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Efficient tetanus toxoid immunization upon vitamin D supplementation

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Running title: Vitamin D and immunization

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Abstract

Objectives: Vitamin D mediates immunomodulatory functions and its deficiency has been associated with an increased prevalence of immunologic diseases. The supplementation of vitamin D might be therapeutically beneficial, e.g. in lupus erythematosus patients. However, its impact on established recall immune responses is undefined.

Subjects and Methods: 32 individuals were randomized in a placebo controlled, double-blind setting and received vitamin D (daily 2000 I.U.) for 10 weeks followed by tetanus toxoid booster immunization.

Results: During vitamin D supplementation the median 25-hydroxyvitamin D serum concentration increased to 80.3 nM, which as expected decreased in the placebo group to 29.1 nM during the UV-deprived winter months. The tetanus toxoid (TT) specific IgG boost efficiency was **marginal** higher in the vitamin D group ($p=0.04$). The increase of the 25-hydroxyvitamin D levels correlated with the increase of TT-IgG serum concentrations. The induction of specific serum IgA and specific antibody secreting cells was comparable between both groups. Accordingly, the tetanus toxoid-specific and polyclonally triggered T cell cytokine profiles were stable as well.

Conclusions: Vitamin D supplementation was **successful** and booster immunization induced efficiently specific antibodies titres.

Key words: vitamin D, supplementation, tetanus toxoid, immunization, humoral recall response

Abbreviations: TT, tetanus toxoid; rTT-C, recombinant tetanus toxoid C-fragment; calcitriol, $1\alpha,25$ -dihydroxyvitamin D₃, SEB, Staphylococcus enterotoxin B

Introduction

The vitamin D system, besides its well-described function in calcium homeostasis, is involved in the fine tuning of immunity (Adams and Hewison 2008, Hayes et al 2003, Holick 2007). The biologically active metabolite $1\alpha,25$ -dihydroxyvitamin D₃ (calcitriol) signals following binding to the nuclear vitamin D receptor and transcriptional regulation of target gene expression (Hayes et al 2003). Calcitriol-synthesis is catalyzed enzymatically from the otherwise inert storage metabolite 25-hydroxyvitamin D₃ (Adams and Hewison 2008, Hayes et al 2003). Thus, the precursor serum concentration determines the vitamin D status (Holick 2007) (Vieth 2007) and 25-hydroxyvitamin D serum concentrations below 50 nM (20 ng/ml) are considered as insufficient (Holick 2007). Acquired vitamin D deficiency has been correlated with the prevalence and/or increased severity of autoimmune diseases, but also infectious diseases and cancer (Adams and Hewison 2008, Adorini and Penna 2008, Hayes et al 2003, Holick 2007). Direct modulation of immune cells by vitamin D is most likely since different immune cell types can produce and perceive calcitriol including monocytes, dendritic cells, T helper cells and activated B cells (Adorini and Penna 2008, Hayes et al 2003). Previously, we demonstrated that activated human B cells can synthesize calcitriol from its precursor, which mediates enhanced expression of interleukin (IL) -10 (Heine et al 2008) and inhibited IgE production (Heine et al 2002). In newborns, cord blood levels of 25-hydroxyvitamin D and the ratio of IL-10 to IgE are both higher in infants born in summer compared to winter (Zittermann et al 2004). Other vitamin D receptor mediated functions are enhanced innate anti-microbial defence, increased tolerance of adaptive immune reactions and altered lymphocyte trafficking (Adams and Hewison 2008, Adorini and Penna 2008, Hayes et al 2003).

Until now, the safety of vitamin D supplementation was assessed by parameters related to calcium mobilization reflecting hypercalcaemic toxicity of endogenously synthesized calcitriol (Vieth 1999) and the no-observed-adverse-event-level (NOAEL) for daily vitamin D supplementation was set at 2000 I.U. (50 µg) (Vieth 2007). Still, no data on immunologic safety, e.g. regarding intact recall responses are available.

Materials and Methods

Study population and design

Individuals were recruited at the Department of Dermatology and Allergy, Charité - Universitätsmedizin Berlin, Germany. The inclusion criteria were scheduled tetanus/diphtheria immunization due to a previous vaccination that dated back at least 5 years. The exclusion criteria were a history for sarcoidosis, hypercalcemia, creatinine serum concentration >1 mg/dL, nephrolithiasis, any chronic disease and planned exposition to UV-light including tanning or travelling to countries at lower latitude (<40°). The study was approved by the Ethical Board of the Charité and written informed consent was obtained from all participants.

Individuals were randomly assigned in a double-blind manner to the vitamin D group receiving orally 2000 I.U. vitamin D₃ oil per day (50 µg, n=20) or to the placebo group (equal volume neutral oil; n=12). The daily calcium intake of 1200 mg by each participant was guaranteed by supplementation. Compliance was assessed by determining the **25-hydroxyvitamin D serum levels** and consumed study medication amount at the end of the supplementation period of 10 weeks. An intramuscular booster immunization with a combined tetanus/diphtheria toxoid vaccine (Td-Merieux[®], Sanofi Pasteur MSD, Strasbourg, France) was performed after 9 weeks supplementation period.

Biochemical analysis

25-hydroxyvitamin D was measured by enzyme immunoassay (EIA) (Immundiagnostik AG, Bensheim, Germany). TT-specific IgG and IgA were measured using an EIA from serially diluted sera (DRG Diagnostics, Marburg, Germany). A long-term protective immunity (5-10 yrs) is assumed >1.0 I.U./ml anti-tetanus IgG. TT-specific IgA concentrations were validated by serially dilution of strongly positive sera and units were calculated based on the absorption

at 405 nm. Tetanus-specific IgE was determined using the ImmunoCAP system (Phadia, Freiburg, Germany). All other parameters were determined by the Charité routine laboratory facility.

Flow cytometry

Peripheral T- and B lymphocytes were quantified from full blood after surface-staining using TruCount beads and fluorochrome-conjugated monoclonal antibodies against CD4 or CD8 and CD19, CD20, CD27 (all from BD, Heidelberg, Germany) and CD38 (Beckman Coulter, Krefeld, Germany), respectively. Tetanus-specific B cells and plasmablasts were stained from isolated peripheral blood mononuclear cells (PBMCs) after density gradient centrifugation (Lymphocyte separation medium, PAA, Cölbe, Germany) exactly 7 days after TT-immunization by intracellular binding to the fluorescence labelled recombinant C-fragment of tetanus toxin (rTT.C), as described (Mei et al 2008, Odendahl et al 2005) following CD38, CD3 and CD14 surface staining. T cell activation was monitored following stimulation of full blood with 2 µg/ml Staphylococcus Enterotoxin B (SEB, Sigma Dreieich, Germany) in the presence of 1 µg/ml Brefeldin A (Sigma) (Frentsch et al 2005). Flow cytometric analyses were performed using a LSRII cytometer equipped with an additional UV laser or FACS Canto II and DivaSoft operation system (all from BD) and FlowJo7 software (TreeStar, Ashland, OR).

Tetanus toxoid-induced cytokine release

The TT-specific T cell cytokine profile was monitored before and exactly 7 days after immunization, at the peak of TT-specific T cell circulation (De Rosa et al 2004). Isolated PBMCs (4×10^5 cells in 200 µl) were cultured in RPMI1640 containing 10% FCS as described (Heine et al 2002), in 96-well round bottom culture plates in the presence or absence of 0.5 µg/ml SEB (Sigma, Dreieich, Germany) or 1% tetanus toxoid (Tetanus-Merieux[®], Sanofi Pasteur MSD, Strasbourg, France) in triplicates. After 24 hours, the supernatants were stored

at -80°C until analysis using the Th1/Th2 cytokine CBA kit (BD), which allows the simultaneous quantification of secreted IL-2, IL-4, IL-5, IL-10, TNF- α (all sensitivity thresholds <3 pg/ml), and IFN- γ (< 8 pg/ml).

Statistical methods

Statistical evaluations and graphic display were performed with Prism software (GraphPad Software, Inc., CA, USA) and SPSS15.0 (Spss, Inc. Chicago, IL, USA). Normal distribution was tested by the Kolmogorov-Smirnov test and the D'Agostino & Pearson omnibus normality test. Normally distributed values were tested by the Student-T test and the mean \pm standard deviation (SD) are shown. Whitney-Mann-U test or Wilcoxon Test was applied for non-normally distributed values for independent or dependent parameters, respectively and median and interquartile ranges are shown.

Results

Efficient increase of 25-hydroxyvitamin D serum concentrations by daily intake of 2000 I.U. cholecalciferol

At study entry, the distribution of age, gender and body-mass-index (Suppl.Tab.1) and vitamin D status did not differ between the two groups (Suppl.Tab.2; Fig.1B). The median 25-hydroxyvitamin D concentrations significantly increased upon daily intake of 2000 I.U. (50 µg) vitamin D from 28.4 nM to 80.3 nM after 10 weeks ($P=0.0001$, Fig.1B). As expected, in the placebo group the median 25-hydroxyvitamin D serum concentration decreased by 13.1 nM to 29.1 nM after the study period ($P=0.0007$). Consequently, the 25-hydroxyvitamin D serum concentrations were strongly different between both groups after the supplementation period ($P=0.0001$). During 10 weeks of daily intake of 2000 I.U. vitamin D (cholecalciferol), the calcium, phosphate and IgA, IgG, and IgM serum concentrations were not altered in any individual (Suppl.Tab.2). The differential blood count was comparable in both groups except the absolute monocyte numbers which decreased in the placebo group (-0.04/nl) and remained constant in the vitamin D group ($P=0.04$, Suppl.Tab.2). Adverse events during the study period occurred in both groups in comparable frequencies (vitamin D group: 30.0% common colds, $n=6$; placebo: 33.3% common colds, $n=4$, data not shown). No treatment-related adverse event occurred during the study.

Efficient immunization-triggered tetanus toxoid-specific antibody induction upon vitamin D supplementation

Before immunization, the tetanus toxoid (TT)-specific IgG concentrations were comparable between both groups (Fig.1B). After 9 weeks of supplementation, a tetanus/diphtheria toxoid immunization was performed intramuscular and the TT-specific IgG concentrations were

strongly boosted by $29.8 \text{ I.U.} \pm 14.8$ upon vitamin D intake and $21.0 \text{ I.U.} \pm 13.3$ with placebo (both intragroup changes $P < 0.0001$). The TT-specific IgG boost efficiency was marginally higher in the vitamin D group ($P = 0.04$). The baseline and immunization-induced TT-specific IgA serum concentrations were independent of vitamin D or placebo treatment ($P = 0.70$; data not shown). Herein, TT-specific IgE was present in 9 participants and comparable changes upon immunization were observed in both groups (vitamin D group: 3 up, 3 down; placebo 2 up, 1 down; data not shown). Additionally, circulating tetanus toxoid-specific antibody secreting plasmablasts (Fig.1C) were analyzed exactly 7 days after immunization, as these cells are only present in the peripheral blood in a very limited time frame (Odendahl et al 2005). As shown in Fig.1D, most recombinant tetanus toxoid C-fragment (rTT.C) -specific plasmablasts were of IgG isotype in both groups (intergroup difference $P = 0.57$) followed by rTT.C-specific IgA plasmablasts ($P = 0.53$). The numbers of rTTC-specific plasmablasts and relative frequency of all plasmablasts showed no statistically significant differences in both groups ($P = 0.51-0.26$, data not shown). The frequencies of peripheral naïve B cells ($\text{CD}27^- \text{CD}19^+$), memory B cells ($\text{CD}27^+ \text{CD}19^+$), or terminally differentiated plasmablasts ($\text{CD}27^{++} \text{CD}20^- \text{CD}38^{\text{hi}} \text{CD}19^+$) were comparable between the vitamin D or placebo group (data not shown). Interestingly, a significant correlation between the increased 25-hydroxyvitamin D concentration and TT-IgG serum concentration was observed ($P = 0.042$, $R = 0.351$, Fig.2).

Stable tetanus toxoid induced cytokine secretion by peripheral immune cells

Whether the tetanus toxoid (TT) -specific and/or polyclonal cytokine response is modulated upon vitamin D intake was analyzed from *ex vivo* stimulated peripheral blood cells before and exactly 7 days after immunization, at the peak of specific T cell circulation (De Rosa et al 2004). Unstimulated PBMCs served as negative control and no significant spontaneous

cytokine release was observed, otherwise the individual data set was excluded due to subclinical concomitant infection (vitamin D group: n=5; placebo n=4). As positive control and reference for the circulating T cell pool polyclonal T cell stimulation by SEB resulted in activation CD4⁺ T helper cells in the vitamin D or placebo group in comparable frequencies (4.7% ± 0.8 or 3.9% ± 0.5 of all lymphocytes, data not shown) as determined by CD154-upregulation (Frentsch et al 2005). These data were paralleled by SEB-induced IFN- γ , TNF α , IL-10, IL-2, IL-4 and IL-5 secretion (intergroup differences P-value range 0.84-0.11, Tab.1). Tetanus toxoid stimulation of PBMCs for 24 h before and one week after immunization induced IL-2, IL-10, IFN- γ , or TNF- α secretion and no significant differences between both groups regarding the respective TT- induced cytokine secretion were observed (P-values 0.76-0.11, Tab.1). TT-induced IL-4 or IL-5 secretion was below the detection threshold in all samples (data not shown).

Discussion

Herein, we show that the daily intake of 2000 I.U. vitamin D over 10 weeks was well tolerated and sufficient to restore the vitamin D status as determined by a median 25-hydroxvitamin D concentration of 80.3 nM which is considered as sufficient for optimal vitamin D function (Adams and Hewison 2008, Holick 2007). The tetanus toxoid boost immunization is fully operational upon vitamin D intake with regard to TT-specific immunoglobulin induction and cytokine expression and more importantly, is not negatively altered in a placebo-controlled setting. In contrast, the data indicate that induction of TT-specific IgG was **marginally** more efficient in vitamin D compared to the placebo group (P=0.04). These data are in line with previous studies (Enioutina et al 1999, Enioutina et al 2008, Ivanov et al 2006)(Van der Stede et al 2001) **and attributed most likely to induction of specific IgG1, which is the dominant subclass induced by the vaccination used herein (>92% of the circulating specific plasmablasts) (Frolich et al 2010). That vitamin D supplementation did not alter the specific IgG-subclass composition is also suggested by our data that vaccination-induced boosted anti-tetanus antibodies of IgA or IgE isotype was independent of vitamin D supplementation, although common cytokines were involved, and further supported by previous studies in animals (Van der Stede et al 2001).** However, our data should be validated by investigation of a larger study cohort, **due to the limited** group size.

The reports on vitamin D-mediated modulation of T helper cell polarization in mice and human are heterogeneous (Hayes et al 2003) and include altered Th1/IFN- γ (Daniel et al 2005), Th2/IL-4- (Hayes et al 2003), but also tolerogenic responses (Adorini and Penna 2008, Schleithoff et al 2006). Herein, we observed a stable recall T helper cell response upon vitamin D regarding the secretion of TT-specific or polyclonally induced IL-2, IL-4, IL-5, IL-10, IFN- γ or TNF α compared to placebo. Interestingly, the blood monocytes counts were

higher in the vitamin D group after the supplementation period, compared to placebo. As differentiation of monocytes/macrophages from progenitor cells is promoted by calcitriol (Adams and Hewison 2008, Hayes et al 2003), our findings indicate that supplemented vitamin D metabolized to calcitriol modulated cellular immunity. However, whether and how vitamin D-induced monocytes contributed to the enhanced TT-IgG boost efficiency, e.g. by altered antigen presentation and IgG promoting costimulation of T and B lymphocytes, is not known yet.

An advantage in our study setting during the winter months was the lack of UV-derived vitamin D synthesis allowing a correlation between vitamin D provided by supplementation with biological effects. As prerequisite, a high prevalence vitamin D deficiency in December was observed herein and confirms a recent epidemiological report from Germany (Hintzpeter et al 2008). As vitamin D supplementation was efficient, differing averagely less than 2% from a calculation by Heaney et al. (Heaney et al 2003), and the 25-hydroxyvitamin D serum levels further decreased in the placebo group during the study period, strongly different 25-hydroxyvitamin D serum concentrations between both groups were observed ($P=0.0001$). In further placebo controlled studies during the winter months, the efficacy of immunological functions of endogenously produced calcitriol following vitamin D supplementation can be examined in different disease settings, e.g. allergy or autoimmunity.

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Conflicts of Interest

All authors declare no financial or personal conflict of interest.

Supplementary information is available at The European Journal of Clinical Nutrition's website.

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Table

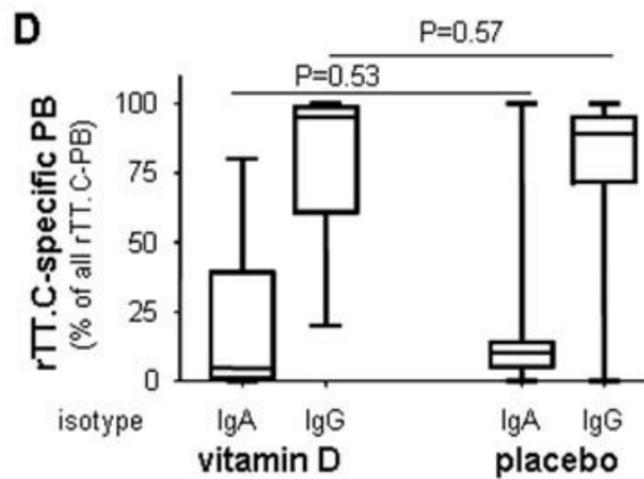
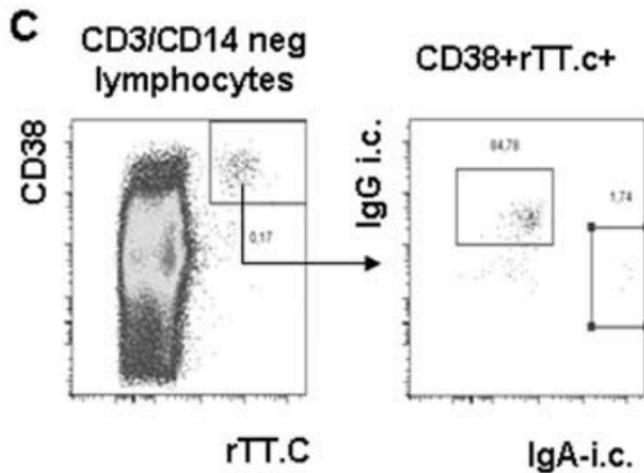
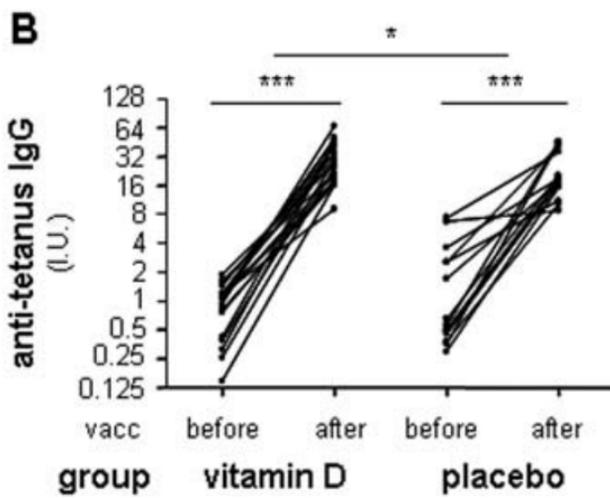
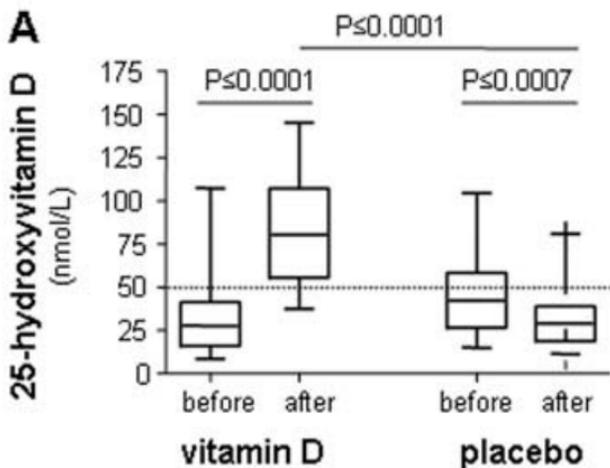
Table 1: Tetanus toxoid-specific cytokine profile upon vitamin D supplementation. PBMCs were activated before and 7 days after a tetanus toxoid immunization for 24 h in the presence of tetanus toxoid (Tet) or Staphylococcus enterotoxin B (SEB) and the secreted cytokines were determined from the supernatants. All values are medians in pg/ml and interquartile ranges obtained from the day of tetanus immunization. ΔP indicates intergroup changes calculated by Whitney-Mann-U-Test. ΔTet indicates the difference in tetanus toxoid-induced cytokine secretion (after – before to immunization).

	Vitamin D (n=15)			Placebo (n=8)			SEB ΔP	$\Delta Tet\Delta P$
	nil	Tet	SEB	nil	Tet	SEB		
IFN- γ	7 (7;7)	512 (45;2786)	44943 (16914;55717)	7 (7;21)	2212 (38;3058)	45968 (17426;63819)	0.56	0.76
TNF- α	3 (3;5)	132 (54;232)	1269 (720;3316)	3 (3;4)	191 (23;472)	1342 (674;1895)	0.49	0.23
IL-10	6 (4;9)	25 (11;184)	1714 (985;2376)	6 (3;10)	20 (8;55)	1858 (724;2939)	0.84	0.11
IL-2	6 (3;10)	116 (26;167)	4957 (3761;7135)	3 (3;8)	137 (4;215)	5267 (2742;6060)	0.45	0.26
IL-4	< 5	< 5	92 (3;144)	< 5	< 5	3 (3;121)	0.11	n.a.
IL-5	< 5	< 5	58 (3;124)	< 5	< 5	3 (3;120)	0.41	n.a.

Legends for Figures

Figure 1: Efficient immunization-triggered tetanus toxoid-specific antibody induction upon vitamin D supplementation. (A) Vitamin D (2000 I.U./day) or placebo was supplemented for 10 weeks during the UV-deprived winter months (December to March) and serum 25-hydroxyvitamin D concentrations were determined by ELISA. The dotted line indicates vitamin D-deficiency. (B) TT-specific IgG concentration in the sera before and 1 month after tetanus toxoid booster immunization. The intragroup changes as efficiency parameter and intergroup differences of the efficiency were statistically tested. (C) Immunoglobulin profile of circulating tetanus toxoid-specific plasmablasts was analyzed 1 week after immunization by staining using fluorescent labelled rTT.C fragments. **Dotblots show the gating strategy from a representative individual staining.** (D) **Analysis of all study participants according to (C). * indicates $P \leq 0.05$; *** indicates $P \leq 0.001$.**

Figure 2: Correlation between vaccination-induced anti-tetanus-IgG. (concentrations after-before immunization) and change of the 25-hydroxyvitamin D serum concentrations during the study (after-before supplementation). *** indicates $P \leq 0.001$.



anti-tetanus-IgG

(after-before vaccination)

