Modulation of *Mycobacterium bovis*-Specific Responses of Bovine Peripheral Blood Mononuclear Cells by 1,25-Dihydroxyvitamin D₃


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Historically, administration of vitamin D has been considered beneficial in the treatment of tuberculosis. The interaction of this vitamin (i.e., 1,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃]) 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)₂D₃ on *M. bovis*-specific responses in vitro. Addition of 1 or 10 nM 1,25(OH)₂D₃ inhibited *M. bovis*-specific proliferative responses of PBMC from *M. bovis*-infected cattle, affecting predominately the CD4⁺ cell subset. In addition, 1,25(OH)₂D₃ 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)₂D₃ to PBMC cultures. These findings support the current hypothesis that 1,25(OH)₂D₃ enhances mycobacterial killing by increasing NO production, a potent antimicrobial mechanism of activated macrophages, and suggest that 1,25(OH)₂D₃ 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 D₃. Vitamin D binding protein aids in the transport of vitamin D₃ from the skin to the liver, where it is converted to 25-hydroxyvitamin D₃ [25(OH)D₃], the predominant circulating form of vitamin D. In response to hypocalcemic states, 25(OH)D₃ is hydroxylated to form 1,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃], the major mediator of the biological activity of vitamin D. In humans, circulating concentrations of 25(OH)D₃ range from 55 to 75 nM, and circulating concentrations of 1,25(OH)₂D₃ 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 D₃ in the skin often results in measurable reductions in 25(OH)D₃ concentrations in plasma. For instance, concentrations of 25(OH)D₃ 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)D₃ 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)₂D₃ 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-

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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 isolated 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)2D3.

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)2D3 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, Agricultural Research Service, Ames, Iowa, according to institutional procedures and 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 Bogovim assay (C5L 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 containment 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 ~109 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 g for 15 min 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 intratracheal inoculation, cattle (n = 2) were restrained and anesthetized with 500 mg of ketamine (Fort Dodge Animal Health, Fort Dodge, Iowa) and 30 ml 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 tracheal cannula of anesthetized cattle as previously described for inoculation of white-tailed deer (45). For aerosol inoculation, cattle (n = 5) 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/in2) was used to jet nebulize the challenge inoculum (2-m1 volume of ~107 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)2D3, solubilized in 100% ethanol or with ethanol alone [i.e., no 1,25(OH)2D3] in a 10-μl volume. Ethanol was then allowed to evaporate, leaving the 1,25(OH)2D3 at the desired concentration (i.e., 0, 1, or 10 nM). Wells were then seeded with 2 × 104 mononuclear cells in a total volume of 200 μl per well. Medium was RPMI 1640 supplemented with 25 mM HEPES buffer, penicillin (10 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; C5L Limited), rESAT-6 (1 μg/ml; kindly provided by F. C. Minow, 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% CO2 in air. After 5 days, 0.5 μCi of [methyl-3H]thymidine (specific activity, 6.7 Ci mmol−1; Amersham Life Science, Arlington, United States) was added, and the cultures were incubated an additional 16 h. At the conclusion of the incubation, 1 ml of medium was harvested into 96-well round-bottom microtiter plates (Falcon, Becton Dickinson; Lincoln Park, N.J.). Incorporation of [3H]thymidine by viable lymphocytes was determined by liquid scintillation spectrometry. From 10 to 50,000 counts per minute were obtained. Experiments were performed in triplicate.
TABLE 1. Addition of 1,25(OH)2D3 to PBMC cultures and decrease in lymphocyte blastogenesis as measured by [methyl-3H]thymidine uptake

<table>
<thead>
<tr>
<th>Animal no.</th>
<th>1,25(OH)2D3 concn (nM)</th>
<th>NS</th>
<th>CF</th>
<th>PPD</th>
<th>rESAT-6</th>
<th>PWM</th>
</tr>
</thead>
<tbody>
<tr>
<td>236</td>
<td>0</td>
<td>8.719</td>
<td>41.094</td>
<td>32.108</td>
<td>20.733</td>
<td>97.870</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>3.225</td>
<td>30.176</td>
<td>26.917</td>
<td>4.672</td>
<td>77.538</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>3.240</td>
<td>27.874</td>
<td>25.103</td>
<td>5.094</td>
<td>78.669</td>
</tr>
<tr>
<td>244</td>
<td>0</td>
<td>7.627</td>
<td>65.617</td>
<td>67.140</td>
<td>56.447</td>
<td>112.487</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>2.935</td>
<td>51.334</td>
<td>25.085</td>
<td>23.735</td>
<td>78.826</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>1.434</td>
<td>34.107</td>
<td>28.907</td>
<td>7.646</td>
<td>80.902</td>
</tr>
</tbody>
</table>

* 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 Ci of [methyl-3H]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.

** Both animals 236 and 244 were infected with 107 CFU of M. bovis strain 1315 by intranasal inoculation (52 days postinoculation).

TABLE 2. Effects of 1,25(OH)2D3 on lymphocyte subset proliferation in response to stimulation with M. bovis PPD

<table>
<thead>
<tr>
<th>Cattle group and 1,25(OH)2D3 concn (nM)</th>
<th>No. of cells that proliferateda</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
</tr>
<tr>
<td>Noninfected (n = 3)</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>502 ± 263</td>
</tr>
<tr>
<td>1</td>
<td>199 ± 83</td>
</tr>
<tr>
<td>10</td>
<td>224 ± 127</td>
</tr>
<tr>
<td>M. bovis infected (n = 5)</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>4,844 ± 500</td>
</tr>
<tr>
<td>1</td>
<td>4,536 ± 696</td>
</tr>
<tr>
<td>10</td>
<td>4,325 ± 625</td>
</tr>
</tbody>
</table>

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-\(\gamma\) 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)\(_2\)D\(_3\) on lymphocyte proliferation. Addition of 1 or 10 nM 1,25(OH)\(_2\)D\(_3\) 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)\(_2\)D\(_3\) 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 predominately CD4\(^+\) and WC1\(^+\) (i.e., \(y8\) 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)\(_2\)D\(_3\) decreased CD4\(^+\) cell proliferation but not WC1\(^+\) cell proliferation in samples from infected animals (Table 2).

Addition of 1,25(OH)\(_2\)D\(_3\) to PPD-stimulated cultures also diminished the number of CD4\(^+\) cells that had proliferated through multiple generations. Addition of 1.25(OH)\(_2\)D\(_3\) 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)\(_2\)D\(_3\), 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)\(_2\)D\(_3\)-treated cultures compared to nontreated, control cultures [e.g., 46, 32, and 32\% for 0, 1, and 10 nM 1,25(OH)\(_2\)D\(_3\), respectively] (Fig. 1). Although this finding was most apparent for the CD4\(^+\) cell subpopulation, WC1\(^+\) cells within 1,25(OH)\(_2\)D\(_3\)-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)\(_2\)D\(_3\) (Table 2).

Effects of 1,25(OH)\(_2\)D\(_3\) on lymphocyte apoptosis. A significant sequela to lymphocyte proliferation and activation is apoptosis (1). To determine the effect of 1,25(OH)\(_2\)D\(_3\) on apoptosis of lymphocytes during an in vitro recall response, PBMC from \(M. bovis\)-infected cattle were incubated with medium alone (e.g., nontreated) 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)\(_2\)D\(_3\) compared to nonsupplemented PPD-stimulated cultures (Table 3). This trend was not detected for nonsupplemented 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)\(_2\)D\(_3\) was similar for each T-cell subset (CD4\(^+\), CD8\(^-\), and WC1\(^+\) cells) tested.

Antigen-specific IFN-\(\gamma\) and NO responses of \(M. bovis\)-infected cattle. Prior to evaluating the effects of 1,25(OH)\(_2\)D\(_3\) on \(M. bovis\)-specific IFN-\(\gamma\) and NO responses, the capacity of PBMC from infected cattle to produce IFN-\(\gamma\) and NO in response to stimulation with \(M. bovis\) antigens was determined. IFN-\(\gamma\) secretion by PBMC from \(M. bovis\)-infected cattle stimulated with either \(M. bovis\) CF, PPD, or rESAT-6 exceeded \((P < 0.05)\) IFN-\(\gamma\) secretion by nonsupplemented, autologous PBMC (Fig. 2a). In addition, the IFN-\(\gamma\) response of PBMC
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)₂D₃ 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)₂D₃. Addition of 1,25(OH)₂D₃ enhanced (*P* < 0.01) nitrite production by PWM-stimulated PBMC from *M. bovis*-infected cattle (Fig. 3). Addition of 1,25(OH)₂D₃ 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)₂D₃ 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)₂D₃ inhibiting (*P* < 0.05) IFN-γ production in response to both CF and PPD (Fig. 4). While 1,25(OH)₂D₃ enhanced nitrite production in response to rESAT-6 (Fig. 3), significant modulation by 1,25(OH)₂D₃ 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)₂D₃ 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)₂D₃ to cultures diminished this correlation, although statistically significant differences were not detected (data not shown).

**DISCUSSION**

Recent evidence suggests that 1,25(OH)₂D₃-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)₂D₃ to 1,25(OH)₂D₃, the most active, naturally occurring form of the vitamin (54). 1,25(OH)₂D₃ induces NOS2 expression and the production of NO by human macrophages, necessary for mycobacterial kill-

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### Table 3. Inhibition of apoptosis by addition of 1,25(OH)₂D₃ to lymphocyte cultures

<table>
<thead>
<tr>
<th>Treatment and 1,25(OH)₂D₃ concn (nM)</th>
<th>% Annexin V positive</th>
<th>% of cells within:</th>
</tr>
</thead>
<tbody>
<tr>
<td>No stimulation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>31.47 ± 1.38</td>
<td>40.95 ± 4.37</td>
</tr>
<tr>
<td>1</td>
<td>30.97 ± 1.70</td>
<td>39.47 ± 3.16</td>
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<tr>
<td>10</td>
<td>31.77 ± 1.68</td>
<td>38.80 ± 2.98</td>
</tr>
<tr>
<td>PPD stimulation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>29.20 ± 1.98</td>
<td>37.17 ± 4.14</td>
</tr>
<tr>
<td>1</td>
<td>26.57 ± 1.79</td>
<td>32.68 ± 2.09</td>
</tr>
<tr>
<td>10</td>
<td>25.95 ± 1.81</td>
<td>30.87 ± 1.99</td>
</tr>
</tbody>
</table>

*Results are from comparison of forward- and side-scatter plots.*

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![Figure 2](http://cv.asm.org/)

**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/ml), 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 cell differ from responses of controls. Symbols: *, *P* < 0.01; **, *P* < 0.05; ***, *P* < 0.001. Error bars, SEM.
In the present study, it was determined that 1,25(OH)2D3 increased NO production and decreased IFN-γ/H9253 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)2D3, has been shown to inhibit IFN-γ/H9253 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/H9253 production by 1,25(OH)2D3 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)2D3 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)2D3 was 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)2D3 on M. bovis-specific IFN-γ production is most likely a direct effect of 1,25(OH)2D3 and not a secondary response to increased NO production.

Mitogen-induced CD4+ T-cell proliferation is inhibited by 1,25(OH)2D3 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)2D3. 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)2D3 inhibited only CD4+ cell proliferation. These findings are in agreement with a previous study in which 1,25(OH)2D3 inhibited mitogen-induced proliferation of CD4+ cells yet had no effect on γδ TCR+ cell proliferation (43). Since PPD is composed of a

FIG. 3. Addition of 1,25(OH)2D3 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)2D3, or 10 nM 1,25(OH)2D3 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.
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 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, 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)2D3 on CD4 cells is that the proliferative response of 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)2D3 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)2D3 affects CD4 cells specifically in regards to inhibition of proliferation in response to PPD.

1,25(OH)2D3 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)2D3 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)2D3 and to determine the underlying mechanisms. Inhibition of apoptosis of M. bovis-specific T cells by 1,25(OH)2D3 at the site of M. bovis infection would likely be beneficial to the host antitubercular response.

It has been postulated that 1,25(OH)2D3 enhances mycobacterial killing via an NO-dependent mechanism. It is also postulated that production of 1,25(OH)2D3 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)_2D3 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.

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individuals infected by Mycobacterium tuberculosis and vaccinated with BCG.

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