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Oral vaccination of badgers (*Meles meles*) against tuberculosis: Comparison of the protection generated by BCG vaccine strains Pasteur and Danish

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ABSTRACT

Vaccination of badgers by the subcutaneous, mucosal and oral routes with the Pasteur strain of *Mycobacterium bovis* bacille Calmette-Guérin (BCG) has resulted in significant protection against experimental infection with virulent *M. bovis*. However, as the BCG Danish strain is the only commercially licensed BCG vaccine for use in humans in the European Union it is the vaccine of choice for delivery to badger populations. As all oral vaccination studies in badgers were previously conducted using the BCG Pasteur strain, this study compared protection in badgers following oral vaccination with the Pasteur and the Danish strains.

Groups of badgers were vaccinated orally with 10^8 colony forming units (CFU) BCG Danish 1331 ($n = 7$ badgers) or 10^8 CFU BCG Pasteur 1173P2 ($n = 6$). Another group ($n = 8$) served as non-vaccinated controls. At 12 weeks post-vaccination, the animals were challenged by the endobronchial route with 6×10^3 CFU *M. bovis*, and at 15 weeks post-infection, all of the badgers were euthanased. Vaccination with either BCG strain provided protection against challenge compared with controls. The vaccinated badgers had significantly fewer sites with gross pathology and significantly lower gross pathological severity scores, fewer sites with histological lesions and fewer sites of infection, significantly lower bacterial counts in the thoracic lymph node, and lower bacterial counts in the lungs than the control group. No differences were observed between either of the vaccine groups by any of the pathology and bacteriology measures. The ELISPOT analysis, measuring production of badger interferon – gamma (IFN-γ), was also similar across the vaccinated groups.

Introduction

Tuberculosis (TB) caused by *Mycobacterium bovis* is present in a range of wildlife species worldwide (Corner, 2006) and the badger (*Meles meles*) has been identified as the principle wildlife reservoir of the disease in Ireland and in the UK (Griffin et al., 2005; Donnelly et al., 2007). As part of a strategy to control infection in this wildlife species, vaccination offers a means to reduce the incidence of infection in the population, thereby reducing the risk of transmission to cattle (Gormley and Costello, 2003).

Currently the only available vaccine against TB is the attenuated *M. bovis* strain bacille Calmette-Guérin (BCG) developed by serial passage of a virulent *M. bovis* isolate from a mastitic cow between 1908 and 1921 (Behr and Small, 1999). From 1924 onwards this strain was distributed by the Institut Pasteur to other institutes worldwide and all current BCG strains are derivatives of this initial single strain. At the Institut Pasteur, BCG (the strain now referred to BCG Pasteur) was maintained by serial passage until lyophilisation after 1173 passages in 1961 (Behr and Small, 1999). The Statens Serum Institut (Denmark) obtained a BCG strain (passage 423) in late 1931. In 1960, passage 1331 was freeze-dried, and in 1966 adopted as the primary seed lot (Oettinger et al., 1999). The BCG Danish 1331 strain is currently the only commercially available BCG vaccine licensed for use in humans in the European Union.

In 1994 a joint WHO/FAO/OIE consultative group recommended that BCG Pasteur strain 1173P2 should be used in animal vaccine efficacy studies (WHO, 1994). Since then this strain has been used successfully in vaccine studies in domestic cattle (*Bos taurus*), red deer (*Cervus elaphus*), ferrets (*Mustela furo*), brushtail possums (*Trichosurus vulpecula*), white-tailed deer (*Odocoileus virginianus*) and

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badgers (*Meles meles*) (Aldwell et al., 1995a, 1995b; Buddle et al., 1995; Griffin et al., 1999; Qureshi et al., 1999; Palmer et al., 2007; Corner et al., 2008). Under conditions of natural transmission of infection, the BCG Pasteur 1173P2 vaccine was shown to induce significant levels of protective immunity and also prevented infection in wild possum populations (Corner et al., 2002; Tompkins et al., 2009). In a comparative study of BCG strains, the BCG Danish 1331 and BCG Pasteur 1173P2 vaccine strains induced similar levels of protection against virulent *M. bovis* challenge in possums and cattle (Wedlock et al., 2007). In badgers, the BCG Pasteur 1173P2 strain has been shown to be protective when delivered by a variety of routes including subcutaneous injection, deposition on mucosal surfaces via the nasal passages and conjunctiva and oral cavity (Corner et al., 2008, 2010). Where the BCG Danish 1331 strain has been used in captive and wild free-ranging badgers and delivered by the intramuscular route, it has been found to be safe and to induce protective immunity (Lesellier et al., 2006; Chambers et al., 2011).

The long term option of vaccination by the oral route is the only likely practical strategy for targeting badger populations on a cost-effective basis. To expedite the development of a licensed oral vaccine it is probable that the BCG Danish strain will be used. As all previous oral vaccination studies in badgers were conducted with the BCG Pasteur strain, we have compared protection in badgers following oral vaccination with the Pasteur and the Danish strains. We assessed the level of protection by endobronchial challenge with *M. bovis* and compared the vaccine strains by measuring the severity of infection using the following six parameters: (1) number of sites with gross lesions; (2) sum of gross lesion severity scores; (3) the number of sites with histological lesions; (4) the number of sites positive for *M. bovis* infection by culture; (5) the bacterial load in thoracic lymph nodes (LN) and (6) the bacterial load in the lungs. The immunological responses (interferon gamma responses) of vaccinated and non-vaccinated badgers were also compared throughout the present study.

Materials and methods

This study was carried out under licences (B100/3187) issued by the Department of Health and Children, and ethical approval was obtained from the University College Dublin (UCD) Animal Research Ethics Committee (AREC-P-04–28).

Badgers

The 21 badgers used in the study were taken from a TB-free wild population and were negative to the badger interferon (IFN)-γ ELISPOT (AHVLA) for tuberculosis prior to the start of the study. The badgers were housed and fed as previously described (Corner et al., 2010). For handling, the badgers were anaesthetised with ketamine hydrochloride (10 mg/kg, Vetalar, Boehringer Ingelheim) and medetomidine hydrochloride (0.1 mg/kg, Domitor, Pfizer) co-administered by intramuscular injection. The badgers were examined at 3 weeks before vaccination, at 0, 5 and 12 weeks post-vaccination, and 6, 10 and 15 weeks after challenge. On each occasion the badgers were weighed and examined for signs of disease or injury, blood was collected by jugular venepuncture and in the post challenge period urine, faeces and a pharyngeal swab were collected as previously described (Corner et al., 2010).

All badgers were allocated to pens based on their social group of origin; badgers were also allocated to treatments based on pen such that each group had a similar distribution of yearling, adult and old badgers. There were three treatment groups: Group 1 received 10^8 colony forming units (CFU) BCG Pasteur 1173P2 ($n = 6$) and Group 2 received 10^8 CFU BCG Danish 1331 ($n = 7$). Both vaccines were presented in an oral lipid formulation comprised of a plant derived lipid (Aldwell et al., 2003). The third group of badgers served as non-vaccinated controls ($n = 8$).

Oral vaccine formulation and vaccination

BCG Pasteur 1173P2 and BCG Danish 1331 were grown and the oral lipid vaccine (Liporale-BCG) was prepared as previously described (Ancelet et al., 2012). Briefly, BCG was grown to mid-log phase in 7H9 liquid medium supplemented with albumin–dextrose–catalase. Bacilli were harvested by centrifugation and washed twice in phosphate buffered saline (PBS) prior to storage at –70 °C. The number of CFU in the frozen suspension of BCG was determined by plating on 7H11 agar plates. The BCG was thawed and suspended in the lipid formulation, the lipid having been liquefied by warming to 37 °C. The lipid-BCG suspension was homogenised by repeated inversion, transferred to 10 mL syringes and with continued gentle mixing allowed to solidify at 4 °C.

For administration, the syringes containing vaccine were warmed to 37 °C, attached to a 22 cm long (5 mm outside diameter, 3 mm internal diameter) plastic cannula which was introduced 5 cm into the oesophagus and 1 mL of vaccine was deposited in the oesophagus. The viable count of BCG in the inoculated vaccine was determined retrospectively.

M. bovis *suspension and experimental infection*

M. bovis strain M2137 was originally isolated from a tuberculous badger. The suspension for inoculation was prepared as previously described (Corner et al., 2010) except that the frozen stock suspension was thawed and incubated for 3 days in 7H9 liquid broth, and then diluted with PBS to the required concentration.

At 12 weeks post-vaccination, all badgers were challenged with *M. bovis* by endobronchial inoculation (Corner et al., 2010). For infection the badgers were anaesthetised, a 3.5 mm endoscope was passed per os into the trachea to the level of the tracheal bifurcation and then into the bronchus of the right middle lung lobe. When in place a small cannula (1.5 mm outside diameter with a dead space of 0.7 mL) was passed down the endoscope, deeper into the bronchus and 1.0 mL of the *M. bovis* suspension was inoculated and the cannula flushed with 1.5 mL of sterile PBS. The badgers were immediately placed in right lateral recumbency and allowed to recover. The suspension inoculated was found to contain *M. bovis* at a concentration of 6×10^3 CFU/mL.

Post-mortem examination

The badgers were euthanased with an intravenous overdose of sodium pentobarbitone at 15 weeks post infection and were subjected to a detailed post-mortem examination. Tissue samples were collected for culture and histopathology as previously described except that tonsils were not collected (Corner et al., 2010). Tracheal and pharyngeal swabs were also collected during the post-mortem examination. Gross lesions were confirmed by culture or had the histological features consistent with tuberculosis. The severity of the gross lesions in lymph nodes and organs were scored according to a scheme described previously and individual scores for lesions were added to generate an overall pathology score (Corner et al., 2007).

Specimens for histopathology were fixed in 10% buffered formalin, sectioned at 3 μm and stained with haematoxylin and eosin for tissue architecture, and by the Ziehl–Neelsen method for acid-fast organisms. Histopathological examination was carried out to detect tuberculous granulomas containing acid-fast bacilli (Gavier-Widen et al., 2001).

Bacterial culture

All samples were cultured separately as described previously (Corner et al., 2007). All tissue samples were weighed before culture so that the concentration of *M. bovis* in the sample could be calculated (CFU/g). Except for lung, urine, faeces and pharyngeal swabs, all other samples were cultured without decontamination. Samples of lung, urine and pharyngeal swabs were decontaminated with 0.075% w/v cetylpyridinium chloride (CPC) and faeces with 0.75% w/v CPC prior to culture. Where primary cultures of tissue samples were contaminated, a stored portion of the macerated sample was decontaminated with 0.075% w/v CPC and media re-inoculated. The mean number of colonies on the 7H11 plates was used to calculate the concentration of *M. bovis* in the sample. Preliminary isolate identification was based on colony morphology, growth rate, pigmentation and cording characteristics, and by the AccuProbe DNA hybridisation probe (Gen-Probe) for the *M. tuberculosis* complex. Isolates were confirmed as *M. bovis* by spoligotyping (Kamerbeek et al., 1997).

Immunological assays

The badger IFN-γ ELISPOT was carried out as described previously (Lesellier et al., 2006). Briefly, peripheral blood mononuclear cells (PBMC) were isolated from whole blood and added to wells on 96-well plates coated with purified protein derivative from *M. bovis* (PPD-B), Con-A or not coated. The spots on the plates were counted using an automated AID ELISPOT reader (Autoimmun Diagnostika) and the net spot forming units (SFU) calculated.

Statistical analysis

Statistical analyses were performed using Prism version 6.00 for Mac OS X (GraphPad Software). Data were tested for normality using D'Agostino and Pearson omnibus normality test. Differences between groups in disease severity parameters and immune responses were compared using non-parametric Kruskal–Wallis test with post hoc testing of selected pairwise comparisons by Dunn's multiple comparison test, or Mann–Whitney test. Significance for all statistical analyses was set at $P < 0.05$, and adjusted where multiple comparisons were performed. Pathology scores and ELISPOT data were compared using the Spearman correlation coefficient r_s.