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NITRIC OXIDE PRODUCTION AS AN INDICATION OF *MYCOBACTERIUM BOVIS* INFECTION IN WHITE-TAILED DEER (*ODOCOILEUS VIRGINIANUS*)

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ABSTRACT: White-tailed deer (*Odocoileus virginianus*) are reservoirs for *Mycobacterium bovis* in northeast Michigan, USA. Production of nitric oxide (NO) by activated macrophages is a potent mechanism of mycobacterial killing. The capacity of macrophages to produce NO, however, varies among mammalian species. The objective of this study was to determine if mononuclear cells from white-tailed deer produce nitrite as an indication of NO production and, if so, is NO produced in response to stimulation with *M. bovis* antigens. Supernatants were harvested from adherent peripheral blood mononuclear cell (PBMC) cultures that had been stimulated with either *Mannheimia haemolytica* lipopolysaccharide (LPS) or media alone (i.e., no stimulation). Nitrite levels within *M. haemolytica* LPS-stimulated culture supernatants exceeded ($P < 0.05$) those detected within supernatants from non-stimulated cultures as well as those detected within supernatants from cultures receiving an inhibitor of NO synthase in addition to *M. haemolytica* LPS. In response to stimulation with *M. bovis* antigens, nitrite production by PBMC from *M. bovis*-infected deer exceeded ($P < 0.05$) the production by PBMC from non-infected deer. The response of PBMC from infected deer to *M. bovis* antigens exceeded ($P < 0.05$) the response of parallel cultures from the same deer receiving no stimulation. The response of PBMC from *M. bovis*-infected deer to *M. avium* antigens did not differ from that of PBMC from *M. bovis*-infected deer to no stimulation or from that of PBMC from non-infected deer to *M. avium* antigens. These findings indicate that adherent PBMC from white-tailed deer are capable of NO production and that mononuclear cells isolated from *M. bovis*-infected white-tailed deer produce NO in an antigen-specific recall response.

Key words: Bovine tuberculosis, lipopolysaccharide, *Mannheimia haemolytica*, mononuclear cells, *Mycobacterium bovis*, nitric oxide, *Odocoileus virginianus*, reactive nitrogen intermediates, white-tailed deer.

INTRODUCTION

White-tailed deer (*Odocoileus virginianus*) are reservoirs of *Mycobacterium bovis* in Michigan, (USA) (Schmitt et al., 1997; Fitzgerald et al., 2000; Palmer et al., 2000). This designation follows an outbreak of *M. bovis* in the northeast portion of the state affecting numerous wildlife species including white-tailed deer, coyotes (*Canis latrans*), raccoons (*Procyon lotor*), red fox (*Vulpes vulpes*), bobcat (*Lynx rufus*), and black bear (*Ursus americanus*) (Bruning-Fann et al., 2001). Other countries with wildlife reservoirs of *M. bovis* have been unable to eradicate tuberculosis from their domestic herds (Barrow and Gallagher, 1981; Coleman, 1988; Buchan and Griffin, 1990). Within these countries, research in vaccination and immunity of reservoir hosts is designed to develop tuberculosis control measures as alternatives to traditional carcass inspection and test and slaughter campaigns used for domestic livestock (Hughes et al., 1996; Griffin et al., 1999). Considering the recent outbreak of tuberculosis in Michigan similar changes in research focus within the United States may enhance control of this zoonotic pathogen.

Generation of nitric oxide (NO) and related reactive nitrogen intermediates by interferon-γ (IFN-γ)- and/or tumor necrosis factor-α (TNF-α)-activated macrophages is a major effector mechanism of intracellular mycobacterial killing (Chan and Kaufmann, 1994; Flynn and Chan, 2001; MacMicking et al., 1997). The production

of reactive nitrogen intermediates by activated macrophages, however, varies among species (Schoedon et al., 1995). The objective of the present study, therefore, was to determine if adherent peripheral blood mononuclear cells (PBMC) from white-tailed deer produce NO and, if so, do PBMC from *M. bovis*-infected deer produce NO in response to *M. bovis* antigen stimulation.

MATERIALS AND METHODS

Experimental animals and *M. bovis* challenge

White-tailed deer (1–1.5 yr old) were either born at the National Animal Disease Center (NADC; Ames, Iowa, USA) and raised within our tuberculosis-free herd or obtained from producers of farmed white-tailed deer with no history of tuberculosis in their herds. Five castrated males and seven non-pregnant females were experimentally infected with *M. bovis* as described (Palmer et al., 1999) and 11 castrated males and two non-pregnant females served as non-infected controls. The strain of *M. bovis* used for the challenge inoculum (strain 1315) was isolated from a white-tailed deer from Michigan in 1994 (Schmitt et al., 1997). The challenge inoculum consisted of 300 colony forming units (cfu) of mid-log-phase *M. bovis* grown in Middlebrook's 7H9 media supplemented with 10% oleic acid-albumin-dextrose complex (OADC, Difco, Detroit, Michigan) plus 0.05% Tween 80 (Sigma Chemical Co., St. Louis, Missouri, USA) as described (Bolin et al., 1997). To harvest tubercle bacilli from culture media, cells were pelleted by centrifugation at 750 × G, washed twice with 1 ml of phosphate-buffered saline solution (PBS, 0.01 M, pH 7.2), and diluted to the appropriate cell density in 2 ml of PBS. Enumeration of bacilli was by serial dilution plate counting on Middlebrook's 7H11 selective media (Becton Dickinson, Cockeysville, Maryland, USA). For intratonsilar inoculation, deer were restrained and anesthetized with ketamine (6 mg/kg, Fort Dodge Animal Health, Fort Dodge, Iowa) and xylazine (2 mg/kg, Bayer Corp., Shawnee Mission, Kansas, USA) given intramuscularly. Effects of xylazine were reversed by intravenous administration of 4 mg/kg tolazoline (Lloyd Laboratories, Shenandoah, Iowa). The challenge inoculum was instilled directly into the tonsilar crypts of anesthetized deer. Infected deer were housed in pens (two-four deer/pen) inside a biosecurity level 3 building with negative air-flow exiting the building through high efficiency particulate air filters. Three of the 13 non-infected deer were housed as for the infected deer but in a separate building and the remainder of the non-infected deer were housed in a paddock of approximately 2 ha. Deer were fed a pelletized ration and alfalfa hay.

Prior to the experiment and 90 days after inoculation, experimentally inoculated deer and three of the control deer were tested for immune reactivity to mycobacterial antigens by the comparative cervical skin test as described (Palmer et al., 1999). Results were used to categorize deer as negative, suspect, or reactor in relation to exposure to *M. bovis* (United States Department of Agriculture, 1999). All *M. bovis*-inoculated deer were euthanized at various time points ranging from 6–11 mo post inoculation by intravenous injection of sodium pentobarbital (Fort Dodge Laboratories). Various tissues were collected for bacteriologic culture and microscopic examination with a detailed description of these results presented in a companion manuscript.

Mononuclear cell culture and antigens

Mononuclear cells were isolated from buffy coat fractions of peripheral blood collected in acid citrate dextrose using standard procedures (Burton and Kehrli, 1996). Wells of 96-well round-bottomed 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 per well. The medium was RPMI 1640 (Gibco, Invitrogen Life Technologies, Frederick, Maryland) supplemented with 25 mM HEPES buffer, 100 units/ml penicillin, 0.1 mg/ml streptomycin, 5×10^{-5} M 2-mercaptoethanol (Sigma Chemical Co., St. Louis, Missouri), and 10% fetal bovine sera (FBS). Wells contained medium plus 5 μg/ml *M. bovis* purified protein derivative (PPDb; CSL Limited, Parkville, Victoria, Australia), 5 μg/ml *M. avium* purified protein derivative (PPDa; CSL Limited), 5 μg/ml *M. bovis* strain 1315 culture filtrate (CF), 10 μg/ml *M. bovis* strain 1315 whole cell sonicate (WCS), 20 μg/ml *M. bovis* strain 1315 proteinase K digested whole cell sonicate (PK), or medium alone (no stimulation). The CF was from 2 wk *M. bovis* strain 1315 cultures (bacteria pelleted, supernatant harvested and filtered [0.22 μm] twice). For the WCS antigen preparation, 2 wk *M. bovis* strain 1315 cultures in Middlebrook's 7H9 media supplemented with 10% OADC were pelleted, sonicated in PBS, and further disrupted with 0.1–0.15 mm glass beads (Biospec Products, Bartlesville, Oklahoma, USA) in a bead beater (Biospec Products) then placed on ice. The preparation was then centrifuged

TABLE 1. Nitrite (ng/ml) detected in culture supernatants of white-tailed deer adherent peripheral blood mononuclear cells stimulated with *Mannheimia haemolytica* LPS.

Treatment	Animal							
	536	542	564	568	540	548	Mean	SEM
Medium	57	13	38	63	69	44	47	8
LPS	126	25	88	114	88	82	87[a]	14
LPS + L-NMMA	25	19	19	25	38	57	30	6

[a] $P < 0.05$, mean response to LPS differs from mean responses to no stimulation (medium) or stimulation with LPS+L-NMMA.

and the supernatant harvested, filtered (0.22 μm), and stored at −20 C. The PK antigen was prepared by digestion of the WCS in a 1 mg/ml proteinase K (Roche Molecular Biochemicals, Indianapolis, Indiana, USA) solution (50 mM Tris, 1 mM $CaCl_2$ buffer, pH 8.0) for 1 hr at 50 C. Protein concentrations of the CF, WCS, and PK antigens were determined using a protein determination kit (Bio Rad, Hercules, California, USA). Mononuclear cell cultures were incubated for 24, 48, or 72 hr at 37 C in 5% CO_2 in air. Supernatants were removed from the cell pellet and stored at −70 C for later analysis.

Monocytes were isolated from buffy coat fractions of peripheral blood of non-infected deer by adherence in 25 cm^2 flasks at 37 C for 1 hr. Cells were cultured for 2 wk at 37 C with 5% CO_2 in RPMI 1640 (Gibco, Invitrogen Life Technologies, Frederick, Maryland) supplemented with L-glutamine, penicillin, streptomycin, 2-Mercaptoethanol, 10% heat-inactivated fetal calf serum, and 10% heat-inactivated white-tailed deer serum. Medium was changed every 3–4 days during the culture period. At the end of the 2 wk culture period, cells were stimulated for 24 hr with or without 1 μg/ml *Mannheimia haemolytica* LPS (Brogden et al., 1995). Supernatants were harvested and stored at −70 C for later analysis.

Nitric oxide assay

Nitrite is the stable oxidation product of NO and the amount of nitrite within culture supernatants is indicative of the amount of NO produced by cells in culture. Nitrite was measured using the Griess reaction (Rajaraman et al., 1998) performed in 96-well microtiter plates (Immunolon 2, Dynatech Laboratories, Inc., Chantilly, Virginia, USA). Culture supernatant (100 μl) was mixed with 100 μl of Griess reagent (0.5% sulfanilamide [Sigma Chemical Co.] in 2.5% phosphoric acid [Mallinckrodt Chemicals, Inc., Paris, Kentucky, USA]) and 0.05% N-(1-naphthyl) ethylenediamine dihydrochloride (Sigma Chemical Co.). The mixture was incubated at 21 C for 10 min. Absorbances of test and standard samples at 550 nm were measured using an automated ELISA plate reader (Molecular Devices, Menlo Park, California). Dilutions of standards were made using supplemented RPMI 1640 media. Absorbances of standards, controls, and test samples were converted to ng/ml of nitrite by comparison with absorbances of sodium nitrite (Fisher Chemicals, Fair Lawn, New Jersey) standards within a linear curve fit. N^G-monomethyl-L-arginine (L-NMMA; Calbiochem, La Jolla, California), a competitive inhibitor of the enzyme nitric oxide synthase (NOS) (1.15 mM; equimolar to the amount of L-arginine in the culture medium) was added to parallel cultures to verify that the nitrite produced was due to the activity of NOS.

Statistics

Data were analyzed by one-way analysis of variance followed by Tukey-Kramer multiple comparisons test. Differences between groups were considered significant if probability values of $P < 0.05$ were obtained.

RESULTS

All *M. bovis*-inoculated deer were classified as negative by the comparative cervical test of delayed type hypersensitivity prior to exposure and as reactors 90 days post inoculation. All non-infected deer tested by the comparative cervical test were classified as negative for skin hypersensitivity to *M. bovis*.

White-tailed deer macrophages produced nitrite in response to stimulation with 1 μg/ml *M. haemolytica* LPS (Table 1). The response to LPS was significantly ($P < 0.05$) higher (87 ± 14 ng/ml nitrite) than the response of non-stimulated cells (47 ± 8 ng/ml nitrite). Nitrite levels de-

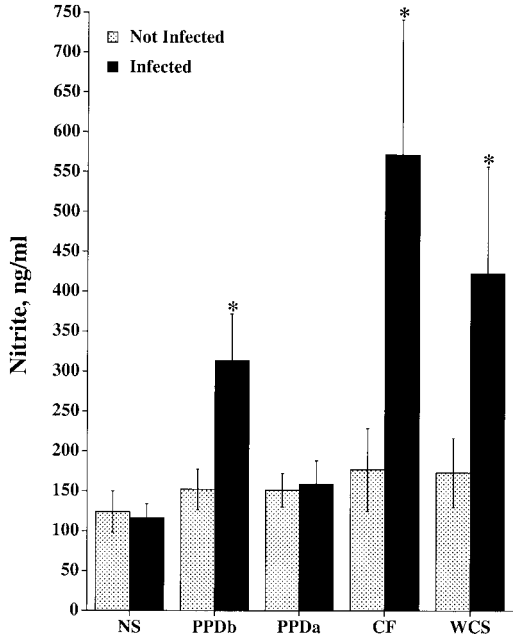

FIGURE 1. Antigen-specific induction of nitrite production by peripheral blood mononuclear cells from *Mycobacterium bovis*-infected white-tailed deer. Dotted bars indicate responses from non-infected deer ($n = 13$ for no stimulation [NS], *M. bovis* purified protein derivative [PPDb], *M. avium* PPD [PPDa], and $n = 10$ for *M. bovis* strain 1315 culture filtrate [CF] and *M. bovis* strain 1315 whole cell sonicate [WCS]. Closed bars indicate responses of *M. bovis*-infected deer ($n = 12$ NS, PPDb, and PPDa and $n = 5$ for CF and WCS). *Responses of deer not infected with *M. bovis* were different ($P < 0.05$) than deer infected with *M. bovis*.

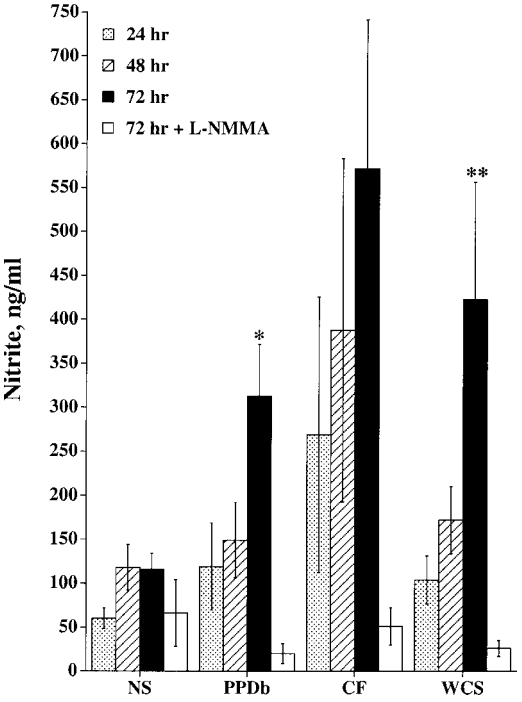

FIGURE 2. Antigen-specific production of nitrite by peripheral blood mononuclear cells from *Mycobacterium bovis*-infected white-tailed deer was cumulative over time in culture and inhibited by presence of a nitric oxide synthase inhibitor. Dotted bars represent responses at 24 hr ($n = 5$), hatched bars represent responses at 48 hr ($n = 5$), closed bars represent responses at 72 hr ($n = 5$) for *M. bovis* strain 1315 culture filtrate [CF] and *M. bovis* strain 1315 whole cell sonicate[WCS], $n = 12$ for no stimulation [NS] and *M. bovis* purified protein derivative [PPDb], and open bars represent responses at 72 hr plus N^G-mononethyl-L-arginine [L-NMMA] ($n = 5$ for CF and WCS, $n = 12$ for NS and PPDb). Response was significantly different * $P < 0.05$, ** $P < 0.01$, compared to responses at 24 hr, 48 hr, or 72 hr plus L-NMMA.

tected in culture supernatants of cells stimulated with LPS in the presence of L-NMMA did not differ ($P > 0.05$) from nitrite levels in supernatants of the non-stimulated cultures.

Nitrite production by PBMC from infected deer in response to stimulation with *M. bovis* antigens exceeded ($P < 0.05$) that of the response of non-infected deer to the respective antigen (Fig. 1). The response to *M. bovis* antigens by PBMC from *M. bovis*-infected deer exceeded ($P < 0.05$) the response of parallel cultures receiving no stimulation. Antigens produced from *M. bovis* strain 1315 (i.e., CF and WCS) tended ($P < 0.1$) to generate a greater response than did non-homologous *M. bovis* antigens (i.e., PPDb). The response of infected deer to *M. bovis* antigens PPDb, CF, and WCS exceeded ($P < 0.05$) the response to stimulation with antigens prepared from a related species of mycobacteria, *M. avium* PPD (i.e., PPDa). Nitrite production by PBMC from infected deer in response to stimulation with *M. bovis* antigens was cumulative over time in culture with responses increasing from 24–72 hr in culture (Fig. 2). Nitrite accumulation within PPDb- and WCS-stimulated cul-

tures incubated for 72 hr exceeded ($P < 0.05$) that of parallel cultures incubated for either 24–48 hr with PPDb or WCS, respectively. Addition of L-NMMA removed this response, indicating that nitrite accumulation within the supernatants results from the activity of NOS (Fig. 2). Stimulation of PBMC from infected deer with a proteinase K-digested *M. bovis* WCS antigen for 72 hr did not induce a significant response (138 ± 48.0 for the proteinase K-digested *M. bovis* WCS stimulation versus 116 ± 18.2 for no stimulation).

DISCUSSION

White-tailed deer are reservoirs of *M. bovis* infection of cattle within northeast Michigan. Environmental factors (e.g., increasing deer populations) and management practices, such as use of feeding stations to enhance deer populations, influence spread of the bacillus among concentrated deer populations (Schmitt et al., 1997). Although not tested in controlled studies, white-tailed deer appear to be highly susceptible to *M. bovis* infection in comparison to other mammalian species, representing another factor likely affecting this recent epizootic (Palmer et al., 2001). Deficits in host immune capability or ability to respond to bacterial infections as compared to other ruminants may influence this presumed susceptibility. Production of NO by infected macrophages is critical for mycobacterial killing (MacMicking et al., 1997; Flynn and Chan, 2001). Macrophages from red deer (*Cervus elaphus*), however, are deficient in their ability to produce NO (Cross et al., 1996). In contrast, adherent mononuclear cells from white-tailed deer are capable of NO production. The discrepancy between these two findings may result from species differences in NO production capability or the assay system. In the present study, LPS derived from *M. haemolytica* was used for stimulation of adherent mononuclear cells whereas LPS derived from *Escherichia coli* was used for stimulation of red deer adherent mononuclear cells (Cross et al., 1996). Regardless, it appears that macrophages from white-tailed deer are not deficient in their ability to produce NO, at least in response to *M. haemolytica* LPS.

Mononuclear cells isolated from *M. bovis*-infected deer produced NO in response to *M. bovis* but not *M. avium* antigens, indicating antigen-specificity. This response required intact *M. bovis* proteins because proteinase K-digestion of the *M. bovis* WCS abrogated significant nitrite production as detected with intact WCS. The proteinase K-digested *M. bovis* WCS antigen does react, however, with antibodies in serum obtained from *M. bovis*-infected deer by western blot analysis and enzyme-linked immunosorbent assay (ELISA) (data not shown). Thus, B cells from infected deer respond to non-proteinaceous antigens or peptides by production of antibody specific to these antigens whereas the cellular response, as detected by NO production, requires intact proteins. It is likely that protein antigens (e.g., *M. bovis* WCS antigens) presented to T cells results in elaboration of cytokines such as TNF-α and/or IFN-γ that in turn induce NO production by macrophages. Unfortunately, antibodies necessary for detection of TNF-α or IFN-γ by ELISA are not available for white-tailed deer. A kit available for detection of cervine IFN-γ (Slobbe et al., 2000) using antibodies developed for red deer failed to react with PBMC culture supernatants from white-tailed deer stimulated with pokeweed mitogen or *M. bovis* antigen (data not shown). Future studies with isolated monocytes/macrophages will be necessary to determine if the NO response to *M. bovis* antigens is, indeed, T cell dependent.

In summary, mononuclear cells isolated from *M. bovis*-infected deer produce NO in response to stimulation with *M. bovis* but not *M. avium* antigens. The response was dependent upon intact *M. bovis* proteins. The response was also abrogated by addition of NOS inhibitor, L-NMMA. While the functional relevance of these findings is unclear, it is apparent that

mononuclear cells from white-tailed deer are capable of producing NO in an in vitro recall response.

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