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Abstract

Wildlife reservoirs of *Mycobacterium bovis* represent serious obstacles to the eradication of tuberculosis in domestic livestock. In Michigan, USA tuberculous white-tailed deer transmit *M. bovis* to cattle. One approach in dealing with this wildlife reservoir is to vaccinate deer in order to interrupt the cycle of deer to deer and deer to cattle transmission. Thirty-one white-tailed deer were assigned to one of three groups; 2 SC doses of 10^7 CFU of *M. bovis* BCG ($n = 11$); 1 SC dose of 10^7 CFU of *M. bovis* BCG ($n = 10$); or unvaccinated deer ($n = 10$). After vaccination, deer were inoculated intratonsilarly with 300 CFU of virulent *M. bovis*. Gross lesion severity scores of the medial retropharyngeal lymph node were significantly reduced in deer receiving 2 doses of BCG compared to unvaccinated deer. Vaccinated deer had fewer lymph node granulomas than unvaccinated deer, and most notably, fewer late stage granulomas characterized by coalescent caseonecrotic granulomas containing numerous acid-fast bacilli. BCG was isolated from 7/21 vaccinated deer as long as 249 days after vaccination. In one case BCG was transmitted from a vaccinated deer to an unvaccinated deer. In white-tailed deer BCG provides measurable protection against challenge with virulent *M. bovis*. However, persistence of vaccine within tissues as well as shedding of BCG from vaccinates remain areas for further investigation.
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Keywords: BCG; Deer; *Mycobacterium bovis*; Tuberculosis; Vaccination

1. Introduction

In 1994, a free-ranging white-tailed deer (*Odocoileus virginianus*) in Michigan was diagnosed with tuberculosis due to *Mycobacterium bovis*, the causative agent of bovine tuberculosis [1]. Subsequent surveys identified a focus of *M. bovis* infection in free-ranging white-tailed deer in northeast Michigan [2,3]. This represents the first known reservoir of *M. bovis* in free-ranging wildlife in the United States and a significant obstacle to the eradication of bovine tuberculosis in domestic livestock. Several factors are thought to have contributed to the establishment and persistence of *M. bovis* in this wildlife reservoir. It is postulated that *M. bovis* was transmitted from cattle to deer at some time during the mid 1900s when a large number of Michigan cattle were infected with *M. bovis* [4]. During this same period, Michigan's deer population was steadily increasing beyond normal habitat carrying capacity to focal concentrations of 19–23 deer/km^2 [1]. Transmission and maintenance of *M. bovis* among deer is thought to have been facilitated by the common practice in Michigan of long-term winter feeding of deer by private citizens to prevent migration and decrease winter mortality in order to keep deer numbers high for hunting purposes [1].

Current disease control measures in Michigan include decreasing deer density through increased hunting and strict control of feeding and baiting of white-tailed deer. Another possible control measure could be vaccination of white-tailed deer to prevent infection, disease, or transmission. Vaccination of captive farmed red deer in New Zealand has shown that vaccination with *M. bovis* BCG can prevent infection and disease (i.e. lesion development) [5,6]. BCG vaccination

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has also been used to control tuberculosis in farmed sika deer (*Cervus nippon*) in China [7].

M. bovis BCG was first used as an anti-tuberculosis vaccine in humans in 1921 and is one of the oldest and most widely used vaccines in the world today [8]. Although reports of efficacy in humans vary widely by geographic region and among different age groups, consistent protection has been observed against miliary tuberculosis and tuberculous meningitis when used as a single dose in neonates. The great variability in efficacy is often attributed to one or more factors including, differences in vaccines strains, prior host sensitization to various non-tuberculous environmental mycobacteria, genetic differences in trial populations, or trial design [8].

Protection against *M. bovis* or *M. tuberculosis* has been shown to be associated with a TH1 immune response; generally characterized by production of cytokines such as IFN-γ, IL-12, and TNF-α. It would appear then that any successful vaccine against tuberculosis would require the induction of a TH1 immune response. The immune response to *M. bovis* BCG has been investigated in white-tailed deer [9]. Upon stimulation with *M. bovis* PPD, CD4+ and γ/δ T cells proliferated in vaccinated but not unvaccinated deer. Likewise, peripheral blood mononuclear cells (PBMC) from vaccinated but not unvaccinated deer produced IFN-γ upon stimulation with *M. bovis* PPD [9]. However, the ability of vaccination with *M. bovis* BCG to protect against infection or disease in white-tailed deer has not been explored. The purpose of the current study was to determine the ability of parenterally administered *M. bovis* BCG to prevent infection or disease in white-tailed deer after intratonsilar challenge with virulent *M. bovis*.

2. Materials and methods

2.1. Animals and bacteria

Thirty-one white-tailed deer (∼1 year old) were obtained from a captive breeding herd (tuberculosis- and paratuberculosis-free) at the National Animal Disease Center (Ames, IA, USA). All deer were housed and cared for according to institutional guidelines. Deer were randomly assigned to one of three groups; 2 SC doses of 10^7 colony-forming units (CFU) of *M. bovis* BCG administered 6 weeks apart ($n = 11$); 1 SC dose of 10^7 CFU of *M. bovis* BCG ($n = 10$); or unvaccinated deer ($n = 10$). The *M. bovis* BCG (Pasteur strain) as well as the challenge strain *M. bovis* 1315 were grown in Middlebrook's 7H9 media supplemented with 10% oleic acid–albumin–dextrose complex (Difco, Detroit, MI) plus 0.05% Tween 80 (Sigma Chemical Co., St. Louis, MO) as described [10]. Mid log-phase growth bacilli were pelleted by centrifugation at 750 × *g*, washed twice with phosphate buffered saline (PBS) (0.01 M, pH 7.2), and diluted to the appropriate cell density in 2 ml of PBS. Bacilli were enumerated by serial dilution plate counting on Middlebrook's 7H11 selective media (Becton Dickinson, Cockeysville, MD). A single vaccine dose consisted of 10^7 CFU *M. bovis* BCG in 1.5 ml PBS and was administered SC. Individual vaccine doses were thoroughly vortexed immediately prior to injection to disperse bacilli in suspension and minimize clumping. In the 2 dose group a 6-week interval separated doses. Unvaccinated and vaccinated deer were housed together in an outdoor paddock prior to challenge with virulent *M. bovis*, at which time they were moved to appropriate biosecurity level 3 (BL-3) animal housing.

Seventy-seven days after the 2 dose group received the second dose of vaccine all deer were inoculated intratonsilarly with approximately 150 CFU of *M. bovis* strain 1315 into each tonsilar crypt for a total dose of 300 CFU, as described previously [11]. Strain 1315 was originally isolated from a white-tailed deer in Michigan. For inoculation deer were anesthetized by IM injection of a combination of xylazine (2 mg/kg) (Mobay Corporation, Shawnee, KS) and ketamine (6 mg/kg) (Fort Dodge Laboratories, Fort Dodge, IA). After inoculation the effects of xylazine were reversed by IV injection of tolazoline (4 mg/kg) (Lloyd Laboratories, Shanandoah, IA). Animals were housed in BL-3 housing, and fed a commercial pelleted feed with free access to water. All procedures were approved by the National Animal Disease Center Institutional Animal Care and Use Committee prior to the beginning of the experiment.

2.2. Interferon-γ enzyme-linked immunosorbent assay

A whole-blood culture system for the determination of recall IFN-γ production as used for cattle [12] and red deer [13] was adapted for use with samples from white-tailed deer [14]. Briefly, 1.5 ml heparinized blood was added to 24-well tissue culture plates. Treatments included 100 μl PBS (i.e., no stimulation), 20 μg/ml *M. bovis* purified protein derivative (PPD), 20 μg/ml *M. avium* PPD, 10 μg/ml rESAT-6:CFP-10, or 20 μg/ml pokeweed mitogen (PWM). Optimal dilutions of PPDs, recombinant proteins and PWM were determined previously [14]. Samples were incubated for 48 h at 37 °C in a humidified chamber with 5% CO_2. Samples were then centrifuged (400 × *g*), and plasma was harvested and stored at −80 °C until analyzed for IFN-γ by enzyme-linked immunosorbent assay (ELISA) with a commercially available kit (Cervigam™, Prionics AG, Schlieren, Switzerland). Interferon-γ concentrations (ng/ml) in test samples were determined by comparing the absorbance of test samples with the absorbance of standards within a linear curve fit.

2.3. ELISA for antibody to lipoarabinomannan (LAM)-enriched mycobacterial antigen preparations

Antigens were prepared from *M. bovis* strain 1315 and antibody measured as described [15]. Bacilli were harvested from 4-week cultures, sonicated in PBS, further disrupted with 0.1–0.15 mm diameter glass beads (Biospec Products, Bartlesville, OK) in a bead beater (Biospec Products), cen-

trifuged, filtered (0.22 μm pore size), and digested in a 1-mg/ml proteinase K (Roche Molecular Biochemicals, Indianapolis, IN) solution (50 M Tris, 1 mM $CaCl_2$ buffer, pH 8.0) for 1 h at 50 °C. Protein concentrations were determined (BioRad, Hercules, CA) and antigens were stored at −20 °C. The antigen concentration used for ELISA was 40 μg/ml.

Immulon II 96 well microtiter plates (Dynatech, Chantilly, VA) were coated with 100-μl/well antigen diluted in carbonate/bicarbonate coating buffer (pH 9.6). Antigen coated plates, including control wells containing coating buffer alone, were incubated for 15 h at 4 °C. Plates were washed three times with 200 μl/well containing 0.05% Tween 20 (i.e., PBST; Sigma), and blocked with 200 μl/well commercial milk diluent/blocking solution (Kirkegaard and Perry Laboratories, Gaithersburg, MD). After incubation for 1 h at 37 °C in the blocking solution, wells were washed nine times with 200 μl/well PBST and test sera added to wells (100 μl/well). Test and control sera were determined by evaluation of the reactivity of twofold serial dilutions ranging from 1:6 to 1:800 (volume of sera/volume of diluent ratio) with antigen. After incubation for 20 h at 4 °C with diluted test sera, wells were washed nine times with 200 μl/well PBST and incubated for 1 h at 37 °C with 100 μl/well horseradish peroxidase (HRP)-conjugated anticervine immunoglobulin G (IgG) heavy and light chains (Kirkegaard and Perry) diluted 1:500 in PBS plus 0.1% gelatin. Wells were washed nine times with 200 μl/well PBST and incubated for 4.5 min at room temperature with 100 μl/well 3,3′,5,5′-tetramethylbenzidine. The reaction was stopped by addition of 100 μl/well 0.18 M sulfuric acid, and the A450 of individual wells was measured with an automated ELISA plate reader (Molecular Devices, Menlo Park, CA). Changes in optical density readings (ΔOD) were calculated by subtracting the mean OD readings for wells receiving coating buffer alone (two replicates) from the mean OD readings for antigen coated wells (two replicates) receiving the same serum sample.

2.4. Necropsy and tissue sampling

One hundred and thirty days after challenge with virulent *M. bovis* all deer were euthanized by IV sodium pentobarbital. At necropsy, the following tissues or fluids were collected and processed for isolation of *M. bovis* and microscopic analysis as described [16]; palatine tonsil, lung, liver, mandibular, parotid, medial retropharyngeal, tracheobronchial, mediastinal, hepatic, mesenteric and prefemoral lymph nodes. Tissues were processed for isolation of *M. bovis* as previously described [17]. Isolates of *M. bovis* were identified by colony morphology, growth, and biochemical characteristics as well as by PCR.

Lymph nodes were cross-sectioned at 0.5 cm intervals and examined. Each lung lobe was examined separately and cross-sectioned at 0.5–1.0 cm intervals. Lungs and lymph nodes were subjected to semi-quantitative scoring of gross lesions adapted from Vordermeier et al. [18]. Lung lobes (left cranial, left caudal, right cranial, right caudal, middle and accessory) were subjected to the following scoring system: (0) no visible lesions; (1) no external gross lesions, but lesions seen upon slicing; (2) <5 gross lesions of <10 mm in diameter; (3) >5 gross lesions of <10 mm in diameter; (4) >1 distinct gross lesion of >10 mm in diameter; (5) coalescing gross lesions. Scoring of lymph node gross lesions was based on the following scoring system: (0) no visible lesions; (1) small focal lesion (1–2 mm in diameter); (2) several small foci; (3) extensive lesions. Tissues collected for microscopic analysis were fixed by immersion in 10% neutral buffered formalin and included all tissues collected for bacteriologic examination. For microscopic examination, formalin-fixed tissues were processed by routine paraffin-embedment techniques, cut in 5 μm sections and stained with hematoxylin and eosin (HE). Adjacent sections were cut from samples containing lesions suggestive of tuberculosis (caseonecrotic granulomata) and stained by the Ziehl-Neelsen technique for identification of acid-fast bacteria (AFB). Numbers of AFB were recorded for each granuloma. Due to an inability to reliably enumerate large numbers of AFB, granulomas with >200 AFB were recorded as >200 AFB. Microscopic tuberculous lesions were staged (I–IV) according to criteria adapted from that described by Rhoades et al. [19]. Stage I (initial) granulomas were characterized by accumulations of epithelioid macrophages with low numbers of lymphocytes and neutrophils. Multinucleated giant cells may be present but necrosis was absent. Acid-fast bacilli, when present, were seen within macrophages or multinucleated giant cells. Stage II (solid) granulomas were characterized by accumulations of epithelioid macrophages surrounded by a thin connective tissue capsule. Infiltrates of neutrophils and lymphocytes may be present as well as multinucleated giant cells. Necrosis when present was minimal. Stage III (necrotic) granulomas were characterized by complete fibrous encapsulation. Necrotic cores were surrounded by a zone of epithelioid macrophages admixed with multinucleated giant cells and lymphocytes. Stage IV (necrotic and mineralized) granulomas were characterized by a thick fibrous capsule surrounding irregular multicentric granulomas with multiple necrotic cores. Necrotic cores contained foci of dystrophic mineralization. Epithelioid macrophages and multinucleated giant cells surrounded necrotic areas and there were often moderate to marked infiltrates of lymphocytes. Acid-fast bacilli were often present in moderate numbers and primarily located within the caseum of the necrotic core.

Additionally, each animal was assigned to one of three classifications according to the scheme by Griffin et al. [6]: (1) uninfected animals with no gross or microscopic lesions and from which *M. bovis* could not be isolated by bacteriological culture from samples collected, (2) infected animals from which *M. bovis* could be isolated by bacteriological culture, but no gross or microscopic lesions were present, and (3) diseased animals with gross or microscopic lesions consistent with tuberculosis and from which *M. bovis* could be isolated by bacteriological culture.

2.5. PCR methods

PCR was used to confirm *M. bovis* infection and to distinguish *M. bovis* from *M. bovis* BCG Pasteur. Region of difference 1 (RD1), while present in virulent *M. bovis* is absent from *M. bovis* BCG [20]. The following primer pairs were used to detect the presence or absence of the RD1 region using a method similar to that described by Talbot et al. [21]; RD1-1 GTTCATGCTCGCGGACTAC, RD1-2 ATACATCGGTGACCCTTTGC and RD1-3 TAGGTTCG-GTTACGCTGGTT. Primers RD1-1 and RD1-3 produce a 542 bp product from *M. bovis* BCG Pasteur while RD-2 and RD-3 produce a 384 bp product from *M. bovis*.

Mycobacteria were transferred to 25 μl of sterile TE using a sterile-disposable inoculation loop. The bacterial suspension was heat inactivated by heating to 80 °C for 10 min, vortexed, then heated for an additional 10 min. One microliter of the bacterial preparation was added to the following PCR master mix: 1 × PCR reaction buffer with $MgCl_2$ (Roche Applied Science), 50 pM of each primer, 200 μM of each dNTP (PCR Nucleotide Mix, Roche Applied Science), 2 U FastStart Taq (Roche Applied Science), 5 μg BSA (Ambion, Austin, TX) in a total reaction volume of 50 μl. Touchdown PCR was performed with an initial preheating step of 2 min at 94 °C followed by denaturation at 94 °C for 45 s, annealing started at 65 °C for 1 min with a 1 °C decrease per cycle followed by extension at 72 °C for 2 min, after the annealing temperature reached 50 °C an additional 29 cycles were performed. A final extension at 72 °C for 10 min was included. Samples were analyzed by electrophoresis on 1.5% agarose gel and visualized with ethidium bromide.

Isolates of acid-fast bacteria that were not identified by PCR as virulent *M. bovis* or *M. bovis* BCG Pasteur were further identified using 16S ribosomal DNA sequencing as described previously [22]. Sequences were then identified through the use of a mycobacterial species sequence database [23].

2.6. Statistical analysis

Mean group values for lesion scores were compared using one-way repeated measures analysis of variance (GraphPad Prism, GraphPad Software, San Diego, CA). Differences between means were then compared using the Bonferonni method. Results of IFN-γ analysis and ELISA were compared using one-way analysis of variance followed by Tukey–Kramer multiple comparisons test using a commercially available statistics program (InStat 2.00, GraphPad Software). A p-value <0.05 was considered significant.

3. Results

Regardless of group the most common site for lesion development was the medial retropharyngeal lymph node (9 of 10 diseased deer). Pulmonary lesions were less common, being present in 4 of 10 diseased deer. Six of 10 (60%) unvaccinated deer developed gross or microscopic lesions consistent with tuberculosis (Table 1) and would be categorized as diseased while 2/10 (20%) and 2/11 (18%) of *M. bovis* BCG vaccinated deer were categorized as diseased in the 1 dose and 2 dose groups, respectively.

Table 1
Summary of gross lesions, microscopic lesions and bacteriological isolation of *Mycobacterium bovis* from white-tailed deer vaccinated with *M. bovis* BCG Pasteur and challenged by intratonsilar inoculation of 300 CFU of virulent *M. bovis*

	Unvaccinated	BCG 1 dose	BCG 2 doses
Gross lesions	5/10	2/10	1/11
Microscopic lesions	6/10	4/10	3/11
Isolation of virulent *M. bovis*	5/10	3/10	5/11
Isolation of *M. bovis* BCG	1/10	4/10	3/11
Isolation of *M. bovis* BCG from tissues with lesions	0/1	0/4	2/3[a]
Isolation of *M. bovis* BCG only and not virulent *M. bovis*	1/10	4/10	2/11
Isolation of non-tuberculous mycobacteria	1/10[b]	2/10[b]	1/11[c]
Deer from which virulent *M. bovis* was not isolated and lesions were not seen	4/10	7/10	6/11

[a] Hepatic lymph node lesion yielded both virulent *M. bovis* and *M. bovis* BCG.
[b] *M. terrae*.
[c] *M. kansasii*.

Gross lesion severity scores of the medial retropharyngeal lymph node were significantly reduced in deer receiving 2 doses of *M. bovis* BCG compared to unvaccinated deer (Fig. 1) but not significantly different compared to deer receiving a single dose of BCG. Lesion severity scores of the lungs did not differ between groups (data not shown). Total pathology scores (sum of scores for lymph nodes and lung) were lower ($p = 0.1$) in deer receiving 2 doses of BCG (Fig. 2) compared to unvaccinated deer or deer receiving a single dose of BCG. Microscopic evaluation of the medial retropharyngeal lymph nodes revealed a greater number of granulomas in unvaccinated deer than in vaccinated deer (Table 2). All deer, regardless of vaccination status, had microscopic lesions in all stages. However, vaccinated deer had fewer stage IV granulomas than unvaccinated deer. Enumeration of AFB within granulomas revealed that stage IV granulomas had a mean of >200 AFB/granuloma while those of stages I–III had a mean of 0–11 AFB/granuloma (Table 3).

Table 2
Numbers of granulomas at different microscopic stages of development[a] in the medial retropharyngeal lymph node of white-tailed deer vaccinated with *M. bovis* BCG and challenged by intratonsilar inoculation of 300 CFU of virulent *M. bovis*

	Stage I	Stage II	Stage III	Stage IV	Total
BCG 2 doses	2	3	5	1	11
BCG 1 dose	1	4	3	2	10
Unvaccinated	18	18	11	7	54

[a] Stages defined in text.

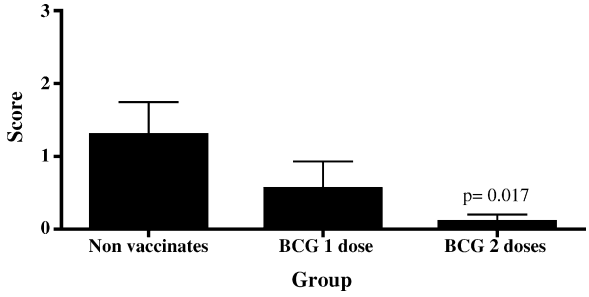

Fig. 1. Gross lesion severity scores from medial retropharyngeal lymph nodes of unvaccinated deer ($n = 10$) or deer vaccinated SC with one ($n = 10$) or two doses ($n = 11$) of 10^7 CFU *Mycobacterium bovis* BCG Pasteur and challenged intratonsilarly with 300 CFU of virulent *M. bovis*. Scores represent the mean ± standard error. Scoring system as follows: (0) no visible lesions; (1) small focal lesion (1–2 mm in diameter); (2) several small foci; (3) extensive lesions.

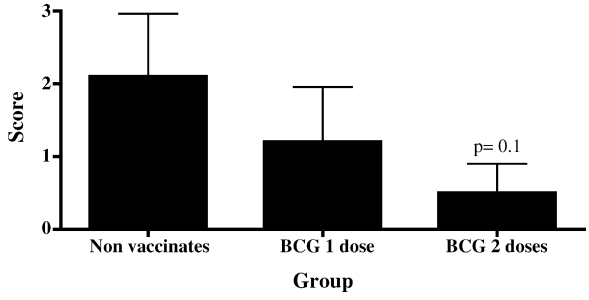

Fig. 2. Total combined gross lesion severity scores from lymph nodes and lungs of unvaccinated deer ($n = 10$) or deer vaccinated SC with one ($n = 10$) or two doses ($n = 11$) of 10^7 CFU *M. bovis* BCG Pasteur and challenged intratonsilarly with 300 CFU of virulent *M. bovis*. Scores represent the mean ± standard error. Scoring system as in Fig. 1 for lymph nodes. Lung scoring system as follows: (0) no visible lesions; (1) no external gross lesions, but lesions seen upon slicing; (2) <5 gross lesions of <10 mm in diameter; (3) >5 gross lesions of <10 mm in diameter; (4) >1 distinct gross lesion of >10 mm in diameter; (5) coalescing gross lesions.

Virulent *M. bovis* was isolated from 4 deer with no gross or microscopic lesions (i.e. infected deer). Infected, but not diseased deer included 1 deer from the 1 dose group and 3 deer from the 2 dose group. Uninfected deer (deer from which virulent *M. bovis* could not be recovered and in which no gross or microscopic lesions were seen) included 4 unvaccinated deer, 7 deer from the 1 dose group and 6 deer from the 2 dose group (Table 1).

Table 3
Mean number of AFB in medial retropharyngeal lymph node granulomas of different developmental stages[a] from white-tailed deer vaccinated with *M. bovis* BCG and challenged by intratonsilar inoculation of 300 CFU of virulent *M. bovis*

	Stage I	Stage II	Stage III	Stage IV
BCG 2 doses	1	0	5.4	>200[b]
BCG 1 dose	0	0	11.3	>200
Unvaccinated	0	0.17	0.18	>200

[a] Stages defined in text.
[b] Due to an inability to reliably enumerate large numbers of AFB, granulomas with >200 AFB were recorded as >200 AFB.

M. bovis BCG was isolated at the time of necropsy from 8 deer, including 1 unvaccinated deer, 4 deer from the 1 dose group and 3 deer from the 2 dose group. Isolations of BCG were made from various tissues including, medial retropharyngeal, tracheobronchial, mediastinal and hepatic lymph nodes. *M. bovis* BCG was isolated from a single site in 5 deer, 2 sites in 2 deer and 3 sites in 1 deer. In 2 deer the isolation of BCG was made from tissues (hepatic and mediastinal lymph nodes) in which microscopic, but not gross lesions, were seen. In one of these 2 deer, virulent *M. bovis* was also isolated from a microscopic lesion in the hepatic lymph node. The mediastinal lymph node was the only tissue from which BCG was isolated from the single unvaccinated deer. In addition to virulent *M. bovis* and *M. bovis* BCG, *M. terrae* and *M. kansasii* were isolated from 4 deer. In all cases *M. terrae* was isolated from tissues not containing gross or microscopic lesions. However, *M. kansasii* was isolated from a mediastinal lymph node in which gross and microscopic lesions were seen. Within that same animal, other tissues contained lesions from which virulent *M. bovis* was isolated.

Prior to challenge with virulent *M. bovis*, vaccinated deer but not unvaccinated deer demonstrated IFN-γ production in response to *M. bovis* PPD stimulation (Fig. 3A). Although not statistically significant, deer receiving 2 doses of BCG had higher levels of IFN-γ production after booster vaccination, than did deer receiving 1 dose of BCG. After intratonsilar challenge with virulent *M. bovis* both vaccinated and unvaccinated deer were shown to produce IFN-γ upon stimulation with *M. bovis* PPD.

When rESAT-6:CFP-10 was used in place of *M. bovis* PPD, neither vaccinated nor unvaccinated deer demonstrated IFN-γ production prior to challenge with virulent *M. bovis* (Fig. 3B). Similar to findings with *M. bovis* PPD, IFN-γ production was evident in all groups in response to rESAT-6:CFP-10 after challenge with virulent *M. bovis*. However, in contrast to responses seen upon stimulation with *M. bovis* PPD, IFN-γ production in response to rESAT-6:CFP-10 was transient as levels returned to pre-challenge values by 80 days after challenge (220 days after vaccination). Prior to initiation of the study all 3 deer from which *M. terrae* was isolated did not show IFN-γ responses to *M. bovis* PPD, *M. avium* PPD or rESAT-6:CFP-10. Following challenge, IFN-γ responses were moderate to *M. bovis* PPD and rESAT-6:CFP-10 compared to other deer in the same groups. The single deer from which *M. kansasii* was isolated demonstrated moderate IFN-γ responses to *M. bovis* PPD, *M. avium* PPD and rESAT-6:CFP-10 prior to the study as well as following vaccination and challenge.

Antibody responses to a LAM-enriched *M. bovis* antigen were greater in vaccinated deer than in unvaccinated deer (Fig. 4). Optical density values were significantly greater in deer receiving 2 doses of BCG 63 and 112 and days after primary vaccination compared to unvaccinated deer or deer receiving a single dose of BCG. A significant boost in anti-

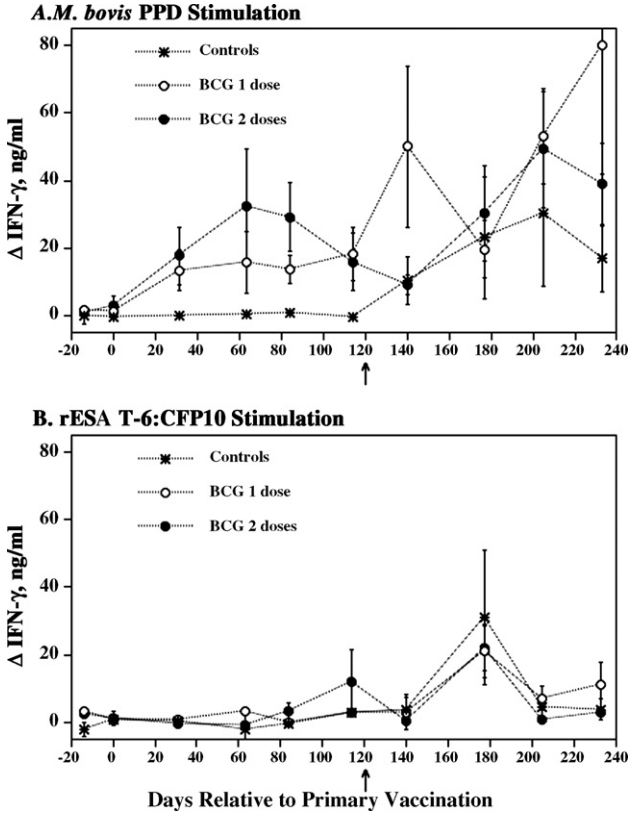

Fig. 3. Interferon-γ production in response to stimulation with *M. bovis* PPD (A) or rESAT-6:CFP10 (B) as measured by optical density (OD, 450 nm). Interferon-γ concentrations (ng/ml) in test samples were determined by comparing the absorbance of test samples with the absorbance of standards within a linear curve fit. Samples from unvaccinated deer ($n = 10$) or deer vaccinated with one ($n = 10$) or two doses ($n = 11$) of 10^7 CFU of *M. bovis* BCG were analyzed for antigen specific IFN-γ production prior to and after vaccination (day 0) and challenge with 300 CFU of virulent *M. bovis* (arrow, day 119). Background OD values (stimulation with PBS alone) have been subtracted from the response to *M. bovis* PPD or rESAT-6:CFP10. Data are represented as means ± standard error.

body was not seen in vaccinated deer after challenge with virulent *M. bovis*.

4. Discussion

Previous studies in red deer established that doses of approximately 5×10^4 to 5×10^7 CFU of *M. bovis* BCG (Pasteur) provided protection against challenge with virulent *M. bovis* [5,6]. These studies used intratonsilar challenge doses of 100–500 CFU of virulent *M. bovis* similar to those used in the present study. Also similar to findings in the current study, two doses of BCG provided superior protection in red deer compared to a single dose [6]. Although a single dose of *M. bovis* BCG did not protect red deer against infection, it did result in fewer animals with tuberculous lesions. The present study design was patterned after extensive studies in red deer that used a similar BCG strain with a similar interval between priming and boosting doses of vaccine as well as a similar interval between challenge and necropsy [5]. In those studies

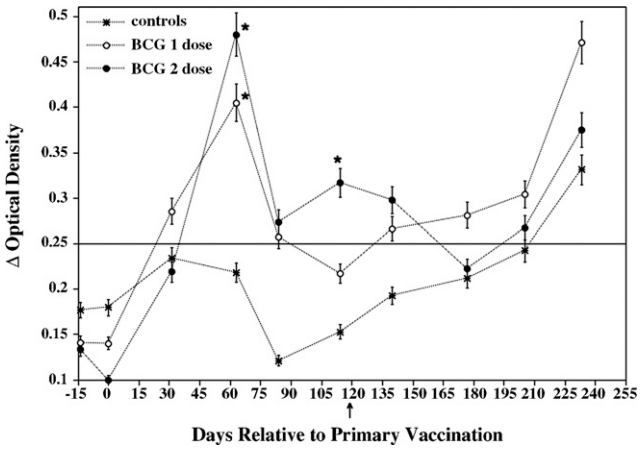

Fig. 4. Response kinetics of serum antibody specific for LAM-enriched antigen. Sera from unvaccinated deer ($n = 10$) or deer vaccinated with one ($n = 10$) or two doses ($n = 11$) of 10^7 CFU of *M. bovis* BCG was analyzed for reactivity to *M. bovis* derived LAM by ELISA prior to and after vaccination (day 0) and challenge with 300 CFU of virulent *M. bovis* (arrow, day 119). Data are represented as means ± standard error. *Significantly different from unvaccinated deer ($p < 0.05$).

the interval from boosting dose to challenge and the interval from challenge to necropsy did not decrease the protective effect of vaccination. In contrast, the interval between priming and boosting doses of vaccine was critical as prolonged intervals (i.e. 43 weeks) resulted in decreased protection [5]. The current findings are also in agreement with vaccination studies in cattle, where vaccination with a similar dose of *M. bovis* BCG (Pasteur) followed by intratracheal challenge with virulent *M. bovis* resulted in less severe lesions in vaccinated cattle compared to unvaccinated cattle [24]. In a separate study, cattle dosed twice with *M. bovis* BCG demonstrated fewer granulomas, less necrosis and fewer acid-fast bacilli than unvaccinated cattle [25]. Studies to date in deer, and most studies in cattle, have used BCG strain Pasteur. It is unknown if other strains of BCG would differ in protective efficacy in white-tailed deer compared to BCG Pasteur. Recently both BCG strains Pasteur and Danish were reported to provide similar levels of protection in vaccinated calves [26].

Similar to previous findings in white-tailed deer and red deer, IFN-γ production in response to *M. bovis* PPD was evident in vaccinated, but not unvaccinated deer. After vaccination, no significant differences were seen in deer receiving 1 dose of *M. bovis* BCG compared to deer receiving 2 doses. IFN-γ production was not detected in vaccinated deer prior to challenge when rESAT-6:CFP-10 was used as antigen instead of *M. bovis* PPD. However, IFN-γ responses to rESAT-6:CFP-10 responses were robust in all deer after challenge with virulent *M. bovis*, suggesting that responses to rESAT-6:CFP-10 could prove useful in differentiating BCG vaccinated from *M. bovis* infected deer. The lack of response to rESAT-6:CFP-10 is due to the absence of the RD1 region of the genome in *M. bovis* BCG compared to virulent *M. bovis*. The genes responsible for ESAT-6 and CFP-10 production are located within this RD1 region. The protein ESAT-6

is co-secreted by members of the *M. tuberculosis* complex in a tight 1:1 complex with CFP-10 [27]. Genes for both ESAT-6 and CFP-10 are absent in many non-tuberculous mycobacteria as well as the vaccine *M. bovis* BCG. However, *esat-6* and *cfp-10* are present in a subset of non-tuberculous mycobacteria such as *M. kansasii*, *M. marinum*, *M. leprae* and *M. smegmatis* [28–30]. The presence of *esat-6* and *cfp-10* in *M. kansasii*, likely explains the pre-vaccination and pre-challenge response to rESAT-6:CFP-10 seen in the deer from which *M. kansasii* was isolated. Lack of response to these recombinant antigens by BCG vaccinated animals has been proposed as a means of differentiating vaccinated from infected animals [31,32] as well as differentiating *M. bovis* infected animals from those infected with other mycobacterial pathogens such as *M. avium* subsp. *paratuberculosis* [33]. Lipoarabinomannan is a cell wall glycolipid of mycobacteria. LAM-based ELISA assays, similar to that used in the present study, have demonstrated utility for diagnosis of mycobacterial diseases including bovine tuberculosis and paratuberculosis [34,35].

Although the present study is the first to examine the efficacy of BCG vaccination of white-tailed deer and demonstrate some protection against challenge with virulent *M. bovis*, successful vaccination of wildlife poses various challenges. It is doubtful that parenteral vaccination, such as that used in the present study, would be feasible with free-ranging wildlife on a large scale. More likely, an oral vaccine such as that currently used to vaccinate wildlife against rabies would be necessary [36]. Oral delivery of live BCG; while posing several challenges, has been shown to be feasible with white-tailed deer [37]. Oral or intragastric vaccination of brushtail possums (*Trichosurus vulpecula*) with *M. bovis* BCG produces inferior protection to intraduodenal vaccination [38–40]. However, lipid based preparations to protect live *M. bovis* BCG from the harmful effects of the gastric environment have proven effective in brushtail possum vaccination trials [41].

In red deer, *M. bovis* BCG Pasteur was still present in tissues from the site of injection as well as the draining lymph nodes 3 months after SC vaccination with 2×10^6 CFU of *M. bovis* BCG Pasteur [42]. Gross lesions in regional lymph nodes and transmission from vaccinated deer to unvaccinated deer have not been features of studies involving red deer [43] or cattle. In the current study, unlike red deer, BCG not only persisted in vaccinated deer, but spread to multiple sites, was associated with microscopic granulomas, and in one case, was shed by vaccinated deer and transmitted to a unvaccinated deer, being recovered from the mediastinal lymph node. Studies in mice demonstrate persistence of *M. bovis* BCG for up to 30 weeks and spread to distant organs after SC vaccination [44,45]. Murine studies further suggest that persistence of *M. bovis* BCG within a vaccinated host is vital in sustaining long-lasting immunological memory. Vaccinated mice receiving chemotherapy to eliminate residual post-vaccinal BCG demonstrated inferior cell-mediated immune responses and inferior protection against challenge with virulent *M. tuberculosis* as measured by colonization of the spleen by *M. tuberculosis* when compared to mice still harboring low numbers of BCG [45,46]. Interestingly, persistence of BCG has not been a feature of human vaccination studies. Even among individuals with symptomatic AIDS, bacteremia following BCG immunization is reported to be uncommon [47]. However, recent analysis shows the risk of disseminated disease from BCG vaccination may be significantly greater in HIV infected infants compared to uninfected infants [48].

Isolation of non-tuberculous mycobacteria from several deer illustrates the challenges posed by exposure to saprophytic non-tuberculous mycobacteria present in the environment. Although exposure of red deer to environmental saprophytic mycobacteria did not influence susceptibility to experimental infection with *M. bovis* [6], the low number of deer similarly exposed in the present study precludes any definitive conclusions; however, all 3 deer from which *M. terrae* was isolated were categorized as uninfected while the single deer from which *M. kansasii* was isolated was categorized as diseased. It is unclear from the current study whether exposure to saprophytic environmental mycobacteria had a positive or negative effect on vaccine efficacy. Studies in cattle have shown that sensitization of calves to environmental saprophytic mycobacteria adversely affects the protective efficacy of BCG vaccination [49]. Likewise, one explanation for the highly variable, and often disappointing, efficacy observed in human vaccine trials has been exposure to saprophytic environmental mycobacteria [50]. Vaccination of neonatal calves has been used as a strategy to avoid prior sensitization by saprophytic environmental mycobacteria. Indeed, vaccination of neonatal calves induces a higher level of immunity than that seen in calves vaccinated at 5–6 months of age [44,51–53]. A similar strategy could be feasible for vaccination of captive white-tailed deer, but would be problematic for vaccination of free-ranging white-tailed deer. Although the ultimate goal of any tuberculosis vaccination program is to eradicate tuberculosis, a more immediate aim is to reduce the rate of transmission between susceptible hosts. Any tuberculosis vaccine considered for use in free-ranging white-tailed deer may not need to protect animals against infection. Disease prevalence could be decreased if vaccinated animals did not develop advanced lesions or shed virulent *M. bovis*, thereby decreasing disease transmission. The present study demonstrates that parenteral vaccination of white-tailed deer with *M. bovis* BCG Pasteur provides some protection against challenge with virulent *M. bovis*. The present study also reveals several points that will require further investigation, not least of which are those related to safety. Further studies will need to investigate the virulence of *M. bovis* BCG in white-tailed deer and the degree and duration of vaccine persistence within tissues. Shedding of vaccine to other deer or non-target species, including humans, also remains in question.

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