Cytokine Profile in Patients with Multiple Sclerosis Following Vitamin D Supplementation.

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Abstract

Multiple sclerosis patients were randomized, in a double blind design, and placed into either a vitamin D supplemented group or a placebo control group. As expected serum 25-hydroxyvitamin D levels increased significantly following 6 month vitamin D supplementation (17 $\pm$ 6 ng/ml at baseline to 28 $\pm$ 8 ng/ml at 6 months). Vitamin D supplementation also significantly increased serum transforming growth factor (TGF)- β1 levels from 230 $\pm$ 21 pg/ml at baseline to 295 $\pm$ 40 pg/ml 6 months later. Placebo treatment had no effect on serum TGF-β1 levels. Tumor necrosis factor–α, interferon-γ, and interleukin (IL)-13 were not different following vitamin D supplementation. IL-2 mRNA levels decreased following vitamin D supplementation but the differences did not reach significance. Vitamin D supplementation of MS patients for 6 months was associated with increased vitamin D status and serum TGF–β1.

Key Words: Vitamin D, Multiple Sclerosis, Transforming Growth Factor-β1.

1. Introduction

Multiple sclerosis (MS) is an autoimmune disease in which the myelin sheath surrounding both peripheral and central nerve axons is targeted and broken down. Without adequate myelin sheaths, neurons fire abnormally, and paralysis or death may result (Scheinberg and Smith, 1987; Steinman, 1996). Approximately 350,000 people are afflicted with MS in the US alone (Scheinberg and Smith, 1987). Despite decades of research, the etiology and pathogenesis of MS is still unknown, though both genetic and environmental components of the disease seem evident. First degree relatives of MS patients are 20-40 times more likely to acquire the disease than others, and the monozygotic twin of a patient is at greater risk (an additional 10 fold risk) (Ebers et al., 1986; Ebers and Sadovnick, 1994). However, the concordance rate between monozygotic twins is only 30% (Ebers et al., 1986), suggesting that environmental components affect the etiology of the disease as well. One potential environmental factor affecting the development of MS may be vitamin D.

The prevalence of MS worldwide increases as one moves further north or south away from the equator (Hayes et al., 1997). Vitamin D is generated in the dermal layer of the skin following irradiation from UV light, but the equator is where the earth receives the most UV irradiation over the course of a year. Epidemiological studies have shown that the prevalence rate for MS is nearly zero for people living at the equator, and increases to over 50 cases per 1,000,000 individuals as one moves 45° north or south of the equator (Hayes, 2000). It seems possible, that vitamin D exposure might explain the curious geographical pattern of MS prevalence.

Vitamin D deficiency or insufficiency has been documented in MS patients. MS patients also display decreased bone mineral densities, and increased risk for bone fractures (Nieves et

al., 1994). MS patients had low levels of circulating 25 hydroxyvitamin D (mean 17 $\pm$ 2 ng/ml) and as many as 64% had levels of less than 20 ng/ml indicating clinical vitamin D deficiency/insufficiency (Cosman et al., 1998). Lumbar spine and femoral neck bone mass densities of MS patients were 1 to 2 standard deviations lower than healthy control subjects (Nieves et al., 1994). As a result MS patients experienced significantly more fractures than normal controls (22% vs. 2%, respectively) (Cosman et al., 1998). It is unclear whether vitamin D insufficiency is a cause or a result of autoimmunity and/or corticosteroid therapies, which are commonly used to treat these patients (Cantorna et al., 1998a; Cosman et al., 1998; Nieves et al., 1994; Sayetta, 1986).

Vitamin D status determines the severity of experimental autoimmune encephalomyelitis (EAE, a well studied animal model of MS)(Cantorna et al., 2000). EAE is mediated by CD4+ T cells, which target the central nervous system and induce inflammation and paralysis of the mice. Supplementation with active vitamin D (1,25 dihydroxyvitamin D_3) has been shown to block the progression and onset of EAE (Cantorna et al., 1996). Conversely, vitamin D deficient mice showed increased susceptibility to EAE (Cantorna et al., 1996). Although compelling, the scientific evidence linking MS and vitamin D in EAE cannot be directly translated to humans with MS.

MS is a disease in which the immune system inappropriately attacks tissues in the body; therefore, any potential MS-vitamin D interaction will likely involve the immune system. MS results in alterations in the immune response that occur with the symptoms of the disease, and correlate with the severity of demyelination (Bertolotto et al., 1999a; Bertolotto et al., 1999b; Clerici et al., 2001; Killestein et al., 2001). MS symptoms are associated with increased production of inflammatory cytokines including, interleukin (IL)-2, tumor necrosis factor (TNF)-

α, and interferon (IFN)- γ, and decreased production of some anti-inflammatory cytokines like transforming growth factor (TGF)- β1 and IL-13 (Bertolotto et al., 1999a; Bertolotto et al., 1999b; Clerici et al., 2001; Killestein et al., 2001).

How vitamin D may be linked to MS in unknown. The discovery of vitamin D receptors in cells of the immune system suggested that vitamin D must be a regulator of immune function (Bhalla et al., 1983; Provvedini et al., 1983). The active metabolite of vitamin D (1,25-dihydroxyvitamin D3) has been shown to reduce the production of inflammatory, and MS associated cytokines, IL-2, TNF-α and IFN-γ (Lemire, 1992; Rigby et al., 1987; Rigby et al., 1985; Rigby et al., 1984). In animal models of MS 1,25-dihydroxyvitamin D3 treatment has been shown to increase TGF-β1 production (Cantorna et al., 1998b). The increased production of TGF-β1 correlated with the inhibition of MS-like symptoms in mice with EAE.

Collectively these data suggest that vitamin D status may be crucial for the development of MS. The targets of vitamin D, in EAE, include both the inhibition of inflammatory cytokines and the increased production of anti-inflammatory cytokines. Here we determined whether vitamin D supplementation of MS patients regulated several vitamin D targets. The effect of short-term vitamin D supplementation on selected inflammatory and anti-inflammatory cytokines was assessed in a group of MS patients.

2. Materials and methods.

2.1. Subjects

All subjects were diagnosed with MS as confirmed by a certified neurologist. Both female and male patients were accepted and independently randomized. MS patients with a baseline 25-hydroxyvitamin D level of <20 ng/ml (about 48% of the MS patients) were accepted into the study. Enrolled patients were selected from a larger ongoing 2 year vitamin D supplementation study. The results from the 39 patients who had both baseline and 6 month follow up samples are reported here.

2.2. Treatment

Male and female patients were randomized independently, in a double blind design, into one of two treatment groups. Controls (n= 22) received 800 mg supplemental calcium plus placebo. Vitamin D treated (n=17) individuals received 800 mg supplemental calcium plus 1000 IU vitamin D. All treatments were repeated daily for 6 months. Since calcium intakes are documented to be below the RDA, supplements were given to all patients to prevent calcium deficiency, which might mask any potential vitamin D effect. Compliance was assessed by counting unused pills.

2.3. Biochemical analyses

Blood samples were collected for both serum and peripheral blood mononuclear cell (PBMC) isolation. 25-hydroxyvitamin D was determined using a radioligand-binding assay exactly as described (Cosman et al., 1998). Serum TGF-β1 was measured using a commercial kit from Promega Corp. (Madison, WI). PBMC were isolate by Ficoll-Isopaque (Sigma Chemical Co. St. Louis, MO) density gradient centrifugation and the washed cells were lysed for RNA analysis

using a commercial kit from Promega. Semi-quantitative PCR was done to analyze the levels of IL-2, TNF-α, IFN-γ, and IL-13 in the samples. Glyceraldehype-3-phosphate dehydrogenase (G3PDH) PCR was done to control for the amount of RNA in each sample. Total RNA was isolated and reverse transcribed using oligo(dT) and the manufacturer's protocol (Promega, Madison, WI). Primers specific for each gene were designed to cross exon/intron borders and optimized prior to running the experiment. Multiple dilutions of each cDNA were made and run to insure that the measurements made were done using the linear portion of the amplification phase. The products were resolved by 1.5% agarose gel electrophoresis and ethidium bromide stained. The gene products were identified by size with respect to molecular weight standards. Densitometry scanning was used to quantitate the intensity of the bands. Values are reported as the intensity (arbitrary units) expressed in the gene of interest per 100 G3PDH units.

2.4. Statistics

The results are presented as means $\pm$ SEM. If the normality or equal variance test failed, data were transformed as required. The data were analyzed with a two-factor repeated-measures analysis of variance, with time as a within subject and treatment as a between subject factor. The Students Newman-Keuls Multiple Comparison Analyses was used to analyze the differences in the means of 25-hydroxyvitamin D3 and TGF-β1 levels in the serum. The level of significance was set at $P < 0.05$. Data were analyzed using StatView (SAS Institute Inc., Cary, NC).

3. Results

Almost half of the MS patients recruited for this study had low circulating levels of 25-hydroxyvitamin D (<20 ng/ml). Vitamin D supplementation at 2.5 times the RDA (1000 IU or 25 μg daily vitamin D3) significantly increased the 25-hydroxyvitamin D levels in these subjects at six months (Fig. 1, baseline, 17 $\pm$ 6 ng/ml, six month supplementation, 28 $\pm$ 8 ng/ml). As

expected placebo treatment did not affect 25-hydroxyvitamin D levels. Vitamin D supplementation also significantly increased TGF-β1 levels from 230 ± 21 ng/ml at baseline to 295 ± 40 ng/ml six months later (Fig. 1). Placebo treatment had no effect on serum TGF-β1 levels. PBMC were collected and analyzed for the levels of mRNA for three inflammatory cytokines (TNF-α, IL-2, and IFN-γ) and one anti-inflammatory cytokine (IL-13). The levels of TNF-α, IFN-γ, and IL-13 were unaffected by vitamin D supplementation. In contrast IL-2 mRNA levels decreased following vitamin D supplementation but the differences did not reach significance (baseline: 79 ± 45 IL-2/100 G3PDH, and six months vitamin D: 14 ± 4, P = 0.072). Placebo treatment was also associated with a decrease in IL-2 mRNA (P= 0.42).

4. Discussion

Six month supplementation of a small group of MS patients was sufficient to increase the levels of 25-hydroxyvitamin D in the blood; however, it should be noted that the patients' vitamin D status was still below normal values. Vitamin D is a hormone/nutrient important in the maintenance of calcium homeostasis. More recently it has become evident that vitamin D functions in numerous other systems as well (DeLuca, 1993). The impact of prolonged vitamin D insufficiency is unknown but the effects are likely to be broad and involve multiple organ systems. It seems probable that MS patients would benefit from increasing their intakes of vitamin D. The dose of vitamin D3 used here should have been enough to bolster the 25-hydroxyvitamin D levels into the optimal range (40-100 ng/ml) in these patients. Perhaps patients with MS have a higher need for vitamin D and/or the disease process in MS alters the metabolism of vitamin D.

TGF-β1 levels increased in the serum of MS patients supplemented with vitamin D for 6 months. The results for IFN-γ, IL-2, TNF-α and IL-13 were variable and inconclusive. Perhaps a

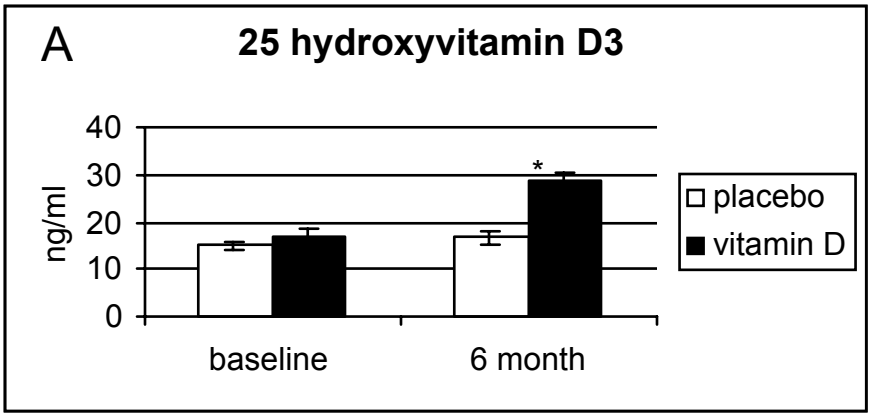
longer study or a larger sample size would have revealed some alterations in these cytokines following vitamin D supplementation. TGF-β1 has been shown to be an important anti-inflammatory cytokine in animal models of MS (EAE) and vitamin D treatment of mice with EAE has been shown to induce TGF-β1, which correlated with the suppression of symptoms in those mice (Cantorna et al., 1998b). TGF-β1 is produced by regulatory T cells, which inhibit the development of EAE (Meyer et al., 2001). Furthermore, exogenous TGF-β1 prevented the development of EAE and neutralization of TGF- β1 increased the severity of the disease (Racke et al., 1992). There have also been associations made between TGF- β1 levels and appearance of symptoms in humans with MS. Human T cell lines derived from patients with active MS produced less TGF- β1 compared to T cells from patients with stable disease (Mokhtarian et al., 1994). High levels of TGF- β1 mRNA were reported in MS patients with no or only slight disabilities (Link et al., 1994). The increased TGF-β1, following vitamin D supplementation, suggests that vitamin D supplementation could potentially improve the symptoms of MS patients.

Six month supplementation with vitamin D3 increased levels of 25-hydroxyvitamin D and TGF-β1 in the sera of MS patients. These two positive beneficial results suggest that vitamin D supplementation could be beneficial in patients with MS. The immune system seems to be one of the systems which require vitamin D at some unknown concentration for optimal performance. The evidence continues to accumulate supporting a link between vitamin D, immune regulation, and MS.

Figure Legends.

Figure 1. Serum levels of 25-hydroxyvitamin D and TGF-β1 in MS patients. * Serum 25-hydroxyvitamin D and TGF-β1 levels from vitamin D supplemented patients were significantly (Students Newman-Keuls Multiple Comparison Analyses) higher than the samples from the same individuals at baseline.

Figure 2. Cytokine mRNA levels in the PBMC of MS patients at baseline and 6 months after vitamin D supplementation or placebo.


A
25 hydroxyvitamin D3
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30
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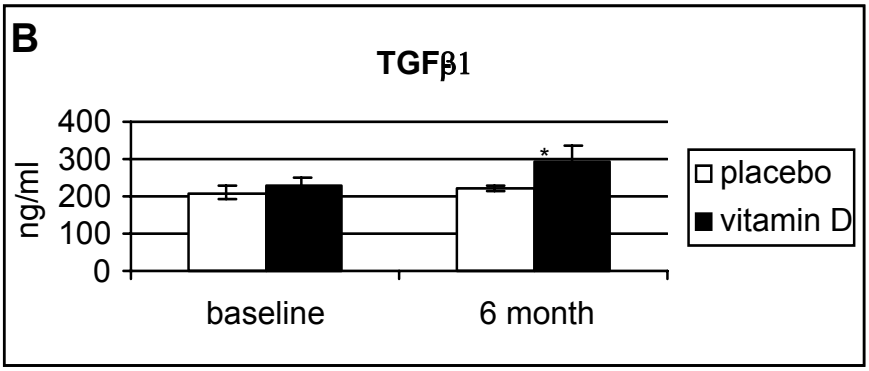
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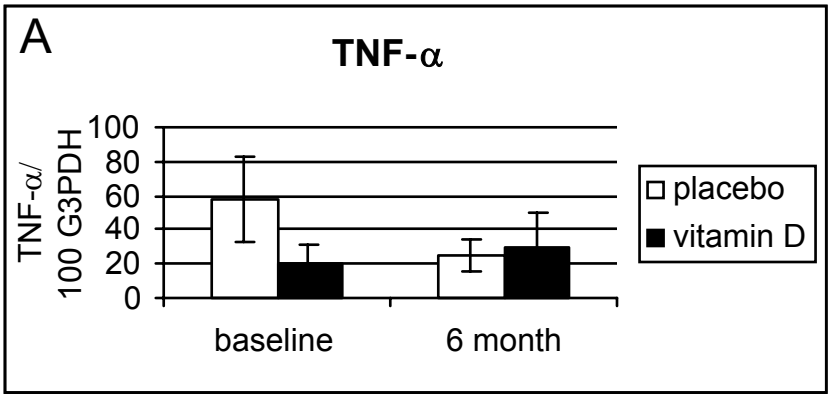
A
TNF-α
TNF-α/
100 G3PDH
100
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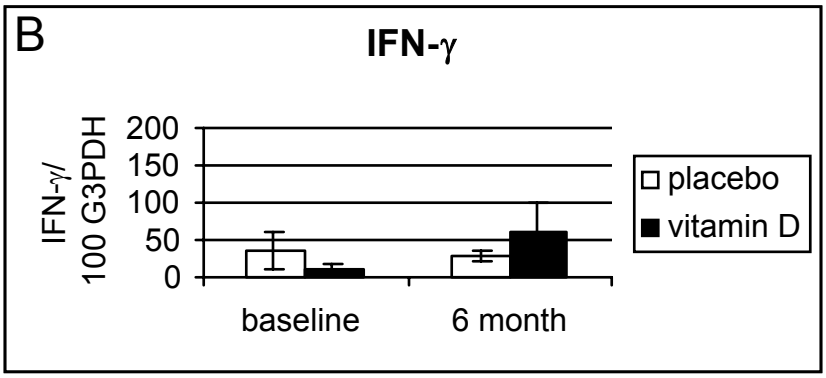
vitamin D


B
IFN-γ
IFN-γ/
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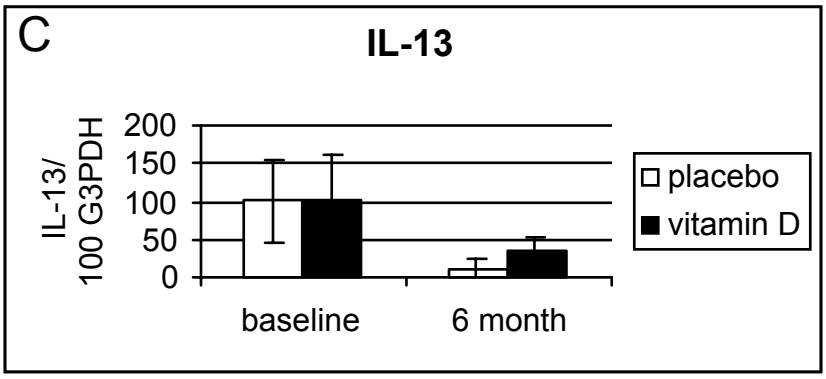
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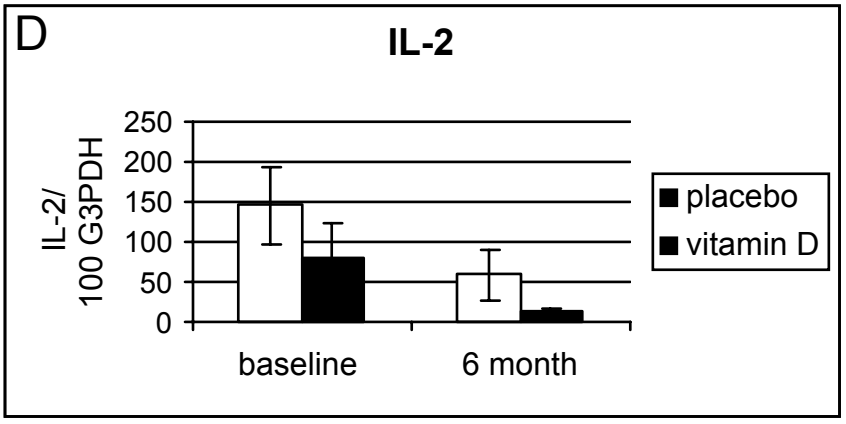
D
IL-2
IL-2/
100 G3PDH
250
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100
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baseline
6 month
placebo
vitamin D

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