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Low calcium diet and 1,25-dihydroxyvitamin D3 infusion modulate immune responses during Mycobacterium paratuberculosis infection in geige mice

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Low calcium diet and 1,25-dihydroxyvitamin D₃ infusion modulate immune responses during *Mycobacterium paratuberculosis* infection in beige mice

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**Abstract**

A 12-month study was conducted to evaluate the effects of feeding a low calcium (Ca) diet or 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃) infusion on the persistence of *Mycobacterium paratuberculosis* infection using a mouse model. Male beige mice 6–8 weeks of age were assigned to one of the following treatments: (1) non-infected, (2) infected, (3) non-infected/1,25(OH)₂D₃, (4) infected/1,25(OH)₂D₃, and (5) infected/low Ca (0.15%) diet. Infected mice were inoculated intravenously with live *M. paratuberculosis*. At 1, 6 and 12 months postinfection, mice in Treatments 3 and 4 were implanted subcutaneously with mini-osmotic pumps to deliver 1,25(OH)₂D₃. Infusion with 1,25(OH)₂D₃ exacerbated *M. paratuberculosis* infection in most tissues at all time points. Mice infused with 1,25(OH)₂D₃ had higher bacterial counts in spleen, liver, and ileum compared with control infected mice after 1 month of infection. In contrast, feeding a low Ca diet reduced the number of viable organisms cultured from the liver and ileum of infected mice. Plasma Ca and 1,25(OH)₂D₃ were increased in mice infused with 1,25(OH)₂D₃ at all time points but values for low Ca mice were not different than for non-infused mice. Splenocyte production of TNF, IL-1 and IL-6 was higher for mice fed the low Ca diet compared with control infected mice after 1 month of infection. Inducible IL-6 activity remained higher for
this treatment at 6 months postinfection. These results suggest that feeding a low Ca diet to mice chronically infected with \textit{M. paratuberculosis} appears to enhance their ability to clear the infection in a manner distinct from any effect of $1,25(\text{OH})_2\text{D}_3$.

\textit{Keywords: Mycobacterium paratuberculosis; Calcium; Vitamin D$_3$}

1. Introduction

Clinical studies have documented perturbations in calcium metabolism in patients with pulmonary tuberculosis. Some of the earliest reports indicated that patients with tuberculosis had reduced serum calcium concentrations (Matz, 1925). Brookbank (1927) observed that patients with acute pulmonary tuberculosis had reduced serum calcium whereas patients in remission had slightly elevated serum calcium levels. More recent and perhaps more consistent findings have indicated that patients with granulomatous diseases such as tuberculosis, sarcoidosis and leprosy have elevated serum calcium concentrations (Shai et al., 1972; Abbasi et al., 1979; Gkonos et al., 1984; Ryzen et al., 1988). In most cases, the hypercalcemia due to mycobacterial infection was indistinguishable from that caused by therapeutic doses of vitamin D and supplemental calcium. Because of its significant regulatory role in calcium homeostasis, vitamin D has often been implicated as a causative factor in this hypercalcemia.

In the mid-1800s, heliotherapy and vitamin D in the form of cod liver oil were employed as possible curatives for tuberculosis (Davies, 1985). Intravenous administration of calcium salts appeared to be beneficial in the treatment of some cases of chronic pulmonary tuberculosis as well (Brookbank, 1927). With the advent of anti-tuberculosis drugs such as rifampicin and isoniazid, other forms of chemotherapy became secondary. Although a connection between vitamin D, calcium and mycobacterial disorders had been validated by these early observations little progress was made in understanding the interplay between these factors. In more recent years, several laboratories have evaluated the use of vitamin D in vitro as an anti-mycobacterial agent. Treatment of human monocyte-derived macrophages with $1,25$-dihydroxyvitamin D$_3$ prior to and during infection effectively inhibited growth of \textit{Mycobacterium tuberculosis} (Crowle et al., 1987). As a hormone with immunomodulatory function, vitamin D could affect certain arms of the immune system which are important in host resistance to mycobacteria. Little, if anything, is known about how vitamin D and calcium affect the growth of \textit{Mycobacterium paratuberculosis}, the causative agent of a chronic enteritis known as Johne’s disease found primarily in ruminants. However, it is well documented that parturition, lactation and dietary deficiencies as well as other stressors precipitate clinical disease in infected animals (Kreeger, 1991). In fact, an increased incidence in Johne’s disease has been documented in areas where the soil is acidic and deficient in calcium content, indicating that calcium perturbations may modulate \textit{M. paratuberculosis} infection in cattle (Kopecky, 1977). This study was designed to evaluate the effects of $1,25$-dihydroxyvitamin D$_3$ infusion and the effects of low dietary calcium on the persistence of \textit{M. paratuberculosis} infection using a beige mouse model. In addition, the interactive effects of $1,25$(OH)$_2$D$_3$, calcium and \textit{M. paratuberculosis} infection on splenocyte function and cytokine secretion were examined.
2. Materials and methods

2.1. Experimental animals

Specific pathogen-free male beige mice (C57BL/6J-bg^+/bg^-; The Jackson Laboratory, Bar Harbor, ME) averaging 6 weeks of age and 20 g in weight were obtained for this study. The beige mouse model we utilized in our study had previously been shown to be suitable for infection studies with *M. paratuberculosis* because the progression of disease followed a pattern typically observed in naturally infected ruminants (Whipple et al., 1991). The animals were housed in polycarbonate cages (four per cage) with stainless steel feeders and allowed access to food and water ad libitum. Random assignment of animals to treatment group and infection period was made at the beginning of the study. Animals were weighed prior to inoculation and at the end of their respective infection period immediately before they were killed.

2.2. Experimental protocol

This study was designed to evaluate the effects of 1,25(OH)_{2}D_{3} infusion and dietary calcium (Ca) on the persistence of *M. paratuberculosis* infection in beige mice. In order to observe temporal effects of these nutrients, mice were assigned to an infection period of either 1, 6 or 12 months duration. Within each period, mice were assigned to one of five treatment groups (*n =* 8-13): (1) non-infected, (2) infected, (3) non-infected/1,25(OH)_{2}D_{3} infusion, (4) infected/1,25(OH)_{2}D_{3} infusion, and (5) infected/low Ca diet. The infected/low Ca diet treatment was included in the study as a positive control for comparison with the 1,25(OH)_{2}D_{3} infusion group since reduced dietary Ca (0.15%) should increase endogenous 1,25(OH)_{2}D_{3} levels. Mouse diets formulated to our specifications were purchased from Harlan Teklad (Madison, WI) and fed to mice throughout the study. Diets were isocaloric and contained the current RDA for vitamin D, and Ca. Mice in Treatments 1-4 were fed a mouse chow containing 5000 IU vitamin D, kg^{-1} and 0.45% Ca and a chow of similar composition except for a reduction in Ca content (0.15%) was fed to mice assigned to Treatment 5.

At the beginning of the experiment, mice in Treatments 2, 4 and 5 were inoculated intravenously in the tail vein with 0.1 ml of *M. paratuberculosis* (Strain 19698, National Animal Disease Center, Ames, IA) in phosphate-buffered saline (PBS) which contained approximately 10^8 viable organisms. The intravenous inoculation route has previously been shown to be the most effective route in establishing intestinal infection for *M. paratuberculosis* in this mouse model (Chandler, 1962; Whipple et al., 1991). Non-infected mice in Treatments 1 and 3 were sham inoculated with the same volume of PBS containing no bacteria. At 1, 6 and 12 months postinfection, mice in Treatments 3 and 4 were implanted subcutaneously with mini-osmotic pumps (Alza Corp., Palo Alto, CA) which delivered 477 pg 1,25(OH)_{2}D_{3} per g BW per day for 14 days. The dose of 1,25(OH)_{2}D_{3} delivered by pumps was determined in two preliminary experiments using elevation of plasma 1,25(OH)_{2}D_{3} as the selection criteria. At the end of the infusion period, mice in all treatment groups were killed after anesthetizing with isoflurane inhalation followed by decapitation by guillotine. Blood was immediately collected into
tubes containing heparin. A ventral mid-line incision was made and tissues were removed from the thoracic and abdominal cavities. Portions of spleen, liver, intestinal tissues, lung and regional lymph nodes were excised and cultured for viable *M. paratuberculosis*. Spleens from four animals in each treatment group were aseptically removed and placed immediately in petri dishes containing 10 ml of RPMI-1640 medium (Gibco, Grand Island, NY). The remaining tissues were weighed and homogenized with a stomacher in 0.75% hexadecylpyridinium chloride solution for 1 min and allowed to decontaminate overnight at room temperature. Serial ten-fold dilutions of individual tissue homogenates were inoculated (100 μl per tube) onto agar slants of Herrold’s egg yolk medium containing 2 mg mycobactin J l⁻¹. After 12 weeks of incubation at 37°C, viable organisms were determined by counting the number of colonies on the agar slants. Data were expressed as colony forming units corrected for wet tissue weights (CFU mg⁻¹).

2.3. **Plasma analyses**

The plasma calcium concentration was determined by atomic absorption spectrophotometry (Perkin-Elmer Corp., Norwalk, CT). Plasma 1,25(OH)₂D₃ concentration was determined by the method of Reinhardt et al. (1984).

2.4. **Cytokine assays**

Spleens were thoroughly minced in RPMI-1640 medium and then passed through a 20 gauge needle with a 10 cc syringe at least five times to attain a single-cell suspension. Cell viability was determined using propidium iodide exclusion. Splenocytes were plated in 48-well tissue culture plates (Costar) at a concentration of 4 × 10⁵ viable cells per well in complete medium (RPMI-1640 containing 10% fetal bovine serum (Hyclone), 2 mM L-glutamine, 100 U of penicillin per ml and 100 μg of streptomycin per ml (Gibco)). Cells were either cultured with medium alone (non-stimulated) or with 14 μg ml⁻¹ of *M. paratuberculosis* whole cell sonicate (MpS) for 24 and 48 h at 37°C in a 5% CO₂, humidified atmosphere. The MpS was prepared by sonication of 1 ml volumes of *M. paratuberculosis* (1 × 10⁹ ml⁻¹) at 25 W for 25 min (Tekmar). Cell-free supernatants were harvested and frozen at −20°C until analyzed for IL-1, IL-6 and TNF activity. Supernatants were analyzed for IL-1 using a commercial kit (mouse IL-1α ELISA kit; Genzyme, Cambridge, MA). Interleukin-6 activity in supernatants was measured using 7TD1.B3 cells, an IL-6-dependent cell line (American Type Culture Collection, Rockville, MD), according to the method of Van Snick et al. (1987). Samples were analyzed for TNF activity using WEHI 164 (Clone 13) cells (CRL 1751; American Type Culture Collection, Rockville, MD) as described by Mosmann (1983) and with a modification by Promega (Madison, WI) which allows for direct addition of their reagents, dimethylthiazol diphenyltetrazolium bromide (MTT) dye and solubilization solution, to sample wells.

2.5. **Lymphocyte blastogenesis**

Splenocytes were added to 96-well microtiter plates (Falcon) at a concentration of 2 × 10⁵ cells per well. Triplicate cultures received medium alone (non-stimulated). 5 μg
ml⁻¹ of concanavalin A (ConA; Sigma), 10 μg ml⁻¹ of phytohemagglutinin-P (PHAP; Sigma), 0.4 μg ml⁻¹ of pokeweed mitogen (PWM; Sigma) and 1 or 10 μg ml⁻¹ of Johnin purified protein derivative (PPD-J) and MpS (National Animal Disease Ctr., Ames, IA). Cultures stimulated with ConA, PHAP or PWM were incubated for 3 days while antigen-stimulated cultures (PPD and MpS) were incubated for 5 days at 37°C in a 5% CO₂, humidified atmosphere. Cells were then pulsed with [³H]methylthymidine (14 kBq per well) for an additional 18 h, harvested onto glass fiber filters using a PhD cell harvester (Cambridge, MA) and [³H]thymidine incorporation was measured with a liquid scintillation counter (Beckman, Irvine, CA).

2.6. Histopathology

Sections of spleen, liver, intestinal tissues and lung were obtained at necropsy and fixed by immersion in zinc formalin. Tissues were routinely processed, embedded in paraffin, cut at 4–6 μm, stained with H&E, and examined by light microscopy. Briefly, splenic and hepatic lesions were scored according to the number of granulomas per section as follows: (1) minimal, less than two granulomas; (2) mild, less than five granulomas; (3) moderate, more than five granulomas but small (< 200 μm in diameter); (4) marked, more than ten granulomas in multifocal, extensive (> 100 μm in diameter), and coalescing patterns; (5) severe, inflammatory process occupying more than 20% of the section.

2.7. Statistical analyses

Statistical analyses were conducted by the Statview program (Abacus Concepts, Inc., Berkeley, CA). Statistical evaluation of all results was performed by two-way analysis of variance (ANOVA) and significant differences between group means were tested by the Fisher’s protected least significant difference test.

3. Results

The effects of 1,25(OH)₂D₃ infusion and feeding a low Ca diet on plasma Ca and 1,25(OH)₂D₃ are shown in Table 1. Infusion of 1,25(OH)₂D₃ resulted in significant (P < 0.01) increases in circulating levels of both parameters at the end of all three infection periods. Plasma 1,25(OH)₂D₃ was increased (P < 0.01) in the infected/1,25(OH)₂D₃ treatment group at 1 month of infection. However, at 6 and 12 months infection mice in the infected/1,25(OH)₂D₃ treatment group had significantly (P < 0.01) lower plasma 1,25(OH)₂D₃ levels than the non-infected/1,25(OH)₂D₃ treated group. Feeding a low Ca diet did not overtly affect either plasma Ca or plasma 1,25(OH)₂D₃ concentrations during any infection period although a slight increase in plasma 1,25(OH)₂D₃ was noted after 12 months infection.

The influence of 1,25(OH)₂D₃ infusion or dietary Ca on the persistence of viable M. paratuberculosis organisms in the spleens of infected mice during 1, 6 and 12 months of infection is summarized in Fig. 1. Infusion with 1,25(OH)₂D₃ increased the number of
Table 1
Effects of a low Ca diet and 1,25(OH)₂D₃ infusion on plasma calcium and 1,25(OH)₂D₃ concentrations in mice infected with *M. paratuberculosis*.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Infection period</th>
<th>1 month</th>
<th></th>
<th></th>
<th>6 months</th>
<th></th>
<th></th>
<th>12 months</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Plasma Ca (mg dl⁻¹)</td>
<td>Plasma 1,25(OH)₂D₃ (pg ml⁻¹)</td>
<td>Plasma Ca (mg dl⁻¹)</td>
<td>Plasma 1,25(OH)₂D₃ (pg ml⁻¹)</td>
<td>Plasma Ca (mg dl⁻¹)</td>
<td>Plasma 1,25(OH)₂D₃ (pg ml⁻¹)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-infected</td>
<td></td>
<td>8.01 ± 0.19</td>
<td>37.25 ± 7.65</td>
<td>7.51 ± 0.18</td>
<td>17.24 ± 2.54</td>
<td>8.27 ± 0.32</td>
<td>40.40 ± 17.90</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Infected</td>
<td></td>
<td>7.92 ± 0.19</td>
<td>36.25 ± 4.70</td>
<td>7.41 ± 0.07</td>
<td>11.11 ± 3.65</td>
<td>8.54 ± 0.75</td>
<td>13.00 ± 5.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-infected/1,25(OH)₂D₃</td>
<td></td>
<td>10.93 ± 0.61</td>
<td>89.03 ± 19.60</td>
<td>11.58 ± 0.23</td>
<td>209.27 ± 22.74</td>
<td>14.74 ± 0.81</td>
<td>195.20 ± 9.90</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Infected/1,25(OH)₂D₃</td>
<td></td>
<td>12.44 ± 0.57</td>
<td>134.40 ± 14.60</td>
<td>11.68 ± 0.31</td>
<td>172.00 ± 36.37</td>
<td>13.37 ± 0.66</td>
<td>130.30 ± 23.30</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Infected/low Ca</td>
<td></td>
<td>8.12 ± 0.32</td>
<td>46.40 ± 3.80</td>
<td>7.28 ± 0.15</td>
<td>11.91 ± 2.53</td>
<td>9.04 ± 0.33</td>
<td>63.80 ± 11.20</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Values are means ± standard error of the mean for eight animals per treatment (except for infected/low Ca group in 12 month period, n = 13). * Significantly different from non-infused groups, *P* < 0.05.*
organisms present compared with infected controls after 1 month of infection although differences were not statistically significant. This pattern was also apparent following 6 and 12 months of infection. Significantly ($P < 0.01$) higher numbers of bacteria were isolated from spleens of infected/1,25(OH)$_2$D$_3$ compared with the mice fed the low Ca diet after 1 month of infection. In contrast, the number of viable organisms isolated from spleen tissue at 6 months of infection were not affected by feeding a low Ca diet to infected mice. Although greater numbers of viable M. paratuberculosis were recovered from spleens of mice fed a low Ca diet compared with infected control mice at 12 months of infection this effect was not statistically significant due to high variability within treatment group.

After 1 month of infection, mice infused with 1,25(OH)$_2$D$_3$ had significantly higher ($P < 0.05$) numbers of viable M. paratuberculosis isolated from their livers compared with infected control mice (Fig. 2). Infusion with 1,25(OH)$_2$D$_3$ continued to be associated with an increase in the number of organisms present in the liver at 6 and 12 months of infection. However, during these same infection periods mice fed the low Ca diet had reduced numbers of organisms compared with both infused and control infected mice. Similar effects on the pattern of infectivity were noted in the ileum of mice after 1, 6 and 12 months of infection (Fig. 3). Higher numbers of viable organisms were recovered from intestinal tissues of mice infused with 1,25(OH)$_2$D$_3$ compared with infected controls and low Ca mice after 1 month of infection. However, total numbers of organisms recovered were low regardless of treatment. By 6 months of infection, the number of isolated viable M. paratuberculosis had increased markedly for all treatment groups signifying more pronounced colonization of the intestinal region. Similar numbers of organisms were isolated from tissues of mice infused with 1,25(OH)$_2$D$_3$ compared with infected controls yet dramatic reductions were noted in tissues from the
low Ca group. At 12 months of infection, the level of infectivity remained fairly constant compared with the 6 month infection period although the pattern for lower \((P < 0.05)\) numbers of organisms in the ileum of mice fed the low Ca diet persisted. There were no significant effects of \(1,25(OH)_2D_3\) infusion or low Ca diet on infectivity levels in the duodenum or jejunum.

The effects of \(1,25(OH)_2D_3\) infusion and a low Ca diet on IL-1 production by splenocytes cultured with \(M. paratuberculosis\) sonicate are presented in Fig. 4. After 1 month of infection, mice fed the low Ca diet had higher \((P < 0.05)\) IL-1 activity in the 24- and 48-h culture supernatants than control infected mice and mice infused with \(1,25(OH)_2D_3\). There were no differences between treatment groups during the 6- and 12-month infection periods. Interleukin-6 activity was significantly increased \((P < 0.05)\) in 24-h supernatants during the early stages of infection in mice fed the low Ca diet (Fig.
Fig. 3. Effect of a low Ca diet and 1,25(OH)₂D₃ (VD) infusion on the pattern of infection in intestinal tissues of mice infected with *M. paratuberculosis* for 1 (A), 6 (B) and 12 (C) months. Values are means ± standard errors of the mean for eight animals per treatment per infection period (except for infected/low Ca group in 12 month period, n = 13). * Significantly different from control infected group within infection period. *P* < 0.05.

5). In contrast, IL-6 activity was significantly lower (*P* < 0.05) in this treatment group following 12 months of infection after 24 h incubation. Infusion with 1,25(OH)₂D₃ after 6 months of infection or feeding a low Ca diet during this period resulted in a significant (*P* < 0.05) increase in IL-6 production compared with control infected mice. The same patterns of IL-6 production for infused mice and mice fed the low Ca diet were apparent in supernatants harvested after 48 h of incubation with *M. paratuberculosis* sonicate
although they were significant \((P < 0.05)\) only for the low Ca group. After 1 month of infection, mice fed a low Ca diet also had increased TNF activity in splenocyte supernatants cultured for 24 and 48 h with \(M. \text{paratuberculosis}\) sonicate (Fig. 6). No other significant effects were observed for other treatments during any infection period. Feeding infected mice a low Ca diet significantly \((P < 0.05)\) reduced proliferative responses of splenocytes to ConA, PHAP and PWM mitogens compared with infected controls after 1 month of infection (Table 2). Reduced splenocyte responsiveness to these mitogens was also observed in infected/1,25(OH)\(_2\)D\(_3\) mice with significant \((P < 0.05)\) reductions only to ConA and PHAP. No significant treatment differences in splenocyte proliferation were noted after 6 months of infection (data not shown). After 1 month of infection, splenocytes were generally unresponsive to antigen (PPD J) regardless of treatment group. However, proliferative responses to PPD-J tended to be higher for all three groups of infected mice compared with non-infected mice by 6 months of
infection (stimulation indices were 5.1 vs. 2.2 for infected and non-infected mice, respectively).

Granulomatous lesions were found predominantly in the liver and spleen of mice infected with *M. paratuberculosis* (Table 3). Lesion scores closely followed patterns of infectivity at each time period. After 1 month of infection, mice infused with 1,25(OH)₂D₃ had higher numbers of granulomas with a greater degree of inflammation compared with the other infected treatment groups. By 6 months of infection, granulomatous lesions were dramatically reduced in tissues of mice fed the low Ca diet while similar numbers were observed for control infected and infected/1,25(OH)₂D₃ mice.

4. Discussion

Clinical studies have suggested that calcium metabolism is affected by granulomatous disorders such as tuberculosis, sarcoidosis and berylliosis, with the most common
Fig. 6. Effect of a low Ca diet and 1,25(OH)\textsubscript{2}D\textsubscript{3} (VD) infusion on the production of TNF by splenocytes isolated from mice infected with \textit{M. paratuberculosis} for 1, 6 and 12 months. Splenocytes were incubated with \textit{M. paratuberculosis} sonicate for 24 (A) and 48 (B) h. Values are means ± standard errors of the mean for four animals per treatment per infection period.

Table 2

Effects of low Ca diet and 1,25(OH)\textsubscript{2}D\textsubscript{3} infusion on splenocyte proliferative responses to mitogens in mice infected with \textit{M. paratuberculosis} for 1 month.\textsuperscript{a}

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mitogen</th>
<th>ConA</th>
<th>PHAP</th>
<th>PWM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-infected</td>
<td></td>
<td>24.9 ± 1.7</td>
<td>5.3 ± 0.6</td>
<td>5.2 ± 1.0</td>
</tr>
<tr>
<td>Infected</td>
<td></td>
<td>88.9 ± 29.5</td>
<td>20.0 ± 4.7</td>
<td>9.2 ± 0.7</td>
</tr>
<tr>
<td>Non-infected/1,25(OH)\textsubscript{2}D\textsubscript{3}</td>
<td></td>
<td>15.1 ± 5.3</td>
<td>3.4 ± 1.5</td>
<td>3.9 ± 1.4</td>
</tr>
<tr>
<td>Infected/1,25(OH)\textsubscript{2}D\textsubscript{3}</td>
<td></td>
<td>36.4 ± 8.5 *</td>
<td>5.7 ± 1.7 *</td>
<td>6.7 ± 2.9</td>
</tr>
<tr>
<td>Infected/low Ca</td>
<td></td>
<td>8.4 ± 6.5 *</td>
<td>4.0 ± 2.5 *</td>
<td>2.7 ± 0.9 *</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Data are presented as stimulation indices (SI) of mitogen-stimulated proliferative responses of mouse splenocytes compared with proliferative responses of non-stimulated cells. Values are means ± standard errors of the mean for four animals per treatment. * Significantly different from control infected group, \( P < 0.05 \). ConA, concanavalin A; PHAP, phytohemagglutinin-P; PWM, pokeweed mitogen.
Table 3
Effects of a low Ca diet and 1,25(OH)$_2$D$_3$ infusion on granulomatous lesions in tissues of mice infected with M. paratuberculosis.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Infection period</th>
<th>1 month</th>
<th>6 months</th>
<th>12 months</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-infected</td>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Infected</td>
<td></td>
<td>2.4 (8/8)</td>
<td>2.5 (8/8)</td>
<td>2.7 (8/9)</td>
</tr>
<tr>
<td>Non-infected/1,25(OH)$_2$D$_3$</td>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Infected/1,25(OH)$_2$D$_3$</td>
<td></td>
<td>3.0 (8/8)</td>
<td>2.7 (7/8)</td>
<td>2.8 (9/9)</td>
</tr>
<tr>
<td>Infected/low Ca</td>
<td></td>
<td>2.6 (6/8)</td>
<td>1.6 (6/8)</td>
<td>2.5 (12/13)</td>
</tr>
</tbody>
</table>

Lesions per section were scored as follows: 1, less than two granulomas; 2, less than five granulomas; 3, more than five granulomas; 4, more than ten granulomas; 5, inflammatory process occupying more than 20% of section.

indicates number of animals with lesions/total number of animals.

Finding being hypercalcemia (Stoeckle et al., 1969; Shai et al., 1972; Reiner et al., 1976). Although an exact mechanism for this hypercalcemia is unknown, granulomas that metabolize vitamin D have been implicated as a causative factor. Elevated serum levels of the active metabolite of vitamin D, 1,25(OH)$_2$D$_3$, have been observed in patients with tuberculosis, sarcoidosis and leprosy (Papapoulos et al., 1979; Peces and Alvares, 1987; Ryzen et al., 1988). Hydroxylation of 25-hydroxyvitamin D in the kidney is generally tightly regulated by PTH which is secreted in response to increased calcium demands. However, extra-renal production of 1,25-dihydroxyvitamin D is not well regulated and has been observed in some patients with granulomatous disease (Barbour et al., 1981; Gkonos et al., 1984). Mononuclear phagocytes, the predominant cell type in granulomatous lesions, have been cited as a potential source for this excess production of 1,25-dihydroxyvitamin D (Adams et al., 1983; Cadranel et al., 1988). These cells express 1α-hydroxylase activity and, under some conditions, are able to metabolize 25-hydroxyvitamin D to 1,25-dihydroxyvitamin D. In patients with tuberculosis, T lymphocytes have also been shown to have this capability (Cadranel et al., 1990).

One potential mode of action for 1,25-dihydroxyvitamin D may lie in its ability to regulate granulomatous reactions via induction of differentiation and maturation of mononuclear phagocytes and lymphocytes. Addition of 1,25-dihydroxyvitamin D to cultures has invoked maturation of myeloid leukemic cells and the monocytic cell line U937 as well as differentiation of a promyelocyte line (HL-60) toward monocytes/macrophages (Gray and Cohen, 1985; Zhou et al., 1989). Shifting the population to a more mature and perhaps more functional cell type may be the key in regulating the ability of cells to respond to intracellular pathogens such as mycobacteria. Studies have shown that incubating human monocyte-derived macrophages with 1,25-dihydroxyvitamin D caused significant stasis in the growth of ingested M. tuberculosis and Mycobacterium avium (Rook et al., 1986; Crowle et al., 1987; Bermudez et al., 1990). To our knowledge no controlled studies have been conducted to examine the effects of exogenous administration of 1,25-dihydroxyvitamin D on the progression of mycobacterial infection in an animal model.
In the present study, although higher numbers of viable bacteria were recovered from tissues of mice infused with 1,25(OH)$_2$D$_3$ in the early stages of infection, growth of *M. paratuberculosis* in ileal tissue was inhibited ($P < 0.12$) somewhat in the latter stages of infection. This is an important point since the major area of bacterial colonization in animals naturally infected with *M. paratuberculosis* is the terminal ileum. In this study, there did not appear to be any benefit of 1,25(OH)$_2$D$_3$ therapy during *M. paratuberculosis* infection. Hernandez-Frontera and McMurray (1993) found that varying dietary levels of vitamin D did not affect the degree of infectivity in guinea pigs experimentally inoculated with *M. tuberculosis*. However, they did not measure serum 1,25-dihydroxyvitamin D and, subsequently, were unable to correlate circulating levels of this active metabolite with infection status. In the present study, feeding a low Ca diet to infected mice improved bacterial clearance at all stages of infection. We originally included the low Ca diet treatment so we could compare effects of increased endogenous 1,25-dihydroxyvitamin D without hypercalcemia to exogenous administration of this metabolite which generally increases blood calcium. Contrary to what we had anticipated, feeding a low level of calcium in the diet (0.15%) did not overtly affect plasma 1,25-dihydroxyvitamin D levels. However, it did reduce the number of viable bacteria cultured from the tissues of mice. With the absence of an obvious rise in 1,25-dihydroxyvitamin D, the reduced level of infectivity suggests a singular effect of calcium or some other modulatory factor involved in calcium homeostasis such as parathyroid hormone.

Feeding a low Ca diet to infected mice had a significant beneficial effect on the capacity of splenocytes to secrete inflammatory cytokines, TNF, IL-1 and IL-6, in vitro after 1 month of infection. The mechanism for enhanced cytokine secretion is unknown although it is clear that the moderate reduction in dietary calcium in this treatment group was enough to invoke a significant response. Although not measured in this study, it is possible that the lower level of dietary calcium may have increased serum levels of parathyroid hormone. Parathyroid hormone (PTH) is an important regulatory factor in calcium homeostasis through its bone-resorptive activity as well as permits the synthesis of 1,25(OH)$_2$D$_3$ by the kidney. To date there is very little evidence to suggest that PTH modulates immune cell function; however, cytokine production does appear to be affected. Interleukin-1 and IL-6 release from osteoblastic cells is stimulated by incubation with PTH (Feyen et al., 1989; Lowik et al., 1989; Li et al., 1991). In addition, treatment with PTH also induces the release of proteins with bone-resorptive activity from mononuclear leukocytes (Perry, 1986). These proteins could be IL-1 and IL-6 since both of these cytokines are known mediators of PTH-modulated bone resorption (Li et al., 1991).

The observed increase in cytokine production in early infection may be responsible for the subsequent decrease in viable numbers of *M. paratuberculosis* in tissues of mice fed the low calcium diet. The cytokines measured in this study, TNF, IL-1 and IL-6, share a common function as T cell activators. In addition, they are classified as pro-inflammatory cytokines because they are responsible for the production of acute-phase proteins and neutrophil activation during an inflammatory response (Wewers, 1992). If the enhanced cytokine production obtained in vitro after exposure of splenocytes to the *M. paratuberculosis* sonicate mimics conditions in the host, it is possible that enhanced release of TNF, IL-1 and IL-6 by mice fed the low calcium diet optimized
macrophage–T-cell interactions, allowing for improved clearance of the organism. Further credence is given to this theory by the reduced numbers of granulomas observed in tissues of mice fed the low Ca diet at 6 months of infection.

The reduced proliferative responses to mitogens noted in the low Ca treatment group after 1 month of infection would support the aforementioned observations. Reduced proliferation combined with increased cytokine secretion suggests that cells had differentiated to a more mature, functional state. Infusion with 1,25(OH)\textsubscript{2}D\textsubscript{3} also decreased responses to ConA, PHAP and PWM during the early stages of infection, however, not to the degree noted for mice fed the low calcium diet. Inhibitory effects of 1,25-dihydroxyvitamin D on cellular proliferative responses have been well documented (Lemire et al., 1984; Rigby et al., 1984). It was interesting to note that splenocytes from infected mice responded very poorly to antigens (PPD-Johnin and M. paratuberculosis sonicate) at 1 month of infection but produced a measurable response by 6 months of infection. This suggests that a more chronic, progressive state of infection may be necessary in order to prime cells to respond to these antigens. In conclusion, acute administration of 1,25(OH)\textsubscript{2}D\textsubscript{3} after various periods of M. paratuberculosis infection does not appear to profoundly affect clearance of the organism. However, feeding a low calcium diet throughout the study significantly reduced the number of viable organisms recovered from tissues of infected mice. The dramatic effect of the low calcium diet on cytokine production during the early stages of infection, followed by the reduction in infectivity level, suggests that a mechanism other than, but perhaps not exclusive of, 1,25-dihydroxyvitamin D may be responsible for decreased persistence of M. paratuberculosis in this treatment group. Currently, studies are being conducted to further investigate effects of dietary calcium on M. paratuberculosis infection in the beige mouse model. Cattle diets can vary widely from one management unit to another, suggesting that dietary calcium level may be correlated with degree of resistance or susceptibility to M. paratuberculosis infection in cattle.

References


