Vaccine 29 (2011) 3782–3790

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Protection of Eurasian badgers (*Meles meles*) from tuberculosis after intra-muscular vaccination with different doses of BCG

Sandrine Lesellier[a], Si Palmer[a], Sonya Gowtage-Sequiera[a], Roland Ashford[a], Deanna Dalley[a], Dipesh Davé[a], Ute Weyer[b], F. Javier Salguero[c], Alejandro Nunez[c], Timothy Crawshaw[d], Leigh A.L. Corner[e], R. Glyn Hewinson[a], Mark A. Chambers[a,*]

[a] TB Research Group, Department of Bacteriology, Veterinary Laboratories Agency Weybridge, Woodham Lane, New Haw, Addlestone, Surrey KT15 3NB, UK
[b] Animal Services Unit, Veterinary Laboratories Agency Weybridge, New Haw, Addlestone, Surrey KT15 3NB, UK
[c] Department of Pathology, Veterinary Laboratories Agency Weybridge, New Haw, Addlestone, Surrey KT15 3NB, UK
[d] Veterinary Laboratories Agency Starcross, Staplake Mount, Starcross, Exeter EX6 8PE, UK
[e] School of Agriculture, Food Science and Veterinary Medicine, University College Dublin, Dublin 4 Belfield, Ireland

ARTICLE INFO

Article history:
Received 10 November 2010
Received in revised form 4 March 2011
Accepted 8 March 2011
Available online 7 April 2011

Keywords:
Wildlife
Vaccination
Tuberculosis
Pathology
Immunology

ABSTRACT

Mycobacterium bovis infection is widespread in Eurasian badger (*Meles meles*) populations in Great Britain and the Republic of Ireland where they act as a wildlife reservoir of infection for cattle. Removal of infected badgers can significantly reduce the incidence of bovine tuberculosis (TB) in local cattle herds. However, control measures based on culling of native wildlife are contentious and may even be detrimental to disease control. Vaccinating badgers with bacillus Calmette-Guerin (BCG) has been shown to be efficacious against experimentally induced TB of badgers when administered subcutaneously and orally. Vaccination may be an alternative or complementary strategy to other disease control measures. As the subcutaneous route is impractical for vaccinating wild badgers and an oral vaccine bait formulation is currently unavailable, we evaluated the intramuscular (IM) route of BCG administration. It has been demonstrated that the IM route is safe in badgers. IM administration has the practical advantage of being relatively easy to perform on trapped wild badgers without recourse to chemical immobilisation. We report the evaluation of the efficacy of IM administration of BCG Danish strain 1331 at two different doses: the dose prescribed for adult humans ($2–8 \times 10^5$ colony forming units) and a 10-fold higher dose. Vaccination generated a dose-dependent cell-mediated immune response characterised by the production of interferon-γ (IFNγ) and protection against endobronchial challenge with virulent *M. bovis*. Protection, expressed in terms of a significant reduction in the severity of disease, the number of tissues containing acid-fast bacilli, and reduced bacterial excretion was statistically significant with the higher dose only.

1. Introduction

The Eurasian badger (*Meles meles*) was first identified as a wildlife host for *Mycobacterium bovis* in Great Britain in 1971 [1]. Since then, epidemiological studies have demonstrated that *M. bovis* infection is widespread in badger populations in Great Britain and the Republic of Ireland [2,3] and that infected badgers excrete *M. bovis* [4], potentially contaminating the environment [5].

Efforts to eradicate tuberculosis (TB) from cattle in these two countries have been thwarted where badgers are a reservoir of *M. bovis* leading to continued re-infection of herds. Removal of infected badgers can significantly reduce the incidence of TB in local cattle herds [6,7] but the effects are variable and may even be detrimental to disease control for a time. To be beneficial, culling must be comprehensive and sustained over relatively large areas [8]. Control of livestock diseases based on culling of native wildlife is contentious.

Vaccinating badgers may be an alternative or complementary strategy to overcome some of the problems associated with culling [9]. *M. bovis* strain BCG vaccination confers a degree of protection to captive badgers against experimental challenge with *M. bovis* [10–12]. In these studies, BCG was delivered either subcutaneously (SC), orally to the back of the throat, or via a combined intranasal/conjunctival route. However, each of these approaches is currently impractical for delivery to wild badgers. For reliable SC vaccination animals would need to be anaesthetised, and whilst the combined intranasal/conjunctival route demonstrated that mucosal vaccination might be feasible, research on oral bait vaccine formulation and the means for delivery to badger populations over large areas has yet to be realised. BCG vaccination

* Corresponding author. Tel.: +44 01932 357494; fax: +44 01932 357260.
E-mail address: m.a.chambers@vla.defra.gsi.gov.uk (M.A. Chambers).

0264-410X/$ – see front matter.
doi:10.1016/j.vaccine.2011.03.028

via the intramuscular (IM) route has not previously been considered, largely because the route is associated with adverse effects in humans [13]. However, in badgers IM administration of BCG is both safe and of equivalent immunogenicity to SC BCG [14] and has the practical advantage of being easily administered to trapped wild badgers without recourse to chemical immobilisation. We recently published the results of the first studies with both captive and wild badgers using IM administration of BCG [15].

Here we describe in more detail the nature of protection against TB induced in badgers by the IM administration of BCG vaccine at two different doses: the dose prescribed for adult humans ($2–8 \times 10^5$ colony forming units (CFU)) and a 10-fold higher dose, which was shown by us to be more immunogenic in captive badgers (unpublished findings) and confer protection against challenge with *M. bovis* [15]. We report that BCG Danish strain 1331, manufactured for use in humans, generated a strong cell-mediated immune response in badgers characterised by the production of interferon-γ (IFNγ) and protection against challenge with virulent *M. bovis*. In the two experiments undertaken, protection was seen as a significant reduction in the severity of disease, the number of tissues containing acid-fast bacilli, and reduced bacterial excretion. Protection was dependent on BCG dose with the lower dose providing less protection than a 10-fold higher dose.

2. Materials and methods

2.1. BCG vaccine

BCG Danish strain 1331 vaccine was supplied by the Statens Serum Institut (SSI), Denmark at $2–8 \times 10^6$ CFU per vial. The higher dose (HD) of vaccine was prepared by adding 1 ml of Sauton diluent (provided by SSI) to each vial. The lower dose (LD) was prepared by diluting 1/10 one HD vial into Sauton diluent. Sufficient vaccine was reconstituted and vials pooled in order to provide a uniform titre of the higher BCG dose for all animals. At the end of each day's vaccination session, residual vaccine was cultured on modified Middlebrook 7H11 agar plates to determine the viable count of the vaccine (Table 1).

2.2. Mycobacterium bovis

The *M. bovis* strain used for challenge was isolated in 1997 from an infected wild badger in the UK (isolate 74/0449/97). It had been stored as a first passage stock culture until expanded and stored as frozen aliquots (−80 °C) for badger infection studies at the VLA. The clonality of the culture was confirmed by demonstrating the spoligotype – SB0140 (VLA type 9) and VNTR type (8-5-5-5-3-3.1) of 27 individual colonies (10% of those grown from a 1:1000 dilution of a culture at $\sim 10^5$ CFU/ml). The stock vials used for the challenge had not been passaged further. Each vial contained $\sim 10^7$ CFU/ml viable *M. bovis*. On the day of challenge, one aliquot was thawed and serially diluted in sterile water + 0.05% (v/v) tween 80 to contain approximately 5×10^3 CFU/ml. The dilutions were vortexed immediately before challenge to diminish the risk of bacterial clumping in the inoculum. The final dilution (challenge inoculum) was made in sterile PBS + 0.05% tween 80. The titre of the challenge inoculum was determined by plating a sample from a syringe kept in the same conditions as those used for challenge on Middlebrook 7H11 agar. In each experiment, infection of the badgers was conducted over two consecutive days and the inoculum used for the second day was kept at 4 to 8 °C over night. The viable count of the inoculum was determined for each day (Table 1).

2.3. Animals and sampling

The first experiment (VES1) involved five adult badgers and the second experiment (VES2) 18 adult badgers (Table 1). The animals were sourced from a county of England with no reported cases of TB in badgers and very few cases of TB in cattle. The badgers were housed in groups of up to four animals, corresponding as close as possible to their originating social group at capture. Badgers were identified by subcutaneous microchip with a unique number (AVID PLC, Lewes, UK) and a cutaneous tattoo. After capture, the badgers were tested for TB at approximately one month intervals using the IFNγ enzyme immunoassay (EIA) [16] and culture for *M. bovis* from clinical samples (tracheal aspirate, laryngeal swab, urine and rectal swab) cultured for 12 weeks. Each animal was deemed to be TB-free on the basis of three consecutive negative results to both tests, spanning a total period of 24–25 weeks.

Each group of badgers was housed in open-air pens of approximately 50 m^2 with large wooden boxes provided as artificial setts. Objects, such as concrete tunnels and wooden pallets, were placed in the pens for environmental enrichment. The badgers received a diet of dog food, peanuts and occasionally eggs. Tap water was supplied *ad libitum* in large trays that allowed both bathing and drinking. Food uptake, weight variations, and blood chemistry and haematology parameters (as described previously [14]) were used to monitor their welfare. The badgers were moved to an Advisory Committee on Dangerous Pathogens (ACDP) Containment Level 3 facility 5–6 weeks before challenge.

Once every 2–3 weeks, the badgers were anaesthetised and examined. Blood was collected by jugular venipuncture into Vacutainer tubes (BD, Plymouth, UK) for immunological, haematological and biochemical analyses. Tracheal mucus was collected by aspirating with an 8Fx50 cm flexible urinary catheter (Arnolds Veterinary Products, Shrewsbury, UK) and dispensed into 3.5 ml Middlebrook 7H9 broth. Laryngeal and rectal swabs were collected and placed into 3.5 ml 7H9 broth and 5 ml PBS, respectively. Urine was collected into sterile 15 ml plastic tubes following compression of the bladder.

Table 1
Vaccination of badgers with BCG: number and sex ratio of animals in each treatment group, and the vaccine and challenge doses.

Experiment	Treatment group[a]	Dose of BCG (CFU[b]/ml)	Dose of *M. bovis* (CFU/ml)	Number of badgers	Sex ratio (M:F)
VES1	HD BCG	5.4×10^6	4.8×10^3	3	1:2
	Non-vaccinated	NA	4.8×10^3 3.7×10^3	1 1	1:1
VES2	HD BCG	3.2×10^6	2.6×10^3 2.8×10^3	4 2	4:2
	LD BCG	3.3×10^5	2.6×10^3 2.8×10^3	4 4	5:3
	Non-vaccinated	NA	2.8×10^3	4	3:1

[a] HD = high dose; LD = low dose.
[b] CFU = colony forming units.

Table 2
Distribution and severity score of visible lesions.

Tissue[a]	Control						HD BCG									LD BCG							
	C037	C029	D118	D123	D547	D811	C071	C067	C094	D101	D313	D546	D564	D591	D862	D126	D264	D304	D343	D346	D619	D779	D816
L cranial lobe											3								1			3	
L caudal lobe											2					1						2	
R cranial lobe			1			2					2								3			3	3
R middle lobe		4	3	4	4	3	2	1		1	3		1	1	1		3	3	3	3	1		4
R caudal lobe	4		1		1	2					3	1				2			3			2	1
Accessory lobe		2						1			2		1									1	
L bronchial	1	1							2							1							
R bronchial	3	3	3	3	4	4	2	2		2	4	2	2	2	1	1	2	2	1	4	2	4	2
Post med	3	1	2		4	4			1		1		1	1		1			1		1		
Mediastinum	2	1				2	1	1			4		2		1	1			4	3	2	2	4
HLN	2																		1				
MLN											1												
Spleen					1															1			
Liver											2												

[a] Post med = posterior mediastinal (LN), HLN = hepatic LN, MLN = mesenteric LN. Other tissues examined but without visible lesions = anterior mediastinal LN, left and right mandibular, parotid, retropharyngeal, axillary, and inguinal LNs, tonsils, heart, and kidney.

Table 3
Distribution and number of CFU (log_{10} rounded up to whole number) per gram of tissue from which *M. bovis* was isolated by culture.

Tissue[a]	Control						HD BCG									LD BCG							
	C037	C029	D118	D123	D547	D811	C071	C067	C094	D101	D313	D546	D564	D591	D862	D126	D264	D304	D343	D346	D619	D779	D816
L cranial lobe		1	1								2											2	
L caudal lobe											3					<1						1	
R cranial lobe		2	1			3					3								2			5	2
R middle lobe		6	5	5		4	3	4		3	4		4	3	4	2	4	4	5	5	3		4
R caudal lobe	4		3		3	6		1			4	4							2			1	3
Accessory lobe	3	2				3		3			2		1									2	
L bronchial	3	5		3		3			6		3					3			3				
R bronchial	4	5	5	5	5	6	5	5		5	5	5	4	5	5	4	5	5	5	6	4	5	5
Ant med		3				5																	
Post med	4	4	4		3	5	4	4	5		5	4	4	4		5		3	4	4	4		5
L parotid						4																	
R parotid						3																	
L&R axillary		3				3														3			
L&R pop											2												
Mediastinum	4	5		2		4	3	2		4	4		1		3	5		3	4	3	4	3	4
HLN	3	3	2	4	4	5	2		2	4	5	4	4		3	4	4	3	4	4	4	4	4
MLN		2				3					3												
Spleen	1		1		2	2					3						2		2	1		1	
Liver						3					3	2	1						2	2			3
Heart																							4

[a] Post med = posterior mediastinal (LN), HLN = hepatic LN, MLN = mesenteric LN. Other tissues cultured but not positive for *M. bovis* = left and right mandibular, retropharyngeal, and inguinal LNs, tonsils, and kidney.

Table 4
Tissues that contained acid-fast bacilli (AFB) on histology (marked with a cross).

Tissue[a]	Control						HD BCG							LD BCG						
	C037	C029	D118	D123	D547	D811	C071	C067	C094	D101	D313	D546	D564	D591	D862	D126	D264	D304	D343	D346	D619	D779	D816
L cranial lobe											X								X				
L caudal lobe						X					X												
R cranial lobe			X	X		X	X				X								X			X	X
R middle lobe	X	X	X	X	X	X	X				X		X	X	X			X	X	X			X
R caudal lobe	X					X					X					X	X	X					
Accessory lobe			X					X			X							X					
L bronchial									X														
R bronchial	X	X	X	X	X	X	X	X		X		X	X	X	X	X	X	X	X	X	X	X	X
Ant med						X																	
Post med	X		X			X		X			X					X		X		X		X
R mandib						X																	
HLN	X		X	X	X	X					X	X					X		X	X	X	X	X
Liver											X												

[a] Post med = posterior mediastinal (LN), HLN = hepatic LN, MLN = mesenteric LN. Other tissues examined histologically but without AFB found = left mandibular LN, left and right parotid, retropharyngeal, axillary, inguinal, and popliteal LNs, tonsils, spleen, heart, and kidney.

2.4. Vaccination

All badgers of a group received the same treatment and groups were allocated to treatments by randomisation. The vaccine was injected in the left lumbar muscle, following shaving and cleaning of the overlying skin. All vaccinated animals received 1 ml of vaccine that had been reconstituted for less than 4 h (as per SSI recommendations). In VES1 only high dose vaccine was used and in VES2 both high and low dose was used (Table 1).

2.5. Experimental infection with M. bovis

Seventeen weeks post-vaccination, all badgers were infected under anaesthesia, by endobronchial instillation of 1 ml of *M. bovis* suspension using a 70 cm fibroscope (Olympus UFR-P2, 3.6 mm × 1.2 mm canal) and targeting the bronchus of the right middle lobe. The *M. bovis* suspension was inoculated via a sterile plastic catheter (1 mm × 1 m) (approx. 0.8 ml dead volume) and the catheter was flushed with 1 ml PBS. Between animals the fibroscope was disinfected with ortho-phthalaldehyde (Cidex-OPA®) and 70% ethanol then rinsed with sterile water.

2.6. Postmortem examination

Twelve weeks after challenge (29 weeks after vaccination), the badgers were killed humanely with an intravenous overdose of sodium pentobarbitone and promptly subjected to postmortem examination. A detailed gross examination of the carcasses for visible lesions was undertaken by experienced pathologists and a pre-determined set of 27 tissues collected using a separate set of sterile instruments for each tissue. The collected tissues were examined for visible lesions by fine slicing. A lesion severity score was derived using a standardised ordinal scoring system of 1–4 (few foci or slight swelling to extensive caseation or areas of coalesced foci) [17,18]. All staff involved in the postmortem examination were blinded to treatment allocation. An overall disease severity score based on the number, size, and distribution of visible lesions was derived from the sum of the highest scoring lung lobe plus the scores from all other tissues. Only visible lesions subsequently confirmed as tuberculous by either isolation of *M. bovis* from the tissue by culture or the appearance of AFB in ZN stained histological sections counted towards the final disease severity score. Each lymph node (LN) was divided for histology and culture, and for the larger organs such as spleen, approx. 3 cm^3 of tissue was submitted for culture and the remainder for histology. Histologically, a TB lesion consisted of one or more granulomas containing acid-fast bacteria (AFB) in Ziehl-Neelsen (ZN) stained sections.

2.7. Culture of M. bovis

Clinical samples (tracheal aspirate, laryngeal swab, urine and rectal swab) were taken every 2–3 weeks from the day of challenge to detect *M. bovis* excretion. All samples were cultured on the day of collection, except for faecal swabs which were cultured on the following working day. The laryngeal swabs and tracheal aspirates in 7H9 broth were inoculated onto six Middlebrook 7H11 slopes and incubated for 12 weeks at 37 °C. Except for faecal swabs, the samples were not decontaminated before culture. Faecal swabs were soaked overnight in 0.85% saline solution. The following day the swab was discarded and the saline solution decontaminated with 5% final volume oxalic acid for 10 min at room temperature. Material for sowing onto Middlebrook 7H11 slopes was retrieved by centrifugation and the oxalic acid removed by a wash step using saline. A sample of positive cultures was identified by spoligotyping [19] and VNTR [20] to confirm that the isolates were the same as the challenge strain.

Tissue samples collected at postmortem were collected aseptically, weighed and frozen at −20 °C on the day of collection. Tissues for culture were thawed to room temperature and each tissue sample was cultured separately. Tissues were homogenised in 10 ml 0.85% saline using IKA® tubes. Samples (100 μl) of tissue homogenate were plated onto each of four plates of Middlebrook 7H11 and each of four plates of Middlebrook 7H11 containing 60 μg/ml cycloserine [21,22] to preferentially select for BCG growth. Homogenates for Middlebrook 7H11 were diluted 1/10, 1/100 and 1/1000 to allow for bacterial enumeration. If contamination occurred, stored homogenate was re-cultured without additional decontamination. Plates were examined after 6 and 12 weeks of incubation. Up to 20 colonies from one plate per tissue were typed by spoligotyping and VNTR.

2.8. IFNγ ELISPOT assay

The kinetics of the immune responses was monitored by measuring the frequency of peripheral blood mononuclear cells (PBMC) producing IFNγ by ELISPOT assay [14]. Antigens used to stimulate PBMC cultures were PPD-B and a mixture of ESAT-6 and CFP-10. The mitogen concanavalin A was used as a positive control.

2.9. Data analysis

Analyses of disease severity scores, visible lesions, culture and histology data were compared using the non-parametric Kruskal–Wallis test and significant differences ($p < 0.05$) further investigated using Dunn's test for multiple pair wise comparisons. A parametric approach to analysis using a general linear model was initially explored to take account of the data arising from two studies and testing for treatment group and study factors. This did indicate no differences between studies but in view of the sample sizes, data not being normally distributed and data recorded on an ordinal scale it was considered necessary to adopt the rank score approach. Comparisons of proportions positive within groups were carried out using Fisher's exact probability test.

Analyses were undertaken using NCSS 2007 Statistical Software (NCSS, Utah, USA) and Minitab Version 15 (Minitab, Coventry, UK).

3. Results

The *M. bovis* challenge strain was recovered from all badgers. Tables 2–4 present for each animal details of where visible lesions were observed, which tissues were culture positive for *M. bovis* and which tissues contained histological lesions.

3.1. Tuberculous lesions postmortem

Tissues were divided to provide samples for the two confirmatory tests and so lesions may not have been equally represented in the two samples. Hence a tissue was deemed diseased and visible lesions "confirmed" if they were either culture positive or histologically positive. Table 2 presents the number of visible lesion sites and their severity scores. Analysis revealed significant differences between treatments with respect to the disease severity score (Fig. 1A, Table 5): non-vaccinated vs HD BCG ($p < 0.05$); LD BCG vs HD BCG ($p < 0.05$); non-vaccinated vs LD BCG ($p > 0.05$). One HD vaccinated badger in D313 exhibited the most extensive pathology of all animals in the studies (Table 2).

Lesions in lung lobes were observed in 22 of the 23 badgers. In 18 animals the right middle lobe contained visible lesions. In two animals the right caudal was the only lobe with visible lesions. In one animal (D313), all six lung lobes contained visible lesions. The left side of the lung contained visible lesions in four badgers; all were in vaccinated groups. Pleurisy on the mediastinum was observed across all groups and no statistically significant difference between groups was detected in this respect. The most frequently and severely affected thoracic LN was the right bronchial (Table 2), followed by the posterior mediastinal. The only extrathoracic LNs with visible lesions were the hepatic (two badgers) and the mesenteric (one badger). No lesions were found in LNs of the head, the body, or in the kidneys of any animal.

3.2. Distribution of disease

The distribution of disease is described using the bacteriology and the histology results shown in Tables 3 and 4. *M. bovis* was isolated from more tissues than had visible lesions. Sites of infection included LNs from the head (parotid) and body (popliteal). *M. bovis* was isolated from the right bronchial LN of all but one animal (Table 3). In 19 out of 23 badgers the highest concentration of *M. bovis* was found in the right bronchial LN, but occasionally in the posterior mediastinal LN. Within the lung, the highest bacterial yields were always from the right side and were generally from those lobes with the highest visible lesion scores. *M. bovis* was isolated from the hepatic LN of 21 (91%) badgers and frequently from the mediastinum. Lower counts of *M. bovis* were found in the liver and spleen. *M. bovis* was isolated from the spleen of four non-vaccinated badgers, four LD vaccinated badgers, and one HD vaccinated badger (D313).

Histological lesions were found in fewer sites than were found to be infected. Most often histological lesions were associated with either visible lesions or the isolation of *M. bovis*, but occasionally microscopy provided the only evidence of infection, for example, the right middle lung lobe of C037 and the accessory lobe of D118. No bacteriological or histological evidence of infection was found in the retropharyngeal or inguinal LNs, tonsils or kidneys of any badger.

Fig. 1 also shows the number of tissues containing either visible lesions (B) or *M. bovis* confirmed by culture (C) or histology (D) for each animal. Data are presented for all positive tissues (filled symbols) or only the extrathoracic sites (open symbols). Visible lesions were rarely observed outside of the thoracic cavity, although several of these tissues were found to contain *M. bovis* (Fig. 1C). Table 5 summarises the results of the statistical tests for differences between treatments for each parameter. A significant reduction was found in the number of tissues at all sites containing AFB in the HD BCG group compared with the control and LD BCG groups. No other significant between-group differences were found, reflecting the low power from the relatively small group sizes and the variation between animals within a treatment group. Nonetheless, a dose-dependent response to the vaccine was evident for all disease severity parameters, with the greatest reductions achieved with the higher dose. Fig. 1 shows a further dose effect for BCG in that the HD BCG groups had less dissemination of *M. bovis* from the thoracic cavity than the LD BCG group.

3.3. Excretion of M. bovis

M. bovis was detected intermittently in the larynx/trachea of non-vaccinated badgers from 4 weeks post-challenge in VES1 and from 2 weeks post-challenge in VES2 (data not shown). All six non-vaccinated and 7/8 badgers vaccinated with LD BCG excreted *M. bovis* at some point in the experiment, whilst only 3/9 animals vaccinated with HD BCG had positive samples. Seventeen of 36 samples submitted for culture from the non-vaccinated animals over the whole study were positive; the LD BCG group were positive on 14 of 48 occasions and the HD BCG group on 7 of 54 occasions. One of the HD BCG badgers, D313, which had extensive lesions in the lung at postmortem, was positive on 5/6 occasions, and two HD BCG badgers (C071

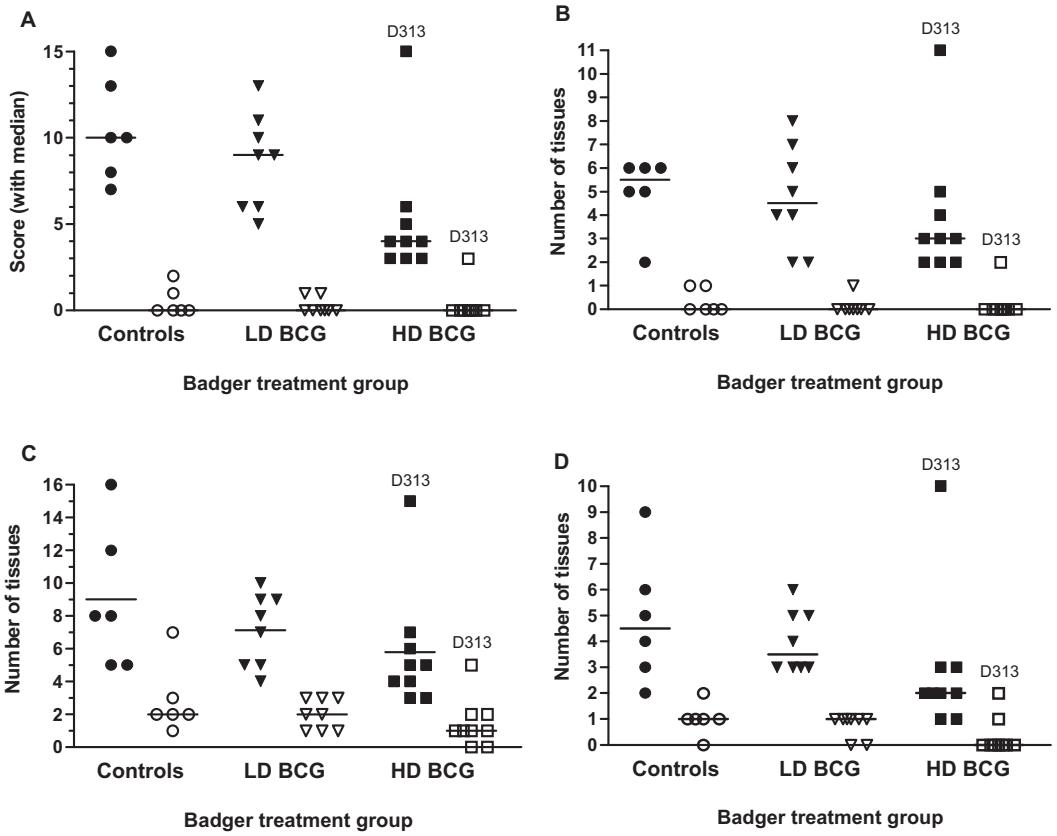

Fig. 1. Vaccination of badgers with BCG and challenged 17 weeks later with endobronchial *M. bovis*. Individual animal disease severity scores (A), and the number of tissues containing visible lesions (B), or *M. bovis* confirmed by culture (C) or histology (D) are shown 12 weeks post-challenge for all sites (filled symbols) or just those outside of the thorax (open symbols). The values for one animal (D313) are indicated where vaccination failed to protect. The median is shown with the exception of the culture (C) data for all sites where the mean is shown, as these data were found to be normally distributed. Badgers were vaccinated with either a low or high dose of BCG (LD, HD, respectively).

Table 5
Results of tests for significance for disease severity scores and in the number of tissues containing either visible lesions (VL), *M. bovis* confirmed by culture or histology.

Parameter	All sites	Extra-thoracic
Disease severity scores		
Control	10.0	0.0
HD BCG	4.0	0.0
LD BCG	9.0	0.0
Chi-square (dof[a])	9.47 (2)	0.13 (2)
p value	0.009[b]	0.938
VL		
Control	5.5	0.0
HD BCG	3.0	0.0
LD BCG	4.5	0.0
Chi-square (dof)	2.74 (2)	0.83 (2)
p value	0.253	0.661
Culture		
Control	8.0	2.0
HD BCG	5.0	1.0
LD BCG	7.5	2.0
Chi-square (dof)	4.61 (2)	3.90 (2)
p value	0.100	0.142
Histology		
Control	4.5	1.0
HD BCG	2.0	0.0
LD BCG	3.5	1.0
Chi-square (dof)	6.67 (2)	5.06 (2)
p value	0.036[b]	0.079

[a] Degrees of freedom.
[b] Kruskal–Wallis Multiple Comparisons test (Dunn's test) indicates HD BCG group significantly lower than Control and LD BCG groups (Control and LD BCG groups not significantly different $p > 0.05$).

in VES1 and D591 in VES2) were positive on one occasion only.

Fewer badgers excreted *M. bovis* in the groups vaccinated with BCG than in the non-vaccinated. Pair-wise comparison of proportions between the three groups showed no significant differences between the non-vaccinated and LD BCG groups ($p = 1.00$, Fisher's exact test). However, the non-vaccinated and the LD BCG groups had significantly higher proportions of positive cultures than the HD BCG group ($p = 0.028$ and 0.050, respectively, by Fisher's exact test). *M. bovis* was not detected in the urine of any badger, and only a single faeces sample yielded *M. bovis*: the postmortem sample from HD BCG vaccinated badger D313.

3.4. IFNγ ELISPOT

The IFNγ ELISPOT assay measures the number of PBMC responding to antigenic stimulation. An IFNγ response was detected by ELISPOT in all badgers after vaccination and after challenge (Figs. 2 and 3). The vaccinated animals were responsive to PPD-B before challenge, but not to a combination of ESAT-6 and CFP-10 antigens, consistent with these latter antigens being expressed by *M. bovis* but not BCG [23]. The magnitude of response following HD BCG vaccination was lower in VES2 (Fig. 3A) than in VES1 (Fig. 2A). The response to LD BCG was lower still, but still clearly above the background responses seen in the non-vaccinates (Fig. 3A), providing evidence that the vaccine generated T-cell memory in these animals. No correlation was found between the pre-challenge IFNγ response and the disease severity score (data not shown).

In both experiments all animals showed an ELISPOT response following challenge and vaccinated badgers had a greater IFNγ

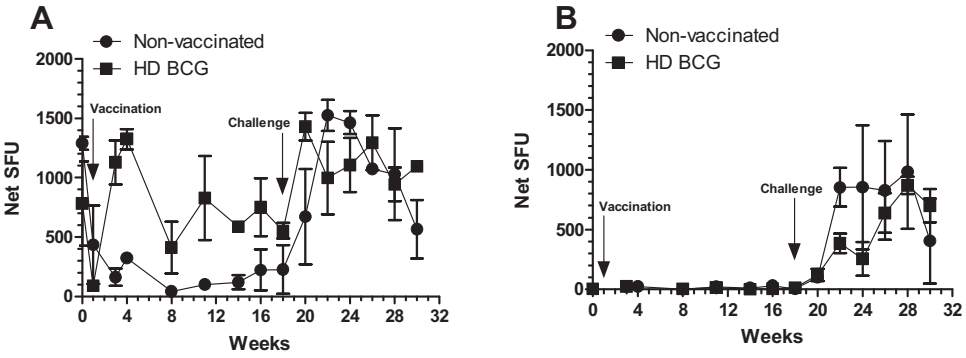

Fig. 2. Vaccination of badgers with BCG and challenged 17 weeks later with endobronchial *M. bovis* (experiment VES1): IFNγ ELISPOT results expressed as the net spot forming units (SFU) calculated by subtracting the response in the absence of antigen from the response to stimulation with PPD-B (A) or ESAT-6/CFP-10 (B). Vaccination with high dose (HD) BCG occurred in week 1 and challenge with *M. bovis* in week 18 following commencement of the study (week 0).

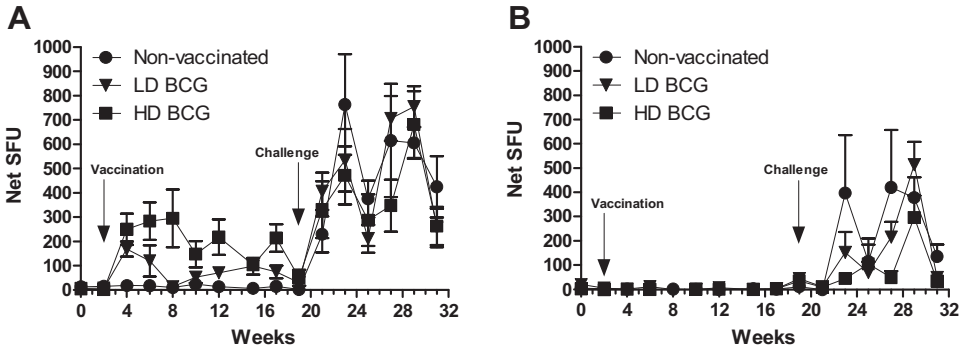

Fig. 3. Vaccination of badgers with BCG challenged 17 weeks later with endobronchial *M. bovis* (experiment VES2): IFNγ ELISPOT results expressed as the net spot forming units (SFU) calculated by subtracting the response in the absence of antigen from the response to stimulation with PPD-B (A) or ESAT-6/CFP-10 (B). Vaccination with either high dose (HD) or low dose (LD) BCG occurred in week 2 and challenge with *M. bovis* in week 19 following commencement of the study (week 0).

response than non-vaccinated at 2 weeks post-challenge; evidence of priming caused by vaccination (Figs. 2A and 3A). At 4 and 6 weeks after challenge in both VES1 and VES2, the mean response of non-vaccinated animals exceeded that of the vaccinated animals. The responses remained elevated in all groups from 8 to 12 weeks after challenge. The earliest distinct response to ESAT-6/CFP-10 antigens was seen at four weeks after challenge (Figs. 2B and 3B). In both experiments the mean response to the combined antigens was greater in the non-vaccinated groups compared with the vaccinated groups between weeks 4 and 8 after challenge; after which there were no consistent differences between groups.

3.5. Animal welfare

The badgers adapted well to captivity with food intake and body weights typical of what we have observed before (ranging from 6.8 to 15.8 kg, depending on season). Haematological and biochemical parameters remained within the normal ranges described previously [14] and did not vary between vaccinated and non-vaccinated groups, or between pre- and post-challenge periods (data not shown). The badgers did not show a reduction in the number of leukocytes or reduced activation of neutrophils (data not shown) that are signs of stress in badgers [24], and in possums [25] after capture.

4. Discussion

We demonstrated that BCG administered IM to badgers elicits an IFNγ response and that BCG vaccination reduced the severity and progression of experimentally induced TB in badgers. These results present a more comprehensive picture of BCG-mediated protection in badgers than published recently [15] and show that vaccination with the higher dose (10 times the human dose) was more immunogenic and gave superior protection than the lower dose (normal human dose).

The endobronchial *M. bovis* infection model is particularly suitable to the experimental evaluation of vaccine efficacy as the respiratory route is considered the primary route of *M. bovis* infection in wild badgers [4,26]. For the efficacy experiments reported here, a target challenge dose of 10^3 to 10^4 CFU *M. bovis* was used (actual dose, $2.6–4.8 \times 10^3$ CFU) and badgers were killed 12 weeks after challenge for assessment of disease severity. We consider the number of viable *M. bovis* organisms used in the challenge inoculum to be substantially higher than encountered in natural aerosol exposure. This makes the challenge system used a stringent test of vaccine induced protection, in terms of protection against a single large dose exposure. Although technically more challenging to replicate experimentally, it would be valuable to also determine the degree of protection afforded by different doses of BCG against repeated low dose exposure to *M. bovis*, as may also occur in the wild. This would allow vaccine-mediated protection to be defined in detail postmortem; something that was not feasible when the vaccine was used in a population of wild badgers [15].

The disease severity parameters we used were based on studies published previously [11,17,27] that showed the disease induced by endobronchial inoculation displayed the characteristics of disease observed in naturally infected badgers [27]: disease mainly occurred in the organs of the thoracic cavity, with limited distribution of visible lesions but infection more widely disseminated on the basis of culture. The parameters used to assess severity of disease have previously been used to assess BCG vaccine in white-tailed deer [28], and cattle [29,30], as well as badgers [11]. As is

reported from naturally infected badgers, the experimental infection resulted in many infected tissues that were without visible lesions [4,18,26].

BCG vaccination reduced the severity of the experimental infection and the frequency of excretion of *M. bovis*. HD BCG vaccination was found to significantly reduce the severity of disease compared to the non-vaccinated controls, with the severity of disease in the LD BCG group between the other two. The protection mediated by IM vaccination in our experiments was similar in scope and magnitude to a study conducted previously in badgers where the SC route of BCG vaccination was compared with a combined mucosal route of vaccination [11]. Disease severity scores and the numbers of sites containing visible lesions or confirmed to contain *M. bovis* by culture or histology were very similar between a SC dose of 5×10^5 CFU and IM HD BCG. An intermediate level of protection was obtained with the combined mucosal route of vaccination [11]; very similar to that seen with LD BCG. Thus, although the dose may need to be optimised for each species and route of administration, BCG vaccination studies in badgers support the findings from possums [31] and deer [32] that protection can be induced with different strains of BCG, delivered by a variety of routes and over a wide range of doses (10^5 to 10^8 CFU).

IM vaccination did not prevent infection, most likely because of the overwhelming size of the challenge dose. With this in mind, whilst HD BCG gave greater protection than LD BCG, the dose effect exhibited clearly in Fig. 1 suggests the protection mediated by the human dose of BCG should not be overlooked. When a population of wild possums were vaccinated via combined mucosal routes or by oral delivery, BCG vaccination significantly reduced the number of cases of TB in the vaccinated groups [33,34], although evidence of such high levels of protection was not seen when TB was experimentally induced [35,36]. Therefore, in settings of natural transmission, BCG vaccination protection may be more profound than in experimental studies, a situation also encountered for BCG in cattle [37]. In wild badger populations the protection induced by the LD or HD BCG vaccination over time may provide complete or adequate protection, either individually or through a herd immunity effect. Further analysis of the data from the field study [15] is underway in this regard but will always be limited by the lack of postmortem assessment of disease.

One badger of the HD group (D313) developed severe generalised tuberculosis; the most severe of all the badgers in the study. The failure of HD BCG to protect this animal was not due to a failure to deliver the vaccine successfully or for it to induce T-cell memory as the response of D313 in the IFNγ ELISPOT was indistinguishable from the other animals in the same treatment group (data not shown). Based on the levels of haematological and biochemical parameters monitored throughout the experiment as markers for the clinical condition of badgers, there was no evidence that D313 had any underlying physiological or pathological condition and at postmortem examination no pathological conditions other than TB were observed. Thus we have no explanation as to why the infection was overwhelming in this animal.

The predominant protective immune response in mycobacterial infection is a type 1 cell-mediated response, typified by production of IFNγ [38], which has been shown to be essential for protection against virulent mycobacteria [39]. Although there is some suggestion that the frequency of IFNγ producing cells might correlate with protection against TB [40,41], the magnitude of *in vitro* (PPD)-specific IFNγ production assessed during the course of TB infection in mice and cattle does not [42,43]. In these experiments, we assessed the IFNγ response to BCG vaccination and challenge with *M. bovis* using ELISPOT, which measured the frequency of IFNγ producing cells. Animals vaccinated with BCG were responsive in the ELISPOT with the number of IFNγ producing cells being higher in animals given the higher dose of BCG. However, we found no correlation between the pre-challenge IFNγ response and the disease severity score (data not shown). We do not have a definitive explanation for why the IFNγ ELISPOT responses were high in all VES1 badgers one week before vaccination. Importantly, this apparent false-positivity had all but disappeared by the time of vaccination a week later. It could be due to exposure to environmental mycobacteria resulting in cross-recognition of antigens in PPD-B. This may explain why the responses to vaccination were generally lower in VES2; as a degree of boosting may have occurred in the first experiment.

All animals in both experiments responded in the IFNγ ELISPOT after they were challenged with *M. bovis*. There was a clear anamnestic response to PPD-B after challenge in VES1, consistent with BCG-mediated immunological memory, although no such response was seen in VES2. Nonetheless, HD BCG vaccination induced the same level of protection in VES2 as it had in VES1. Responses to ESAT-6/CFP-10 antigens were only seen after challenge, consistent with these antigens being expressed by *M. bovis* but not BCG [23]. The quantity of IFNγ production post-challenge has been correlated with disease severity, for example in experimental *M. bovis* infection of cattle [29]. The same association was found in these studies regarding the number of IFNγ-producing cells: fewer cells responding to ESAT-6/CFP-10 were seen in the vaccinated groups compared to non-vaccinated; most notably in the HD BCG groups in both studies.

In conclusion, IM administration of BCG was shown to confer a significant level of protection to badgers against experimental inoculation with *M. bovis*. As IM BCG is both protective and safe for badgers [14] and can be administered to trapped conscious animals, it makes field vaccination of badgers feasible for the first time; representing a new intervention tool for the control of TB in badgers. The vaccine is currently in use within the UK [44].

Acknowledgements

All animal procedures were covered by a licence issued under the Animals (Scientific Procedures) Act 1986 and following local ethical approval. This manuscript was prepared in accordance with the ARRIVE Guidelines for reporting animal research [45].

We greatly appreciate the input of Professor George Gettinby, Veterinary Informatics and Epidemiology, The University of Strathclyde in Glasgow, UK for advice on experimental design, statistical analysis and interpretation. We thank the staff at the Royal Veterinary College, Diagnostic Laboratories, Hatfield, UK for performing haematological and biochemical tests.

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