosis, caused by infection with *Mycobacterium bovis*, is a major economic problem in several countries and constitutes a serious public health risk in a number of developing countries. The implementation of national bovine tuberculosis programmes in many industrialised countries, based on regular tuberculin testing and removal of infected animals, has led to successful eradication or a major reduction in the incidence of bovine tuberculosis in cattle and farmed deer herds. Bovine tuberculosis continues to be a major problem in countries that cannot afford such programmes, and these control measures have been only partially effective in countries such as the United Kingdom (UK), the Republic of Ireland and New Zealand, which have a wildlife reservoir of infected animals (108). In these three countries, the eradication of bovine tuberculosis and fulfilment of the Office International des Epizooties (OIE) international standards for freedom from tuberculosis (less than 0.2% herds infected) will require the implementation of alternative strategies, including the development of effective vaccines for control of tuberculosis in both domestic livestock and wildlife.

**Reasons for vaccination of domestic livestock and wildlife**

Vaccination of domestic animals in developing countries is likely to be a feasible option for both public health and economic reasons. The widespread occurrence of bovine tuberculosis in such countries and the potential contribution to the prevalence of tuberculosis in humans is a major
problem. In developing countries, the epidemic of human immunodeficiency virus (HIV) infection, together with the widespread incidence of M. bovis infection in domestic and wild animals and conditions that favour zoonotic transmission, provide ample opportunity for zoonotic tuberculosis to pose a serious public health problem (38, 99). The use of ‘test and slaughter’ programmes to eradicate tuberculosis would be economically and socially unacceptable in many countries of Africa.

Eradication of bovine tuberculosis has proved to be difficult in countries that have wildlife reservoirs of M. bovis infection. In New Zealand, the brushtail possum (Trichosurus vulpecula) population has expanded to approximately seventy million and is a major vector of bovine tuberculosis for domestic and feral animals (39). In the British Isles, the badger (Meles meles) has been associated with tuberculosis in cattle and with an increase in the incidence of tuberculosis in the south-west of England as well as in the Republic of Ireland and Northern Ireland (73). Traditional control measures for possums using poisoning are effective in reducing numbers, but areas in which bovine tuberculosis is endemic can be rapidly re-colonised by possums when poisoning is discontinued. Despite the implementation of culling at rates calculated to be adequate to eliminate tuberculosis in possums, tuberculosis persists within possum populations (82). Furthermore, public concern has also been raised over the continual use of poisons. The most promising options for eliminating tuberculosis in possums are the development of strategies for biological control of these animals and the development of an improved tuberculosis vaccine. An effective tuberculosis vaccine for cattle and farmed deer would protect domestic stock against infection by M. bovis-infected possums.

An improved understanding of the role of badgers as a source of M. bovis infection should be provided by a large culling trial currently being undertaken by the Ministry of Agriculture, Fisheries and Food in the UK (52). Although the control of tuberculosis in badgers by culling may be effective in reducing outbreaks of tuberculosis in cattle herds, such an approach is non-discriminatory and badgers are a protected species in the UK, under the Protection of Badgers Act 1992. Vaccination of badgers has been considered as a feasible approach for the control or eradication of bovine tuberculosis in the UK and Ireland (105, 155, 159). The development of an effective vaccine for cattle, along with associated diagnostic tests to differentiate between vaccinated and infected animals, was recommended by a recent independent scientific panel, as the best long-term strategy for the control of bovine tuberculosis in the UK (83). The report also recommended that a tuberculosis vaccine for badgers should be considered for control of tuberculosis, although this would pose greater technical problems in terms of development and delivery.

The spread of bovine tuberculosis amongst wildlife in game parks in Africa is becoming a serious problem. Bovine tuberculosis has been diagnosed in African buffalo (Syncerus caffer) in the Queen Elizabeth National Park (Ruwenzori) in Uganda (156) and in the Hluhluwe/Umfolosi Park and Kruger National Park (KNP) in South Africa (R. Bengis, unpublished observation). Bovine tuberculosis is widespread in the buffalo herds of the southern KNP and has been detected in several spill-over species, including baboon, greater kudu, lion, cheetah and leopard (R. Bengis, personal communication). The presence of tuberculosis is a serious threat to biodiversity in these parks and also poses a regulatory dilemma in translocation exercises, as sensitive and specific ante-mortem tests are only available for a few species. The development of an effective tuberculosis vaccine and an appropriate method of delivery would contribute to tuberculosis management strategies in these populations.

Requirements of vaccines

The qualities required for vaccines intended for use in domestic animals and wildlife are summarised in Table I. The goal of vaccinating cattle and farmed deer against tuberculosis is to prevent the establishment of infection in these animals. If these vaccinated animals were subsequently exposed to M. bovis, the animals would resist infection and would not react in a tuberculin skin test. The vaccine would need to be safe and use of the vaccine in cattle would have to be acceptable to countries importing beef and dairy products. In developing countries, the goal of vaccinating cattle may be less

<table>
<thead>
<tr>
<th>Species</th>
<th>Vaccine characteristics required</th>
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<tbody>
<tr>
<td>Cattle and deer (domestic)</td>
<td>Prevention of the establishment of infection in vaccinated animals</td>
</tr>
<tr>
<td></td>
<td>High levels of protection induced</td>
</tr>
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<td></td>
<td>Animals not sensitised to the tuberculin skin test</td>
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<tr>
<td></td>
<td>Minimal reaction at the site of injection</td>
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<tr>
<td></td>
<td>Safe</td>
</tr>
<tr>
<td></td>
<td>Acceptable to countries importing meat and dairy products</td>
</tr>
<tr>
<td>Wildlife (possums, badgers, ferrets and African buffalo)</td>
<td>Prevention of infection of animals not necessary</td>
</tr>
<tr>
<td></td>
<td>Prevention of transmission of tuberculosis to other wildlife and to domestic livestock</td>
</tr>
<tr>
<td></td>
<td>Protective immunity induced when formulated in an appropriate target-specific delivery system</td>
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<td></td>
<td>Cost-effective delivery</td>
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<td></td>
<td>Preferably life-long immunity induced with a single dose of vaccine</td>
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</table>
stringent, with the principal requirement being to reduce the spread of bovine tuberculosis. The ultimate goal of tuberculosis vaccination programmes for wildlife is to eradicate infection from wildlife reservoirs. A more immediate, and perhaps more achievable aim, is to reduce or prevent the excretion of tuberculous bacilli from wildlife, thus breaking the chain of infection from wildlife to cattle and farmed deer. The vaccine does not necessarily have to prevent primary infection of wildlife, but rather to reduce the bacillary load to a level which is insufficient to sustain tuberculosis in the wildlife population.

Development of challenge models to assess vaccine efficacy

Preliminary screening of tuberculosis vaccines in small animal models such as mice and guinea-pigs is a cost-effective strategy. An aerosol challenge of mice or guinea-pigs with M. tuberculosis has been accepted as the most appropriate small animal model for evaluation of human tuberculosis vaccines (94, 111). These models have now been adapted for the evaluation of vaccines for control of bovine tuberculosis by using an aerosol challenge with M. bovis (12; G.W. de Lisle, unpublished observations).

The vaccine candidates identified as the most promising in small animal models must subsequently be evaluated in the target species. This evaluation should be performed initially by experimental challenge of the target species with M. bovis to optimise vaccination strategies. As field trials are expensive, with large group sizes required due to the low prevalence or patchy distribution and the slow progression of disease, these trials should be used only at the final testing stage. Control of host factors and uptake of vaccine by wildlife are also difficult. A requirement in a challenge model is that the lesions produced should mimic those observed in the natural disease. The challenge model must be adjusted to the target species, as the location of lesions in the natural disease varies according to the species infected, and is a reflection of the source of infection. In cattle, possums and badgers, lesions are predominantly located in the lungs or lymph nodes of the thorax (60, 101); in badgers, lesions may also be associated with infections of bite wounds. In deer, lesions are most commonly found in the retropharyngeal lymph node (63) and in ferrets, in lymph nodes associated with the buccal cavity and alimentary tract (118). To mimic the type of lesion observed in the natural disease, the challenge dose needs to be as low as possible. Host factors may also influence the response of the animal to vaccination. The stress of capture of wild animals such as possums and ferrets has been demonstrated to weaken cellular immune responses (17, 40) and a period of acclimatisation to captivity is required before vaccination trials commence. Other factors, including the condition of the animals and sensitisation to environmental mycobacteria, can affect the response to a tuberculosis vaccine.

A low-dose respiratory challenge model has been established in cattle using an intra-tracheal challenge of 10^5 colony forming units (CFU) of virulent M. bovis (21). When the animals are euthanised and examined, four to five months after challenge, tuberculous lesions are almost entirely restricted to the thoracic cavity with lesions in the lungs and thoracic lymph nodes. The lung lesions consist of small nodules of 3 mm-5 mm in diameter and the lymph node lesions vary in size from 2 mm to 30 mm in diameter. These types of lesions are typical of those observed in the natural disease. Cattle challenged with doses of 10^5-10^6 CFU of M. bovis develop disease with multiple lymph node involvement and large lung lesions, which is atypical of that generally reported in the natural disease (18).

A low-dose challenge model has also been established in deer. A small volume (0.2 ml) of inoculum containing 100-500 CFU of M. bovis is instilled into the tonsillar crypt (64). When the animals are killed six months later, lesions are detected predominantly in the retropharyngeal lymph nodes draining the tonsils.

A useful challenge model for comparing different vaccination regimes and vaccines in possums has been the intra-tracheal inoculation of 10-100 CFU of M. bovis (1, 2, 19). Lesions are confined to the lungs and lymph nodes of the thorax, except in the advanced stage of disease (36). Possums develop a severe pneumonia six to eight weeks after challenge. Recently, possums have been infected by exposure to a low aerosol dose of M. bovis using an aerosol-generating chamber. A dose of 10^6 CFU in the nebuliser consistently produced eight to fifteen primary tuberculous lung nodules in each animal and the development of the disease was slower than that seen following intra-tracheal challenge (B.M. Buddle, unpublished observations). To simulate a natural challenge, vaccinated and non-vaccinated possums were housed with possums that had been experimentally infected with M. bovis. However, transmission of disease was variable during these studies, with infection rates for in-contact possums ranging from 10% to 60%, rendering interpretation of results difficult (L.A. Corner and B.M. Buddle, unpublished observations).

Reports of a challenge model for badgers are few. Studies by Pritchard et al. demonstrated that the intradermal challenge route, which mimics bite-wound infection, is suitable for establishing M. bovis infection in badgers and may be suitable for studying the efficacy of vaccines (116). In contrast, badgers inoculated intra-tracheally with M. bovis showed no evidence of infection.
Ingestion is considered to be the natural route of entry leading to disease in ferrets, and a challenge model has been established by feeding ferrets pieces of deer lung inoculated with $10^6$ CFU of *M. bovis* (117). At twenty weeks after ingestion of the *M. bovis*-inoculated tissues, the mesenteric lymph nodes of all of the animals were culture-positive for *M. bovis* and visible tuberculous lesions were present in 50% of the animals.

**Processes involved in immunity to mycobacterial infections**

An understanding of the processes involved in the protective immune response to mycobacteria should assist the design of improved vaccines. The interacting network of immune responses to mycobacteria is complex. The principal cells involved in protective immunity are the macrophage and the T lymphocyte. Mycobacteria are initially phagocytosed by macrophages and can survive in the phagosome. Activation of macrophages by exposure to mycobacteria and subsequently, by stimulation by cytokines, results in a reduction in the intra-phagosomal pH, exposure of the mycobacteria to reactive oxygen and nitrogen products, followed by death of the mycobacteria. The macrophage is the principal effector cell of protective immunity, whereas the T lymphocyte is the major inducer of the protective acquired immune response (32). Current understanding of protective immunity against tuberculosis has been established using mouse models of the disease and by analysing the responses of human patients with tuberculosis and other mycobacterial infections. The studies in mice have the advantages of the availability of numerous reagents, the possibility of performing cell transfer experiments and the accessibility of mice with gene deletions which allow assessment of susceptibility when a particular cell or cytokine is absent. More recently, immunological studies have been performed in cattle experimentally infected with *M. bovis*.

The lung is a frequent portal of entry of the pathogen in tuberculosis and is commonly affected by the disease in many species. Putative models of events that occur are presented in Figures 1 and 2. Following exposure to mycobacteria, macrophages in the interstitium of the lung become activated, via cell surface receptors, in response to molecules of the mycobacterial cell wall. These molecules, which are often referred to as pattern recognition molecules, alert the macrophage to ‘danger’ (97), thereby initiating the early innate immune responses (98). If the macrophage becomes infected, it may contain the infection, but these macrophages appear to be insufficiently equipped for microbial killing. The activated macrophage is programmed to secrete a number of interleukins (IL), molecules involved in communication between cells of the immune system. This leads to the production of gamma interferon (IFN-γ), a cytokine which is crucial for the activation of macrophage effector function (53).

Mice and humans with a deficiency in the ability to produce or respond to IFN-γ are very susceptible to tuberculosis (37, 79). As part of the early innate response, IL-12 secretion can lead to natural killer (NK) T cell activation and NK T cells may be important in the early secretion of IFN-γ (49). The inflammatory cytokines, tumour necrosis factor alpha (TNF-α) and IL-1, IL-6 and IL-10, are also produced together with chemokines, which are molecules involved in attracting specific cell types into the site of infection (121). One well-characterised chemokine thought to be important is monocyte chemoattractant protein 1 (MCP-1) (88), a chemokine involved in the recruitment of monocytes, memory T lymphocytes and NK T cells. However, mice that are deficient in MCP-1 do not have a reduced ability to clear a tuberculosis infection (92).

Three populations of T lymphocytes have an important role in protection against tuberculosis, namely: Ty6 cells (141) and T cells characterised by the expression of cluster differentiation antigen (CD) 4 or CD8 referred to as CD4+ and CD8+ T cells (32). The Ty6 cells are involved in the early response, providing a rich source of chemokines (34), and can also secrete IFN-γ (140). T cells expressing the γδ receptor accumulate in mycobacterial lesions (65), but the contribution of these cells to protective immunity is not yet

![Fig. 1](image)

**Fig. 1**

*A model of early events in the lung following infection with mycobacteria via the airways*

Mycobacteria are phagocytosed by alveolar macrophages. Following exposure to mycobacteria, macrophages are activated. The interaction of mycobacteria and NK T cells with macrophages may lead to IL-12 production which stimulates early production of IFN-γ from NK T cells and Ty6 cells. Early IFN-γ stimulates further activation of macrophages, leading to the death of some mycobacteria and the production of more IL-12 and other cytokines important in acquired immune responses. The production of TNF-α and other cytokines and chemokines leads to recruitment and activation of macrophages, lymphocytes and neutrophils, to the site of infection.
CD : cluster of differentiation antigen
CD4+ : T cells belonging to CD4+ lineage
CD8+ : T cells belonging to CD8+ lineage
IFN-g : gamma interferon
IL : interleukin

Fig. 2
Events leading to protective acquired immune responses against mycobacteria

Mycobacterial antigens from killed mycobacteria are taken up by dendritic cells and presented to CD4+ and CD8+ T cells. Macrophages can also present mycobacterial antigens but are not so well equipped as dendritic cells. Under the influence of IL-12, CD4+ T cells mediate a T helper 1 type response and secrete IFN-g. This is required for activation of macrophage intracellular pathways that lead to the death of mycobacteria. CD4+ T cells also produce IL-2, which aids in the multiplication of T cells and the generation of memory cells. CD8+ T cells develop into cytotoxic cells and can kill macrophages infected with mycobacteria. Cytotoxic T cells may also contain molecules that kill mycobacteria directly.

north (106) was the first to demonstrate that mice lacking T cells are highly susceptible to infection with M. tuberculosis. Subsequently, lefford demonstrated that T cells could transfer protective immunity (86). Since then, the importance of different T cell sub-populations of CD4+ and CD8+ T cells has been established. The CD4+ T cells capable of transferring protective immunity appear early after infection in the mouse (109). These cells recognise mycobacterial antigens in the context of major histocompatibility complex (MHC) class II cell surface molecules and become activated. Under the influence of IL-12 (54) and IL-18 (58) from the antigen-presenting cell (macrophages and DC), CD4+ T cells producing a Th1 type immune response are stimulated. The IFN-g produced by these cells activates the macrophages to enhance the ability to kill the mycobacteria that they harbour (53). Another important function of CD4+ T cells, a proportion of which is long-lived, is to mediate immunological memory (110).

The role of CD8+ T cells is less well understood. These cells can prolong the survival of acutely challenged mice, and mice are more susceptible to infection when CD8+ T cells are depleted (109). The CD8+ T cells recognise mycobacterial antigen in the context of MHC class I, release IFN-g in response to mycobacterial antigens and can be cytolytic towards cells expressing mycobacterial antigens (85, 131). Molecules released from CD8+ cytotoxic T cells during the cytolytic process may be anti-microbial (4). The CD8+ T cells appear to be more crucial in the later stages of response and may be important in controlling the chronic phase of the disease.
Activated macrophages are a rich source of TNF-α and evidence suggests that TNF-α contributes to bactericidal action (32). However, the principal role of TNF-α in vivo appears to be the local organisation of granulomas (123). The TNF-α drives the delayed type hypersensitivity (DTH) response by inducing chemokines which in turn recruit blood-borne monocytes into the lesion. The influx of macrophages and the formation of the granulomas at sites of infection may be similar to the DTH reaction that occurs in the skin after injection with tuberculin. Persistent TNF-α production, resulting in continued recruitment of macrophages into the granuloma, leads to consolidation of the lung tissue and reduction in lung function.

Evidence is accumulating to suggest that induction of the DTH response to tuberculin and protection against tuberculosis is dissociated. Studies in IFN-γ-knockout mice demonstrate that although these mice lose the ability to secrete IFN-γ and to be protected against M. tuberculosis, the ability to produce a tuberculin DTH response is retained (37).

Protective immunity is driven by cytokines, and the balance of interacting cytokines is critical. Vaccination against bovine tuberculosis must aim to induce a Th1 type immune response and the cytokines, IL-12, IL-18 and IFN-γ will act in feedback loops to maintain a Th1 type immune response. Production of IL-10 may switch the cytokine profile of the T cell response from the Th1 type immune response to the Th2 type response, which is not protective (139). Transforming growth factor beta (TGF-β) may also reduce the protective Th1 response (122). Any event that upsets the ability of the individual to mount a Th1 type response may seriously compromise protective immunity. The dose of vaccine may be important, as this can influence the Th type of immune response stimulated (114); other important factors include exposure to parasites and other infectious agents, and age at exposure (126).

These preliminary studies in mice have enabled selected experiments in cattle to be undertaken, and immune responses have been followed during vaccination and challenge trials. Results from these studies have been used to determine the types of immune responses that are protective in cattle and the suitability of different types of vaccines.

Vaccines for the control of Mycobacterium bovis infection

Historical perspectives

The BCG strain of M. bovis, developed by Calmette and Guérin by attenuation of a virulent M. bovis strain, was originally isolated by Nocard from a cow with tuberculous mastitis. In 1906, subcultures of this strain were initiated on ox bile glycerine-potato medium. After thirty-nine passages, a change in colony morphology was noted and the virulence of this variant strain was tested. Over a period of thirteen years, infection of cattle, guinea-pigs, mice, rhesus monkeys and chimpanzees with this strain produced no evidence of reversion to virulence, but instead conferred resistance, after thirty days, to challenge with the virulent bovine or human tubercle bacillus. Calmette and Guérin concluded that immunisation with a single dose of between 50 mg and 100 mg BCG protected cattle from artificial and natural infections, but protection was not long-lived. In addition, intradermal immunisation was deemed to be preferable to immunisation by the intravenous route (31).

In the 1920s, Calmette and Guérin (31) attempted to eliminate tuberculosis from an infected herd by vaccination of young calves with BCG. Although many of the cattle subsequently slaughtered were found to be free of tuberculosis, definite proof of the efficacy of BCG in this situation was lacking, due to the absence of appropriate controls. Attempts to confirm these findings during the next decade gave variable results (70, 157). The contribution of factors such as the dose of challenge and susceptibility to infection in different individuals and in different herds were realised. Intradermal or oral routes of BCG administration were demonstrated to give no better protection than those routes previously tested. Particularly disappointing were results in field trials (151) and the observation that re-vaccination had no effect.

In 1940, a vole bacillus, M. microti, was discovered to induce better immunity, in the guinea-pig, to both the human and bovine tubercle bacillus, compared to BCG (154). Subsequently, calves were vaccinated, and in a limited trial, protection was considered to be better than expected with BCG (68). In a series of trials, Young and Paterson reported that calves vaccinated intravenously with 5 mg of viable M. microti developed a relatively high degree of immunity to M. bovis (160). Immunity was evident one year after vaccination, but declined thereafter. No further field trials were performed with this vaccine, probably because some strains of M. microti were more virulent than BCG and could cause disease in calves, and the immunity produced was not demonstrated to be markedly different to that produced by BCG (160).

During the same period, a BCG vaccination study was performed on a heavily-infected herd of approximately 150 cattle (45). The cattle were vaccinated with BCG within ten days of birth, at six months and then yearly with BCG over a period of up to five years. Thereafter, vaccination was given only to new-born calves. The losses from infection and re-infection and the problem of interference with skin testing led to the conclusion that the use of a 'test and slaughter' programme was more economical than proceeding with vaccination for tuberculosis control. During the 1950s, Govorov used BCG on farms in which tuberculosis had been apparent for a long time and obtained some protection with

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4 mg dried BCG administered in two intradermal inoculations one month apart (61). After several decades of vaccination trials in Canada (152), the United States of America (USA) (70), Australia (157), Great Britain (45), Africa (11) and other countries referred to in the references summarised in Table II, BCG was concluded to be ineffective as a vaccine in the field against natural infection, although the vaccine could protect cattle from experimental infections. The inability of BCG to confer absolute immunity was generally recognised by that time. Virulent M. bovis were retained in the nearest lymph node to the point of entry, formed a tubercle, but did not show a tendency to form extensive lesions in other parts of the body. The implementation of test and slaughter programmes for control of bovine tuberculosis in industrialised countries in the 1960s and 1970s achieved dramatic results, and vaccination studies were largely curtailed.

### Table II

**Vaccination using BCG in cattle (review of references)**

<table>
<thead>
<tr>
<th>Time period</th>
<th>References (in chronological order)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-1930s</td>
<td>30, 143, 103, 142, 5, 138, 120</td>
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<tr>
<td>1930-1939</td>
<td>70, 148, 6, 8, 27, 66, 67, 149, 77, 150, 151, 31, 26, 142, 130, 28, 29</td>
</tr>
<tr>
<td>1940-1949</td>
<td>55, 78</td>
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<tr>
<td>1950-1959</td>
<td>60, 57, 62, 128, 87, 125, 45, 56, 74, 156</td>
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<tr>
<td>1960-1969</td>
<td>—</td>
</tr>
<tr>
<td>1970-1979</td>
<td>129, 46, 146, 33, 47, 10, 100</td>
</tr>
<tr>
<td>1980-1989</td>
<td>11, 132, 107, 115</td>
</tr>
</tbody>
</table>

The apparent failure of BCG to protect cattle in the field trials may be attributed to a number of factors. High doses of BCG (equivalent to $10^6$-$10^{10}$ CFU) were generally used for subcutaneous or intradermal vaccination of cattle; these doses are now known to be less effective than lower doses in stimulating protective immunity against tuberculosis (64). Vaccination was often performed in areas with a very high prevalence of bovine tuberculosis (11) and calves may have been exposed to M. bovis prior to vaccination, through the consumption of milk from cows with tuberculous mastitis. In vaccination trials in humans, BCG has been less effective when used in the tropics where exposure to environmental mycobacteria is greater. Accumulating evidence in humans and cattle suggests that prior exposure to environmental mycobacteria may lower the efficacy of BCG vaccine (20, 51), therefore this type of exposure needs to be considered in the evaluation of cattle trials.

Recent advances in immunology and the molecular biology of mycobacteria have greatly increased the choices of candidate vaccines. The two principal groupings are live attenuated vaccines such as BCG and other avirulent mycobacteria of the *M. tuberculosis* complex, and sub-unit vaccines based on either mycobacterial protein or deoxyribonucleic acid (DNA). Live attenuated vaccines promote the strong cellular immune responses which are required for protective immunity against tuberculosis and may only need a single dose to induce life-long immunity. This latter feature is particularly important for vaccinating wildlife, as animals may only take up vaccine bait on one occasion, or be trapped once for vaccine administration. Sub-unit vaccines typically require a number of booster immunisations to obtain good levels of immunity. However, the use of sub-unit vaccines in domestic animals has some advantages. Fewer regulatory requirements are associated with the use of these vaccines and animals may not react in a tuberculin skin test following vaccination. A comparison of different types of vaccines against *M. bovis* is summarised in Table III.

### Bacillus Calmette-Guérin

The existing BCG vaccine may not be sufficiently effective, but has many properties that are desirable for a good vaccine. The vaccine is cheap to produce, safe, relatively stable, derived from *M. bovis*, can be administered via a number of routes and has efficacy in reducing haematogenous spread of virulent mycobacteria. The latter observation is important for wildlife tuberculosis vaccines, since prevention of haematogenous spread of infection could be sufficient to prevent the spread of disease to other animals. A drawback of BCG is that cattle exposed to BCG may become sensitised for a positive tuberculin reaction and on this basis, would be assumed to have bovine tuberculosis and hence slaughtered.

An understanding of the mechanisms associated with protection against bovine tuberculosis can now be gained from investigating cytokine responses and the activation of different T cell subsets in animals vaccinated with BCG and challenged with *M. bovis*. The production and supply of immunological reagents to measure immune responses in domestic and wildlife animal species has expanded greatly during the 1990s and BCG vaccination studies should assist in the rational design of improved tuberculosis vaccines. In addition, studies with BCG help to determine the optimal conditions for use of improved attenuated *M. bovis* vaccines and serve as a benchmark for comparison with other tuberculosis vaccines.

### Cattle

The early field vaccination trials in cattle used high doses of BCG and produced disappointing results. Recent information suggests that lower doses of BCG may preferentially stimulate cellular immunity to mycobacterial antigens and may have minimal effect, in the long term, on skin testing (14). To determine whether low doses of BCG could protect cattle against bovine tuberculosis, three vaccination/challenge trials were undertaken using doses ranging from $10^4$ CFU-$10^6$ CFU of BCG. In the first trial, groups of calves were vaccinated subcutaneously with $10^4$ CFU (low dose) or $10^6$ CFU (medium dose) of BCG Pasteur (21). Eight weeks after vaccination, fifteen animals from each group, together with sixteen non-vaccinated calves were challenged intratracheally with virulent *M. bovis*. All animals were killed and necropsied twenty-two weeks after challenge. The proportion of animals...
Calves had high IFN-γ responses to purified protein derivative from culture of *M. avium* (PPD-A), suggesting exposure to environmental mycobacteria. Pre-exposure to environmental mycobacteria, BCG multiplication in the environment, and release of high levels of IFN-γ and IL-2 from the whole blood cultures stimulated with purified protein derivative from culture of *M. bovis* (PPD-B), peaking at two to four weeks after vaccination (21, 22). In the trial in which BCG did not induce protection, a delay occurred in the IFN-γ response to PPD-B, with no increase in the response by week two following vaccination and a small increase in the response by week four (Fig. 3). In animals which have been exposed to environmental mycobacteria, BCG multiplication in the animals following vaccination may have been suppressed, whereas no suppression occurred in non-sensitised animals.

One drawback of the BCG vaccine is that vaccination may induce tuberculin skin-test reactivity, even using low doses of BCG. A number of mycobacterial antigens were recently evaluated in a whole blood IFN-γ test for differentiating BCG-vaccinated cattle from those infected with bovine tuberculosis (24). In concurrence with previous findings (112), the protein ESAT-6, which is produced by virulent *M. bovis* strains, but not by BCG, was demonstrated to be a very useful reagent for specific diagnosis of bovine tuberculosis, and was a suitable antigen for differentiating between BCG-vaccinated and *M. bovis*-infected cattle.

### Deer

The efficacy of BCG vaccination has been assessed in deer following an intratracheal challenge with *M. bovis* (64). A single dose of BCG vaccine 

\[10^6\ \text{CFU}\] administered subcutaneously induced significant protection against disease (presence of lesions), but was not against infection (isolation of *M. bovis*), whereas two doses of BCG eight weeks apart produced significant protection against infection and disease. The level of protection was similar when the dose of BCG ranged from \[10^4\ \text{CFU}\] to \[10^5\ \text{CFU}\], but a high dose of \[10^6\ \text{CFU}\] BCG induced less protection. No difference in the vaccine efficacy of freshly cultured or lyophilised BCG was reported. A long acting dose of dexamethasone given at the time of vaccination ablated the protective response of a single dose of BCG against disease. No protection was induced when animals were vaccinated with two doses of heat killed BCG 

\[5 \times 10^7\ \text{CFU}\] in an oil adjuvant.

The studies in deer concur with the results in cattle that low doses of BCG can induce protection against bovine tuberculosis. Animals vaccinated with low doses of BCG remained protected, despite the diminishing of the DTH reactivity, indicating that DTH reactivity is not a requirement

#### Table III

Comparison of different types of vaccines for protection against experimental challenge with *Mycobacterium bovis*

<table>
<thead>
<tr>
<th>Type of vaccine</th>
<th>Protection against <em>M. bovis</em> infection</th>
<th>Requirement for multiple vaccinations</th>
<th>Vaccine-induced tuberculin skin test reactivity</th>
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<tbody>
<tr>
<td></td>
<td>Small animal model</td>
<td>Target species</td>
<td></td>
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<tr>
<td>Bacillus Calmette-Guérin (BCG)</td>
<td>+</td>
<td>+</td>
<td>No</td>
</tr>
<tr>
<td>Modifications of BCG</td>
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</tr>
<tr>
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<td>+</td>
<td>No</td>
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<td>±</td>
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</tr>
<tr>
<td>DNA vaccines</td>
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</table>
| a) protection defined as significant reduction in bacterial counts in lungs or spleen, or in size and distribution of lesions
| b) target species: cattle, deer, possums, ferrets or badgers
| + protection
| - no protection
| ± variable protection compared to BCG
| NT not tested

with tuberculous lesions in the low-dose BCG, medium-dose BCG and non-vaccinated groups were 2/15, 4/15 and 10/16, respectively. The BCG induced a significant level of protection against development of tuberculous lesions compared to the non-vaccinated controls.

In a second trial, groups of calves were vaccinated with low to medium doses of BCG, either by the subcutaneous or intratracheal route, and challenged as in the first trial (22). Vaccination with BCG resulted in fewer animals developing lesions and a reduction in the number of lesions in the diseased animals compared to the non-vaccinated group. An advantage of vaccinating by the respiratory route was that non-challenged animals from this group produced a minimal reaction in the tuberculin skin test in contrast to the corresponding animals from the group vaccinated subcutaneously. In the third trial, a year later, the calves were of the same age and were sourced from the same farm as in the previous two trials, but subcutaneous vaccination with a dose of \[10^5\ \text{CFU}\] BCG induced no protection (20). Initially, the calves had high IFN-γ responses to purified protein derivative from the culture of *M. avium* (PPD-A), suggesting exposure to environmental mycobacteria. Pre-exposure to environmental mycobacteria has been proposed as an explanation of the failure of BCG to induce protection in many tuberculosis trials in humans (50) and may be the reason for the failure of BCG to protect the cattle in this trial.

A feature of the immunological profile of BCG-vaccinated cattle is the release of high levels of IFN-γ and IL-2 from whole blood cultures stimulated with purified protein derivative from culture of *M. bovis* (PPD-B), peaking at two to four weeks after vaccination (21, 22). In the trial in which BCG did not induce protection, a delay occurred in the IFN-γ response to PPD-B, with no increase in the response by week two following vaccination and a small increase in the response by week four (Fig. 3). In animals which have been exposed to environmental mycobacteria, BCG multiplication in the
Fig. 3
Interferon-γ released from peripheral blood lymphocytes stimulated with purified protein derivative from culture of *Mycobacterium bovis*, in cattle vaccinated with bacillus Calmette-Guérin (BCG)

Cattle were vaccinated with 10^6, 10^5 or 10^4 colony forming units (CFU) of BCG or were non-vaccinated. Three vaccination trials performed in sequential years are presented in a), b) and c). In trials a) and b), BCG vaccination induced a significant level of protection against challenge with virulent *M. bovis*, while no protection was observed in trial c). Data are expressed as mean optical density (OD) units (x 1000)

for protective immunity. An interesting finding from the deer studies was that two doses of BCG, within an eight-week interval, were better than one dose in reducing the subsequent infection. This is in contrast to data from humans which show that booster doses of BCG administered to individuals, when primary responses were inadequate, did not increase the protective efficacy of BCG (158).

Analysis of IL-4 messenger ribonucleic acid (mRNA) expression in lymphocytes from deer vaccinated with live and killed BCG has provided some insights into possible immune mechanisms associated with protection (73). Following primary immunisation, elevated levels of IL-4 mRNA were detected in only a proportion of vaccinated animals and this did not correlate with the use of either live BCG or killed BCG in oil. After boosting, all animals vaccinated with killed BCG showed elevated levels of IL-4 mRNA expression, whereas none of the animals vaccinated with live BCG showed elevated levels. The data suggest that boosting with live BCG may down-regulate IL-4 production and this could be important in induction of protective immunity. High lymphocyte proliferation responses to PPD-B did not correlate with protection, as deer vaccinated with a high dose of BCG (10^8 CFU) had higher proliferation responses, but a lower level of protection than those vaccinated with a low dose of BCG (10^6 CFU) (64).

Possums

A number of vaccination studies have been undertaken in possums using live BCG, demonstrating a significant level of protection against intratracheal challenge with virulent *M. bovis* (1, 2, 23). These studies have concluded that the route used to administer the vaccine affects the protective efficacy of BCG. In the first study (2), groups of possums were vaccinated with 10^6 CFU BCG by three different routes: subcutaneous, intratracheal and intragastric. Vaccination by the subcutaneous or intratracheal route resulted in a marked reduction in the severity of tuberculosis compared with non-vaccinated animals and those vaccinated intragastrically. In a second trial, the delivery of BCG by two routes applicable to the field situation, intranasal (aerosol) and oral, were compared with subcutaneous vaccination (1). Possums vaccinated intranasally by aerosol spray with 10^6 CFU BCG or by the subcutaneous route with 10^6 CFU BCG had a marked reduction in severity of disease compared to animals vaccinated orally with 10^8 CFU BCG. In an earlier study, a lower dose (10^6 CFU BCG) administered by the intragastric route was reported to confer no protection against infection. Protection against tuberculosis by aerosol spray or subcutaneous vaccination was characterised by a minimal change in body weight following challenge, fewer lung lesions and microscopic lesions in the liver and spleen, in addition to lower bacterial counts in the lungs and spleen. Vaccination of possums with BCG influences the immunopathology observed after challenge. In comparison with the predominantly necrotic lesions present in non-vaccinated animals, the lung lesions in BCG-vaccinated possums were mostly granulomas with minimal necrosis and few acid-fast bacilli (2).
For possums, the preferred route of vaccine delivery is oral administration. A study compared the level of protection against tuberculosis in intraduodenally BCG-vaccinated possums with that in possums vaccinated by the intragastric route, to determine whether degradation of BCG in the stomach lowers vaccine efficacy (23). Possums administered BCG directly into the gastrointestinal tract, at a site immediately past the stomach, had significantly greater peripheral blood lymphocyte blastogenic responses to PPD-B and 100-fold lower lung bacterial counts compared to animals vaccinated by the intragastric route. The enhanced CMI responses and improved protection against tuberculosis in the intraduodenally BCG-vaccinated possums indicated that protection of the BCG vaccine from degradation in the stomach should improve efficacy.

Vaccination with BCG does not induce complete protection of possums against experimentally-induced challenge with *M. bovis*, although BCG vaccination induces a marked reduction in the dissemination of *M. bovis* to the spleen and liver after challenge. Vaccination with BCG may be more effective in protecting possums against a naturally-induced *M. bovis* infection, as the time course of the disease is longer than that observed in experimentally-challenged animals. A more natural challenge system has been developed using an aerosol-generating chamber to expose possums, to a low aerosol dose of *M. bovis*. While the course of tuberculosis was slower in possums challenged by this system in comparison with animals infected by the intratracheal route, the level of protection conferred by BCG vaccination was similar for both types of challenge (B.M. Buddle and G.W. de Lisle, unpublished observations).

Another important issue is the longevity of protection conferred by vaccination of possums with a live vaccine such as BCG. To investigate this issue, groups of possums were vaccinated with BCG by aerosol spray and challenged with *M. bovis* at intervals of two, six and twelve months (L.A. Corner, unpublished observations). The results of this study have demonstrated that BCG vaccination induced moderate protection from challenge with intervals of two or six months between vaccination and challenge but a lower level of protection when this interval was increased to twelve months.

Badgers

Reports of vaccination studies on badgers are few. In one study, badgers vaccinated with $10^6$ CFU BCG by the intradermal route had higher lymphocyte blastogenic responses to PPD-B than non-vaccinated animals (137). Bacillus Calmette-Guérin was considered non-pathogenic to badgers and the animals did not excrete bacilli. Seven of the twelve badgers in this study were challenged by intradermal inoculation with $10^3$ CFU *M. bovis* at five to twenty-five months after vaccination. Animals vaccinated with BCG lived longer and shed fewer bacilli than non-vaccinated control animals, suggesting that protective immunity was induced by vaccination.

A recent study in Ireland measured immunological responses in a resident population of badgers which had been vaccinated subcutaneously with a low dose of BCG and compared these responses with a non-vaccinated group (E. Gormley, unpublished observations). Cellular immune responses were observed in badgers vaccinated with multiple doses of BCG, characterised by lymphocyte blastogenic responses to PPD-B, but antibody responses to *M. bovis* antigen were minimal.

Ferrets

Ferrets orally vaccinated with live BCG incorporated into dietary meat were partially protected against oral challenge with virulent *M. bovis* (117). Vaccination of ferrets with BCG by the subcutaneous route also induced protection against challenge and resulted in reduced severity of disease (41). Protection against tuberculosis was characterised by a significant reduction in viable bacterial load, the prevention of gross lesions in mesenteric lymph nodes and reduced bacterial spread to thoracic lymph nodes. In comparison with the favourable results in possums (23), vaccination by intraduodenal inoculation of BCG was not effective in reducing disease.

Modified bacillus Calmette-Guérin

Based on the good safety record of BCG, a number of researchers considered that an effective way of developing an improved tuberculosis vaccine could be to enhance the efficacy of BCG by altering the gene complement. Cytokine genes have been added to amplify specific immune responses (104) and a lysteriolysin gene has been added to promote MHC class I presentation of antigens (71). Modification by inactivation or alteration of the genes of BCG has also been performed in attempts to produce safer vaccines for immunocompromised individuals or to reduce skin test responses (93). Although these modified forms of BCG have some advantages, as yet, none have induced greater protection against tuberculosis in small animal models than the conventional form of BCG.

Preliminary studies have been undertaken to test two of these types of vaccine in deer and cattle. A recombinant BCG, which constitutively secretes deer IL-2, has been developed and used to immunise deer (133). Compared to normal BCG, no differences were noted in lymphocyte stimulation responses or IL-2 and IFN-γ mRNA expression, whereas the level of IL-4 expression was lower in the group inoculated with the recombinant BCG. A smaller DTH response to BCG antigens was observed in the group vaccinated with recombinant BCG. Although the recombinant appeared safe and may have improved the quality of the immune response to antigens of *M. bovis*, the ability of this vaccine to induce a protective immune response against *M. bovis* has not been tested.
Two auxotrophic mutants of BCG have been produced which have mutations in the genes involved in the metabolism of leucine and methionine (96). These mutants are not able to grow in minimal medium unless the medium is supplemented with the appropriate amino acid. Ability to grow in vivo is also reduced and these mutants have been considered to be safer vaccines for immunocompromised individuals. Vaccination of mice with these auxotrophic mutants has induced protection against M. tuberculosis infection (69). Vaccination of cattle with a leucine auxotroph of BCG did not induce a skin test response to PPD-B (H.M. Vordermeier, unpublished observation), but protection against bovine tuberculosis has yet to be evaluated.

Attenuation of virulent strains of Mycobacterium bovis

Bacillus Calmette-Guérin was conceived in an empirical manner. Recent genetic analysis has indicated that all strains of BCG have a 9.5 kilobase (kb) deletion, involving nine genes, when compared to virulent strains of M. bovis and M. tuberculosis (95). Improvement of BCG should be possible by deletion, from virulent strains of M. bovis or M. tuberculosis, of specific genes which are involved in virulence or encode enzymes for essential metabolic pathways. These mutants may resemble virulent strains more closely than BCG in terms of antigenic profile and hence may have enhanced vaccine efficacy. Molecular biological techniques including transposon mutagenesis, illegitimate recombination and allelic exchange have now been developed to inactivate genes in M. bovis and screening techniques have been established to identify attenuated mutants (35). An attenuated vaccine for field use would be required to have a deletion in two different genes, either of which could cause attenuation, to avoid any possibility of reversion to a virulent strain through mutation. Development of an immunological screening test to distinguish between vaccinated animals and those infected with M. bovis would be advantageous. If the new vaccine strain also had one or more gene deletions, the products of which induce DTH or another immunological reaction that could be tested, then an immunological test could be developed to distinguish between vaccinated and infected animals. In preliminary studies, the esat-6 gene of a wild-type M. bovis strain has been deleted. Guinea-pigs inoculated with this mutant did not react in a skin test to ESAT-6 protein, but reacted strongly to PPD-B. In contrast, animals inoculated with the wild-type M. bovis strain reacted strongly to both ESAT-6 and PPD (148).

Auxotrophy or the inability to grow in minimal medium indicates that a strain has lost some metabolic function. This approach has been successfully used for several bacterial pathogens to develop attenuated strains with vaccine properties (72). In initial attempts to determine the usefulness of this approach, several attenuated strains of M. bovis were developed by chemical mutagenesis of a liquid culture with nitrosoquandine. Following the screening of strains for auxotrophy, the auxotrophs were tested for virulence in guinea-pigs (147). Two of these auxotrophic M. bovis strains, which were shown to be attenuated in guinea-pigs, were tested for protection of cattle against bovine tuberculosis (20). Prior to vaccination, the calves in this trial had high IFN-γ responses to PPD-A, suggesting exposure to environmental mycobacteria. Vaccination with either of the two auxotrophic M. bovis strains resulted in development of tuberculous lesions in significantly fewer animals compared to BCG-vaccinated and control groups. The failure of BCG to protect cattle in this experiment may have been a result of previous exposure of the calves to environmental mycobacteria. The colony count of live bacteria in the vaccines prepared from the auxotrophic strains was $1-2 \times 10^6$ CFU/dose, approximately $1 \log_{10}$ higher than that in the BCG vaccine. This is unlikely to have contributed to the improvement in vaccine efficacy, as doses of $10^5-10^6$ CFU BCG can induce similar levels of protection (21). Overall, the success of the newly-derived attenuated M. bovis strains in this situation is encouraging. Despite exhaustive efforts, genetic alterations in these two auxotrophic strains could not be identified.

To produce attenuated strains of M. bovis with defined deletions, a number of mutants of M. bovis have been produced by illegitimate recombination. Four mutants that were selected for inability to grow in minimal medium were shown to be attenuated in guinea-pigs. When challenged with virulent M. bovis, two of these mutants induced a level of protection similar to that induced by BCG (43). One of these mutants had a 2 bp chromosomal deletion while the other had a large DNA deletion of 15 kb, containing twelve genes. These two strains have recently been tested for vaccine efficacy in possums (B.M. Buddle and G.W. de Lisle, unpublished observations). The strains were inoculated subcutaneously at a dose of $10^6$ CFU and the possums were challenged eight weeks later by aerosol with virulent M. bovis. When the possums were necropsied eight weeks following challenge, those vaccinated with either of the auxotrophic strains or with BCG had significantly fewer lung lesions and a lower loss in body weight than the non-vaccinated controls. However, only those vaccinated with the auxotrophic strains had a significantly lower spleen bacterial count than the controls. Few, if any, new tuberculosis vaccines tested in mouse or guinea-pig models show more protection against tuberculosis than BCG, therefore the possum model could be useful in identifying improved tuberculosis vaccines.

Use of live vectors

Live vectors such as vaccinia virus (161) and attenuated Salmonella strains (71) have been used to express genes for mycobacterial antigens, but these vaccines have not been assessed for protection against M. bovis. The use of live vectors is attractive for wildlife vaccination, as most vectors are amenable to both the oral and parenteral route of delivery. Oral vaccination of badgers with a live recombinant vaccine protected 50% of the animals against rabies (15). The use of live virus vectors expressing mycobacterial genes could be an
Killed mycobacteria

Killed mycobacterial vaccines have perceived advantages in being safer to use than live attenuated M. bovis vaccines. However, traditional killed mycobacterial vaccines mixed in an oil adjuvant stimulate a Th2 type immune response and are not protective (64). A killed M. vaccae has been advocated for the immunoprophylaxis of tuberculosis (135). The protective effects of M. vaccae are considered to arise from stimulation of CMI responses to common mycobacterial antigens and from switching off the tissue-necrotising aspects of the Koch phenomenon. To assess vaccine efficacy of killed M. vaccae, a group of calves were vaccinated intradermally with 10^6 CFU killed M. vaccae and subsequently challenged with M. bovis (22). No protection against tuberculosis was noted in comparison to calves vaccinated with BCG. Vaccination of badgers with killed M. vaccae also induced no protection against tuberculosis (136). In a preliminary trial in possums, killed M. vaccae alone, administered intranasally and intraconjunctivally, induced no protection against a subsequent challenge with M. bovis. However, a combination of killed M. vaccae and live BCG administered in a similar manner induced protection against the M. bovis challenge and the spleen bacterial count was significantly lower than with BCG alone (M.A. Skinner, unpublished observations). This raises the possibility that killed M. vaccae may enhance the vaccine efficacy of BCG.

Mycobacterial protein vaccines

An alternative approach focuses on the use of protective protein antigens released from live mycobacteria. Potentially, such sub-unit vaccines may not compromise diagnostic tests and efficacy may not be affected by prior sensitisation of animals to environmental mycobacteria. Culture filtrates prepared from M. tuberculosis have been shown to contain proteins that are highly stimulatory to T cells of human tuberculosis patients (44), and both mice (3, 124) and cattle (113) that have been experimentally infected with tuberculosis. Several studies using small animal models have demonstrated the protective potential of antigens contained in culture filtrates. Immunisation of mice and guinea-pigs with culture filtrate proteins (CFP) from M. tuberculosis afforded high levels of protection against aerogenic challenge with M. tuberculosis (7, 124). Similarly, a CFP vaccine derived from M. bovis has been demonstrated to induce significant protection in mice against aerogenic challenge with virulent M. bovis (12). In collaboration with P. Andersen (Statens Serum Institute, Copenhagen) and I. Orme (Colorado State University), the authors have tested in cattle, vaccines prepared from culture filtrates of M. tuberculosis or M. bovis, which have induced a significant level of protection in mice. Use of adjuvants such as dimethyldeoctadecylammonium chloride (DDA) with M. tuberculosis CFP induced cellular immunity to mycobacterial antigens in mice, but induced no response in cattle. Although the use of a diethylaminoethyl (DEAE) dextran adjuvant in cattle helped induce strong antigen-specific antibody and IL-2 responses, a minimal IFN-γ response was detected (D.N. Wedlock and B.M. Buddie, unpublished observations). The incorporation of a recombinant cytokine, bovine IL-2, in a M. bovis culture filtrate protein vaccine with lipid A adjuvant, markedly enhanced antigen-specific antibody responses and induced weak antigen-specific IFN-γ responses in cattle (153). This vaccine reduced the mean tuberculous lung lesion score in challenged cattle and induced no tuberculin skin-test reactivity, but these animals had a higher prevalence of extrathoracic spread of disease than non-vaccinated animals.

These studies highlight the difficulty of stimulating strong antigen-specific IFN-γ responses using mild adjuvants in cattle. Use of similar adjuvants in small animal models and cattle have not produced the same results. To date, in cattle, none of the sub-unit vaccines have induced antigen-specific IFN-γ responses comparable to those induced by attenuated M. bovis vaccines. Improvements could be made by formulation with better adjuvants and incorporating other cytokines such as IL-12 or IL-18 to promote antigen-specific IFN-γ responses.

DNA vaccines

The general principal of DNA vaccination is quite simple, but this type of technology has only been in development since the beginning of the 1990s. A DNA vaccine consists of an expression plasmid, into which a piece of DNA coding for a microbial antigen has been inserted. This plasmid DNA containing the gene for the microbial antigen is amplified in a transformed bacteria and the purified plasmid DNA, containing the gene for the antigen, is used to immunise the host. The plasmid DNA directly transfects a living cell. The gene for the antigen is transcribed to RNA in the nucleus and translated to protein in the cytoplasm. Finally, the host becomes immunised by the microbial protein produced in its own cells, thus strong humoral and cell-mediated responses that are long-lasting and protective can be induced (76).

In small animal models, DNA vaccines have shown considerable promise in inducing protection against tuberculosis (7, 90). Features of tuberculosis DNA vaccines are the ability to stimulate IFN-γ and cytotoxic T cell responses, elicit strong memory responses and protect when encoding a single protein or epitope. Protection is enhanced if the DNA vaccine encodes for several proteins or epitopes (81) and if adjuvant molecules such as DNA CpG motifs are incorporated in the vaccine (84). A recent study in a mouse model has shown that tuberculosis DNA vaccines can cure an existing infection (91), which would be advantageous for use in wildlife. A disadvantage is that multiple inoculations appear to be necessary. In a recent study in cattle, two of these vaccines induced CD4+ T cell responses and immunoglobulin G1 (IgG1)-biased humoral responses, but only weak IFN-γ
responses to M. bovis antigens. Encouragingly, no tuberculin skin test reactivity was observed in vaccinated animals (145). Further studies are in progress to assess the protective efficacy of these vaccines.

Vaccine delivery strategies

Cattle and farmed deer

The most likely method for vaccination of domestic stock will be parenteral, with delivery of vaccines by subcutaneous or intramuscular injection, depending on the type of vaccine (Table IV). The relative ease of access to animals would, in most cases, make multiple vaccination or re-vaccination a feasible option, although ideally, a single-shot vaccine would be preferable.

Wildlife

Several strategies exist for the delivery of vaccines to wildlife. One approach is to trap the target species and administer the vaccine to the restrained animal. This method allows the greatest control over the route of delivery and dose of vaccine administered. Alternatively, the vaccine can be offered in some form of bait or food. A number of bait delivery systems have been evaluated for the delivery of rabies vaccine to semi-domesticated dogs, wild foxes and jackals (89, 96). The vaccination of possums by delivery of an aerosol to the face as the animal approaches a bait placed within an automated vaccine delivery device has been suggested (R.S. Morris, personal communication). Possums exposed to an intranasal aerosol spray of BCG have a similar level of protection against experimentally-induced M. bovis infection as animals vaccinated subcutaneously (1). The relative merits of each vaccine delivery system are summarised in Table IV.

An oral vaccination strategy for wildlife has several merits. Both badgers and possums are known to readily accept oral baits. For example, studies have shown that the biomarkers isophenoxic acid and tetracycline are readily taken up by badgers (134). Badgers do not display competitive behaviour when feeding, therefore uptake of baits should not be limited to a few dominant individuals within a territory (75). Badgers normally chew baits and this should ensure the vaccine is delivered to the oral cavity and not directly into the stomach.

In humans, BCG was used as an oral vaccine until 1976, but was discontinued because of the unacceptable number of cases of lymphadenitis in children (9, 59). A review of BCG as a recombinant, multivalent vector concluded that oral administration of BCG could be a simple and effective method of vaccination (9).

The delivery of a live vaccine such as BCG in bait form requires the stability of the vaccine under diverse environmental conditions for prolonged periods. Vaccination of wildlife by oral delivery of the glycoprotein of rabies virus expressed in a live recombinant vaccinia virus has been successfully implemented in several countries of Europe for foxes and in the USA for vaccination of racoons and coyotes (16). Oral vaccination of badgers with the vaccine protected 50% of the animals against rabies (15). The success of such programmes demonstrates that stabilisation of a live vaccine in bait is possible.

The efficacy of a live vaccine such as BCG may be severely reduced by exposure of the vaccine to gastric secretions. Nevertheless, an oral BCG vaccine could possibly be effective if combined with a technique such as micro-encapsulation to protect the bacteria from the bactericidal effects of the stomach contents. The enhanced immune responses and protection against bovine tuberculosis reported when possums were administered BCG by the intraduodenal route (23) has provided encouragement, in that an orally-administered tuberculosis vaccine can induce protective immunity, suggesting that oral vaccination is a feasible strategy. Sub-unit vaccines (peptide, protein or DNA) are less effective when delivered orally and will require the

Table IV
Relative merits of different vaccine delivery strategies

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<th>Method</th>
<th>Advantages</th>
<th>Disadvantages</th>
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<td>Direct administration to animal during routine handling</td>
<td>Route of delivery, dose of vaccine and age of animal vaccinated can be controlled</td>
<td>Feasible only for domestic livestock Labour intensive</td>
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<td></td>
<td>Re-vaccination possible</td>
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<td></td>
<td>Test for protective immunity may be possible</td>
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<tr>
<td>Direct administration following trapping of target species</td>
<td>Specific delivery to target species (e.g. badgers) Ability to control vaccine dose and route of delivery</td>
<td>Labour intensive Stress may adversely affect immune response Fewer animals reached and an age/sex bias may occur</td>
</tr>
<tr>
<td>Bait delivery to wildlife</td>
<td>Least labour intensive Large areas can be covered more animals reached</td>
<td>Vaccine may be taken by non-target species Difficult to control dose of vaccine and number of animals vaccinated Effect of environment may limit life of vaccine</td>
</tr>
<tr>
<td>Automated vaccination of wildlife</td>
<td>Less labour intensive A number of delivery routes are theoretically possible</td>
<td>No attested equipment currently available Vaccine may be taken by non-target species Difficult to control dose of vaccine and number of animals vaccinated Effect of environment may limit life of vaccine</td>
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addition of a suitable mucosal adjuvant or some form of enteric coating. A potential problem with the delivery of vaccines in oral baits is the possibility that the vaccine in the bait will be ingested by non-target species, and if taken by domestic cattle or deer, this could compromise the use of diagnostic tests for tuberculosis.

A number of other live vectors are being developed which could be used for delivery of vaccines to wildlife. Bacteria such as Salmonella which have been transformed with plasmid DNA vaccines, have been used as recombinant delivery vehicles to transfer plasmid DNA to the host (42). The use of a self-transmitting vaccine such as a species-specific virus or parasite in which genes from M. bovis have been cloned is theoretically possible. While the use of transmissible vaccines would enable large populations of possums or other wildlife to be vaccinated, the major obstacles would be approval from authorities and public acceptance before the field release of such vaccines could occur.

Field vaccination trials

Following extensive testing of vaccine candidates under experimental challenge conditions, the most promising candidates need to be tested against natural challenge in field trials. Immunological correlates of protection, such as IFN-γ responses, are not sufficiently predictive to be used as the sole criterion to assess protection. Vaccinated and control animals must be slaughtered and animals examined for presence of disease and M. bovis infection after a defined period which could be between two and five years. Prior to vaccination, animals should be tested in an immunological assay, such as skin test or IFN-γ assay, for responses to PPD-A and PPD-B. Lack of prior exposure to M. bovis is a critical attribute of animals selected for the trial, and lack of exposure to environmental mycobacteria, as indicated by the immunological responses of the animals, is preferable. The group size is dependent on the expected prevalence of bovine tuberculosis in the control group at the end of the trial, with a large group size required when prevalence of disease is low. Detection of tuberculous lesions, particularly in cattle, can be difficult and trained personnel should be involved in the necropsies. Bacterial culture of lesions and pooled samples of lymph nodes from non-lesioned animals provide valuable information on protection from M. bovis infection. Additional procedures must be undertaken during field trials of tuberculosis vaccines in wildlife, as mentioned in a recent review (25). When the vaccine is to be delivered as a bait, estimates of the uptake of vaccine by target and non-target species should be obtained. If a biomarker or immunological marker cannot readily identify vaccinated animals, comparable areas must be selected for the vaccinated and control groups. Success of these trials will be dependent on a thorough epidemiological investigation of the disease prevalence prior to vaccination and at various time points during the trials. Vaccine efficacy can be determined by examination following slaughter of sub-sets of the vaccinated and control populations at the conclusion of the trial.

Prospects for the future

The increase in knowledge of mycobacterial genetics and understanding of immune responses since the early 1990s suggest that the development of an effective vaccine for bovine tuberculosis is a realistic goal. Studies using relatively low doses of BCG in cattle and farmed deer indicate that this vaccine has the potential to reduce economic losses in developing countries, although protection may only be partial. Trials should be performed in these countries to determine whether a low dose BCG vaccination strategy could reduce the spread of bovine tuberculosis. Vaccination is the most humane and cost-effective method of disease control in wildlife vectors, particularly in the UK where the badger is a protected species. A live vaccine is more suitable for wildlife as the vaccine is cheap to produce, can be delivered in a single dose and gives long-term protection. Novel attenuated strains of M. bovis currently being developed for wildlife may also be suitable for cattle, once new differentiating diagnostic tests are developed.

Vaccines have traditionally consisted of either live attenuated pathogens, whole inactivated organisms or inactivated toxins, but in the past decade, several new approaches to vaccine development have emerged. New-generation vaccines, such as sub-unit protein vaccines and recombinant DNA vaccines, may provide a vaccine for bovine tuberculosis. Many candidate molecules are now available. However, these are poorly immunogenic when administered alone and there is a lack of appropriate adjuvants to stimulate the cellular immune response in cattle required for protective immunity. A requirement exists for immunological adjuvants that are potent, safe and compatible with new-generation vaccines. Future adjuvants will probably exploit more site-specific delivery mechanisms, to enable immune responses to be targeted to specific organs, cells or sub-cellular compartments involved in antigen presentation. A better understanding of overlapping immune networks involved in the host response to tuberculosis is likely to drive further developments in novel vaccination strategies for bovine tuberculosis in both cattle and wildlife. The future promises to be positive, with vaccination programmes leading to the control and eventual eradication of bovine tuberculosis.

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Vaccination des animaux contre *Mycobacterium bovis*

M.A. Skinner, D.N. Wedlock & B.M. Buddle

Résumé
La vaccination peut être un moyen pratique de contrôler la tuberculose bovine dans les pays où les animaux sauvages constituent le réservoir de la maladie ainsi que dans ceux qui ne disposent pas des ressources suffisantes pour la mise en œuvre de stratégies de prophylaxie classiques. L'élaboration de nouveaux vaccins et leur évaluation supposent une bonne compréhension des processus liés à la réponse immunitaire vis-à-vis de la tuberculose. Les auteurs font le point sur les connaissances actuelles concernant cette réponse, essentiellement des résultats obtenus sur souris. L'applicabilité éventuelle de ces résultats à l'élaboration d'un vaccin pour les bovins, les cervidés et les animaux sauvages est discutée. Des modèles expérimentaux ont désormais été mis au point pour tester les vaccins sur plusieurs espèces domestiques et sauvages et sont utilisés notamment pour évaluer les vaccins de la tuberculose. La plupart des études sur l'efficacité des vaccins de la tuberculose chez les animaux cibles portent sur le bacille bîlé de Calmette-Guérin (BCG), une souche atténuée de *Mycobacterium bovis*. Les récents progrès de l'immunologie et de la biologie moléculaire concernant les mycobactéries ont permis d'élargir considérablement le choix des vaccins candidats ; à l'avenir, d'autres types de vaccins seront encore testés, y compris de nouvelles souches atténuées de *M. bovis*, des vaccins à sous-unité protéique et des vaccins à acide désoxyribonucléique recombinant. Plusieurs de ces vaccins ont donné des résultats encourageants sur de petits animaux de laboratoire. Malgré les progrès réalisés dans l'amélioration des systèmes d'administration de vaccins à usage vétérinaire, la vaccination de la faune sauvage pose encore des problèmes techniques non résolus.

Mots-clés

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**Vacunación de animales contra Mycobacterium bovis**

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Resumen
La vacunación puede llegar a convertirse en un instrumento práctico para luchar contra la tuberculosis bovina en países que albergan reservorios salvajes de la enfermedad o que no pueden costear programas clásicos de control. Para que el proceso de elaboración y ensayo de nuevas vacunas resulte coherente, es deseable que antes se entiendan bien los procesos que intervienen en la respuesta inmunitaria protectora frente a la tuberculosis. Los autores pasan revista a los conocimientos actuales en la materia, fruto en buena parte de estudios realizados con ratones. Después examinan, a la luz de esos conocimientos, la posibilidad de utilizar vacunas para inducir inmunidad protectora en ganado vacuno, ciervos y animales salvajes. Existen ahora modelos de confrontación, elaborados para ensayar posibles vacunas en muchas especies domésticas y salvajes, que se están utilizando para evaluar vacunas contra la tuberculosis. La mayoría de los estudios para determinar la eficacia de esas vacunas en los animales diana se han concentrado en el uso de la vacuna BCG (bacilo de Calmette y Guérin), que es una cepa atenuada de *Mycobacterium bovis*. Los recientes avances en el campo de la inmunología y la biología...
molecular micobacteriana han ampliado mucho el repertorio de posibles vacunas. Entre los nuevos tipos de vacuna que en el futuro van a probarse figuran las de nuevas cepas atenuadas de *M. bovis*, las de subunidades proteicas y las de ácido desoxirribonucleico recombinante. Sometidas a prueba en pequeños animales, varias de esas vacunas han ofrecido ya resultados prometedores. Pese a los progresos realizados en la concepción de sistemas para administrar vacunas a los animales, siguen por resolver problemas técnicos ligados a la vacunación de la fauna salvaje.

**Palabras clave**

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**References**


