CLINICAL AND DIAGNOSTIC LABORATORY IMMUNOLOGY, Nov. 2001, p. 1204–1212 Vol. 8, No. 6
1071-412X/01/$04.00+0 DOI: 10.1128/CDLI.8.6.1204–1212.2001

Modulation of *Mycobacterium bovis*-Specific Responses of Bovine Peripheral Blood Mononuclear Cells by 1,25-Dihydroxyvitamin D_3

W. R. WATERS,[1]* B. J. NONNECKE,[2] T. E. RAHNER,[1] M. V. PALMER,[1]
D. L. WHIPPLE,[1] AND R. L. HORST[2]

Bacterial Diseases of Livestock Research Unit[1] and Periparturient Diseases of Livestock Research Unit,[2]
National Animal Disease Center, Agricultural Research Service, United States
Department of Agriculture, Ames, Iowa 50010-0070

Received 8 May 2001/Returned for modification 17 July 2001/Accepted 7 August 2001

Historically, administration of vitamin D has been considered beneficial in the treatment of tuberculosis. The interaction of this vitamin {i.e., 1,25-dihdroxyvitamin D_3 [1,25$(OH)_2D_3$]} with the antitubercular immune response, however, is not clear. In the present study, in vitro recall responses of peripheral blood mononuclear cells (PBMC) from cattle infected with *Mycobacterium bovis* were used to study the immune-modulatory effects of 1,25$(OH)_2D_3$ on *M. bovis*-specific responses in vitro. Addition of 1 or 10 nM 1,25$(OH)_2D_3$ inhibited *M. bovis*-specific proliferative responses of PBMC from *M. bovis*-infected cattle, affecting predominately the $CD4^+$ cell subset. In addition, 1,25$(OH)_2D_3$ inhibited *M. bovis*-specific gamma interferon (IFN-γ) production yet enhanced *M. bovis*-specific nitric oxide (NO) production. Lymphocyte apoptosis, measured by flow cytometry using annexin-V staining, was diminished by addition of 1,25$(OH)_2D_3$ to PBMC cultures. These findings support the current hypothesis that 1,25$(OH)_2D_3$ enhances mycobacterial killing by increasing NO production, a potent antimicrobial mechanism of activated macrophages, and suggest that 1,25$(OH)_2D_3$ limits host damage by decreasing *M. bovis*-induced IFN-γ production.

Before the discovery of effective antimycobacterial drugs, vitamin D therapy in the form of cod liver oil and exposure to sunlight (e.g., heliotherapy) were used to treat human tuberculosis (18). Vitamin D is derived from two sources: dietary intake and by the conversion of 7-dehydrocholesterol to cholecalciferol (i.e., pre-vitamin D) in the skin by a reaction catalyzed by UV light. At body temperature cholecalciferol spontaneously converts to vitamin D_3. Vitamin D binding protein aids in the transport of vitamin D_3 from the skin to the liver, where it is converted to 25-hydroxyvitamin D_3 [25$(OH)D_3$], the predominant circulating form of vitamin D. In response to hypocalcemic states, 25$(OH)D_3$ is hydroxylated to form 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$], the major mediator of the biological activity of vitamin D. In humans, circulating concentrations of 25$(OH)D_3$ range from 55 to 75 nM, and circulating concentrations of 1,25$(OH)_2D_3$ range from 0.062 to 0.082 nM (55). Vitamin D metabolites play a key role in short-term calcium homeostasis in humans and other animals. Experimental evidence suggests that vitamin D metabolites also modulate specific aspects of immune function (52).

Impaired formation of vitamin D_3 in the skin often results in measurable reductions in 25$(OH)D_3$ concentrations in plasma. For instance, concentrations of 25$(OH)D_3$ in plasma are lower in Asians living in Great Britain (48) and Zairians living in Belgium (35) compared to control individuals with less pigmented skin, presumably due to diminished synthesis. Tuberculosis, likewise, is common among individuals with heavily pigmented skin that relocate from equatorial regions to higher latitudes, in part due to deficiencies in vitamin D synthesis within the skin (65). In addition, patients with untreated tuberculosis often have lower concentrations of 25$(OH)D_3$ in plasma than do healthy subjects, and tuberculosis tends to occur during the winter when exposure to sunlight is reduced and production of cholecalciferol within the skin is diminished (16). Evidence for a clear correlation between vitamin D deficiency and susceptibility to tuberculosis, however, remains controversial. In vitro studies, however, provide more compelling evidence linking vitamin D status to susceptibility to tuberculosis.

Addition of 1,25$(OH)_2D_3$ to monocyte-macrophage cultures infected with *Mycobacterium tuberculosis* suppresses bacterial growth and viability (17, 53, 54). The mechanism of this suppression is mediated, at least partially, by nitric oxide (NO) (53). Induction of inducible NO synthase of macrophages and subsequent generation of reactive nitrogen intermediates (RNI) toxic to mycobacteria is a potent mechanism of killing (14, 15, 21, 22, 34). Cytokines (e.g., tumor necrosis factor alpha and gamma interferon [IFN-γ]) from antigen-specific T cells and/or from macrophages stimulated directly with mycobacterial antigens is responsible for RNI-mediated antimycobacterial defense (24, 60). Production of RNI is crucial for controlling acute as well as latent infections in the mouse model of virulent *M. tuberculosis* infection (14, 15, 25, 34). The role of RNI in mycobacterial killing within human macrophages is less clear. Alveolar macrophages of tuberculosis patients express high levels of inducible NO synthase, suggesting a role for RNI in disease pathogenesis and/or host defense (42). Nevertheless, recent evidence suggests that human but not mouse macro-

* Corresponding author. Mailing address: United States Department of Agriculture, Agricultural Research Service, National Animal Disease Center, Bacterial Diseases of Livestock Research Unit, 2300 Dayton Ave., P.O. Box 70, Ames, IA 50010-0070. Phone: (515) 663-7756. Fax: (515) 663-7458. E-mail: rwaters@nadc.ars.usda.gov.

phages utilize NO-independent mechanisms (e.g., via Toll-like receptors) for intracellular killing of the tubercle bacilli (60). This species-specific difference in mycobacterial killing may reflect coevolutionary pressures between *M. tuberculosis* and its natural host, humans.

Primary infection of mice with *M. tuberculosis* results in the generation of highly reactive IFN-γ-producing, $CD4^+$ cells that provide long-lived immunologic memory (5). This T-cell subset, however, is not sufficient for clearance of the primary infection, as antibiotic therapy is necessary for resolution of the initial infection. Upon reexposure to the pathogen, the recall response of the IFN-γ-producing $CD4^+$ cells is greatly accelerated and infection is controlled without the use of antibiotics. Immediate production of IFN-γ by $CD4^+$ cells upon exposure to the bacilli, therefore, appears essential for immune-mediated protection in the secondary response (13). AIDS patients with depressed $CD4^+$ cell counts are remarkably susceptible to tuberculosis, further demonstrating the essential role of $CD4^+$ cells in the host response to infection (7). $CD8^+$ and γδ T cells are also involved in the antituberculous immune response in mice, humans, and cattle (28, 29, 30, 32, 50, 57). Mice depleted of $CD8^+$ cells by treatment with monoclonal antibodies to murine CD8 as well as mice genetically deficient in $CD8^+$ cells are more susceptible to *M. tuberculosis* infection than are mice with intact $CD8^+$ cell populations (23, 36). Mycobacterium-specific $CD8^+$ and γδ T-cell clones have been established from infected individuals, demonstrating a potential role for these subsets in the host response to *M. tuberculosis* (20, 38). γδ T cells also respond to various mycobacterial antigens and accumulate at infection sites (6, 9, 27, 59). Production of IFN-γ by $CD4^+$, $CD8^+$, and/or γδ T-cell receptor positive (TCR^+) cells leads to activation of macrophages and enhanced killing of intracellular mycobacteria (11, 12, 28, 32, 41). Mycobacterium-specific cytotoxic T cells (both $CD4^+$ and $CD8^+$) may also be important in the clearance of *M. bovis* (39, 44, 47). Together, these studies demonstrate the complexity and redundancy of the host response during tuberculosis as well as potential sites for immune modulation with compounds such as $1,25(OH)_2D_3$.

Tuberculosis in humans results from infection with any one of the tubercle bacilli included within the *M. tuberculosis* complex (e.g., *M. tuberculosis*, *M. bovis*, *M. africanum*, and *M. microti*). *M. bovis*, unlike *M. tuberculosis*, has a wide host range and is the species most often isolated from tuberculous cattle. The wide host range of *M. bovis* has made its eradication difficult due to the presence of wildlife reservoir hosts. An outbreak of *M. bovis* in 1994 in white-tailed deer in Michigan has seriously threatened *M. bovis* eradication efforts in the United States, renewing research interests of this zoonotic agent and economically important pathogen of domestic livestock. In addition to the animal health issues of *M. bovis* infections of cattle, this infection also represents a potentially useful animal model for *M. tuberculosis* infection of humans. In the present study, the capacity of $1,25(OH)_2D_3$ to modulate recall proliferative, NO, and IFN-γ responses of peripheral blood mononuclear cells (PBMC) from cattle experimentally infected with *M. bovis* was investigated.

MATERIALS AND METHODS

Animals, bacterial culture, and challenge procedures. Eight Hereford-cross cattle (four males and four females) approximately 6 months old were obtained from herds with no history of tuberculosis and were housed at the National Animal Disease Center, U.S. Department of Agriculture, Agriculture Research Service, Ames, Iowa, according to institutional guidelines for animal care. At the initiation of the study, all animals were tested and confirmed negative for *M. bovis* exposure using both the comparative cervical test (CCT) (61) for delayed-type hypersensitivity and the Bovigam assay (CSL Limited, Parkville, Victoria, Australia) for detection of IFN-γ production in response to *M. bovis* antigen stimulation. Cattle received water ad libitum and a balanced ration consisting of pelleted alfalfa and grain during the study. Infected cattle were housed in temperature- and humidity-controlled rooms (one to two animals/room) within a biosafety level 3 confinement facility with negative airflow exiting the building through high-efficiency particulate air (HEPA) filters. Directional airflow assured that air from animal pens was pulled towards a central corridor and passed through HEPA filters before exiting the building. Airflow velocity was 10.4 air changes/minute. Noninfected control cattle were housed similarly in a separate building. Personnel in contact with *M. bovis*-infected animals wore full-face, HEPA-filtered respirators.

The strain of *M. bovis* used for the challenge inoculum (strain 1315) was isolated from a white-tailed deer in Michigan in 1994 (56). The challenge inoculum consisted of $\sim 10^5$ CFU of mid-log-phase *M. bovis* cultures grown in Middlebrook's 7H9 medium supplemented with 10% oleic acid-albumin-dextrose complex (OADC; Difco, Detroit, Mich.) plus 0.05% Tween 80 (Sigma Chemical Co., St. Louis, Mo.) as previously described (8). To harvest tubercle bacilli from the culture medium, cells were pelleted by centrifugation at $750 \times g$, washed twice with 1 ml of phosphate-buffered saline solution (0.01 M, pH 7.2) (PBS), 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 medium (Becton Dickinson, Cockeysville, Md.). For intratonsillar inoculation, cattle ($n = 2$) were restrained and anesthetized with 500 mg of ketamine (Fort Dodge Animal Health, Fort Dodge, Iowa) and 30 mg of xylazine (Bayer Corp., Shawnee Mission, Kans.) given intravenously. Effects of xylazine were reversed by intravenous administration of yohimbine (0.2 mg/kg; Lloyd Laboratories, Shenandoah, Iowa). The challenge inoculum was instilled directly into the tonsillar crypts of anesthetized cattle as previously described for inoculation of white-tailed deer (45). For aerosol inoculation, cattle ($n = 3$) were restrained and lightly sedated with 5 to 10 mg of xylazine (Bayer Corp.), and the challenge inoculum was delivered by nebulization into a mask covering the animal's nostrils and mouth. The nebulization apparatus consisted of a compressed air tank, jet nebulizer, holding reservoir, and mask (Trudell Medical International, London, Ontario, Canada). Compressed air (25 lb/in^2) was used to jet nebulize the challenge inoculum (2-ml volume of $\sim 10^5$ CFU of *M. bovis* in PBS) directly into the holding reservoir. Upon inspiration, the nebulized inoculum was inhaled through a one-way valve into the mask and directly into the nostrils. A rubber gasket sealed the mask securely to the muzzle, preventing leakage of inoculum around the mask. Expired air exited through one-way valves on the sides of the mask. The nebulization process was continued until all of the inoculum, a 1-ml PBS wash of the inoculum tube, and an additional 2 ml of PBS were delivered (~12 min). Strict biosafety level 3 protocols were followed to protect personnel from exposure to *M. bovis*. At the conclusion of the experiment, cattle were euthanatized by intravenous administration of sodium pentobarbital (Sleepaway; Fort Dodge Laboratories). Lesions typical of *M. bovis* infection were detected in *M. bovis*-inoculated animals, and infection was confirmed by isolation of *M. bovis* from tissues of *M. bovis*-inoculated cattle. Pathological and bacteriologic findings will be presented elsewhere (M. V. Palmer, W. R. Waters, and D. L. Whipple, submitted for publication).

Lymphocyte blastogenesis. Mononuclear cells were isolated from buffy coat fractions of peripheral blood collected in 2× acid citrate dextrose (10). Wells of 96-well round-bottom microtiter plates (Falcon, Becton Dickinson; Lincoln Park, N.J.) were preloaded with $1,25(OH)_2D_3$ solubilized in 100% ethanol or with ethanol alone [i.e., no $1,25(OH)_2D_3$] in a 10-μl volume. Ethanol was then allowed to evaporate, leaving the $1,25(OH)_2D_3$ at the desired concentration (i.e., 0, 1, or 10 nM). Wells were then seeded with 2×10^5 mononuclear cells in a total volume of 200 μl per well. Medium was RPMI 1640 supplemented with 25 mM HEPES buffer, penicillin (100 U/ml), streptomycin (0.1 mg/ml), 50 μM 2-mercaptoethanol (Sigma), and 10% (vol/vol) fetal bovine serum (FBS). Wells contained medium plus *M. bovis* purified protein derivative (PPD) (5 μg/ml; CSL Limited), rESAT-6 (1 μg/ml; kindly provided by F. C. Minion, Iowa State University), *M. bovis* strain 1315 culture filtrate (CF) (5 μg/ml), pokeweed mitogen (PWM) (2 μg/ml), or medium alone (no stimulation). The CF was from 2-week *M. bovis* strain 1315 cultures (bacteria were pelleted, and supernatant was harvested and filtered [0.22-μm pore size] twice). Leukocyte cultures were incubated for 5 days at 37°C in 5% CO_2 in air. After 5 days, 0.5 μCi of [*methyl*-^{3}H]thymidine (specific activity, 6.7 Ci $mmol^{-1}$; Amersham Life Science, Arling-

TABLE 1. Addition of $1,25(OH)_2D_3$ to PBMC cultures and decrease in lymphocyte blastogenesis as measured by [*methyl*-^{3}H]thymidine uptake[a]

Animal no.[b] and $1,25(OH)_2D_3$ concn (nM)	[*methyl*-^{3}H]thymidine uptake (cpm) in cultures with:				
	NS	CF	PPD	rESAT-6	PWM
236					
0	8,719	41,094	32,108	20,733	97,870
1	3,225	30,176	26,917	4,672	77,538
10	3,240	27,874	25,103	5,094	78,669
244					
0	7,627	65,617	67,140	56,447	112,487
1	2,935	51,334	25,085	23,735	78,826
10	1,434	34,107	28,907	7,646	80,902

[a] PBMC were cultured with no stimulation (NS) or with *M. bovis* strain 1315 CF (5 μg/ml), *M. bovis* PPD (5 μg/ml), rESAT-6 (1 μg/ml), or PWM (2 μg/ml) for 5 days; pulsed with 0.5 μCi of [*methyl*-^{3}H]thymidine for 20 h; and harvested onto fiber filters; and incorporated radioactivity was measured. Results are means of triplicates. The experiment was done twice, with similar results obtained in both experiments.

[b] Both animals 236 and 244 were infected with 10^5 CFU of *M. bovis* strain 1315 by intratonsillar inoculation (52 days postinoculation).

ton Heights, Ill.) in 50 μl of medium was added to each well, and cells were incubated for an additional 20 h. Well contents were harvested onto fiber filters with a 96-well plate harvester (EG & G Wallac, Gaithersburg, Md.), and the incorporated radioactivity was measured by liquid scintillation counting. Treatments were run in triplicate, and results are presented as mean counts minute^{-1}.

PKH67 proliferation assay. The PKH67 proliferation assay was performed according to manufacturer instructions (Sigma) and as previously described (62). Briefly, 2×10^7 PBMC were centrifuged (10 min, 400 × *g*), supernatants were aspirated, and cells were resuspended in 1 ml of diluent provided in the PKH67 kit (Sigma). Diluted cells were added to 1 ml of PKH67 green fluorescent dye (2 μM; Sigma) and incubated for 5 min, followed by a 1-min incubation with 2 ml of FBS to stop the reaction. Cells were then washed (10 min, 400 × *g*) three times with RPMI 1640. Wells of 96-well round-bottom microtiter plates were precoated with $1,25(OH)_2D_3$ as described for the blastogenesis procedure. PKH67-stained cells were then added to wells (2×10^5/well; six replicates per treatment [e.g., no stimulation or PPD]) of 96-well round-bottom microtiter plates in medium (no stimulation) or medium plus *M. bovis* PPD (5 μg/ml; CSL Limited). Cultures were incubated for 6 days at 37°C in a humidified chamber with 5% CO_2.

Flow cytometry. At the conclusion of the incubation period, cells were analyzed by flow cytometry (FACScan; Becton Dickinson, San Jose, Calif.) for PKH67 staining as well as cell surface marker expression. Modfit Proliferation Wizard (Verity Software House Inc., Topsham, Maine) and CellQuest software (Becton Dickinson) were used for cell proliferation and phenotype analyses. Proliferation profiles were determined as the number of cells proliferating in PPD-stimulated wells minus the number of cells proliferating in nonstimulated wells for both gated (i.e., CD4$^+$, CD8$^+$, or γδ TCR$^+$) or ungated (total PBMC) populations. Appropriate isotype control antibodies were used for both the nonstimulated and PPD-stimulated wells as a control for nonspecific binding of lymphocyte subset antibodies to activated cells. Data are presented as the mean (± standard error of the mean [SEM]) number of cells that had proliferated per 10,000 PBMC.

Mononuclear cells were analyzed for PKH67 staining (FL1), cell surface antigen expression (FL3), and annexin V staining (FL2) by flow cytometry. Cells (2×10^6/ml) in 100 μl of balanced salt solution with 1% FBS and 0.1% sodium azide (FACS buffer) were stained with 100 μl of primary antibody to leukocyte surface antigens (CACT138A, anti-CD4; CACT80C, anti-CD8α; and BAQ4A, anti-WC1 [VMRD, Pullman, Wash.]). After a 15-min incubation, cells were centrifuged (400 × *g*, 2 min) and resuspended in 100 μl of peridinin chlorophyll protein-conjugated goat anti-mouse immunoglobulin G1 (Becton Dickinson). Cells were then incubated for an additional 15 min, centrifuged (400 × *g*, 2 min), resuspended in 200 μl of 1× annexin V binding buffer (Pharmingen, San Diego, Calif.), and stained with 4 μl of annexin V-phycoerythrin (Pharmingen). Cells were then analyzed using a Becton Dickinson FACScan flow cytometer (10,000 events, live gate, three-color analysis, 488-nm laser).

IFN-γ ELISA. Wells of 96-well round-bottom microtiter plates were preloaded with $1,25(OH)_2D_3$ as described for the blastogenesis procedure. Isolated mononuclear cells were then added to wells (2×10^5/well, six replicates) with PPDb (5 μg/ml), rESAT-6 (1 μg/ml), CF (5 μg/ml), PWM (1 μg/ml), or medium alone. Plates with cells were then incubated at 37°C in a 5% CO_2 humidified chamber. Supernatants were harvested after 24, 48, and 72 h of culture and analyzed for IFN-γ using a commercial enzyme-linked immunosorbent assay (ELISA)-based kit (Bovigam; CSL Limited).

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 (49) performed in 96-well microtiter plates (Immunolon 2; Dynatech Laboratories, Inc., Chantilly, Va.). Culture conditions were as described for the lymphocyte blastogenesis assay. Culture supernatant (100 μl) was mixed with 100 μl of Griess reagent (0.5% sulfanilamide; Sigma) in 2.5% phosphoric acid (Mallinckrodt Chemicals, Inc., Paris, Ky.) and 0.05% *N*-(1-naphthyl) ethylenediamine dihydrochloride (Sigma). 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, Calif.). All dilutions were made using culture medium (RPMI 1640 medium with 2 mM L-glutamine and 10% [vol/vol] FBS). Absorbances of standards, controls, and test samples were converted to nanograms per milliliter of nitrite by comparison with absorbances of sodium nitrite (Fisher Chemicals, Fair Lawn, N.J.) standards within a linear curve fit. N^G-Monomethyl-L-arginine (L-NMMA) (Calbiochem, La Jolla, Calif.), a competitive inhibitor of the enzyme NO 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.

Statistical analysis. Data were assessed for normality prior to statistical analysis. Arithmetic or $\log_{10}$-transformed data were analyzed as a split plot with repeated measures analysis of variance using Statview software (version 5.0; SAS Institute, Inc., Cary, N.C.). Concentrations of $1,25(OH)_2D_3$ in unstimulated and CF-, rESAT6-, PPD-, and PWM-stimulated cultures and their interactions constituted the main plot, and incubation period (in hours) was the repeated mea-

TABLE 2. Effects of $1,25(OH)_2D_3$ on lymphocyte subset proliferation in response to stimulation with *M. bovis* PPD

Cattle group and $1,25(OH)_2D_3$ concn (nM)	No. of cells that proliferated[a]		
	Total	CD4$^+$	WC1^{+b}
Noninfected (n = 3)			
0	502 ± 263	0 ± 0	450 ± 241
1	199 ± 83	62 ± 45	73 ± 73
10	224 ± 127	33 ± 26	90 ± 46
M. bovis infected (n = 5)			
0	4,844 ± 500	3,281 ± 671	710 ± 502
1	4,536 ± 696	2,928 ± 247	797 ± 318
10	4,325 ± 625	2,682 ± 346	964 ± 420

[a] Data represent the number of cells that had proliferated in response to stimulation with *M. bovis* PPD (5 μg/ml) minus the response to no stimulation and are presented as means ± SEM per 10,000 PBMC.

[b] WC1 is a scavenger receptor present on >95% of peripheral blood γδ TCR$^+$ cells of cattle (26, 33).

sure or the split plot. Fisher's protected-least-significant-difference test was applied when treatment effects ($P \leq 0.05$) were detected by the model. Pearson's product-moment correlations were computed between IFN-γ and NO concentrations in supernatants from unstimulated and CF-, rESAT-6-, PPD-, and PWM-stimulated cultures and were considered significant at $P < 0.1$.

RESULTS

Effects of $1,25(OH)_2D_3$ on lymphocyte proliferation. Addition of 1 or 10 nM $1,25(OH)_2D_3$ decreased DNA synthesis in PBMC isolated from *M. bovis*-infected animals. Responses to unstimulated cultures (background proliferation) and cultures stimulated with *M. bovis* antigens (PPD, rESAT-6, and CF) and PWM are shown in Table 1. Analysis of PBMC proliferation using a flow cytometry-based assay (e.g., the PKH67 assay) demonstrated that addition of $1,25(OH)_2D_3$ decreased proliferation of PBMC from *M. bovis*-infected animals in response to *M. bovis* PPD (Table 2) (e.g., the mean proliferative responses of total cells from *M. bovis* infected animals). Flow cytometry-based proliferation assays are more informative than the blastogenesis assay because they allow simultaneous characterization of proliferative responses of individual lymphocyte subsets and evaluation of proliferation throughout the culture period (not just the terminal 20-h period, as with radiometric techniques) (2). As previously reported (64), cells responding to PPD from *M. bovis*-infected cattle were predominantly $CD4^+$ and $WC1^+$ (i.e., γδ TCR^+) cells. Responses of $CD8^+$ cells from *M. bovis*-infected cattle to PPD were negligible (data not shown). While proliferative responses were detected for both $CD4^+$ and $WC1^+$ T-cell subsets, addition of $1,25(OH)_2D_3$ decreased $CD4^+$ cell proliferation but not $WC1^+$ cell proliferation in samples from infected animals (Table 2).

Addition of $1,25(OH)_2D_3$ to PPD-stimulated cultures also diminished the number of $CD4^+$ cells that had proliferated through multiple generations. Addition of $1,25(OH)_2D_3$ decreased the percentage of cells in generations of PPD-stimulated cultures that had gone through the most divisions [e.g., 32, 22, and 11% for 0, 1, and 10 nM $1,25(OH)_2D_3$, respectively, for a representative *M. bovis*-infected animal] (Fig. 1). The generation that had proceeded through the next greatest number of divisions was also decreased in $1,25(OH)_2D_3$-treated cultures compared to nontreated, control cultures [e.g., 46, 32, and 32% for 0, 1, and 10 nM $1,25(OH)_2D_3$, respectively] (Fig. 1). Although this finding was most apparent for the $CD4^+$ cell subpopulation, $WC1^+$ cells within $1,25(OH)_2D_3$-treated cultures also had a decreased percentage of cells within elder generations compared to control cultures (data not shown). The number of $WC1^+$ cells that had proliferated, however, was not lower in the presence of $1,25(OH)_2D_3$ (Table 2).

Effects of $1,25(OH)_2D_3$ on lymphocyte apoptosis. A significant sequela to lymphocyte proliferation and activation is apoptosis (1). To determine the effect of $1,25(OH)_2D_3$ on apoptosis of lymphocytes during an in vitro recall response, PBMC from *M. bovis*-infected cattle were incubated with medium alone (e.g., nonstimulated) or incubated with PPD for 6 days and analyzed for annexin V staining. Although not statistically significant ($P > 0.05$), there was a trend for a lower percentage of annexin V-positive cells and a concurrent lower percentage of cells located in the "apoptotic gate" and a higher percentage of cells located in the "live gate" in PPD-stimulated cultures supplemented with $1,25(OH)_2D_3$ compared to nonsupplemented PPD-stimulated cultures (Table 3). This trend was not detected for nonstimulated PBMC. The apoptotic and live gates were based upon forward and side light scatter properties as well as 7-amino-actinomycin and annexin V staining properties (data not shown). Inhibition of apoptosis by $1,25(OH)_2D_3$ was similar for each T-cell subset ($CD4^+$, $CD8^+$, and $WC1^+$ cells) tested.

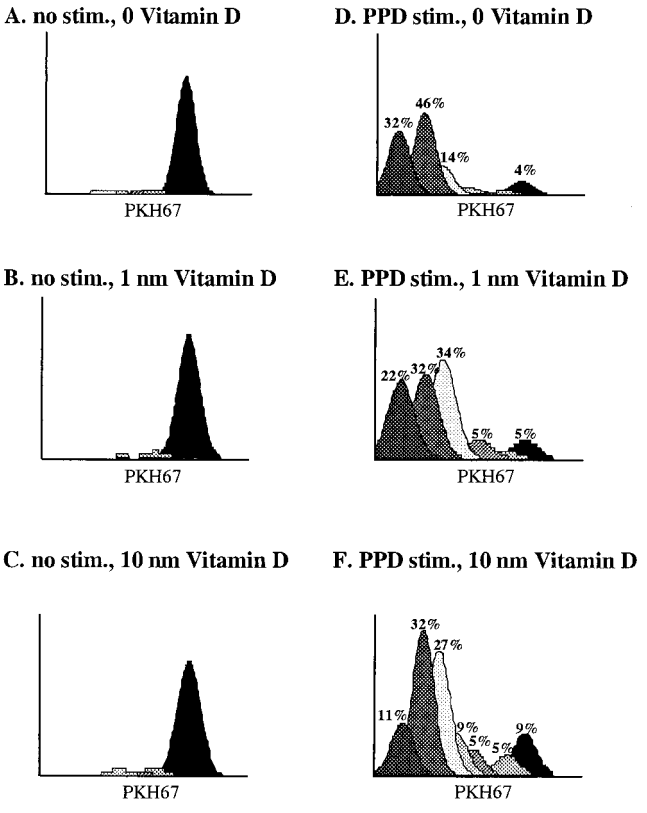

FIG. 1. Addition of $1,25(OH)_2D_3$ decreases the percentage of proliferating $CD4^+$ cells within the eldest generations. PBMC were cultured with no stimulation (A to C) or with *M. bovis* PPD (5 μg/ml) (D to F). In addition, cultures received either 0 (A and D), 1 (B and E), or 10 (C and F) nM $1,25(OH)_2D_3$. After a 6-day incubation, cells were harvested, stained with either CACT138A, anti-CD4; CACT80C, anti-CD8α; or BAQ4A, anti-WC1 and analyzed by flow cytometry for PKH67 intensity and cell surface marker expression. After flow cytometric analysis, data were analyzed by using the Modfit Proliferation Wizard to determine the number of cells that had proliferated (grey peaks). A representative response from a single *M. bovis*-infected animal is depicted. Gates for this particular sample were set on live (e.g., based upon light scatter properties) and $CD4^+$ cells. Black peaks depict the parent generations (e.g., PKH67 bright), whereas daughter generations are depicted with peaks in various shades of grey.

Antigen-specific IFN-γ and NO responses of *M. bovis*-infected cattle. Prior to evaluating the effects of $1,25(OH)_2D_3$ on *M. bovis*-specific IFN-γ and NO responses, the capacity of PBMC from infected cattle to produce IFN-γ and NO in response to stimulation with *M. bovis* antigens was determined. IFN-γ secretion by PBMC from *M. bovis*-infected cattle stimulated with either *M. bovis* CF, PPD, or rESAT-6 exceeded ($P < 0.05$) IFN-γ secretion by nonstimulated, autologous PBMC (Fig. 2a). In addition, the IFN-γ response of PBMC

TABLE 3. Inhibition of apoptosis by addition of $1,25(OH)_2D_3$ to lymphocyte cultures[a]

Treatment and $1,25(OH)_2D_3$ concn (nM)	% Annexin V positive	% of cells within[b]:	
		Apoptotic gate	Live gate
No stimulation			
0	31.47 ± 1.38	40.95 ± 4.37	55.85 ± 4.80
1	30.97 ± 1.70	39.47 ± 3.16	57.84 ± 2.99
10	31.77 ± 1.68	38.80 ± 2.98	58.34 ± 2.84
PPD stimulation			
0	29.20 ± 1.98	37.17 ± 4.14	59.94 ± 2.50
1	26.57 ± 1.79	32.68 ± 2.09	65.29 ± 1.98
10	25.95 ± 1.81	30.87 ± 1.99	66.98 ± 1.85

[a] PBMC from *M. bovis*-infected cattle were cultured with no stimulation or with *M. bovis* PPD (5 μg/ml). Values are presented as means ± SEM ($n = 5$ animals).
[b] Results are from comparison of forward- and side-scatter plots.

from *M. bovis*-infected cattle that were stimulated with CF or PPD was greater ($P < 0.01$) than the response of PBMC from noninfected cattle stimulated with CF or PPD, respectively. Greater ($P < 0.05$) concentrations of nitrite were also detected in supernatants from CF- or PPD-stimulated samples from *M. bovis*-infected cattle compared to concentrations of nitrite in supernatants from CF- or PPD-stimulated samples from noninfected cattle or nonstimulated samples from *M. bovis*-infected cattle (Fig. 2b). Nitrite levels in rESAT-6-stimulated samples from infected cattle were greater ($P < 0.01$) than levels in nonstimulated samples from infected cattle. IFN-γ and NO responses of PBMC from control, noninfected cattle to *M. bovis* antigens (PPD, CF, or rESAT-6) were not greater ($P > 0.05$) than responses to medium alone (no stimulation). IFN-γ and NO responses of PWM-stimulated PBMC were always greater ($P < 0.05$) than corresponding responses of nonstimulated PBMC regardless of infection status.

Effect of $1,25(OH)_2D_3$ on *M. bovis*-specific IFN-γ and NO responses. Nonstimulated and stimulated (i.e., *M. bovis* antigens and PWM) PBMC from *M. bovis*-infected and noninfected cattle were cultured with 0, 1, or 10 nM $1,25(OH)_2D_3$. Addition of $1,25(OH)_2D_3$ enhanced ($P < 0.01$) nitrite production by PWM-stimulated PBMC from *M. bovis*-infected cattle (Fig. 3). Addition of $1,25(OH)_2D_3$ also enhanced ($P < 0.05$) CF-, rESAT-6-, and PPD-specific production of nitrite by PBMC from infected cattle. As determined previously (3, 4), $1,25(OH)_2D_3$ inhibits IFN-γ production by antigen (ovalbumin)-stimulated PBMC from ovalbumin-sensitized cattle. A similar trend was also detected for *M. bovis*-specific responses by PBMC from *M. bovis*-infected cattle, with $1,25(OH)_2D_3$ inhibiting ($P < 0.05$) IFN-γ production in response to both CF and PPD (Fig. 4). While $1,25(OH)_2D_3$ enhanced nitrite production in response to rESAT-6 (Fig. 3), significant modulation by $1,25(OH)_2D_3$ of IFN-γ responses to rESAT-6 stimulation of PBMC from infected cattle was not detected (Fig. 4).

To evaluate relationships between IFN-γ and NO production in response to *M. bovis* infection, Pearson's product-moment correlations were determined for responses to *M. bovis* antigens. The overall correlations, regardless of $1,25(OH)_2D_3$ concentration and time, were positive (for CF, $r = 0.452$, $P < 0.1$; for rESAT-6, $r = 0.507$, $P < 0.001$; for PPD, $r = 0.505$, $P < 0.001$), with increases in IFN-γ associated with concurrent increases in NO. A strong positive correlation between IFN-γ and NO production was also detected for PWM-stimulated cells ($r = 0.664$, $P < 0.001$). Addition of $1,25(OH)_2D_3$ to cultures diminished this correlation, although statistically significant differences were not detected (data not shown).

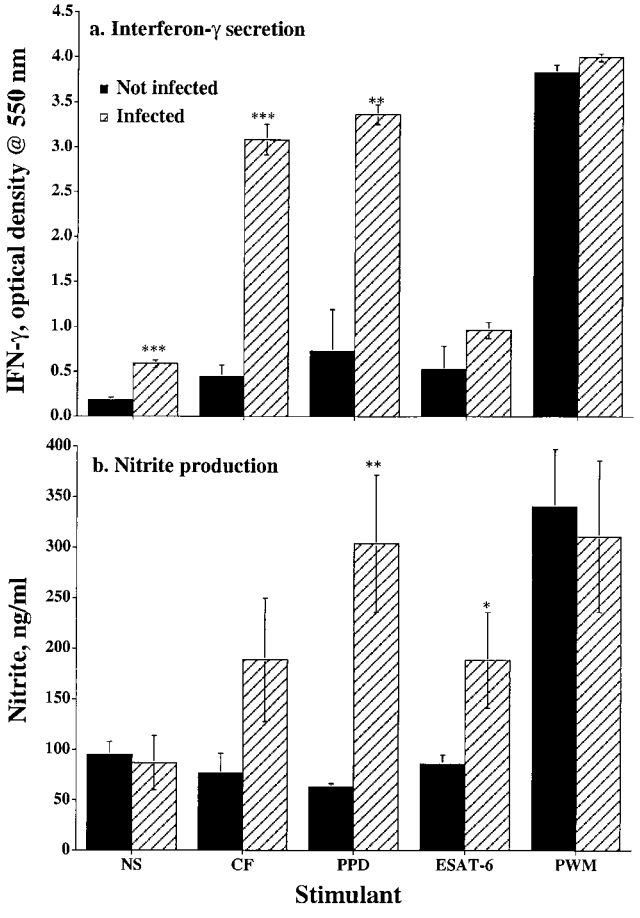

FIG. 2. Antigen-specific IFN-γ and NO responses of *M. bovis*-infected cattle. PBMC were cultured with no stimulation (NS) or with either *M. bovis* strain 1315 CF (5 μg/ml), *M. bovis* PPD (5 μg/ml), ESAT-6 (1 μg/m), or PWM (2 μg/ml). Supernatants were harvested after 72 h for detection of IFN-γ by ELISA (a) and detection of nitrite by Griess reaction (b). PBMC were obtained from noninfected cattle ($n = 3$ [closed bars]) and *M. bovis*-infected cattle ($n = 2$ [hatched bars]). Addition of L-NMMA (at a concentration equimolar to the amount of L-arginine in the culture medium), a competitive inhibitor of the enzyme NOS, inhibited nitrite production to levels detected in medium alone (e.g., background levels; data not shown). For a specific stimulant, responses of infected cattle differ from responses of controls. Symbols: *, $P < 0.01$; **, $P < 0.05$; ***, $P < 0.001$. Error bars, SEM.

DISCUSSION

Recent evidence suggests that $1,25(OH)_2D_3$-induced NO limits replication of *M. tuberculosis* within human macrophages (53). Activation of alveolar macrophages by IFN-γ results in an increased rate of conversion of $25(OH)D_3$ to $1,25(OH)_2D_3$, the most active, naturally occurring form of the vitamin (54). $1,25(OH)_2D_3$ induces NOS2 expression and the production of NO by human macrophages, necessary for mycobacterial kill-

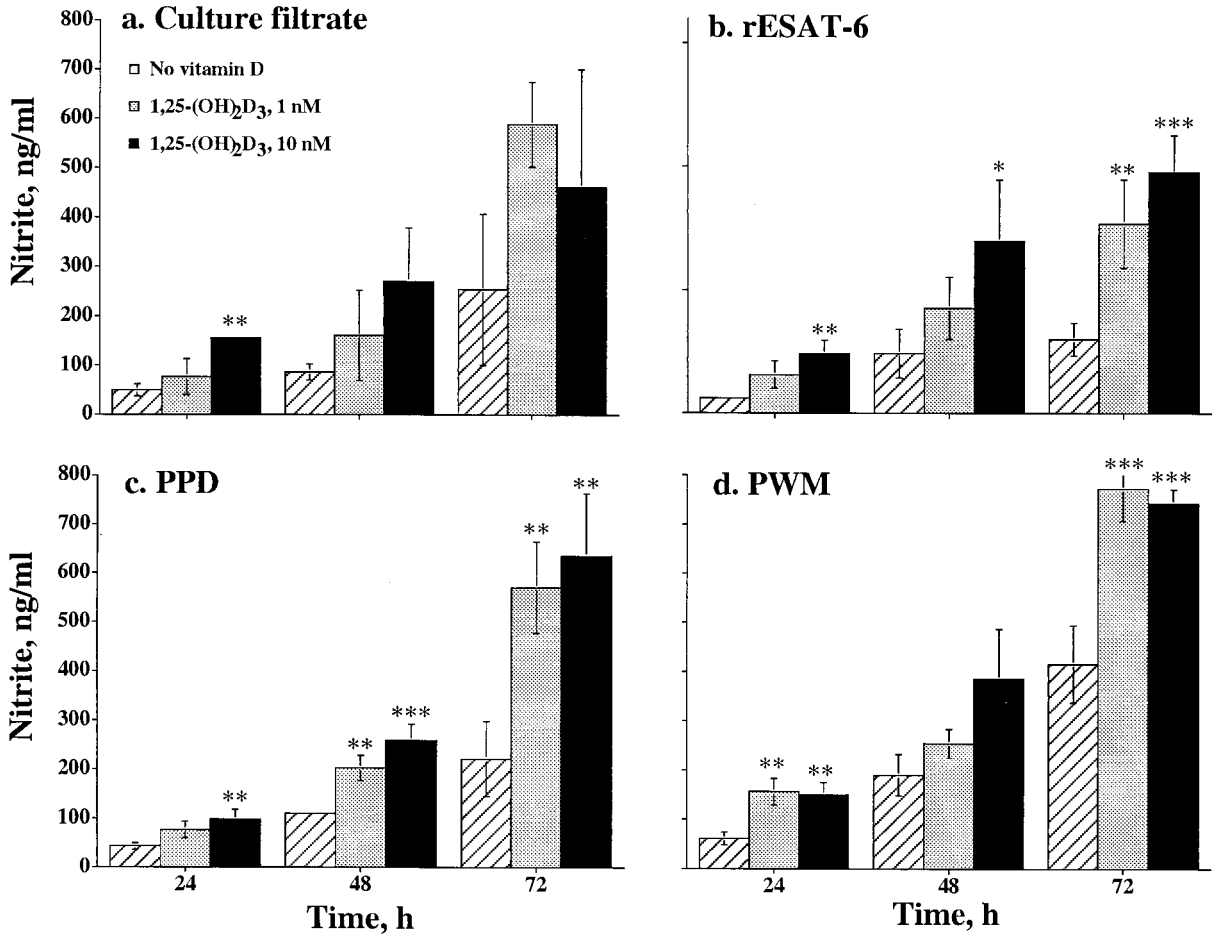

FIG. 3. Addition of $1,25(OH)_2D_3$ increases *M. bovis*-specific and PWM-stimulated production of nitrite by PBMC from *M. bovis*-infected cattle ($n = 5$). Mononuclear cells were cultured or with either *M. bovis* strain 1315 CF (5 μg/ml) (a), rESAT-6 (1 μg/ml) (b), *M. bovis* PPD (5 μg/ml) (c), or PWM (2 μg/ml) (d). To each of these treatments either no vitamin D, 1 nM $1,25(OH)_2D_3$, or 10 nM $1,25(OH)_2D_3$ was added as described in Materials and Methods. Supernatants were harvested after 24, 48, or 72 h for detection of nitrite by the Griess reaction as an indication of NO production. Symbols: *, $P < 0.1$; **, $P < 0.05$; ***, $P < 0.001$ (differs from unsupplemented [no vitamin D] cultures at specific times). Error bars, SEM.

ing (16, 53). In the present study, it was determined that $1,25(OH)_2D_3$ increased NO production and decreased IFN-γ production by antigen (i.e., PPD, CF, and rESAT-6)-stimulated PBMC from *M. bovis*-infected cattle. The biologically active form of vitamin D, $1,25(OH)_2D_3$, has been shown to inhibit IFN-γ production by in vitro-activated PBMC from several different species, including cattle (3, 4, 19, 37). Although speculative, it is plausible that inhibition of IFN-γ production by $1,25(OH)_2D_3$ functions as a negative feedback mechanism to inhibit tissue damage once the antimycobacterial response (i.e., that mediated by NO) is elicited. Another possibility is that the increased NO produced by macrophages as a result of $1,25(OH)_2D_3$ addition to the cultures inhibits IFN-γ production by T cells. Production of NO by splenic macrophages from *M. tuberculosis*-infected mice inhibits $CD4^+$ T-cell mycobacterium-specific proliferative responses; IFN-γ responses, however, are not affected (40). In the present study, addition of a competitive inhibitor of NOS (L-NMMA) abolished NO production yet did not affect IFN-γ responses. The production of IFN-γ in response to antigen stimulation and the suppression elicited by $1,25(OH)_2D_3$ were similar in L-NMMA-treated cultures compared to the cultures that did not receive L-NMMA (data not shown). Thus, the inhibitory effects of $1,25(OH)_2D_3$ on *M. bovis*-specific IFN-γ production is most likely a direct effect of $1,25(OH)_2D_3$ and not a secondary response to increased NO production.

Mitogen-induced $CD4^+$ T-cell proliferation is inhibited by $1,25(OH)_2D_3$ in mice, humans, and cattle (31, 43). Likewise, we determined that *M. bovis*-specific $CD4^+$ cells were the primary target of inhibition by $1,25(OH)_2D_3$. Antigen selection, however, may have biased the observed suppressive effect. Both $CD4^+$ and γδ TCR^+ cells but not $CD8^+$ cells proliferate in response to *M. bovis* PPD (64). Although both subsets responded in the present study, $1,25(OH)_2D_3$ inhibited only $CD4^+$ cell proliferation. These findings are in agreement with a previous study in which $1,25(OH)_2D_3$ inhibited mitogen-induced proliferation of $CD4^+$ cells yet had no affect on γδ TCR^+ cell proliferation (43). Since PPD is composed of a

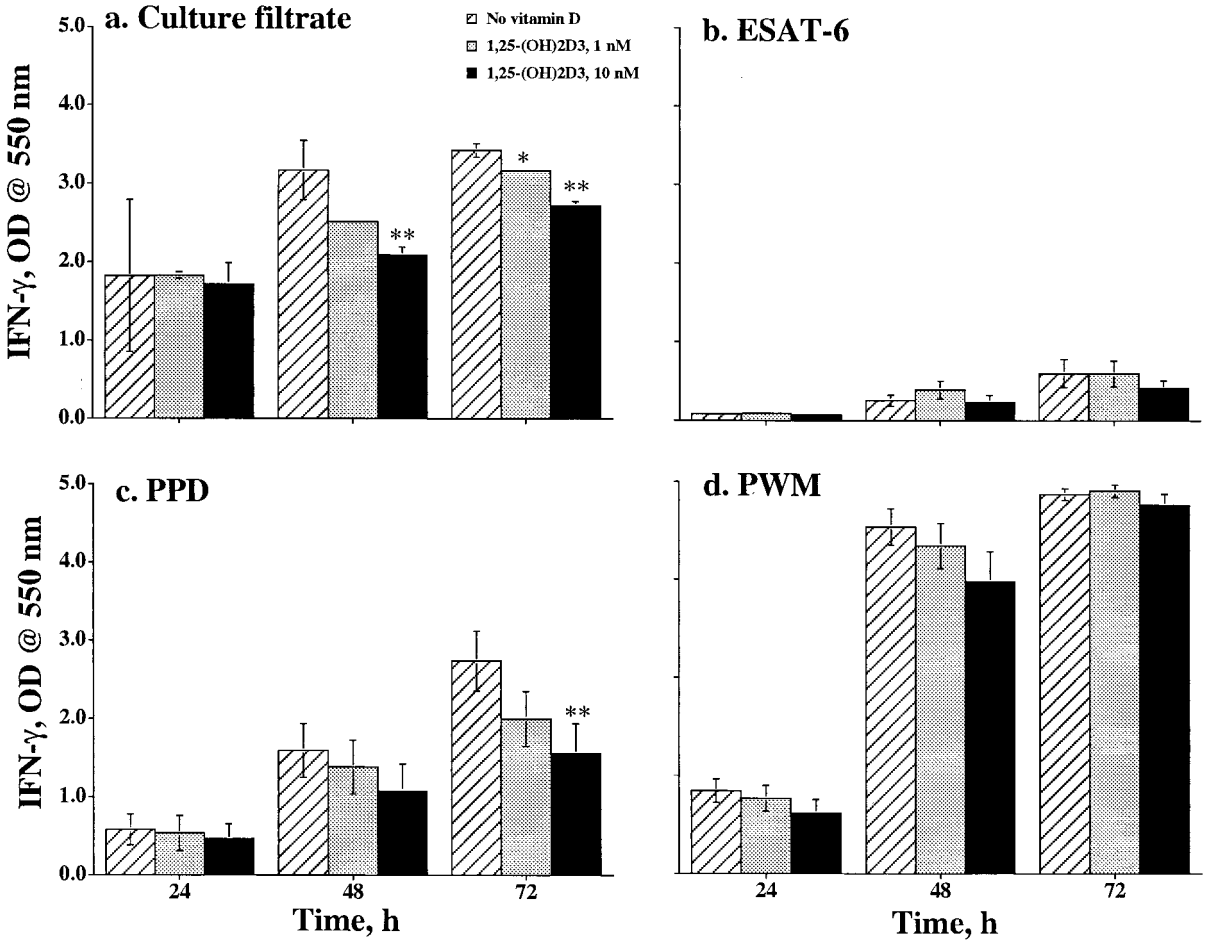

FIG. 4. Addition of $1,25(OH)_2D_3$ decreases *M. bovis*-specific production of IFN-γ by PBMC from *M. bovis*-infected cattle ($n = 5$). Mononuclear cells were cultured with either *M. bovis* strain 1315 CF (5 μg/ml) (a), rESAT-6 (1 μg/ml) (b), *M. bovis* PPD (5 μg/ml) (c), or PWM (2 μg/ml) (d). To each of these treatments either no vitamin D, 1 nM $1,25(OH)_2D_3$, or 10 nM $1,25(OH)_2D_3$ was added as described in Materials and Methods. Supernatants were harvested after 24, 48, or 72 h for detection of IFN-γ by ELISA (Bovigam assay; CSL Limited). Symbols: *, $P < 0.1$; **, $P < 0.05$ (differs from unsupplemented [no vitamin D] cultures at specific times). Error bars, SEM.

mixture of soluble antigens, these antigens are likely processed and presented in association with major histocompatibility complex class II for $CD4^+$ cells or directly without processing for γδ TCR^+ cells. Major histocompatibility complex class II restricted $CD4^+$ cells are the predominant cell type responding to PPD-stimulated PBMC from *M. bovis*-infected white-tailed deer (63). Unlike those of white-tailed deer, γδ TCR^+ cells of *M. bovis*-infected cattle do respond to soluble *M. bovis* antigens (51, 58, 64). One interpretation of the specific effects of $1,25(OH)_2D_3$ on $CD4^+$ cells is that the proliferative response of γδ TCR^+ cells is dependent upon cytokine production by other cells (e.g., bystander proliferation in response to interleukin 2 [IL-2] produced by antigen-specific $CD4^+$ cells). $1,25(OH)_2D_3$ inhibits IL-2 production by human T cells (37) and IL-2 receptor expression by activated bovine PBMC (43). Rhodes et al. (50, 51), however, have clearly demonstrated that γδ TCR^+ cells (isolated and enriched by magnetic bead sorting) from *M. bovis*-infected cattle proliferate in response to soluble *M. bovis* antigens, including *M. bovis* PPD and rESAT-6. Thus, it appears that $1,25(OH)_2D_3$ affects $CD4^+$ cells specifically in regards to inhibition of proliferation in response to PPD.

$1,25(OH)_2D_3$ also induces apoptosis of mitogen-stimulated human T cells by inhibiting IL-2 production (46). In contrast, results from the present study suggest that $1,25(OH)_2D_3$ inhibits apoptosis of antigen-stimulated cells. This discrepancy may reflect the role of the stimulus (mitogen versus antigen) used to determine the effects of vitamin D on apoptosis. Species differences (cattle versus humans) may also influence the outcome of the response. Additional studies are needed to confirm the inhibition of apoptosis of bovine T cells stimulated with mycobacterial antigens by $1,25(OH)_2D_3$ and to determine the underlying mechanisms. Inhibition of apoptosis of *M. bovis*-specific T cells by $1,25(OH)_2D_3$ at the site of *M. bovis* infection would likely be beneficial to the host antitubercular response.

It has been postulated that $1,25(OH)_2D_3$ enhances mycobacterial killing via an NO-dependent mechanism. It is also postulated that production of $1,25(OH)_2D_3$ at the site of infection dampens cell-mediated responses to mycobacterial an-

tigens through antiproliferative and IFN-γ-inhibitory actions. Our findings that $1,25(OH)_2D_3$ enhances *M. bovis*-specific NO production, inhibits *M. bovis*-specific IFN-γ production, and inhibits *M. bovis*-specific $CD4^+$ cell proliferation are consistent with these hypotheses. Future studies are planned to further evaluate the relevance of these findings.

ACKNOWLEDGMENTS

We thank Rebecca Lyon, Jody Mentele, Lori Dethloff, Nancy Eischen, and Darrel Hoy for excellent technical support. We also thank Katherine Lies and Terry Krausman for excellent animal care.

REFERENCES

1. **Ahmed, R., and D. Gray.** 1996. Immunological memory and protective immunity: understanding their relation. Science **272:**54–60.
2. **Allsopp, C. E. M., S. J. Nicholls, and J. Langhorne.** 1998. A flow cytometric method to assess antigen-specific proliferative responses of different subpopulations of fresh and cryopreserved human peripheral blood mononuclear cells. J. Immunol. Methods **214:**175–186.
3. **Ametaj, B. N., D. C. Beitz, T. A. Reinhardt, and B. J. Nonnecke.** 1996. 1,25-Dihydroxyvitamin D3 inhibits secretion of interferon-gamma by mitogen- and antigen-stimulated bovine mononuclear leukocytes. Vet. Immunol. Immunopathol. **52:**77–90.
4. **Ametaj, B. N., B. J. Nonnecke, R. L. Horst, and D. C. Beitz.** 2000. Effects of retinoic acid and 1,25-dihydroxyvitamin D3 on IFN-γ secretion by mononuclear leukocytes form nulliparous and postparturient dairy cattle. Int. J. Vit. Nutr. Res. **70:**92–101.
5. **Andersen, P., A. B. Andersen, A. L. Sorensen, and S. Nagai.** 1995. Recall of long-lived immunity to *Mycobacterium tuberculosis* infection in mice. J. Immunol. **154:**3359–3372.
6. **Augustin, A., R. T. Kubo, and G. K. Sim.** 1989. Resident pulmonary lymphocytes expressing the γ/δ T-cell receptor. Nature **340:**239–241.
7. **Barnes, P. F., A. B. Bloch, P. T. Davidson, and D. E. Snider.** 1991. Tuberculosis in patients with human immunodeficiency virus infection. N. Engl. J. Med. **324:**1644–1650.
8. **Bolin, C. A., D. L. Whipple, K. V. Khanna, J. M. Risdahl, P. K. Peteron, and T. W. Molitor.** 1997. Infection of swine with *Mycobacterium bovis* as a model of human tuberculosis. J. Infect. Dis. **176:**1559–1566.
9. **Boom, W. H., K. A. Chervenak, M. A. Mincek, and J. J. Ellner.** 1992. Role of the mononuclear phagocyte as an antigen-presenting cell for human γδ T cells activated by live *Mycobacterium tuberculosis*. Infect. Immun. **60:**3480–3488.
10. **Burton, J. L., and M. E. Kehrli.** 1996. Effects of dexamethasone on bovine circulating T lymphocyte populations. J. Leukoc. Biol. **59:**90–99.
11. **Carpenter, E., L. Fray, and E. Gormley.** 1997. Cellular responses and *Mycobacterium bovis* BCG growth inhibition by bovine lymphocytes. Immunol. Cell Biol. **75:**554–560.
12. **Carpenter, E., L. Fray, and E. Gormley.** 1998. Antigen-specific lymphocytes enhance nitric oxide production in *Mycobacterium bovis* BCG-infected bovine macrophages. Immunol. Cell Biol. **76:**363–368.
13. **Caruso, A. M., N. Serbina, E. Klein, K. Triebold, B. R. Bloom, and J. L. Flynn.** 1999. Mice deficient in CD4 T cells have only transiently diminished levels of IFN-γ, yet succumb to tuberculosis. J. Immunol. **162:**5407–5416.
14. **Chan, J., Y. Xing, R. Magliozzo, and B. R. Bloom.** 1992. Killing of virulent *Mycobacterium tuberculosis* by reactive nitrogen intermediates produced by activated murine macrophages. J. Exp. Med. **175:**1111–1122.
15. **Chan, J., K. Tanaka, D. Carroll, J. Flynn, and B. R. Bloom.** 1995. Effects of nitric oxide synthase inhibitors on murine infection with *Mycobacterium tuberculosis*. Infect. Immun. **63:**736–740.
16. **Chan, T. Y.** 2000. Vitamin D deficiency and susceptibility to tuberculosis. Calcif. Tissue Int. **66:**476–478.
17. **Crowle, A. J., E. J. Ross, and M. H. May.** 1987. Inhibition by $1,25(OH)_2$-vitamin D_3 of the multiplication of virulent tubercle bacilli in cultured human macrophages. Infect. Immun. **55:**2945–2950.
18. **Davies, P. D. O.** 1985. A possible link between vitamin D deficiency and impaired host defense to *Mycobacterium tuberculosis*. Tubercle **66:**301–306.
19. **Daynes, R. A., and B. A. Araneo.** 1992. Natural regulators of T-cell lymphokine production in vivo. J. Immunother. **12:**174–179.
20. **De Libero, G., I. Flesch, S. H. Kaufmann.** 1988. Mycobacteria-reactive Lyt-2+ T cell lines. Eur. J. Immunol. **18:**59–66.
21. **Denis, M.** 1991. Interferon-gamma-treated murine macrophages inhibit growth of tubercle bacilli via the generation of reactive nitrogen intermediates. Cell. Immunol. **132:**150–157.
22. **Flesch, I. E., and S. H. Kaufmann.** 1991. Mechanisms involved in mycobacterial growth inhibition by gamma interferon-activated bone marrow macrophages: role of reactive nitrogen intermediates. Infect. Immun. **59:**3213–3218.
23. **Flynn, J. L., M. M. Goldstein, K. J. Triebold, B. Koller, and B. R. Bloom.** 2017. 1992. Major histocompatibility complex class I-restricted T cells are required for resistance to *Mycobacterium tuberculosis* infection. Proc. Natl. Acad. Sci. USA **89:**12013–12017.
24. **Flynn, J. L., M. M. Goldstein, J. Chan, K. J. Triebold, K. Pfeffer, C. J. Lowenstein, R. Schreiber, T. W. Mak, and B. R. Bloom.** 1995. Tumor necrosis factor-alpha is required in the protective immune response against *Mycobacterium tuberculosis* in mice. Immunity **2:**561–572.
25. **Flynn, J. L., C. A. Scanga, K. E. Tanaka, and J. Chan.** 1998. Effects of aminoguanidine on latent murine tuberculosis. J. Immunol. **160:**1796–1803.
26. **Hein, W. R., and C. R. Mackay.** 1991. Prominence of γδ T cells in the ruminant immune system. Immunol. Today **12:**30–34.
27. **Janis, E. M., S. H. E. Kaufmann, R. H. Schwartz, and D. M. Pardoll.** 1989. Activation of γδ T cells in the primary immune response to *Mycobacterium tuberculosis*. Science **244:**713–716.
28. **Kaufmann, S. H. E.** 1995. Immunity to intracellular microbial pathogens. Immunol. Today **16:**338–342.
29. **Kaufmann, S. H. E.** 1997. The roles of conventional and unconventional cells in antibacterial immunity. ASM News **63:**251–255.
30. **Lalvani, A., R. Brookes, R. J. Wilkinson, A. S. Malin, A. A. Pathan, P. Andersen, H. Dockrell, G. Pasvol, and A. V. S. Hill.** 1998. Human cytolytic and interferon γ-secreting $CD8^+$ T lymphocytes specific for *Mycobacterium tuberculosis*. Proc. Natl. Acad. Sci. USA **95:**270–275.
31. **Lemire, J. M., J. S. Adams, V. Kermani-Arab, A. C. Bakke, R. Sakai, and S. C. Jordan.** 1985. 1,25-Dihydroxyvitamin D3 suppresses human T helper/inducer lymphocyte activity in vitro. J. Immunol. **134:**3032–3035.
32. **Liebana, E., R. M. Girvin, M. Welsh, S. D. Neill, and J. M. Pollock.** 1999. Generation of $CD8^+$ T-cell responses to *Mycobacterium bovis* and mycobacterial antigen in experimental bovine tuberculosis. Infect. Immun. **67:**1034–1044.
33. **Machugh, N. D., J. K. Mburu, C. J. Carol, C. R. Wyatt, J. A. Orden, and W. C. Davis.** 1997. Identification of two distinct subsets of bovine gamma delta T cells with unique cell surface phenotype and tissue distribution. Immunology **92:**340–345.
34. **MacMicking, J. D., R. J. North, R. LaCourse, J. S. Mudgett, S. K. Shah, and C. F. Nathan.** 1997. Identification of nitric oxide synthase as a protective locus against tuberculosis. Proc. Natl. Acad. Sci. USA **94:**5243–5248.
35. **M'Buyamba-Kabangu, J. R., R. Fagard, P. Lijnen, R. Bouillon, W. Lissens, and A. Amery.** 1987. Calcium, vitamin D-endocrine system, and parathyroid hormone in black and white males. Calcif. Tissue Int. **41:**70–74.
36. **Muller, I., S. P. Cobbold, H. Waldmann, and S. H. E. Kaufmann.** 1987. Impaired resistance to *Mycobacterium tuberculosis* infection after selective in vivo depletion of $L3T4^+$ and $Lyt2^+$ T cells. Infect. Immun. **55:**2037–2041.
37. **Muller, K., K. Rieneck, M. B. Hansen, and K. Bendtzen.** 1992. 1,25-Dihydroxyvitamin D3-mediated suppression of T lymphocyte functions and failure of T cell-activating cytokines to restore proliferation. Immunol. Lett. **34:**37–44.
38. **Munk, M. E., A. J. Gatrill, and S. H. E. Kaufmann.** 1990. Target cell lysis and IL-2 secretion by γ/δ T lymphocytes after activation with bacteria. J. Immunol. **145:**2434–2439.
39. **Mutis, T., Y. E. Cornelisse, and T. H. Ottenhoff.** 1993. Mycobacteria induce $CD4^+$ T cells that are cytotoxic and display Th1-like cytokine secretion profile: heterogeneity in cytotoxic activity and cytokine secretion levels. Eur. J. Immunol. **23:**2189–2195.
40. **Nabeshima, S., M.Nomoto, G. Matsuzaki, K. Kishihara, H. Taniguchi, S. Yoshida, and K. Nomoto.** 1999. T-cell hyporesponsiveness induced by activated macrophages through nitric oxide production in mice infected with *Mycobacterium tuberculosis*. Infect. Immun. **67:**3221–3226.
41. **Ng, K. H., F. E. Aldwell, D. N. Wedlock, J. D. Watson, and B. M. Buddle.** 1997. Antigen-induced interferon-γ and interleukin-2 responses of cattle inoculated with *Mycobacterium bovis*. Vet. Immunol. Immunopathol. **57:**59–68.
42. **Nicholson, S., M. Bonecini-Almeida, J. R. Silva, C. Nathan, Q. W. Xie, R. Mumford, J. R. Weidner, J. Calaycay, J. Geng, N. Boechat, C. Linhares, W. Rom, and J. L. Ho.** 1996. Inducible nitric oxide synthase in pulmonary alveolar macrophages from patients with tuberculosis. J. Exp. Med. **183:**2293–2302.
43. **Nonnecke, B. J., S. T. Franklin, T. A. Reinhardt, and R. L. Horst.** 1993. In vitro modulation of proliferation and phenotype of resting and mitogen-stimulated bovine mononuclear leukocytes by 1,25-dihydroxyvitamin D3. Vet. Immunol. Immunopathol. **38:**75–89.
44. **Orme, I. M., A. D. Roberts, J. P. Griffin, and J. S. Abrams.** 1993. Cytokine secretion by CD4 T lymphocytes acquired in response to *Mycobacterium tuberculosis* infection. J. Immunol. **151:**518–525.
45. **Palmer, M. V., D. L. Whipple, and S. C. Olsen.** 1999. Development of a model of natural infection with *Mycobacterium bovis* in white-tailed deer. J. Wildl. Dis. **35:**450–457.
46. **Pintado, C. O., J. Carracedo, M. Rodriguez, R. Perez-Calderon, and R. Ramirez.** 1996. 1 α, 25-Dihydroxyvitamin D3 (calcitriol) induces apoptosis in stimulated T cells through an IL-2 dependent mechanism. Cytokine **5:**342–345.
47. **Pithie, A. D., M. Rahelu, D. S. Kumararatne, P. Drysdale, J. S. Gaston, P. B. Iles, J. A. Innes, and C. J. Ellis.** 1992. Generation of cytolytic T cells in

individuals infected by *Mycobacterium tuberculosis* and vaccinated with BCG. Thorax **47:**695–701.
48. **Preece, M. A., W. B. McIntosh, S. Tomlinson, J. A. Ford, M. G. Dunnigan, and J. L. O'Riordan.** 1973. Vitamin-D deficiency among Asian immigrants to Britain. Lancet **i:**907–910.
49. **Rajaraman, V., B. J. Nonnecke, S. T. Franklin, D. C. Hammell, and R. L. Horst.** 1998. Effect of vitamins A and E on nitric oxide production by blood mononuclear leukocytes from neonatal calves fed milk replacer. J. Dairy Sci. **81:**3278–3285.
50. **Rhodes, S. G., D. Gavier-Widen, B. M. Buddle, A. O. Whelan, M. Singh, R. G. Hewinson, and H. M. Vordermeier.** 2000. Antigen specificity in experimental bovine tuberculosis. Infect. Immun. **68:**2573–2578.
51. **Rhodes, S. G., B. M. Buddle, R. G. Hewinson, and H. M. Vordermeier.** 2000. Bovine tuberculosis: immune responses in the peripheral blood and at the site of active disease. Immunology **99:**195–202.
52. **Rigby, W. F. C.** 1988. The immunobiology of vitamin D. Immunol. Today **9:**54–58.
53. **Rockett, K. A., R. Brookes, I. Udalova, V. Vidal, A. V. S. Hill, and D. Kwiatkowski.** 1998. 1,25-Dihydroxyvitamin D_3 induces nitric oxide synthase and suppresses growth of *Mycobacterium tuberculosis* in a human macrophage-like cell line. Infect. Immun. **66:**5314–5321.
54. **Rook, G. A., J. Steele, L. Fraher, S. Barker, R. Karmalli, J. O'Riordan, and J. Stanford.** 1986. Vitamin D_3, gamma interferon, and control of proliferation of *Mycobacterium tuberculosis* by human monocytes. Immunology **57:**159–360.
55. **Salle, B. L., E. E. Delvin, A. Lapillonne, N. J. Bishop, and F. H. Glorieux.** 2000. Perinatal metabolism of vitamin D. Am. J. Clin. Nutr. **71:**1317S–1324S.
56. **Schmitt, S. M., S. D. Fitzgerald, T. M. Cooley, C. S. Bruning-Fann, L. Sullivan, D. Berry, T. Carlson, R. B. Minnis, J. B. Payeur, and J. Sikarskie.** 1997. Bovine tuberculosis in free-ranging white-tailed deer from Michigan. J. Wildl. Dis. **33:**749–758.
57. **Smith, R. A., J. M. Kreeger, A. J. Alvarez, J. C. Goin, W. C. Davis, D. L. Whipple, and D. M. Estes.** 1999. Role of $CD8^+$ and WC-1^+ γ/δ T cells in resistance to *Mycobacterium bovis* infection in the SCID-bo mouse. J. Leukoc. Biol. **65:**28–34.
58. **Smyth, A. J., M. D. Welsh, R. M. Girvin, and J. M. Pollock.** 2001. In vitro responsiveness of $\gamma\delta$ T cells from *Mycobacterium bovis*-infected cattle to mycobacterial antigens: predominant involvement of $WC1^+$ cells. Infect. Immun. **69:**89–96.
59. **Tanaka, Y., C. T. Morita, Y. Tanaka, E. Nieves, B. B. Brenner, and B. R. Bloom.** 1995. Natural and synthetic non-peptide antigens recognized by human $\gamma\delta$ T cells. Nature **375:**155–158.
60. **Thoma-Uszynski, S., S. Stenger, O. Takeuchi, M. T. Ochoa, M. Engele, P. A. Sieling, P. F. Barnes, M. Rollinghoff, P. L. Bolcskei, M. Wagner, S. Akira, M. V. Norgard, J. T. Belisle, P. J. Godowski, B. R. Bloom, and R. L. Modlin.** 2001. Induction of direct antimicrobial activity through mammalian toll-like receptors. Science **291:**1544–1547.
61. **United States Department of Agriculture.** 1999. Bovine tuberculosis eradication uniform methods and rules. APHIS 91-45-011. U.S. Government Printing Office, Washington D.C.
62. **Waters, W. R., J. R. Stabel, R. E. Sacco, J. A. Harp, B. A. Pesch, and M. J. Wannemuehler.** 1999. Antigen-specific B-cell unresponsiveness induced by chronic *Mycobacterium avium* subsp. paratuberculosis infection of cattle. Infect. Immun. **67:**1593–1598.
63. **Waters, W. R., M. V. Palmer, B. A. Pesch, S. C. Olsen, M. J. Wannemuehler, and D. L. Whipple.** 2000. MHC class II-restricted, $CD4^+$ T cell proliferative responses of peripheral blood mononuclear cells from *Mycobacterium bovis*-infected white-tailed deer. Vet. Immunol. Immunopathol. **76:**215–229.
64. **Waters, W. R., M. V. Palmer, B. A. Pesch, S. C. Olsen, M. J. Wannemuehler, and D. L. Whipple.** 2000. Lymphocyte subset proliferative responses of *Mycobacterium bovis*-infected cattle to purified protein derivative. Vet. Immunol. Immunopathol. **77:**257–273.
65. **Wilkinson, R. J., M. Llewelyn, Z. Toossi, P. Patel, G. Pasvol, A. Lalvani, D. Wright, M. Latif, and R. N. Davidson.** 2000. Influence of vitamin D deficiency and vitamin D receptor polymorphisms on tuberculosis among Gujarati Asians in west London: a case-control study. Lancet **19:**618–621.