Dietary Vitamin D Affects Cell-Mediated Hypersensitivity but Not Resistance to Experimental Pulmonary Tuberculosis in Guinea Pigs

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Outbred, Hartley strain guinea pigs were fed purified diets varying only in their levels of vitamin D. The amounts of vitamin D in the diets were adjusted to represent 0, 25, 50, 100, or 200% of the recommended level (1,180 IU/kg of body weight) for guinea pigs. In some experiments, half of the animals in each diet group were vaccinated with Mycobacterium bovis BCG vaccine at the time the diets were introduced. Six weeks later, all guinea pigs were infected by the respiratory route with a low dose of virulent M. tuberculosis H37Rv. Vitamin D-deficient animals exhibited marked reductions in levels of the major vitamin D metabolite, 25-hydroxyvitamin D₃, in plasma. Altered vitamin D intake was accompanied by changes in antigen (purified protein derivative)-induced, cell-mediated immune responses both in vivo (tuberculin hypersensitivity) and in vitro (lymphoproliferation). Dermal tuberculin reactivity developed more slowly in vitamin D-deficient guinea pigs but eventually achieved normal levels. The proliferation of splenocytes cultured with purified protein derivative was suppressed by both deficiency and excess of dietary vitamin D. Vitamin D status did not affect the abilities of naive guinea pigs to control primary, pulmonary tuberculosis, nor did it influence the protective efficacy of BCG vaccination. We conclude that changes in dietary vitamin D are associated with alterations in some cellular immune functions but may not be an important determinant of disease outcome in pulmonary tuberculosis, as has been suggested previously.

There is a strong epidemiologic association between malnutrition and infection with Mycobacterium tuberculosis in humans (24). Among the dietary deficiencies which have been implicated in the impaired response to tuberculosis in humans are protein and vitamins A and C (12). Tuberculosis is a major cause of morbidity and mortality in malnourished children, which compose more than half of the pediatric population in many developing countries (37). The reactivation of previous tuberculous infections in the elderly may also be related to the deteriorating nutritional status of these individuals (34). Additional populations at increased risk of developing tuberculosis in which malnutrition is an underlying condition include alcoholics, the homeless, and human immunodeficiency virus-infected individuals (7, 31).

A potential role for vitamin D in resistance to tuberculosis was suggested by several anecdotal observations. In the preantibiotic era, tuberculosis patients were sent to high elevations in Europe or to the sunny southwestern part of the United States. Clinical improvement may have been the result of increased exposure to sunlight leading to enhanced synthesis of vitamin D in the skin (9). Vitamin D (either ingested or synthesized in the skin) is transported to the liver, where it is hydroxylated by vitamin D-25 hydroxylase, forming 25-hydroxyvitamin D₃ (25-OH-D), the most abundant metabolite found in the circulation (23). Further metabolism of 25-OH-D by several types of cells, including activated macrophages, results in the production of 1,25 (OH)₂D₃, or calcitriol. Calcitriol is now well recognized as a potent immunoregulatory hormone (26, 39). Increased levels of calcitriol in serum have been demonstrated with patients with both tuberculosis and hypercalcemia (4) and with anephric patients with tuberculosis (11). Alveolar lavage cells from a patient with pulmonary tuberculosis produced 1,25(OH)₂D₃ (6), and high concentrations of calcitriol and gamma interferon (IFN-γ) were reported in pleural fluid of patients with tuberculous pleuritis (2). Pleural fluid-based macrophages could be the source of calcitriol production which may be regulated by IFN-γ (1)

Recently, investigators have examined the role of vitamin D in resistance against tuberculosis by employing an in vitro model involving cultured macrophages infected with M. tuberculosis (8). Two groups have documented an inhibitory effect of calcitriol in vitro on the replication of mycobacteria within cultured human monocytes (10, 29, 30). In contrast, a similar protective effect of calcitriol was not observed in cultured murine macrophages infected with M. tuberculosis (27, 28). In addition to these effects in vitro, altered macrophage functions have also been reported for vitamin D-deficient mice and humans (3, 36). The effect of dietary vitamin D, as either a deficiency or a supplement, on resistance to tuberculosis has not been determined.

For several years, we have studied the impact of dietary protein deficiency on M. tuberculosis BCG vaccine efficacy and tuberculosis immunity in a guinea pig model involving infection by the respiratory route with virulent M. tuberculosis (16-18). This model has been previously demonstrated to mimic important aspects of the pathogenesis of tuberculosis in humans (33). Recently, we examined the role of dietary vitamin D in the pathogenesis of pulmonary tuberculosis in this animal model.

(This study was previously presented in part [15a]).
VITAMIN D AND TUBERCULOSIS

MATERIALS AND METHODS

Experimental animals. Specific-pathogen-free, outbred albino guinea pigs (Hartley-COBs, Crl:(HA)Br; Charles River Breeding Laboratories, Inc., Wilmington, Mass.) weighing initially between 250 and 400 g were obtained for this study. The animals were housed individually in polycarbonate cages with stainless-steel grid floors and feeders and were allowed food and water ad libitum. Each animal was randomly assigned to an experimental diet and vaccination treatment group when appropriate. Body weights were recorded weekly throughout the experiment.

Experimental diets. The five experimental diets, representing different concentrations of vitamin D, were obtained from a commercial source (Dyets, Inc., Bethlehem, Pa.) and formulated to our specifications. The diets were isocaloric and identical in nutrient content except for their concentrations of vitamin D. The composition of the basal control diet has been published previously (18), and it meets the recommended nutrient requirements for guinea pigs (21). The level of vitamin D in the diets varied from none (0 IU/kg of body weight) to 25% (295 IU/kg), 50% (590 IU/kg), 100% (1,180 IU/kg), or 200% (2,360 IU/kg) of the recommended dietary content for guinea pigs. Prior to the initiation of experimental diets, guinea pigs were weaned from commercial chow by being fed a mixture of 50% powdered control diet and 50% ground chow (Ralston Purina, St. Louis, Mo.) for 2 weeks. Animals were given fresh food daily.

BCG vaccination. In some experiments, 1 week prior to the implementation of experimental diets, half of the guinea pigs in each diet treatment were vaccinated with M. bovis BCG vaccine (Copenhagen 1331; Statens Serum Institut, Copenhagen, Denmark). The vaccine was prepared by reconstituting a vial of lyophilized BCG vaccine in sterile physiological saline. Each animal was injected subcutaneously in its left inguinal region with 0.1 ml of saline suspension which contained approximately 10^9 viable organisms.

Respiratory infection. Virulent M. tuberculosis H37Rv (ATCC 27294) was obtained from the American Type Culture Collection (Rockville, Md.) and stored as single-cell suspensions at −70°C (15). A single lot of culture frozen since 1988 was used for all of these experiments. The challenge inoculum was thawed and diluted just prior to infection. All animals were infected via the respiratory route with an aerosol chamber described previously (40). The infecting inoculum of viable M. tuberculosis H37Rv introduced into the nebulizer was adjusted empirically to result in the inhalation and retention of 5 to 10 viable organisms per animal. The infection was performed in a BL3 biohazard facility designed for use with class 3 human pathogens. Exposure of groups of guinea pigs, selected randomly from the diet groups, resulted in uniform, reproducible infections of all animals with virulent mycobacteria. Following infection, guinea pigs were maintained in individual stainless-steel cages with water bottles and filter bonnets in the BL3 biohazard facility.

Delayed hypersensitivity skin test. One day before being sacrificed, the animals were skin tested with purified protein derivative (PPD) (PPD-RT23; Statens Serum Institut). The 2 doses used consisted of 0.1 ml containing 5 or 100 tuberculin units (TU) of PPD. Each animal was injected intradermally on a shaved area on the right side of the abdomen. Twenty-four hours later, the skin reactions were measured with a plastic ruler, and the mean diameter of induration was recorded in millimeters.

Necropsy procedure. Guinea pigs were killed at intervals following pulmonary infections by the intraperitoneal injection of approximately 2 ml of pentobrobital sodium solution (Sleepaway; Fort Dodge Laboratories, Inc., Fort Dodge, Iowa). A sample of blood was taken immediately by cardiac puncture. The thoracic and abdominal cavities were opened, and the spleen and the entire right lower lung lobe were aseptically removed. Exactly one-half of the spleen (by weight) of each animal was placed in a sterile culture tube containing 3 ml of RPMI 1640 medium (Irvine Scientific, Santa Ana, Calif.) supplemented with 10% fetal bovine serum (Hazleton, Lenexa, Kans.), 100 U of penicillin per ml, 100 μg of streptomycin per ml, 2 mM L-glutamine (Irvine Scientific), and 10 μM 2-mercaptoethanol (Aldrich Chemical Company, Inc., Milwaukee, Wis.). The other half of the spleen (by weight) and the lung lobe were homogenized separately in 4.5 ml of sterile saline with Teflon-glass homogenizers. The number of viable M. tuberculosis H37Rv organisms in each organ was determined by inoculating appropriate dilutions of individual organ homogenates onto duplicate plates of Middlebrook 7H10 agar (Difco Laboratories, Detroit, Mich.). The colonies were counted after being incubated for 3 weeks at 37°C. Data were expressed as the mean log_{10} number of viable organisms present in each portion of tissue.

Lymphocyte blastogenesis. Half of the spleen was homogenized in RPMI 1640 (supplemented as described above) in a Ten Broeck glass homogenizer. The cells were passed through a stainless-steel wire mesh, resulting in a single-cell suspension. Cell viability was determined by trypan blue exclusion. A 100-μl sample of lymphocyte suspension containing 2 × 10^5 viable spleen cells per well was cultured in 96-well microtiter plates (flat-bottom Microtest III plates; Becton Dickinson & Co., Oxord, Calif.). Triplicate cultures received 100 μl of one of the following: 10 μg of concanavalin A per ml (Sigma, St. Louis, Mo.), 25 or 12.5 μg of PPD (PPD-RT40; Statens Serum Institut) per ml, or medium. The cell cultures were incubated for 4 days at 37°C in an atmosphere of 5% CO_2 in air. After incubation, 50 μl of tissue culture medium containing 1.0 μCi of tritiated thymidine (New England Nuclear, North Billerica, Mass.) was added per well, and the cells were cultured for an additional 6 h. The cultures were harvested onto fiberglass filters with a multiple automated cell harvester. The incorporated radioactivity was measured with a liquid scintillation counter (LS 8000; Beckman Instruments, Inc., Irvine, Calif.). The results were expressed as the net uptake of tritiated thymidine, defined as the difference between the mean counts per minute in triplicate stimulated cultures and those in triplicate unstimulated cultures of the same animal’s cells.

25-hydroxyvitamin D assay. Two milliliters of the milliliter of the blood sample was centrifuged separately to obtain the plasma used to measure vitamin D metabolite levels. A commercial 25-OH-D3H assay system was used (Amersham Corporation, Arlington Heights, Ill.). The assay is based on competition between unlabelled 25-OH-D (in the serum sample) and tritium-labelled 25-OH-D for binding to the 25-hydroxyvitamin D-binding protein from sheep serum. The assay was performed exactly as defined in the supplier’s instructions, which accompanied the kit. A standard curve was constructed with concentrations of purified 25-OH-D ranging from 2 to 32 ng/ml. The results were expressed as nanograms per milliliter of guinea pig plasma.

Analysis of variance was utilized to test the effects of dietary vitamin D content on the dependent variables measured. When significant treatment effects were indicated, differences between means were assessed by the new mul-
Table 2. Influence of dietary vitamin D on delayed hypersensitivity to PPD in guinea pigs challenged by the respiratory route with virulent M. tuberculosis H37Rv

<table>
<thead>
<tr>
<th>Conc of dietary vitamin D (IU/kg)</th>
<th>3 wk</th>
<th>6 wk</th>
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<tr>
<td></td>
<td>5 TU</td>
<td>100 TU</td>
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<tr>
<td>2,360</td>
<td>10.7 ± 2.2**</td>
<td>14.2 ± 2.9**</td>
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<tr>
<td>1,180</td>
<td>13.6 ± 1.0*</td>
<td>18.5 ± 1.2*</td>
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<tr>
<td>590</td>
<td>ND</td>
<td>12.1 ± 1.9**</td>
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<tr>
<td>295</td>
<td>ND</td>
<td>10.3 ± 2.0**</td>
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<tr>
<td>0</td>
<td>10.0 ± 1.0**</td>
<td>13.8 ± 1.8**</td>
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* Data are the means ± standard errors of the mean for four to six animals per treatment per interval. Values in the same column with different numbers of asterisks are significantly different from one another (P < 0.05).

** ND, not determined.

RESULTS

Table 1 illustrates the influence of dietary vitamin D levels on growth and the concentration of the major circulating metabolite, 25-OH-D, in plasma in guinea pigs maintained for 6 weeks on their different diets. Diet had no effect on weight gain, indicating that food intake was essentially the same in all groups. Likewise, body weight was unaffected by diet in guinea pigs infected with virulent M. tuberculosis for the last 6 weeks of a 12-week diet treatment (data not shown). However, dietary vitamin D levels did exert a significant influence on the concentration of 25-OH-D in the circulation. Guinea pigs maintained for 6 weeks on the three deficient diets exhibited statistically significant reductions in levels of 25-OH-D in plasma. Supplementation with twice the recommended level did not result in a significant effect on this vitamin D metabolite.

The effect of dietary vitamin D on the abilities of BCG-vaccinated guinea pigs to mount delayed hypersensitivity reactions is presented in Table 2. Animals vaccinated 6 weeks prior to pulmonary challenge with virulent M. tuberculosis H37Rv were skin tested with 2 doses of PPD either 3 or 6 weeks postchallenge. As expected, tuberculin reactions were larger in response to the 100-TU dose and increased between 3 and 6 weeks after infection in all groups. Both the vitamin-deficient and -supplemented groups exhibited significantly reduced skin reaction sizes at both intervals. This dietary effect was most striking with the 5-TU dose of tuberculin.

This suppressive effect of altered vitamin D intake on PPD-induced immune reactions in vivo was mirrored by the proliferative responses of lymphocytes cultured with PPD in vitro (Fig. 1). Splenocytes were prepared from BCG-vaccinated and nonvaccinated guinea pigs fed diets containing normal or twice the normal level of vitamin D or no vitamin D for 12 weeks. All animals had been infected with virulent M. tuberculosis H37Rv by the respiratory route for the last 6 weeks of the dietary treatment. Because of their previous sensitization to mycobacterial antigens, splenocytes from BCG-vaccinated animals consuming the control diet proliferated more extensively than did cells from animals who were developing reactivity to their primary, pulmonary infections. Within the BCG-vaccinated group, vitamin D supplementation resulted in a slight but statistically significant decline in PPD-induced proliferation. Absence of vitamin D also resulted in cells capable of responding at only one-third the normal levels (P < 0.05). In contrast, the reverse trend was observed among previously nonvaccinated guinea pigs, with vitamin D-deficient animals exhibiting increased proliferation. The dietary effect did not reach statistical significance in the nonvaccinated group.

The influence of supplementation with or absence of...
dietary vitamin D on BCG vaccine-induced or innate resistance to pulmonary infection with virulent *M. tuberculosis* was assessed by quantitative culture of viable mycobacteria from the lungs and spleens of guinea pigs at 3 and 6 weeks following infection. These intervals were chosen, on the basis of a detailed knowledge of the time course of infection in this model (40), to represent a point early after the onset of acquired resistance (3 weeks) and a second point after the maximum level of resistance had been attained (6 weeks). Figure 2 demonstrates that BCG vaccination allowed guinea pigs in all three diet groups to effectively control the accumulation of viable mycobacteria in their lungs between 3 and 6 weeks postchallenge. The vaccine effect was highly statistically significant (*P* < 0.01). However, dietary vitamin D levels exerted no demonstrable effect on the level of pulmonary infection in either vaccinated or nonvaccinated groups. The protective effect of BCG was even more striking in the spleen (Fig. 3), in which a 4-log₁₀ difference in infection was observed (*P* < 0.001). Again, dietary vitamin D was not a significant determinant of disease resistance in either vaccinated or nonvaccinated guinea pigs.

**DISCUSSION**

The role of vitamin D in the pathogenesis of tuberculosis remains controversial. On the basis of epidemiological relationships and largely anecdotal clinical observations, one can build a circumstantial case for a protective role of vitamin D in tuberculosis (9, 14). It is clear that the metabolically active form of vitamin D, 1,25(OH)₂D₃, or calcitriol, is capable of synergizing with cytokines such as IFN-γ to endow cultured human macrophages with the ability to retard the intracellular growth of *Mycobacterium avium* and *M. tuberculosis* in vitro (5, 10, 29, 30). In contrast, similar experiments conducted with murine macrophages infected with *M. tuberculosis* failed to demonstrate a protective effect for calcitriol (27, 28). Recent studies have begun to unravel the interactions between calcitriol and cytokines such as interleukin 2 (IL-2), IL-6, tumor necrosis factor alpha, and others at the cellular and molecular levels (19, 20, 25, 38). However, no study published to date has examined the effect of manipulating vitamin D levels in vivo on resistance to pulmonary tuberculosis.

The guinea pig model chosen for these experiments has been devised to mimic human pulmonary tuberculosis in many fundamental ways, ensuring the biological relevance of the observations made with this system (33). The model has been employed previously to study the impact of dietary protein and zinc deficiencies on cellular immune reactivity and acquired resistance to infection with virulent *M. tuberculosis* (16–18). In the present study, we were able to produce significant reductions in circulating concentrations of the principal metabolite of vitamin D, 25-OH-D, by lowering the dietary content to 50% or less of the recommended allowance for guinea pigs (Table 1). Although one might argue that 25-OH-D is not the immunologically active form, levels of this metabolite in plasma are widely accepted as being the best measure of vitamin D status (23). In fact, the reductions in the levels of 25-OH-D in plasma in this study are quite similar to the levels in tissue published recently by Sergeev et al. (32) for vitamin D-depleted guinea pigs. Since we did not actually assess the levels of 1,25(OH)₂D₃ in the plasma of our guinea pigs, any conclusion regarding the effect of our diets on this active metabolite must be considered tentative. However, we believe that the vitamin D-deficient diets, especially the diet containing no vitamin D, had significant biological effects on vitamin D status in our guinea pigs. On the other hand, doubling the recommended level of this vitamin had no measurable effect on concentrations of 25-OH-D in plasma (Table 1).

Although much of the previous research on the relationship between vitamin D and tuberculosis in vitro has focused on the macrophage, there is evidence that vitamin D status can influence lymphocytes directly and indirectly (26, 38, 39). We observed that antigen (PPD)-induced lymphoprolif-
eration was significantly affected by both excesses of and deficiencies in dietary vitamin D (Fig. 1) in previously BCG-vaccinated guinea pigs challenged with virulent M. tuberculosis H37Rv. Interestingly, no such dietary effect was observed in animals responding to mycobacterial antigens for the first time. Calcitriol has been observed to depress both antigen- and mitogen-driven lymphoproliferation when added to cultures in vitro (26, 38). This inhibition could be due to either suppression of the autocline IL-2 loop (25) or reduced levels of IL-1 produced by accessory cells (20). These mechanisms could explain the modest but significant reduction in PPD-induced proliferation in guinea pigs fed a twofold excess of vitamin D. This requires the assumption that levels of calcitriol in tissue and, more importantly, calcitriol receptors on lymphocytes are increased in the excess group. No published studies have reported the effect of vitamin D deficiency on lymphoproliferation, but our results suggest that the dose-response curve has an optimum, with proliferation decreasing on both sides.

The effects of dietary vitamin D excess and depletion on the delayed hypersensitivity response to PPD (Table 2) mirrored the proliferation data. At both intervals postchallenge with virulent mycobacteria (3 and 6 weeks) and with both doses of PPD (5 and 100 TU), the size of the dermal reaction was significantly greater in the group fed the optimal vitamin D level (1,180 IU/kg) than in either the group fed a twofold excess of or the group fed no vitamin D. Of course, more than just antigen-induced lymphocyte proliferation is involved in the development of delayed hypersensitivity. A variety of nonspecific inflammatory mediators are also required for the expression of an indurated dermal response. In addition to the effects of calcitriol on IL-1 and IL-2 noted above, investigators have reported that the production and function of IL-6 is inhibited by high levels of calcitriol in vitro (19). IL-6 is a proinflammatory cytokine produced principally by monocyte-macrophages, and its inhibition in the vitamin D excess group could explain the reduction in PPD skin test size observed by us. On the other hand, treatment with calcitriol at physiological levels in vitro augments the chemotactic response of human monocytes (13). Reduced reduction of IL-6 in the vitamin D-deprived group might have impaired the migration of monocytes into the developing PPD reaction and resulted in a smaller response.

The most important implication from the previous work with vitamin D in vitro in human phagocytes infected with mycobacteria is that vitamin D metabolites synergize with cytokines (e.g., IFN-γ) to induce resistance in cultured macrophages (5, 8, 10, 29, 30). Calcitriol has been previously demonstrated to contribute to the in vitro activation of murine alveolar macrophages and their fusion into multinucleated giant cells, a hallmark of the granulomatous response in tuberculosis (38). However, other investigators have observed no protective role for calcitriol, with or without cytokines, in the antimycobacterial activities of cultured murine macrophages (27, 28). It has been previously suggested that this apparent discrepancy between the responses of murine and human macrophages to calcitriol must represent a fundamental biological difference in the roles of vitamin D in mice and humans (28). However, there are a number of other differences between these two studies which could account for the observed variation in calcitriol effect, including the source and state of maturation of the macrophages employed. Since we did not test the effect of vitamin D on the antimycobacterial properties of guinea pig cells in vitro, it is impossible to know whether the results would have approximated those observed in human or murine cells. Nevertheless, the extrapolation of these in vitro observations to the living host must be considered quite tenuous. Some of the antimycobacterial effects in vitro required levels of calcitriol which were clearly orders of magnitude higher than physiological concentrations (8). The in vitro culture systems employed were admittedly somewhat artifactual. The clinical reports that dietary vitamin D might influence resistance to tuberculosis were not well founded in carefully controlled trials with humans or animals (9, 12, 14).

No previous studies had addressed the potential effect of vitamin D on the ability to protect against tuberculosis by BCG vaccination. Thus, we believe that the experiments described here represent the first rigorous test of the hypothesis in a highly relevant model of pulmonary tuberculosis. Our results are reasonably unequivocal. No difference in resistance to primary, pulmonary infections with a low dose of virulent M. tuberculosis H37Rv was observed in guinea pigs fed a twofold excess of or deprived of dietary vitamin D. No dietary effect was seen either on the accumulation of virulent mycobacteria in the lungs (Fig. 2) or on the bacillary phase of the disease, as reflected in mycobacterial loads in the spleen (Fig. 3). Note that in this guinea pig model, levels of viable mycobacteria in the lungs represent events occurring at the primary lesions, while spleen counts result both from hematogenous dissemination from the primary lung tubercles and from growth of the organism with the spleen itself. This mimics exactly what is known to occur in human pulmonary tuberculosis. Bacterial loads in the spleen were no higher than counts in the lung (Fig. 2 and 3). Previous experiments with this model have demonstrated convincingly that the ultimate outcome in guinea pigs (i.e., percent mortality and mean time to death) correlates statistically with bacterial loads in the lungs (40). Furthermore, guinea pigs dying from such infections several months postchallenge apparently die of pulmonary complications. For these reasons, we believe that this model has a high likelihood of predicting events which may occur in human pulmonary tuberculosis (33). Guinea pigs vaccinated with BCG but challenged with virulent M. tuberculosis H37Rv exhibited solid resistance, as evidenced by 100-fold reductions of viable mycobacteria in the lungs (Fig. 1) and 10,000-fold reductions in splenic bacterial loads (Fig. 3). Altered dietary vitamin D intake had no effect on BCG vaccine efficacy in either tissue.

In conclusion, diets varying widely in vitamin D content exerted significant effects on the principal circulating metabolite, 25-OH-D, and on selected antigen (PPD)-induced immune functions in vivo and in vitro. However, these diets did not alter the resistance of either naive or BCG-vaccinated guinea pigs to pulmonary infections with virulent M. tuberculosis H37Rv. It is possible that levels of the crucial immunomodulatory metabolite, calcitriol, in tissue were not influenced significantly by our diets, although we believe that this is unlikely. We are currently testing that hypothesis with a guinea pig model of tuberculous pleurisy by measuring vitamin D metabolites in pleural fluid (2, 22). Likewise, the dietary insult may have been too short to affect disease resistance significantly. However, we have previously observed marked alterations in disease resistance after a similar period of moderate protein deficiency with this model (16, 17). On the basis of the results reported here, we must conclude that vitamin D status appears not to be a determinant of resistance to pulmonary tuberculosis in the guinea pig model.
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