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IMMUNE RESPONSES OF WHITE-TAILED DEER (*ODOCOILEUS VIRGINIANUS*) TO *MYCOBACTERIUM BOVIS* BCG VACCINATION

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ABSTRACT: The objective was to evaluate cellular immune response of captive white-tailed deer (*Odocoileus virginianus*) to live *Mycobacterium bovis* bacille Calmette Guerin (BCG) vaccination and to determine diagnostic implications of these responses. In vitro proliferative and interferon-γ (IFN-γ) responses to *M. bovis* purified protein derivative (PPD) were detected beginning 9 days postvaccination. Responses to *Mycobacterium avium* PPD, however, generally exceeded responses to *M. bovis* PPD. Interferon-γ responses to *M. avium* PPD were not detected prior to vaccination nor in nonvaccinated deer, suggesting that vaccination with BCG boosted prior quiescent *M. avium*–sensitized cells. Both $CD4^+$ and γδ T cells from vaccinated deer proliferated in response to *M. bovis* PPD stimulation. Intradermal administration of *M. bovis* PPD resulted in increases in skin thickness of vaccinated deer beginning 24 hr postinjection. Such early reactions were characterized by edema and minimal mononuclear cell infiltration, whereas later reactions (i.e., 72 hr postinjection) were more typical of delayed type hypersensitivity. Upon in vitro activation with pokeweed mitogen, CD44 expression increased and CD62L expression decreased on lymphocytes from deer regardless of vaccination status. Likewise, *M. bovis* PPD stimulation of lymphocytes from vaccinated deer resulted in increases in CD44 expression and decreases in CD62L expression. These findings demonstrate the potential of BCG vaccination to elicit strong cell-mediated immune responses and appropriate alterations in CD44 and CD62L expression with in vitro stimulation of white-tailed deer lymphocytes. In relation to *M. bovis* diagnosis, vaccination of white-tailed deer with BCG can induce skin test responses that classify the animal as a tuberculosis reactor. In contrast, BCG vaccination will likely not interfere with tuberculosis testing by the IFN-γ assay.

Key words: $CD4^+$ T cells, CD44, CD62L, interferon-γ, *Mycobacterium avium, Mycobacterium bovis* BCG, γδ T cells, white-tailed deer.

INTRODUCTION

Mycobacterium bovis infection is endemic in white-tailed deer (*Odocoileus virginianus*) in northeastern Michigan, USA (Schmitt et al., 1997; Fitzgerald et al., 2000; Palmer et al., 2000). Supplemental feeding practices have contributed to spread of the disease among deer and to domestic livestock (Kaneene et al., 2002; Miller et al., 2003). This outbreak was first detected in 1994 and has affected numerous wildlife species (Bruning-Fann et al., 2001). Other developed countries with wildlife reservoirs of *M. bovis* have been unable to eradicate tuberculosis from their domestic herds, presumably because of continued transmission by reservoir hosts to their domestic livestock (Barrow and Gallagher, 1981; Coleman, 1988). In these countries, vaccination protocols for use in both captive and free-ranging wildlife are being explored as a means to limit the spread of infection (Hughes et al., 1996; Griffin et al., 1999; Chambers et al., 2001; Corner et al., 2001, 2002a, b). Likewise, vaccines might prove necessary for the control of tuberculosis in white-tailed deer within the United States.

Development of effective tuberculosis vaccines, especially those that evoke immune responses distinguishable from virulent infection, will likely require a precise understanding of the target host response to both the infectious bacillus and to the candidate vaccine. The current standard for determination of tuberculosis vaccine efficacy is comparison to that of live *M. bovis* bacille Calmette Guerin (BCG). The efficacy of BCG in prevention of disease,

however, varies depending on multiple factors, especially exposure of the host to other mycobacterial species (Palmer and Long, 1996). Mechanisms explaining how prior exposure to other mycobacterial species alters the immune response to BCG are unclear and controversial (Stanford et al., 1981; Fine, 1995; Palmer and Long, 1996; Howard et al., 2002). Additionally, pre-existing responses to other mycobacterial species (e.g., *Mycobacterium avium*) often confound interpretation of immune responses to the tubercle bacillus. The initial objective of this study was to evaluate the cellular immune response of white-tailed deer to BCG vaccination. A key component of this objective was to compare recall interferon-γ (IFN-γ) responses to other standard measures of mycobacterial-specific responses such as lymphocyte proliferation and delayed type hypersensitivity (DTH). The assay for detection of cervid IFN-γ, originally developed for use with samples from red deer (*Cervus elaphus*; Slobbe et al., 2000), has recently been adapted for use with samples from white-tailed deer (Palmer et al., 2004). Interferon-γ responses to BCG vaccination, however, have not been determined. Another component of this objective was to evaluate activation-associated changes in CD44 and CD62L expression by white-tailed deer lymphocytes. Alterations in expression of these two molecules are useful for identification of distinct activation/memory lymphocyte phenotypes of mouse and human lymphocytes and are particularly relevant for the response to tuberculosis infection (Peters and Ernst, 2003). Expression patterns of CD44 and CD62L on white-tailed deer lymphocytes have yet to be determined. The second objective was to evaluate diagnostic ramifications of BCG vaccination on cellular immune response–based tests of tuberculosis infection in white-tailed deer.

MATERIALS AND METHODS

Animals and *M. bovis* BCG

White-tailed deer (~1 yr old) were obtained from the breeding herd (tuberculosis- and paratuberculosis-free) at the National Animal Disease Center (Ames, Iowa, USA). All deer were housed and cared for according to the Association for Assessment and Accreditation of Laboratory Animal Care International and institutional guidelines. The study was performed from May to November 2002. Pasteur strain BCG was grown in Middlebrook's 7H9 medium supplemented with 10% oleic acid-albumin-dextrose complex (OADC, Difco, Detroit, Michigan) plus 0.05% Tween 80 (Sigma Chemical Co., St. Louis, Missouri, USA). Mid–log-phase growth bacilli were pelleted by centrifugation at 750×G, washed twice in phosphate-buffered saline (PBS; 0.01 M, pH 7.2), and diluted to the appropriate cell density in PBS. Enumeration of bacilli was by serial dilution plate counting on Middlebrook's 7H11 selective media (Becton-Dickinson, Cockeysville, Maryland, USA). The vaccine consisted of $\sim 5\times10^7$ colony forming units (CFU) of *M. bovis* BCG in 1.5 ml PBS and was administered subcutaneously ($n=5$, two doses, 6-wk interval between doses). In addition, five deer were not vaccinated and served as nonvaccinated controls for assay comparisons. Control and vaccinated deer were housed together in an outdoor paddock.

Mononuclear cell culture and lymphocyte blastogenesis

Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coat fractions of peripheral blood collected in 2× acid citrate dextrose. Wells of 96-well round-bottom microtiter plates (Falcon, Becton-Dickinson, Lincoln Park, New Jersey, USA) were seeded with 2×10^5 mononuclear cells in a total volume of 200 μl/well. Medium was RPMI 1640 supplemented with 25 mM *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid) (HEPES) buffer, 100 units/ml penicillin, 0.1 mg/ml streptomycin, 50 μM 2-mercaptoethanol (Sigma), and 10% (v/v) fetal bovine sera (FBS; National Veterinary Services Laboratory [NVSL], Ames, Iowa). Wells contained medium plus 10 μg/ml *M. bovis* purified protein derivative (PPD; CSL Animal Health, Parkville, Victoria, Australia), 10 μg/ml *M. avium* PPD (CSL Animal Health), 1 μg/ml pokeweed mitogen (PWM, Sigma), or medium alone (no stimulation). For assessment of CD44 and CD62L expression on stimulated PBMCs, cells were incubated for 3 or 5 days at 37 C in 5% CO_2 in air, harvested, and analyzed by flow cytometry for activation marker expression. For measurement of DNA synthesis as an indication of proliferation, PBMCs were incubated for 6 days at 37 C in 5% CO_2 in air with 0.5 μCi of methyl-[^{3}H]thymidine (specific activity 6.7 Ci/

mmol, Amersham Life Science, Arlington Heights, Illinois, USA) in 50 μl of medium added to each well for the terminal 20 hr of incubation. Well contents were harvested onto fiber filters with a 96-well plate harvester (EG&G Wallac, Gaithersburg, Maryland), and the incorporated radioactivity was measured by liquid scintillation counting. Treatments were run in triplicate, and data were presented as stimulation indices (SI, mean counts/min PPD-stimulated cultures divided by mean counts/min nonstimulated cultures).

PKH67 proliferation assay

The PKH67 proliferation assay was performed according to manufacturer instructions (Sigma) and as described (Waters et al., 2000, 2002). Briefly, 2×10^7 PBMCs were centrifuged (10 min, 400×G), supernatants aspirated, and cells resuspended in 1 ml of diluent provided in the PKH67 kit. Diluted cells were added to 1 ml of PKH67 green fluorescent dye (2 μM, Sigma) and incubated 5 min, followed by a 1-min incubation with 2 ml of FBS to adsorb excess dye. Cells were washed (10 min, 400×G) three times with RPMI 1640. PKH67-stained cells were then added to wells (2×10^5 cells/well, six replicates per treatment) of 96-well round-bottom microtiter plates in medium (no stimulation) or medium plus 10 μg/ml *M. bovis* PPD. Cultures were incubated for 7 days at 37 C in a humidified chamber with 5% CO_2 and then harvested for flow cytometric measurement of phenotype and proliferation.

Interferon-γ enzyme-linked immunosorbent assay

A whole-blood culture system for the determination of recall IFN-γ production as used for cattle (Wood and Rothel, 1994) and red deer (Slobbe et al., 2000) was adapted for use with samples from white-tailed deer (Palmer et al., 2004). 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* PPD, 20 μg/ml *M. avium* PPD, or 20 μg/ml PWM, with optimal dilutions of PPDs and PWM determined previously (Palmer et al., 2004). Samples were incubated for 48 hr 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™, CSL Animal Health). Data are presented as change in optical density (ΔOD) readings at 450 nm (i.e., OD of PPD-stimulated samples − OD of nonstimulated samples, average of duplicates).

Flow cytometry

Cultured mononuclear cells (~2×10^6 cells/ml) in 100 μl of balanced salt solution with 1% FBS and 0.1% sodium azide were stained with 100 μl of primary antibody to leukocyte surface antigens (17D1, anti-CD4; ST8, anti-CD8; GB21A, anti-γδ TCR; BAQ4A, anti-WC1; BAT31A, anti-CD44; BAQ92A, anti-CD62L [primary antibodies all obtained from VMRD Inc., Pullman, Washington, USA]) designed for use with samples from sheep or cattle, yet also cross-reactive with surface antigens on deer leukocytes (Buchan and Griffin, 1990; Buchan et al., 1992; Cross et al., 1996; Waters et al., 2000; B. Davis, pers. comm.). Following a 15-min incubation, cells were centrifuged (400×G) for 2 min and resuspended in 100 μl of appropriate secondary antibody (phycoerythrin [PE]-conjugated goat anti-mouse immunoglobulins IgM, IgG1, or IgG2b [Southern Biotechnology Associates, Inc., Birmingham, Alabama, USA] or peridinin chlorophyll protein [PerCP]-conjugated rat anti-mouse IgG1 [Becton-Dickinson, San Jose, California, USA]). Cells were then incubated for an additional 15 min, centrifuged (400×G) for 2 min, resuspended in FACS buffer, and analyzed with a Becton-Dickinson FACScan flow cytometer (488 nm laser, two- or three-color according to the assay; Becton-Dickinson) with at least 10,000 events analyzed from each sample. Modfit Proliferation Wizard (Verity Software House Inc., Topsham, Maine, USA) and CellQuest software (Becton-Dickinson) were used for cell proliferation and phenotype analyses, respectively. Proliferation profiles were determined for gated populations (i.e., $CD4^+$, $CD8^+$, γδ TCR^+, and $WC1^+$ cells) and presented as the mean (±SEM) number of cells that had proliferated to *M. bovis* PPD stimulation minus the response to no stimulation (per 10,000 PBMCs). Expression of CD44 and CD62L are presented as geometric mean fluorescence intensities (mfi).

In vivo responsiveness to mycobacterial antigens

Eighteen weeks after primary vaccination, deer were tested for in vivo responsiveness to mycobacterial antigens (i.e., skin test) with a modified comparative cervical test (CCT), enabling collection of biopsies of the dermal reactions to PPDs at 24, 48, and 72 hr after injection. The cervical region was clipped, and animals were injected intradermally in three separate locations for *M. bovis* PPD (100 μg PPD administered at each location, NVSL) and a single location for *M. avium* PPD (40 μg, NVSL). Prior to and at 24, 48, and 72 hr after administration of the PPDs, dermal reactions

were measured, and punch biopsies were obtained from a *M. bovis* PPD injection site daily with a 6-mm skin punch biopsy instrument. The biopsy specimen was placed in 10% neutral buffered formalin, processed routinely, and stained with hematoxylin and eosin (H&E). Deer were classified as either negative, suspect, or reactors by plotting 72-hr measurements on a graph (Veterinary Services form 6-22D) developed by the US Department of Agriculture (USDA) for interpretation of the CCT for Cervidae (USDA, 1999; Palmer et al., 2001). Data are also presented as measurements of skin thickness (means ± SEM) for each treatment group.

Statistical analysis

Data were analyzed by either one-way analysis of variance followed by Tukey-Kramer multiple comparisons test or Student's *t*-test (either unpaired or pairwise comparisons) with a commercially available statistics program (InStat 2.00, GraphPAD Software, San Diego, California).

RESULTS

Proliferative responses to mycobacterial antigens

Lymphocyte blastogenic responses were measured at 9, 28, 70, and 126 days after primary vaccination (Fig. 1). Using a standard cutoff SI of 2 as a positive response to *M. bovis* PPD, 17 of 19 (89%) responses from BCG-vaccinated deer and five of 20 (25%) responses from nonvaccinated deer were considered positive. Four of the five (80%) positive responses to *M. bovis* PPD by nonvaccinated deer were accompanied by a greater response in replicate cultures to *M. avium* PPD (data not shown). Five of the 19 (26%) positive responses to *M. bovis* PPD from BCG-vaccinated deer were also accompanied by a greater response in replicate cultures to *M. avium* PPD. Evaluation of the kinetics of the response to *M. bovis* PPD in individual deer did not reveal a clear trend; however, two of the BCG-vaccinated deer (i.e., indicated by closed circles and diamonds in Fig. 1) were high responders throughout the study. As with *M. bovis*–infected deer (Waters et al., 2000), $CD4^+$ cells from BCG-vaccinated deer were the predominant subset of T cells proliferating in response to *M. bovis* PPD stimulation (Table 1). A significant response ($P<0.05$) by $WC1^+$ cells, a major subset of circulating γδ T cells of ruminants (Hein and Mackay, 1991; Machugh et al., 1997), was also detected. Strong proliferative responses to PWM stimulation, as measured by PKH67 analysis and methyl-[^{3}H]thymidine uptake, were detected by cells from each deer at all time points (data not shown). Pokeweed mitogen treatment was included to

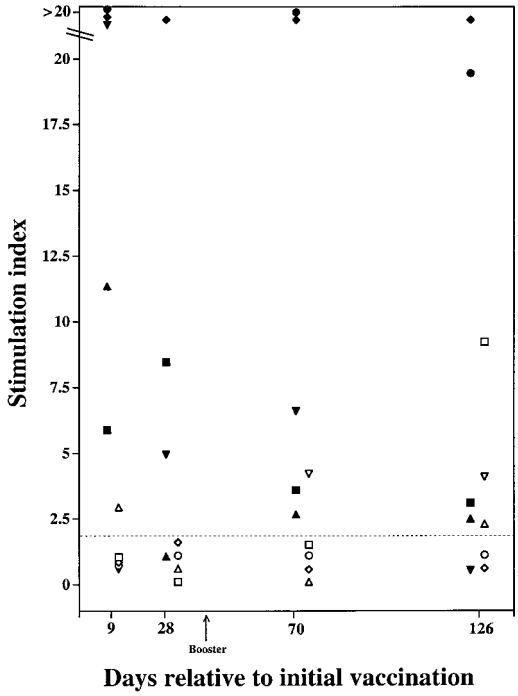

FIGURE 1. Lymphocyte blastogenic responses to *M. bovis* purified protein derivative (PPD) on bacille Calmette Guerin (BCG) vaccination. Mononuclear cells were harvested from buffy coat fractions and incubated for 6 days with medium alone or medium plus 10 μg *M. bovis* PPD/ml. Methyl-[^{3}H]thymidine was added for the terminal 18 hr of culture and incorporated radioactivity measured by liquid scintillation counting. Stimulation indices were determined by dividing *M. bovis* PPD–stimulated cultures by nonstimulated cultures (counts/min). Responses of vaccinated white-tailed deer are indicated by closed symbols; those of nonvaccinated deer are indicated by open symbols. Each symbol represents the response of an individual deer. Responses greater than 2 (dashed line) are traditionally considered positive. A booster dose of BCG was given 42 days after primary vaccination.

TABLE 1. Lymphocyte subset proliferative responses of bacille Calmette Guerin (BCG)–vaccinated white-tailed deer.[a]

Group	$CD4^+$	$CD8^+$	$\gamma\delta$ TCR^+	$WC1^+$
Vaccinates (n = 5)	1,572±287**[b]	510±255	826±374	851±91*
Controls (n = 4)	174±98	177±106	299±257	236±174

[a] White-tailed deer received two doses (42-day intervals) of 5×10^7 colony-forming units (CFU) *M. bovis* BCG subcutaneously (vaccinates) or no vaccine (controls). Mononuclear cells were harvested from buffy coat fractions (56 days after primary vaccination), stained with PKH67, incubated with medium alone or medium plus 10 μg *M. bovis* purified protein derivative (PPD)/ml for 7 days, stained with cell surface markers for flow cytometry, and analyzed for proliferation (i.e., decreased PKH67 staining intensity) in conjunction with lymphocyte subsets. Dead cells (i.e., as determined by light scatter properties) were excluded from analysis. Data represent the mean (±SEM) number of cells that had proliferated to *M. bovis* PPD stimulation minus the response to no stimulation per 10,000 peripheral blood mononuclear cells). Values differed significantly (* $P<0.05$, ** $P<0.01$) from the responses for the respective subsets of control deer (i.e., vertical comparisons).

[b] Value exceeded ($P<0.05$) the response of other subsets (i.e., horizontal comparisons, vaccinates).

assure cell viability and proliferative capability.

As indicated, blastogenic responses to *M. avium* PPD were detected. Prior exposure of cattle to *M. avium* primes the immune system for subsequent responses to BCG vaccination (Howard et al., 2002). Both proliferative (Fig. 1) and IFN-γ (Fig. 2) responses by BCG-vaccinated deer were detected immediately (i.e., 9 days) after vaccination. This immediate response was unexpected, possibly because the deer were previously sensitized to other mycobacterial antigens (e.g., *M. avium*). Although not statistically significant, a comparison of early blastogenic and IFN-γ responses in vaccinated deer (9 days post-vaccination) revealed that responses to *M. avium* PPD generally exceeded those to *M. bovis* PPD (Table 2). Prevaccination IFN-γ responses (i.e., ΔOD = response to PPD stimulation − response to no stimulation) to *M. avium* PPD, however, were not detected (mean ΔOD=0.0, n=10). Blastogenic responses to *M. avium* PPD prior to vaccination were not determined.

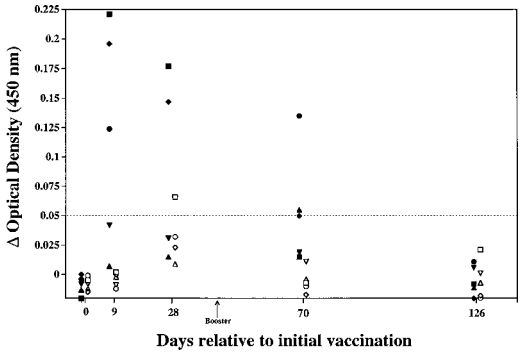

FIGURE 2. Interferon-γ responses to *M. bovis* purified protein derivative (PPD) on bacille Calmette Guerin (BCG) vaccination. Vaccinated deer received ~5×10^7 colony forming units *M. bovis* BCG subcutaneously (n=5, two doses, 6-wk interval between doses). In addition, five deer were not vaccinated and served as nonvaccinated controls. Control and vaccinated deer were housed together in an outdoor paddock. Whole-blood cultures were incubated for 48 hr with medium alone or medium plus 20 μg *M. bovis* PPD/ml. Plasma was collected and IFN-γ concentrations determined by enzyme-linked immunosorbent assay (Cervigam). Data are presented as the change in optical density (ΔOD) readings (i.e., OD of PPD-stimulated samples − OD of nonstimulated samples) and represents responses by individual deer. Responses of ΔOD>0.05 (dashed line) are considered positive.

Interferon-γ production to mycobacterial antigens

Interferon-γ responses were measured at 0, 9, 28, 70, and 126 days after primary vaccination (Fig. 2). Prior to BCG vaccination (i.e., day 0), IFN-γ responses by deer from either group (i.e., vaccinates and nonvaccinates) to either *M. avium* or *M. bovis* PPD were less than 0.01. Nine days after primary vaccination, the mean IFN-γ response in BCG-vaccinated deer to *M. bovis* PPD exceeded ($P<0.02$) prechallenge responses (i.e., 0.12±0.04 versus −0.01±0.005, mean ± SEM ΔOD readings) and the mean response in nonvaccinated deer at the same time point (i.e., 0.12±0.04 versus 0±0.003). With a cutoff

TABLE 2. Lymphocyte blastogenesis and interferon (IFN)-γ production: comparison of responses to *M. bovis* purified protein derivative (PPD) with responses to *M. avium* PPD 9 days after primary vaccination with bacille Calmette Guerin.

	LBT (SI)[a]		Δ IFN-γ[b]	
Deer number	*M. bovis* PPD–stimulated	*M. avium* PPD–stimulated	*M. bovis* PPD–stimulated	*M. avium* PPD–stimulated
476	5.89	1.06	0.221	0.782
546	22.12	65.50	0.196	0.375
563	179.04	194.71	0.124	0.134
566	11.35	22.88	0.007	0.059
596	58.64	22.34	0.042	0.051
Mean	55.41	61.30	0.12	0.28
SEM	32.25	34.96	0.04	0.14

[a] Lymphocyte blastogenesis. Mononuclear cells were harvested from buffy coat fractions and incubated for 6 days with medium alone, medium plus 10 μg *M. avium* PPD/ml, or medium plus 10 μg *M. bovis* PPD/ml. Methyl-[^{3}H]thymidine was added for the terminal 18 hr of culture and incorporated radioactivity measured by liquid scintillation counting. Stimulation indices (SI) were determined by dividing *M. bovis* PPD–stimulated cultures by nonstimulated cultures (counts/min).

[b] Whole-blood cultures were incubated for 48 hr with no stimulation, 20 μg *M. avium* PPD/ml, or 20 μg *M. bovis* PPD/ml. Plasma was collected and IFN-γ concentrations were determined by enzyme-linked immonosorbent assay (Cervigam). Data are presented as change in optical density (ΔOD) readings (i.e., OD of PPD-stimulated samples − OD of nonstimulated samples).

ΔOD value of 0.05 considered indicative of a positive response, three of five BCG vaccinates and none of the five controls were determined positive for having a response to *M. bovis* PPD at both 9 and 70 days after primary vaccination (Fig. 2). At 126 days postvaccination, all IFN-γ responses were less than 0.05, regardless of vaccine treatment. Throughout the study, IFN-γ responses to *M. bovis* PPD were accompanied by responses of similar magnitude to *M. avium* PPD (Table 2). If the criteria for a tuberculosis positive response suggested with the Cervigam kit (i.e., *M. bovis* PPD–stimulated − nonstimulated > 0.05 and *M. bovis* PPD–stimulated − *M. avium* PPD–stimulated > 0.05) is used, only 8% (2/25) of the responses throughout the study in BCG-vaccinated deer and none of the responses in nonvaccinated deer were considered positive.

In vivo responsiveness to mycobacterial antigens

Eighteen weeks after primary vaccination, deer were tested for in vivo reactivity to mycobacterial antigens by a modified CCT. If the criteria established by the USDA for interpretation of the CCT for Cervidae (USDA, 1999; Palmer et al., 2001) is used, four nonvaccinates were classified as negative and one nonvaccinate as suspect, whereas three vaccinates were classified as reactors, one vaccinate as suspect, and one vaccinate as negative. To determine the kinetics of the in vivo response, measurements (Table 3) and biopsies were collected daily for 3 days after injection. The response of vaccinates to *M. bovis* PPD exceeded that of controls at 24, 48, and 72 hr after injection of PPDs (Table 3; $P=0.03$, 0.06, and 0.03, respectively, for each time point). The response of vaccinates to *M. avium* PPD did not exceed the response by controls to *M. avium* PPD at any time point after injection of PPDs. Although not statistically different ($P>0.05$), the response of vaccinates to both *M. bovis* and *M. avium* PPD decreased from 24 to 48 to 72 hr after injection of PPDs. Responses of vaccinates to *M. avium* PPD at 24 hr after injection exceeded ($P<0.05$) preinjection readings. Responses of nonvaccinated deer to *M. avium* PPD at 48 and 72 hr after injection of PPDs exceeded ($P<0.05$) preinjection readings.

Microscopic examination of skin test biopsies

At 24 and 48 hr after intradermal injection of PPDs, biopsies of the injection site from vaccinates were characterized by mild to moderate superficial dermal edema (Fig. 3) and mild to moderate multifocal perivascular infiltrates of inflammatory cells. Inflammatory cells were primarily neutrophils at 24 hr after injection, whereas at 48 hr, the infiltrates were a mixture of neutrophils, macrophages, and lymphocytes. By 72 hr after injection, superficial dermal edema had decreased to minimal or mild severity. Moderate multifocal infiltrates of macrophages and lym-

TABLE 3. Longitudinal response to intradermal administration of mycobacterial antigens (purified protein derivative [PPDs]) by the comparative cervical test (CCT) of delayed type hypersensitivity.

	Preinjection	24 hr	48 hr	72 hr
A. Measurement of skin thickness at *M. bovis* PPD administration site[a]				
Vaccinates	5.2±0.3	11.0±1.6**	10.8±0.9**	9.0±1.0
Controls	4.8±0.2	6.4±0.5	8.2±0.8**	6.3±0.3
P value[b] (vaccinates vs. controls)	0.30	0.03	0.06	0.03
B. Measurement of skin thickness at *M. avium* PPD administration site[a]				
Vaccinates	5.4±0.5	8.1±1.1*	7.8±0.4	7.6±0.2
Controls	4.8±0.4	6.4±0.5	7.7±0.7*	8.0±1.0*
P value[b] (vaccinates vs. controls)	0.46	0.20	0.87	0.73

[a] Eighteen weeks after primary vaccination, controls and vaccinates were injected with PPDs (i.e., *M. avium* PPD, one injection site; *M. bovis* PPD, three injection sites) for evaluation of in vivo responsiveness to mycobacterial antigens. Injection sites were measured for reactivity to antigens immediately before administration and 24, 48, and 72 hr after administration of PPDs. Data are presented as skin thickness (mm) for each treatment group, with responses to *M. bovis* PPD representing averages of three individual measurements (preinjection and 24 hr), two individual measurements (48 hr), or a single measurement (72 hr). Data are presented as means (±SEM, $n=5$ per treatment group). Value differs significantly (* $P<0.05$, ** $P<0.01$; analysis of variance, followed by Tukey-Kramer multiple comparison test) from preinjection response for each treatment group (i.e., horizontal comparisons).

[b] Comparisons (Student's *t*-test) between response of vaccinates and controls at indicated time points (i.e., vertical comparisons).

phocytes surrounded vessels in the superficial and deep dermis (Fig. 4).

Biopsies from nonvaccinated deer at all time points ranged from normal to mild superficial edema and mild multifocal perivascular infiltrates of lymphocytes and fewer macrophages. Biopsies at one or more time points from four of five nonvaccinates and two of five vaccinates contained focal to diffuse subcorneal pustules.

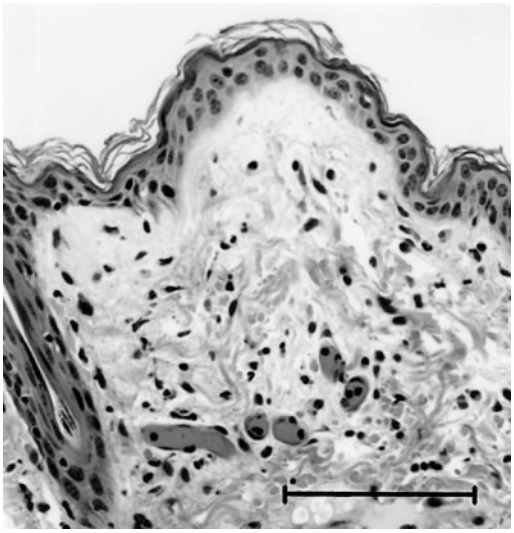

FIGURE 3. Photomicrograph of section of skin from tuberculin skin test site 24 hr after intradermal injection of *M. bovis* purified protein derivative (PPD). Note superficial dermal edema (clear space below epidermis). H&E stain. Bar=100 μm.

Activation-induced alterations in CD44 and CD62L expression

CD44 expression was increased and CD62L expression decreased on PWM-

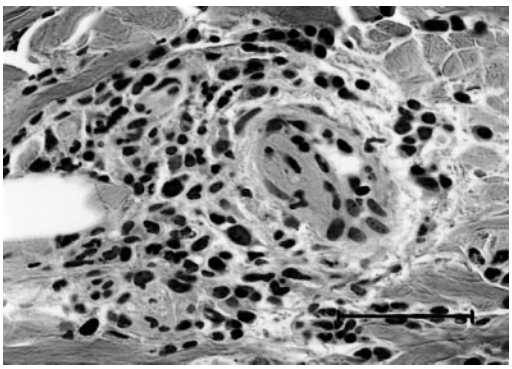

FIGURE 4. Photomicrograph of section of skin from tuberculin skin test site 48 hr after intradermal injection of *M. bovis* purified protein derivative (PPD). Note perivascular infiltrates of inflammatory cells. H&E stain. Bar=50 μm.

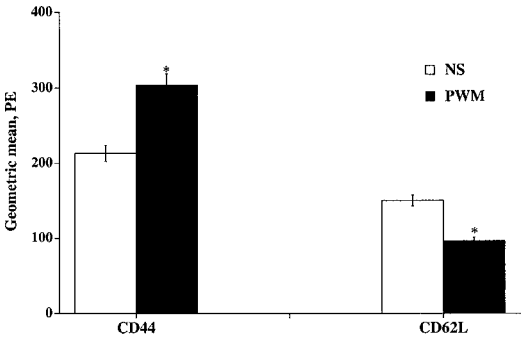

FIGURE 5. Alterations in CD44 and CD62L expression (mean fluorescence intensity [mfi]) in response to pokeweed mitogen (PWM) stimulation. Geometric mean fluorescence intensity (phycoerythrin, PE) of CD44 and CD62L on peripheral blood mononuclear cells from cultures incubated for 3 or 5 days with medium alone (NS, open bars) or 5 μg PWM/ml (closed bars). For flow cytometric analysis, gates were set on cells with light scatter properties of live cells, and the mfi of CD44 and CD62L were determined. Data are presented as means (± SEM, n=18 per treatment group) and represent responses of vaccinated deer (n=4) and nonvaccinated deer (n=5) after 3 days (n=9) and 5 days (n=9) of culture. An asterisk (*) indicates that a value differs significantly (P<0.01, Student's t-test) from nonstimulated samples. Differences as a result of culture duration (i.e., 3 days versus 5 days) or vaccination status were not detected.

stimulated cultures compared with nonstimulated cultures (Fig. 5). Differences as a result of vaccination status or duration of culture (i.e., 3 versus 5 days) were not detected for this response to PWM. Antigen-specific activation of white-tailed deer lymphocytes (56 days postvaccination) also resulted in a significant (P<0.05) decrease in CD62L expression (Table 4). The decrease in CD62L expression on *M. bovis* PPD stimulation was considerably less for one vaccinated deer (no. 476); however, this deer also had a concurrent low blastogenic response (SI=0.53, 56 days postvaccination) to *M. bovis* PPD. One of the nonvaccinates (no. 429) exhibited a decrease in CD62L expression on *M. bovis* PPD stimulation similar to that of vaccinates. This deer had a concurrent high blastogenic response to *M. avium* PPD (SI=12.45, 56 days postvaccination), but not to *M. bovis* PPD (SI=0.09), and a delayed type hypersensitive response to *M. avium* PPD (5-mm increase in skin thickness, 126 days postvaccination). Significant (P<0.05) CD62L antigen-specific responses were detected for cells stimulated for 3 days but not 5 days. CD44 expression was increased (P<0.05, pairwise comparisons) on *M. bovis* PPD–stimulated PBMCs compared with nonstimulated PBMCs (Table 5). This CD44 response was not detected for nonvaccinated deer (P=0.88, pairwise comparisons of responses to *M. bovis* PPD–stimulated versus nonstimulated). The increase in CD44 expression on *M. bovis* PPD stimulation for vaccinates, however, did not exceed (P=0.2, unpaired Student's t-test) the response by nonvaccinates because of large variation in the response by nonvaccinates. Mononuclear cells from two of the nonvaccinated deer (nos. 538 and 588) had increases in CD44 expression on *M. bovis* PPD stimulation similar to that of vaccinated deer. Unlike the outlying CD62L responses by the nonvaccinated deer, neither of these two deer had a concurrent blastogenic response to either *M. bovis* or *M. avium* PPD.

DISCUSSION

Mycobacterium bovis infection, in addition to the on-going outbreak in free-ranging white-tailed deer in Michigan, has been diagnosed in at least 37 captive cervid herds in 18 states within the US since 1990. Currently, the only approved and available antemortem test for tuberculosis diagnosis in deer is the CCT. As previously described for red deer (Griffin et al., 1993), BCG vaccination of white-tailed deer resulted in skin test responses to *M. bovis* PPD that exceeded the response to *M. avium* PPD (Table 3). The change in skin thickness associated with BCG vaccination (i.e., ~3.8 mm, n=5) was comparable to the response reported for BCG-vaccinated red deer (i.e., ~2.0 mm, n=5; Griffin et al., 1993) yet was only one third of the response detected with experimentally infected white-tailed deer (i.e., 12.1 mm, n=60; Palmer et al., 2001). Despite

TABLE 4. Reduction in CD62L expression (mean fluorescence intensity)[a] by peripheral blood mononuclear cells (PBMCs) from bacille Calmette Guerin–vaccinated white-tailed deer on stimulation with *M. bovis* purified protein derivative (PPDb).

Group/ deer number	No stimulation	PPDb stimulation	ΔCD62L[b]	Comments[c]
Vaccinates				
476	73.65	67.38	−6.27	Minimal blastogenic response to *M. bovis* PPD
563	173.03	137.19	−35.84	
566	170.20	120.49	−49.71	
596	214.50	178.17	−36.33	
Mean	157.85	125.81	−32.04*	
SEM	29.84	22.94	9.17	
Controls				
429	167.42	148.55	−18.87	Marked blastogenic response to *M. avium* PPD
438	137.05	153.80	16.75	
538	116.40	133.44	17.04	
567	180.31	176.76	−3.55	
588	73.55	87.19	13.64	
Mean	134.95	139.95	5.00	
SEM	19.01	14.91	7.07	

[a] Isolated mononuclear cells (56 days after primary vaccination) were incubated with media alone (i.e., no stimulation) or media plus 10 μg/ml of *M. bovis* PPD for 3 days and analyzed for CD62L expression. For analysis, the geometric mean fluorescence intensity (phycoerythrin) of CD62L was determined for cells with light scatter properties of live cells.

[b] Mean fluorescence intensity (mfi) of cells responding to *M. bovis* PPD stimulation minus mfi of cells responding to no stimulation.

[c] Comments concerning concurrent proliferative (i.e., blastogenic) responses of deer exhibiting outlying activation marker responses. * Value differs significantly ($P=0.01$, Student's *t*-test) from responses by control deer. Pairwise comparisons of CD62L nonstimulated versus PPDb-stimulated PBMCs (mfi): $P=0.04$ for vaccinates and $P=0.52$ for nonvaccinates.

the weak response compared with experimental infection, four of five vaccinated deer were classified as reactors or suspect by the scatter plot provided by the USDA for interpretation of the CCT for Cervidae (USDA, 1999) and the New Zealand method (Griffin et al., 1993). One nonvaccinated deer was determined suspect by CCT. In contrast, only 8% (2/25) of IFN-γ responses (i.e., throughout the study) in BCG-vaccinated deer were considered positive by the criteria suggested by the manufacturer of the Cervigam assay (i.e., *M. bovis* PPD–stimulated − nonstimulated > 0.05 and *M. bovis* PPD–stimulated − *M. avium* PPD–stimulated > 0.05). Additionally, none of the IFN-γ responses in nonvaccinated deer were considered positive by these same criteria. These findings indicate that for detection of tuberculous deer, the Cervigam assay is less affected than the CCT by BCG vaccination.

In this study, vaccinated deer responded within 9 days of primary immunization to mycobacterial antigen stimulation, as indicated by SI for blastogenic responses exceeding 2 and ΔOD IFN-γ responses exceeding 0.05 (Table 2). Such a rapid response is indicative of prior sensitization with antigens that are cross-reactive with those contained within or produced by BCG (Howard et al., 2002). Because responses to *M. avium* PPD generally exceeded responses to *M. bovis* PPD, it is likely that the deer used in the study had been previously sensitized to *M. avium* antigens. Significant ($P<0.05$) skin test responses of nonvaccinated deer to *M. avium* PPD (Table 3) are also suggestive of *M. avium* sensitization. Although *M. avium* subsp. *paratuberculosis* has never been detected in this herd, on rare occasions, isolates of *M. avium* and other atypical *Mycobacteria* spp. have been cultured from tissues (Palmer et al., 2001). Interferon-γ responses to *M. avium* prior to

TABLE 5. Increase in CD44 expression (mean fluorescence intensity)[a] by peripheral blood mononuclear cells bacille Calmette Guerin (BCG)–vaccinated white-tailed deer on stimulation with *M. bovis* purified protein derivative (PPDb).

Group/ deer number	No stimulation	PPDb stimulation	ΔCD44[b]
Vaccinates			
476	185.35	201.45	16.10
563	260.23	271.68	11.45
566	147.79	154.56	6.77
596	159.29	164.87	5.58
Mean	188.17	198.14*	9.97
SEM	25.27	26.50	2.40
Controls			
429	262.15	257.52	−4.63
438	248.69	229.43	−19.26
538	220.14	232.15	12.01
567	237.03	201.70	−35.33
588	92.98	108.94	15.96
Mean	212.20	205.95	−6.25
SEM	30.59	25.81	9.60

[a] Isolated mononuclear cells (56 days after primary vaccination) were incubated with media alone (i.e., no stimulation) or media plus 10 μg/ml of *M. bovis* PPD for 3 days and analyzed for CD44 expression. For analysis, the geometric mean fluorescence intensity (phycoerythrin) of CD44 was determined for cells with light scatter properties of live cells.

[b] Mean fluorescence intensity (mfi) of cells responding to PPDb stimulation minus mfi of cells responding to no stimulation. * Value differs significantly ($P=0.02$, pairwise comparisons) from the response to vaccinated deer with no stimulation. The response by control deer did not differ ($P=0.88$).

vaccination were not observed, suggesting that detectable in vitro responses required boosting with BCG vaccination. In contrast, experimental inoculation with virulent *M. bovis* of similarly aged deer from the same herd resulted in IFN-γ responses to *M. bovis* PPD exceeding responses to *M. avium* PPD (Palmer et al., 2004). Additionally, IFN-γ responses of experimentally infected deer (Palmer et al., 2004) were of greater magnitude than those detected from BCG-vaccinated deer (this study). Thus, it is likely that antigen stimulation induced by infection with virulent *M. bovis* greatly exceeds that induced by BCG vaccination, thereby resulting in responses to *M. bovis* antigens that exceed any cross-reactive responses to *M. avium* antigens, albeit cross-reacting *M. avium* responses often confound interpretation of immune tests (e.g., IFN-γ and CCT) of tuberculosis infection (Amadori et al., 2002). Present findings with BCG (as a potential model to mimic low-grade infection) further demonstrate the need for improved antigens that enhance the specificity of tests to determine *M. bovis* exposure of white-tailed deer. It could be argued that *Mycobacterium*-naïve deer should have been used for the present study. Because of the ubiquitous nature of nontuberculous mycobacteria (including *M. avium*), truly *Mycobacterium*-naïve deer are likely rare and not representative of candidates subject to tuberculosis diagnostic tests or vaccines.

Extravasation and subsequent trafficking of lymphocytes to sites of inflammation is mediated by differential expression of multiple surface adhesion molecules. L-selectin (CD62L) mediates specific adhesion to peripheral lymph node vascular addressins (PNAds; e.g., GlyCAM-1 and MAdCAM-1) targeting resting lymphocytes ($CD62L^{hi}$) to areas of antigen concentration within lymph nodes (Dailey, 1998). After lymphocyte activation by proinflammatory cytokines, CD62L expression is down-regulated, and integrin (e.g., α4β1, α4β7) expression and binding affinity is rapidly increased, inducing tight adhesion conducive for extravasation of lymphocytes to sites of inflammation. CD44 expression, as with integrin expression, is up-regulated on lymphocytes on activation, thereby promoting their movement through the extracellular matrix via interactions with hyaluronic acid and fibronectin (Dailey, 1998). Actions of these adhesion molecules are modeled in vitro by stimulation of lymphocytes with antigens, anti-CD3, or mitogens (Hamann et al., 1988; Jung et al., 1988; Chao et al., 1994; Dailey, 1998; Waters et al., 2003). As detected with mouse, human, and bovine lymphocytes, stimulation of PBMCs from white-tailed deer with PWM resulted in down-regulation of CD62L expression and up-regulation of CD44 expression. Similar

changes were detected with mycobacterium antigen–stimulated PBMCs from vaccinated deer. These alterations in vivo would affect the functional status and trafficking of mononuclear cells responding to antigen (i.e., in lesions of infected animals and skin test sites). Further studies are needed to understand the ramifications of such changes in adhesion molecule expression as it relates to *M. bovis* vaccination and infection of deer.

Responses at skin test injection sites were evaluated as a measure of in vivo reactivity to cognate antigen by sensitized white-tailed deer. Early edema was a consistent finding at skin test sites of vaccinated deer. Superficial dermal edema was greatest 24 hr postinjection of *M. bovis* PPD. Edema is detected as early as 12 hr after tuberculin skin testing in sensitized cattle (Feldman and Fitch, 1937) and at 48 hr in BCG-vaccinated dogs (Thilstead and Shifrine, 1978). Edema is also detected as early as 4 to 8 hr after injection of dinitrochlorobenzene in a model of DTH in humans (Dvorak et al., 1974). In another study with naturally infected cattle, the peak of edema for skin testing was 72 hr after injection of PPD (Doherty et al., 1996). Mechanisms contributing to inflammatory edema in DTH reactions, however, are not completely understood. With mouse models of tuberculosis, it is suggested that lymphokines released from activated T cells play an important role in edema formation (Van Loveren and Askenase, 1984). Interferon-γ produced by antigen-specific CD4 cells is responsible for more than 50% of the edema in the murine DTH reaction to keyhole limpet hemocyanin (Fong and Mosmann, 1989), and T cell–derived IL-2 might also increase vascular permeability (Rosenstein et al., 1986). Further studies are necessary to evaluate potential mechanisms of edema formation resulting from PPD injection of sensitized deer.

Findings in this study demonstrate the potential of BCG vaccination to evoke cell-mediated immune responses in captive white-tailed deer, the use of an IFN-γ–based assay to detect mycobacterial sensitization, the presence of cross-reactive *M. avium* responses that confound interpretation of *M. bovis* responses, and expected alterations in CD44 and CD62L expression by lymphocytes on activation. Further studies are necessary to evaluate the efficacy of BCG in the prevention of *M. bovis* infection of white-tailed deer.

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