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Title	Current Research at the Badger Vaccine Research Laboratory
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Publication date	2000-10
Publication information	Gormley, Eamonn, and Angela Southey. Current Research at the Badger Vaccine Research Laboratory. Edited by John D. Collins and Robert F. Hammond. University College Dublin. Centre for Veterinary Epidemiology and Risk Analysis, October, 2000.
Series	Selected Papers, 1999
Publisher	University College Dublin. Centre for Veterinary Epidemiology and Risk Analysis
Item record/more information	http://hdl.handle.net/10197/8860

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Appendix V

Current Research at the Badger Vaccine Research Laboratory

Development of a vaccine against tuberculosis for use in badgers *(Meles meles)*

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Acknowledgements: All of this work was carried out with the advice and assistance of Dr Paddy Sleeman (Dept. of Zoology, UCC). We would also like to thank Mr Eamon Costello (Abbotstown), staff at the Veterinary Laboratories Agency UK, Mr Tom Partridge VI (Waterford), Duchas, Dr Ursula Fogarty (Equine Centre), Prof. Dan Collins (UCD), staff at Little Island (Waterford), regional veterinary laboratories, Mr. Ian O'Boyle SVI and Mr Michael Sheridan DCVO. This work was funded by Dept. of Agriculture, Food & Rural Development.

Summary

Studies leading to the development of a vaccine for use in badgers have been in progress for two years. During this period of time considerable advances have been made in analysing immune responses in BCG-vaccinated and *M. bovis*-infected badgers. The nature of these responses are crucial determinants of the outcome of infection. We have established that BCG-vaccinated badgers generate an antigen-specific T lymphocyte response to PPD-bovine and that this response is consistent with the development of protective immunity. The responses in *M. bovis*-infected badgers are more varied but significantly greater when compared to vaccinated animals suggesting that the lymphocyte transformation assay can be used to distinguish between these groups of animals.

At the beginning of the project, one of the major constraints to developing an understanding of the tuberculosis disease process was the lack of suitable reagents and tests for detecting infection in live badgers. We have used two tests, the lymphocyte transformation assay and Brock test (both developed at Veterinary Laboratories Agency UK) in conjunction with conventional *post-mortem* histology and bacterial culture to demonstrate that the blood based immunological tests have potential as diagnostic tests for infection in live badgers. We have found that the sensitivity of combined tests is greater than any individual test. In addition, we have also developed a badger interleukin-2 cytokine assay that provides an additional tool for diagnosis. To date, very little data has been gathered relating to non-specific disease responses in badgers. We have tested serum samples from *M. bovis*-infected and control animals for inflammatory markers of disease and found significantly elevated levels of haptoglobin and Serum Amyloid A (SAA) in some of the *M. bovis*-diseased badgers. These tests add to the current battery of diagnostic assays that are now available for use.

The success of any vaccination program is contingent on the development of an effective delivery strategy to a target population. We have addressed this issue by incorporating biomarkers, sulfadimethoxine and rhodamine B into oral Dupont baits and measuring uptake rates in an island population of badgers. Analysis of the data revealed that uptake of the baits was high (>90%) and we could readily detect the presence of biomarkers in serum and hair samples. We concluded that it was possible to determine which individuals, or the proportion of the population, that had consumed baits, and the numbers of baits eaten by individuals. Studies are currently underway to examine the survival of BCG in a semi-solid matrix that may be incorporated into the baits. In collaboration with Warwick University, we are also working on sensitive methods for detection of BCG and *M. bovis* in environmental samples.

Additional studies are being carried out to compliment the vaccine strategy which will serve to increase our overall understanding of the nature of disease progression in badgers and factors that may influence this process. When requested, we make available samples to other groups investigating, *Neospora sp.* (Dr Grace Mulcahy, UCD), trypanosomes (Dr Paul Voorheis, TCD), IFN-γ and IgA immune responses (VLA, UK), antibodies (Dr Jim McNair, DANI, NI) and tissue samples (Dr Ian Orme, Colorado). In collaboration with Dr Giovanna Batoni (University of Pisa), we have received large quantities of an antigen, SA-5K, which has the potential to be a highly sensitive and specific T cell antigen marker for detecting exposure to *M. bovis* at a very early stage post-infection.

With the construction of the BROC research facility the project enters a new phase. We are confident that all of the progress made in the past two years can be applied to the studies carried out at the facility and will lead to large scale field vaccine trials and implementation of a vaccine program.

The development of a badger vaccine for eradication of tuberculosis in cattle in Ireland

The existence of tuberculosis in wildlife species represents both a human health and animal health hazard for persons and animals who come into contact with them in the course of their normal activities. However, shortcomings in the sensitivity of tuberculin test have allowed residual infection to continue to exist within some herds. The problem has been exacerbated by the chronic persistence of tuberculosis in wildlife species such as the badger which share the same environment as cattle. This renders the eradication of this infection in cattle very difficult under these conditions. While continued tuberculin testing will serve to maintain tuberculosis in cattle at a low level, there is a growing consensus that the problem of *M. bovis* infection in badgers and, consequently, in cattle, will not be solved without a vaccine. As a long term control measure, the aim of vaccination will be to decrease the incidence of infection in susceptible hosts or reduce development of disease pathology in infected individuals to the extent that the disease will eventually be eliminated in badger populations. In conjunction with improvements in sensitivity and specificity of the diagnostic tests used in cattle, this could directly facilitate the completion of bovine tuberculosis elimination in areas so affected.

Given the complexities of progression of disease and the immune responses directed to counteract virulent *in vivo* growth of *M. bovis* in the host, there are many challenges to developing an effective

vaccine strategy to prevent transmission of the bacterium from a population of badgers. Chief among these would be issues relating to the following:

1. Defining the type and formulation of the vaccine to be used.
2. Uptake of vaccine by large percentage of target population
3. Achieving the desired level of protection
4. Identifying and measuring correlates of protection
5. Assessing a large number of target host factors relevant to the generation of protective immunity in a population.

However, in spite of these formidable challenges there are grounds for optimism that vaccination is an option worth pursuing. With increasing advances in molecular biology and immunology, a greater range of vaccine candidates have become available that may have future potential for use with wildlife. Live vaccines such as *M. bovis* BCG or other attenuated strains may be advantageous if they can be delivered as a single dose and persist in the host to continuously prime the cellular immune response. Alternatively, the development of DNA or sub-unit vaccines that induce long term protective immunity may be more cost beneficial, and considered more safe, in the long term. The efficacy of these new generation vaccines and testing of delivery systems need to be evaluated in wildlife. In addition, specific diagnostic tests that can distinguish vaccinated from *M. bovis*-infected hosts need to be developed in parallel.

The sole existing vaccine for tuberculosis, *M. bovis* BCG, was developed in the early 1900's from a virulent *M. bovis* isolate and has since been widely used to control tuberculosis in humans, with varying degrees of success. Many explanations have been put forward to explain the wide range in protection levels obtained in human trials. These include age at vaccination, type of BCG vaccine used, exposure to environmental non-tuberculous mycobacteria, virulence of challenge strains and genetic make up of the target populations. It is likely that all these factors would be equally important when considering vaccinating wildlife hosts. Many studies have been carried out to elucidate the cellular basis for generation of a protective immune response, following vaccination with *M. bovis* BCG. It has been established that development of acquired immunity to tuberculosis is mediated through a specific T cell-mediated immune response in which the co-operative action of antigen-specific T cells and macrophage ultimately control infection by inhibiting intracellular growth of the tubercle bacilli. In contrast, the presence of high titres of antibody is usually associated with chronic or progressive disease.

Despite the widespread use of BCG vaccine in human populations, there is still a paucity of information for defining and optimising parameters necessary for maximising protective immunity. Studies in New Zealand with vaccination of cattle and deer have demonstrated that the BCG vaccine, when delivered at an appropriate low dose can generate significant protective immunity against experimental challenge with virulent *M. bovis*. In similar studies with possums (*Trichosurus vulpecula*) and ferrets (*Mustela fero*) vaccination with BCG did not prevent establishment of infection following virulent challenge, but did lead to a reduction in the severity of the disease. It is considered that suppression of lesion development and reduction of bacillary load might result in decreased shedding of *M. bovis* by infected animals, with subsequent reduction in transmission to other hosts.

In a previous vaccination study the protective effect of the BCG vaccine was investigated in twelve badgers. An intradermal inoculation of 10^6 cfu of BCG was found to be non-pathogenic, was not excreted by the badgers and was not transmitted to in-contact animals. There was an increase in the lymphocyte transformation response (LTR) but no antibody increase to PPD-bovine in all twelve vaccinated badgers. When seven of these badgers were subsequently challenged intradermally with 10^4 *M. bovis*, between 5 and 25 months after vaccination, the LTR response tended to fall and the antibody response rose. The vaccinated badgers were shown to live longer, shed fewer tubercle bacilli and their inoculation sites healed more rapidly after challenge than a group of three control badgers. Thus, although only small numbers of badgers were involved, cell-mediated immunity did seem to be enhanced by BCG vaccination, leading to prolonged survival of the badgers and delayed excretion of tubercle bacilli.

More recent studies have investigated the immune responses in *M. bovis*-infected badgers through the development of an indirect ELISA and lymphocyte transformation assay for the rapid detection of infection in live animals. In contrast, relatively little information has been gained on how badgers might respond immunologically to BCG vaccination pre- or post infection with virulent *M. bovis*. With this in mind, the studies described in this report have been initiated to determine such responses and evaluate immune parameters that might be associated with the generation of a protective immune response. It is predicted that the development of additional tests and baiting strategies will rapidly lead to large scale field trials where the efficacy of vaccines can be tested in natural populations of badgers. These studies are expected to continue for several years and progress to the development of an oral vaccine.

Immunological responses of Eurasian Badgers (*Meles meles*) vaccinated with *Mycobacterium bovis* BCG

Introduction

The principal maintenance host for *Mycobacterium bovis* is the infected bovine animal. However, in Ireland and Britain, wildlife species such as badgers act as reservoir hosts for *M. bovis* and contribute to the spread and persistence of tuberculosis in associated cattle populations. Epidemiological evidence demonstrates a high prevalence of tuberculosis in badgers and controlled studies in Ireland involving comprehensive badger removal have shown that this strategy can serve to significantly reduce cattle reactor rates in the targeted areas. However, as the badger is a protected wildlife species, alternative strategies are required to combat the disease. Targeted vaccination of badgers against tuberculosis is an option which, if successfully employed, could directly facilitate the advancement of bovine tuberculosis eradication in affected areas. Until recently there were few reagents for evaluating badger immune responses directed against tuberculosis. An ELISA system (BrockTest) has since been developed which measures antibody responses to MPB83, a glycosylated lipoprotein which appears to be a major target of the antibody response in *M. bovis* infected badgers. A comparative lymphocyte transformation assay (LTA) has also been developed using bovine and avian tuberculin as the source of antigens to detect cell mediated responses in infected badgers. With the availability of such tests, the present study was carried out to determine to immune responses of a population of badgers vaccinated with *M. bovis* BCG and compare these responses with a control non-vaccinated population.

Methods & Results

The immunological responses of a group of badgers vaccinated subcutaneously with low doses of *Mycobacterium bovis* BCG were measured *in vitro* and compared with non-vaccinated control animals over a period of 42 weeks. When cultured with purified protein derivative (PPD-bovine), proliferation of peripheral blood mononuclear cells (PBMC) was detected in badgers which had received repeated booster injections of BCG. When the background response to PPD-avian was taken into consideration, the specific response to PPD-bovine by PBMC from vaccinated badgers was found to be significantly greater than that observed with control animals. In an ELISA system (Brock test), no animals showed seroconversion to the serodominant *M. bovis* antigen, MPB83. The results demonstrated that repeated vaccination of badgers with *M. bovis* BCG induced a specific population of T lymphocytes responsive to antigens in PPD-bovine.

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Development of an interleukin-2 cytokine assay to detect tuberculosis in badgers

Introduction

The dynamics of *M. tuberculosis/M. bovis* infection leading to disease are different to those of other bacteria causing acute infections of short duration. Following infection, progression to disease is relatively slow suggesting that during the early phase of infection the immune response is able to maintain the bacterial density at low numbers, and it is only at later stages, associated with disease, that the density increases to high levels. The outcome of the infection depends, in part, on the regulation of the $CD4^+$ T cell subsets, Th1 and Th2. In murine and bovine systems it has been demonstrated that the Th1 cells secrete IFN-γ, IL-2 and TNF and are associated with the acquired cell-mediated immune (CMI) response. Proliferation of antigen activated T lymphocytes following culture with PPD-bovine is mediated by production of IL-2 and when measured, is an indicator of a specific response to *M. bovis* infection.

Methods and Results

In this study we set out to investigate if badger lymphocytes produced interleukin-2 and whether we could use this as the basis for development of a diagnostic test. In a series of experiments, T lymphocytes from *M. bovis* infected badgers and control badgers were stimulated with PPD-bovine and conA (a non-specific stimulatory mitogen). After two days, the supernatants were removed and

added to fresh Con A stimulated blast lymphocytes that were isolated from badgers with no evidence of *M. bovis* infection. The results demonstrated that proliferation occurred only when the added supernatants were derived from the *M. bovis*-infected badgers and the conA stimulated samples. To further demonstrate that IL-2 was responsible for the stimulation, a monoclonal antibody derived from human IL-2 was added into the cultures. In the presence of this antibody, the proliferation of the T lymphocytes was inhibited. In parallel experiments, a monoclonal antibody derived from human IFN-γ was added to cultures. In this case, however, there was no effect on the proliferation of T lymphocytes.

Conclusions

These results demonstrated that (a) lymphocytes from *M. bovis* infected badgers produce IL-2 in response to stimulation by PPD-bovine, (b) commercially available monoclonal antibodies to human IL-2 are cross-reactive with badger IL-2 (c) the detection of IL-2 can be used as a diagnostic test for exposure to *M. bovis*.

Acute phase proteins as markers of inflammation in badgers

Introduction

The acute phase proteins play a major role in the inflammatory response during disease progression. They are produced by hepatocytes in response to activation by inflammatory cytokines. Haptoglobin and Serum Amyloid A (SAA) are two of a series of acute phase proteins that are found in the blood of both humans and other animals. Under normal conditions they are either absent from the blood or present at very low levels, depending on the species. However, their levels can increase significantly in response to acute infection, inflammation or trauma. The rise in serum haptoglobin or SAA and their continuous monitoring during the acute phase response can provides valuable information on the prevailing disease state. In this study, we set to determine if the acute phase proteins, haptoglobin and serum amyloid A (SAA) and other haematological markers (fibrinogen, haemoglobin, etc.) can be detected in the sera of badgers.

Methods & Results

Serum assays were carried out on samples taken from 19 badgers (Little Island) which are known to be TB-free, to measure the baseline levels of haptoglobin. In addition, sera was tested from 13 badgers captured in an area with endemic TB (south Louth). Haptoglobin was measured using a commercially available assay kit (Tridelta) which is non-species specific. Haptoglobin baseline levels were found to range from 0.17 - 3.36 mg/ml in 19 TB-free badgers. Haptoglobin levels ranged from 0.14 - 9.63 mg/ml in the 13 badgers captured in the TB endemic area. Three of these badgers had visible lesions at *post-mortem* and were culture positive. Two of these lesion positive badgers had the highest levels of haptoglobin, 5.32 and 9.63 mg/ml respectively. Haptoglobin levels in the other 11 badgers were less than 1.02 mg/ml. Therefore, it appears that haptoglobin is a potential inflammatory marker for infected badgers, though the assays need to be validated on a larger number of infected badgers at different stages of the disease process. Fibrinogen, SAA and other haematological markers will be measured in the next series of experiments.

Evaluation of a bait-marking system for badgers (*Meles meles*) using Sulfadimethoxine and Rhodamine B as biomarkers

Introduction

The use of biomarkers play an important role in the evaluation of bait uptake in field studies assessing oral vaccine strategies. The technique relies on using non-toxic biomarker substances incorporated into baits to 'mark' the animals that consume the baits. In a previous study (Southey and *et al.*, 2000), we showed that iophenoxic acid and tetracycline were effective biomarkers for determining the bait contact status of individual badgers. However, analysis of the data obtained suggested that they are unsuitable for large-scale use in vaccine delivery programmes; iophenoxic acid is expensive and blood samples must be irradiated prior to analysis. In addition, measurement of uptake of tetracycline only provides a retrospective record of bait uptake as it is based on *post-mortem* examination of sectioned teeth samples. In the present study we set out to evaluate the potential usefulness of sulfadimethoxine (SDM) and rhodamine B as biomarkers for live badgers. SDM is a broad-spectrum anti-microbial agent that has previously been evaluated as a serum marker for rabies vaccine bait consumption by racoons and dogs. The duration of marking with SDM appears to be short-term normally persisting in the blood stream for up to 7 days following delivery. However, SDM has the advantage that it can be easily detected and qualitatively measured using fresh blood samples and a commercially available rapid card test. Alternatively, it can be quantitatively measured in a laboratory-based ELISA test.

Rhodamine B is an intense fluorescent dye detectable in hair samples with the unaided eye at high concentrations and under UV light at low concentrations. It has previously been used as a topical marker of bait consumption in animals such as dogs and coyotes (*Canis latrans*). It has been reported that dogs stain bright red on the tongue, oral mucosa, mouth, nose and other parts of the body after contacting baits containing rhodamine B. Systemic marking by rhodamine B has also been observed in coyotes and beavers (*Aplodontia rufa*). In these animals, systemic marking is characterised by UV-fluorescent deposits in the hair and claws, which persists for several months following rhodamine B consumption.

In this study we conducted a field experiment in which badger groups were exposed to baits containing SDM and rhodamine B. The aims of the study were to assess the palatability and acceptance of biomarked-baits by badgers, and to investigate test systems for measuring SDM and rhodamine B in serum and hair samples, respectively.

Methods and Results

A field study was carried out on Little Island (Co. Waterford) in June 2000 to evaluate the potential of a bait-marking system for use in badgers. Two biomarkers, sulfadimethoxine (SDM) and rhodamine B were incorporated into oral baits and distributed at main setts in five test territories for three consecutive days. In parallel, non-biomarked baits were distributed at a single control territory. The objectives of the study were to (1) assess the effects of SDM and rhodamine B on palatability and bait acceptance, (2) investigate the marking capacity of SDM and rhodamine B in serum and hair samples taken from badgers. Trapping was subsequently carried out in each territory for five consecutive days. Analysis of the data revealed that 90-100% of baits were removed in four of the test territories and from the control territory. In the fifth test territory, 61% of baits were removed. Of the badgers (n=26) trapped in the test territories, 18 (69%) were positive when tested for both biomarkers. In contrast,

the remaining eight animals and those captured in the control territory (n=6 badgers) were negative. In the biomarked animals, the highest levels of SDM were recorded in serum samples taken early after bait distribution. Thereafter, the levels declined in each badger over the course of the study. In contrast, rhodamine B was readily detectable by fluorescence microscopy of hair samples throughout the period of study. The results indicate that SDM and rhodamine B act as systemic markers in badgers and have potential future applications for monitoring of oral vaccine uptake.

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Survival of *M. bovis* BCG under partial anaerobiosis

Introduction

When *M. tuberculosis / M.bovis BCG* is rapidly shifted from an aerobic to an anaerobic environment, bacterial replication ceases and cell viability is severely restricted. Bacterial death occurs from about 24 hours and within days >90% of cells are non-culturable. The development of a BCG-based oral vaccine strategy may require survival of immobilised BCG in a semi-solid matrix for up to six months. It is likely that during this period of time, the bacterial cells will be subjected to partial anaerobiosis. Many bacterial species, including mycobacteria have evolved metabolic pathways that allow for survival in a limited oxygen environment (the Krebs cycle). The substrates (e.g. pyruvate) produced by this pathway can then be used in the citric acid cycle to produce ATP, the energy currency of the cell. The efficiency of these reactions can vary depending on the nutrients available to the bacteria. It is feasible that specific nutrients can be incorporated into a growth medium that will allow for long-term survival of mycobacteria under partial anaerobic conditions.

Objectives

To evaluate the survival time of BCG when shifted from aerobic to partial anaerobic growth conditions. Evaluate different growth substrates and nutrients that will enhance viability of BCG in baits.

Experimental Plan

1. Grow *M. bovis* BCG in liquid medium (7H9) and transfer to medium containing gelatine.
2. Incubate cultures at 4°C and room temperature.
3. Determine CFU's at T_0, T_0, T_{2weeks}, T_{4weeks} and T_{8weeks}.

Construct survival curves for BCG.

Complimentary projects (Ongoing)

Development of an *M. bovis* infection model in captive badgers.

Development of novel assays e.g. IFN-γ and IgA ELISA, as diagnostic tools for *M. bovis* infection in badgers (VLA, Weybridge).

Detection and distribution of *M. bovis* and related mycobacteria in environmental samples (Prof. Liz Wellington, Warwick University).

Detection of *Neospora spp.* (Grace Mulcahy, UCD), and trypanosome immunology (Dr Paul Voorheis, TCD) in badgers.

Comparative histopathology of tissues from *M. bovis* infected mammals (Prof. Ian Orme, Colorado).

Evaluation of T cell antigen SA-5k as a diagnostic reagent for *M. bovis* infection (Dr Giovanna Batoni, University of Pisa)

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