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What is This?
A pilot study of the immunological effects of high-dose vitamin D in healthy volunteers

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Abstract
Although vitamin D deficiency is considered an environmental factor in multiple sclerosis (MS), the immunological and clinical effects of vitamin D supplementation remain unclear. We performed a pilot study of the immunomodulatory effects of vitamin D in healthy individuals (n=4), who took 5000–10,000 IU/day of vitamin D over 15 weeks. After 15 weeks of vitamin D supplementation, serum 25(OH) vitamin D levels rose significantly from baseline, with a corresponding increase in IL-10 production by peripheral blood mononuclear cells and a reduced frequency of Th17 cells. These data provide a strong rationale for randomised trials to assess the clinical effects of vitamin D supplementation in MS.

Keywords
disease modifying therapies, immunology, multiple sclerosis

Introduction
There is increasing evidence that vitamin D deficiency may be a significant environmental factor in both susceptibility to, and disease activity in, multiple sclerosis (MS).¹,² Although the anti-inflammatory effects of vitamin D in vitro (and in vivo in the experimental allergic encephalomyelitis (EAE) model) are well established, significant in vivo immunomodulatory effects of vitamin D supplementation in humans have not yet been demonstrated. In planning a prospective study of vitamin D supplementation in patients with clinically isolated syndrome (CIS) we aimed to assess the immunological effects of vitamin D in vivo in a pilot study in healthy participants. We found vitamin D supplementation significantly increased production of the anti-inflammatory cytokine IL-10 and reduced the frequency of pathogenic Th17 cells.

Participants and methods
In December 2010, four healthy control participants took 5000 IU vitamin D (Vigantol® tablets 1000 IU) daily for 10 weeks. Then two participants increased the dose to 10,000 IU daily and the other two participants continued taking 5000 IU daily for 5 weeks. The study was terminated at 15 weeks. Blood samples were taken at baseline, six, 10 and 15 weeks to determine serum concentrations of 25(OH) vitamin D [25(OH)D] and for immunological analysis. Serum 25(OH)D levels were measured by liquid chromatography-mass spectrometry.

Immunological methods
Peripheral blood mononuclear cells (PBMC) were isolated, cryopreserved and immunological analyses were performed upon completion of the study. PBMC cultured at 1×10⁶ cells/ml in complete RPMI medium, were stimulated with
1 μg/ml purified protein derivative (PPD; Serum Staten Institute), 1 μg/ml each of tetanus toxoid (TT) and diphtheria toxoid (DT), 1 μg/ml phytohaemagglutinin (PHA; Sigma) or medium alone. In some experiments, CD4+ and CD4- cells were sorted using magnetic beads (Miltenyi Biotech). After 3–5 days, cell culture supernatants were removed and analysed for IL-17 and IL-10 by enzyme-linked immunosorbent assay (ELISA) (eBioscience). On day 5, PHA-stimulated cells were restimulated with 50 ng/ml phorbol myristate acetate (PMA), 500 ng/ml Ionomycin and 5 μg/ml of Brefeldin A for 6 h (Sigma), followed by staining for cell-surface CD3 and CD8 and intracellular IL-17 (eBioscience). Cells were analysed by flow cytometry using a CyanADP (DakoCytomation) cytometer and FlowJo software.

Statistics

A paired t-test was used to compare data from T1 to T4.

Results

Vitamin D concentrations

Serum 25(OH)D concentrations at baseline were suboptimal in all participants (mean 25(OH)D: 38 nmol/l, range 27–53 nmol/l) and increased steadily throughout the study period. For the two participants who remained on 5000 IU over the 15-week period the maximum concentrations achieved were 152 and 191 nmol/l, while those subjects taking the increased dose of 10,000 IU daily had peak 25(OH)D levels of 152 and 223 nmol/l.

Increased IL-10 after vitamin D supplementation

PBMC were isolated from volunteers at baseline and after 15 weeks of supplementation and subsequently activated in vitro. There was a significant increase in the amount of IL-10 produced by PBMC in response to the recall antigens PPD, TT/DT (p<0.0001) as well as to the polyclonal stimulus PHA (p<0.001) (Figure 1(a)).

Reduction in the frequency of Th17 cells after vitamin D supplementation

Cells stimulated with PHA were restimulated on day 5 with PMA/Ionomycin and analysed by flow cytometry for intracellular cytokines. We observed a progressive decrease in the frequency of IL-17-producing CD4+ T (Th17) cells from baseline to 15 weeks post-vitamin D supplementation for the four participants (Figure 1(b), p=0.012, paired t-test on log transformed data). On the other hand, there was no overall change in the frequency of IL-10 producing CD4+ or CD8+ T cells (p>0.05; data not shown).

Non-CD4+ T cells are the source of increased IL-10 after vitamin D supplementation

We observed a significant increase in the concentration of IL-10 produced in vitro by PBMC after vitamin D supplementation (Figure 1(a)); however the frequency of IL-10-producing CD4+ T cells did not change significantly (data not shown). This suggested that the increased IL-10 was not derived from CD4+ T cells. To investigate this further, CD4+ T cells were positively selected using magnetic beads, and the negative fraction was designated CD4-depleted PBMC. Total PBMC, CD4+ T cells and CD4-depleted PBMC were stimulated with PHA for 3 days and the cell culture supernatants were analysed by ELISA for IL-17F and IL-10. As shown in Figure 1(c), there was a significant increase from baseline to 15 weeks in the concentration of IL-10 produced by both total PBMC and CD4-depleted PBMC. On the other hand, IL-17 production by either PBMC or CD4+ PBMC declined after vitamin D supplementation, whereas no IL-17F was produced by CD4-depleted PBMC. These data indicated that PHA-induced IL-17F produced by CD4+ T cells was inhibited by vitamin D supplementation.

Discussion

In this pilot study we examined the in vivo immunological effects of 15 weeks of high-dose vitamin D in four healthy participants. Following vitamin D supplementation, there was a striking increase in the amount of IL-10 produced by PBMC in response to a range of stimuli. The induction of IL-10 measured by ELISA was not observed when cytokine production by T cells was measured by flow cytometry, suggesting that the increased IL-10 was not derived from CD4+ T cells and the cells responsible remained within the CD4-depleted PBMC. On the other hand, IL-17 production by either PBMC or CD4+ PBMC declined after vitamin D supplementation, whereas no IL-17F was produced by CD4-depleted PBMC. These data indicated that PHA-induced IL-17F produced by CD4+ T cells was inhibited by vitamin D supplementation.

We found that pro-inflammatory Th17 responses decreased significantly after vitamin D supplementation. Our findings are in agreement with those from studies in the EAE model, where vitamin D supplementation has been shown to directly inhibit IL-17 transcription and translation.5 Th17 cells, which produce IL-17, play an important role in autoimmune inflammation during EAE; increased IL-17 has also been detected in MS lesions and cerebrospinal fluid, and has been associated with disease.
Figure 1. Anti-inflammatory effects of vitamin D supplementation. Cryopreserved peripheral blood mononuclear cells (PBMC) from baseline (T1) and 15 weeks (T4) post-vitamin D supplementation were stimulated with PPD, TT/DdT or PHA. After 3–5 days the concentration of IL-10 in the cell culture supernatants was measured by ELISA. M=male, F= female; 5000 IU vitamin D per day, or 5000 IU for 10 weeks and 10,000 IU for the final 5 weeks (A). In (B), PBMC were stimulated with PHA for 5 days then restimulated with PMA/ionsomycin and intracellular IL-17 expression by CD4+ T cells was analysed by flow cytometry; the data for M 5000 IU is plotted on the right Y axis. In (C), the effect of depleting CD4+ T cells on cytokine production was examined. PBMC, CD4+ T cells or PBMC-CD4 depleted were stimulated with PHA and the concentration of IL-10 or IL-17F in the supernatants was measured on day 3. ***=p<0.0001, **=p<0.001, *=p<0.05 for T1 versus T4; paired t-test.

PPD, purified protein derivative; TT, tetanus toxoid; DT, diphtheria toxoid; PHA, phytohaemagglutinin; PMA, phorbol myristate acetate
In summary, vitamin D supplementation increased innate IL-10 production and decreased the frequency of Th17 cells. Both of these effects could have a beneficial effect on the autoimmune inflammation in MS.

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**Conflict of interest**

The authors declare that they have no conflicts of interest.

**References**


