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Effect of Dietary Vitamin D_3 on T Helper-Related Activities of Lupus-Prompt New Zealand Black/New Zealand White F1 Mice

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ABSTRACT

We investigated the effect of dietary vitamin D_3 on T helper-related activities and progress of lupus in NZB/NZW Fl mice. Mice were fed with 0.4 or 2 μg vitamin D_3 dissolved in 50 μL olive oil every other day. Expression of T cell-related cytokine genes including those of IL-2, IL-4, IL-5, IL-6, IL-10, IL-12 and IFN-γ was monitored during the disease course by semi-quantitative reverse transcription polymerase chain reaction (RT-PCR). In addition, total serum IgG1 and IgG2a and anti-dsDNA antibody were measured. We observed a prolonged survival of mice receiving 2μg of vitamin D_3 as compared with control mice. However, vitamin D_3 did not significantly affect the anti-DNA antibody formation, IgG subtype proportion, or cytokine expression profile.

Key words: **vitamin D_3, cytokines, lupus.**

INTRODUCTION

It has been well recognized that vitamin D_3 may act as a steroid hormone to interfere with immune system after converting to 1α,25-dihydroxyvitamin D_3[1,2] which could enhance the expression of IL-4, IL-5, and IL-10 in murine T cells *in vitro*[3,4]. By contrast, it reduced the IL-2 and IFN-γ expressed in activated human T cells and monocytes[5,6]. Based on those observations, it is speculated that vitamin D_3 may stimulate Th2 T cell differentiation which in turn favors the humoral responses. However, this potent immunomodulating effect had not been fully substantiated *in vivo*.

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by the production of polyclonal autoantibodies against a variety of cellular components. Upregulation of both IL-10 and IFN-γ has been observed in SLE patients [7,8]. In SLE-prone MRL-lpr/lpr mice, overexpression of IL-l0 and IFN-γ transcripts could be detected in lymphocytes during the active stage of the disease[9]. Animals treated with anti-IL-l0 specific antibodies displayed significant remission, while injection of IL-10 cytokine accelerated the disease process. Recently, we have demonstrated a sequential expression of IL-10 followed by IFN-γ in peripheral blood mononuclear cells (PBMC) of NZB/NZW F1 mice that was associated with the

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formation of autoantibody and disease progression [10]. In this study, we tested whether dietary vitamin D_3 would alter the expression of T cell-derived cytokines, especially IL-10 and IFN-γ in NZB/NZW Fl and thus modify the T helper activities.

MATERIALS AND METHODS

I. *Mice*

Female, 2- to 3-mo-old NZB/NZW Fl mice were purchased from the Laboratory Animal Center, National Taiwan University and were shipped to the animal facilities at National Cheng-Kung University in sterile container. Mice were maintained in Microisolator cages (Laboratory Product Inc., Maywood, NJ, USA) and provided with sterile food and water *ad libitu*m before the experiment.

Commencing at 3 mo of age, the mice were randomly divided into four groups. The mice (n=5, treatment groups) were oral-fed with 0.4 or 2 μg vitamin D_3, corresponding to 16 IU and 80 IU, respectively. (Sigmal Chemical Co., St. Louis, Mg USA) dissolved in 50 μL olive oil[11,12] every other day. The third group (n=6, control group) were mock fed with olive oil. The fourth group (n=6, untreated group) did not received either vitamin D_3 or olive oil. The animals were then inspected every other day and disease symptoms were recorded including facial edema, footpad paleness, paralysis, diarrhea, and hunched posture. Mice were weighed every two weeks and 50-150 μL of blood sample were obtained from the retrobulbar venous plexus under light anesthesia with ether. PBMC were isolated and handled separately for individual mouse. Plasma was separated and stored at -70°C until measurement of anti-double stranded (ds) DNA antibodies and IgG subtypes. Two mice in the control group at about 5 mo of age died from unknown cause apparently not associated with autoimmunity. Data derived from them were not included for statistical analysis.

II. *Semiquantitative Reverse Transcription-Polymerase Chain Reaction (RT-PCR)*

Total cellular RNA of PBMC was extracted by the RNeasy Total RNA kit according to the manufacturer's instruction (Qiagen, Hilden, Germany). Total RNA was converted to cDNA by StrataScript™ II-reverse transcriptase (Stragene, CA, USA) using oligo dT as a primer. PCR primers selected for genes of IL-2, -4, -5, -6, 10, -12, IFN-γ and β-actin in individual mouse were used as described previously[12]. β-actin served as a quantitative control for RNA. PCR products were fractionated by agarose electrophoresis, stained with ethedium bromide and visualized under UV light. Optical density of DNA bands were scanned with a densitometer (Molecular Dynamic, Sunnyvale, CA, USA).

III. *Enzyme-Linked Immunosorbent Assay (ELISA) for Anti-dsDNA Antibodies and IgG Subtypes*

Serum anti-dsDNA and IgG antibodies were determined by ELISA. In brief, purified calf thymus DNA (2.6 μg/mL; Sigma) was adsorbed on microtiter plates precoated with methylated bovine serum albumin (BSA; 10 μg/mL, Sigma). One hundred microliter of mouse serum diluted in 1% PBS/BSA (50- to 1000-dilution) were added per well and incubated at 40°C for 18 hr. After washing out unbound immunoglobulin, the plates were incubated with peroxidase-conjugated goat anti-mouse IgG antibody (Boehringer Mannheim, Mannheim, Germany). Binding was detected by the addition of substrate solution containing 0.1M sodium citrate, 1% 2,2'-azino-bis (3-ethylbenzthiazoline-6sufonic acid), and 0.03% H_20_2. Absorbency was then determined at 405 nm. Polyclonal antibody against dsDNA served as positive control for anti-DNA autoreactive antibody was kindly provided by Dr. BL Chiang (National Taiwan University, ROC). For IgG subtype determination, a similar protocol was used except that goat anti-mouse κ-light chain monoclonal antibody (2 μg/mL; Boehringer Mannheim) was used as capture antibody for total serum IgG.

The levels of IgG subtypes were then determined with peroxidase-conjugated rat anti-mouse IgG1 or IgG2a monoclonal antibody (PharMingen, San Diego, CA, USA). Results are expressed as ELISA unit, the optical adsorption of test samples over control.

IV. *Statistical Analysis*

Results were analyzed either by nonparametric Kruskal-Wallis test (ANOVA) or Mann-Whitney U test. Differences with $p<0.05$ were judged significant.

RESULTS

I. *Effect of Vitamin D_3 on Mortality and Anti-dsDNA Antibody Formation*

Both the untreated and control mice began to succumb to lupus at about 5-6 mo of age and died at 7.5 mo of age. Vitamin D_3 at the dose of 2 μg delayed appearance of symptoms and death. Specifically, those 2 μg vitamin D_3-treated mice did not show edema and other signs of autoimmunity at ages of 7 mo while most of untreated and control mice were either sick or dead. Treatment with 0.4 μg of vitamin D_3, however, did not significantly affect the disease onset and life span. Figure l presents the cumulative mortality of mice as a function of age. Between 5-6 mo of age, serum anti-dsDNA autoantibody titers started to increase regardless of vitamin D_3 treatment (Fig. 2). There was no significant difference in kinetics and the quantity of autoantibody among experiment groups.

II. *Expression of Helper T Cells-Related Cytokines*

Semiquantitative RT-PCR analysis showed that there was a sequential expression of a variety of cytokines in PBMC of NZB/NZW Fl mice during the disease course. Summaries of reproducible data of cytokine expression in samples having success in PCR analysis were shown in Table 1. Collectively, the onset of lupus-like disease in NZB/NZW Fl mice was correlated with an early-enhanced expression of IL-l0 mRNA followed by the IFN-γ and IL-6. Spontaneous expression of IL-5 and IL-10 was observed around 4-5 mo of age. The IL-I0 expression sustained at a high level

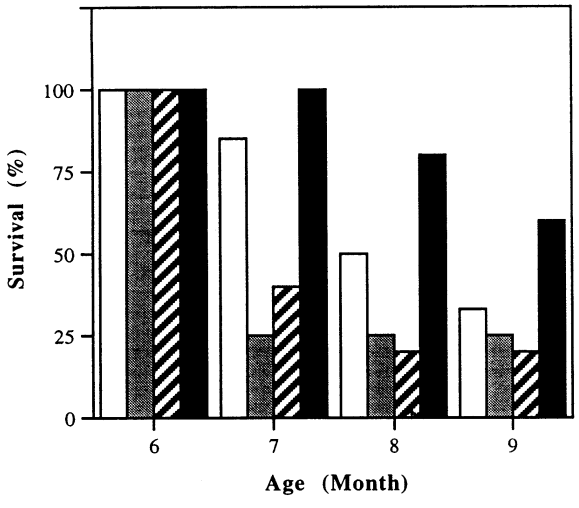

Figure 1. Survival of NZB/NZW Fl mice.
Groups of mice were fed with standard laboratory chow (blank bar), 50 μL olive (dotted bar) oil or 0.4 μg/50 μL olive oil (hatched bar) or 2 μg/50 μL olive oil (filled bar) at 3 mo of age. Mortality was recorded for up to 9 mo of age.

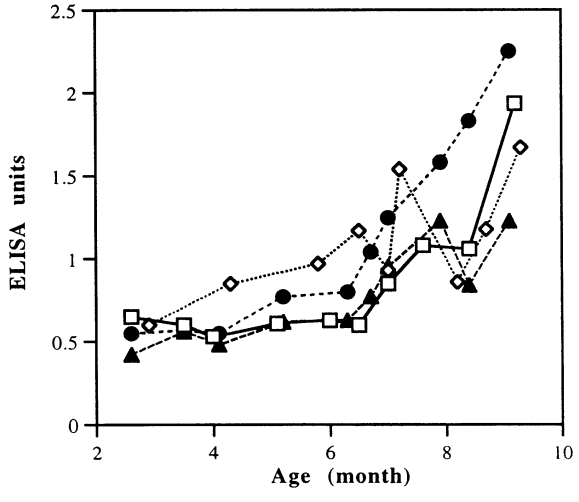

Figure 2. Anti-dsDNA IgG production in NZB/NZW Fl mice.
Mice were fed with standard laboratory chow only (□), with 50 μL olive oil (◇), 0.4 μg/50 μL olive oil (▲), 2 μg/50 μL olive oil (●). Serum anti-ds DNA IgG titer was determined by ELISA. Polyclonal anti-ds DNA antibody served as a positive control. Mean ELISA values are shown.

Table 1. Cytokine gene expression in NZB/NZW Fl mice

		Age in months				
		4	5	6	7.5	9
IL-5	R	+(2/6)	-(0/6)	-(0/6)	-(0/6)	-(0/3)
	C	+(4/4)	+(3/4)	-(0/4)	-(0/2)	-(0/1)
	LV	+(4/5)	+(3/3)	-(0/4)	-(0/2)	-(0/2)
	HV	+/-(2/4)	+(3/5)	-(0/5)	-(0/4)	-(0/2)
IL-6	R	-(0/6)	-(0/6)	-(0/6)	+(5/6)	+(1/3)
	C	-(0/4)	+/-(1/4)	+/-(3/4)	-(0/2)	?
	LV	-(0/5)	-(0/3)	-(0/4)	-(0/2)	+(1/1)
	HV	-(0/4)	-(0/3)	+/-(1/5)	+/-(1/4)	+(2/2)
IL-10	R	+(5/6)	+(2/6)	++(4/6)	+(5/6)	+(2/4)
	C	-(0/4)	+(2/4)	++(4/4)	?	?
	LV	+(4/5)	?	++(3/4)	?	+(1/1)
	HV	+/-(2/4)	+(1/3)	+(3/5)	-(0/4)	+(1/2)
INF-γ	R	-(0/6)	-(0/6)	-(0/6)	+(4/6)	?
	C	-(0/4)	-(0/4)	+(4/4)	+(1/2)	+(1/1)
	LV	-(0/5)	-(0/3)	+/-(1/4)	+/-(2/2)	+(1/2)
	HV	-(0/4)	-(0/3)	+/-(3/5)	+(4/4)	+(2/2)

Blood samples were obtained from mice every two weeks up to 9 mo of age. The transcripts of IL-5, IL-6, IL-10 and IFN-γ of individual mouse were detected by semiquantitative RT-PCR as described. The data shown in parentheses are number of mice being positive in cytokine expression over number of mice having success in RT-PCR analysis for β-actin; PCR amplification was shown with average intensity: - = no signal;+/- = weak signal; + = signal < β-actin; ++ = signal ≧ β-actin. ?: Data were not interpretable. R: untreated; C: 50 μL olive oil; LV: mice fed with 0.4 μg vitamin D_3/50 μL olive oil; HV: mice fed with 2 μg vitamin D_3/50 μL olive oil.

thereafter. IFN-γ was detected around 6 mo and reached maximal level around 8 mo. When the transcripts of IL-6 rose around 7-8 mo, death occurred in untreated and control mice. Administration of mice with vitamin D_3 did not change the cytokine profile compared with those of control and untreated mice. Transcripts of IL-2 and IL-4 were not detected in this experiment. The two survival mice that receiving 2 μg of vitamin D_3 expressed high levels of IL-12, IL-10, IL-6 and IFN-γ at age older than 8 mo.

III. *Serum IgG Subclasses*

Figure 3 shows the serum IgG1 and IgG2a levels in NZB/NZW Fl mice. In control mice, The level of IgG1 was one third lower than that of IgG2a. The proportion did not change throughout the course of experiment. Vitamin D_3 supplementation also did not modify the serum levels of IgG1 and IgG2a.

DISCUSSION

Disregulated expression of several T cell-related cytokines has been observed in SLE. For instance, serum levels of IL-10 and IFN-γ are elevated in human SLE[8,14,15]. Overexpression of IL-10 and IFN-γ mRNA are also demonstrable in the lymph nodes of MRL-lpr/lpr and BXSB male mice[9]. We have recently reported that the disease progression as well as autoantibody formation in NZB/NZW Fl mice were correlated to a sequential expression of a variety of cytokines[10]. Specifica-lly, IL-10 was detected around 4-5 mo of age. The IFN-γ and IL-6 were elevated at 6-8 mo of age while anti-dsDNA antibody formation, severe disease symptoms and frequent death occurred. In this study we demonstrated that force-feeding vitamin D_3 dissolved in olive oil could prolong the life span of NZB/NZW Fl mice. However, it did not affect the overall expression of serum IgG subtypes, autoantibody formation, and cytokine genes that are related to the disease process.

Certain diets such as a low-fat diet and fish oil may modify immune response and have some beneficial effect on improvement of clinical course of the murine lupus[16,17,18,19]. However, among the immune activities we analyzed, only the expression of IL-5 was slightly enhanced by olive oil (Table 1). Olive oil by itself did not influence the expression and outcome of the disease. Although the survival rate of untreated group was

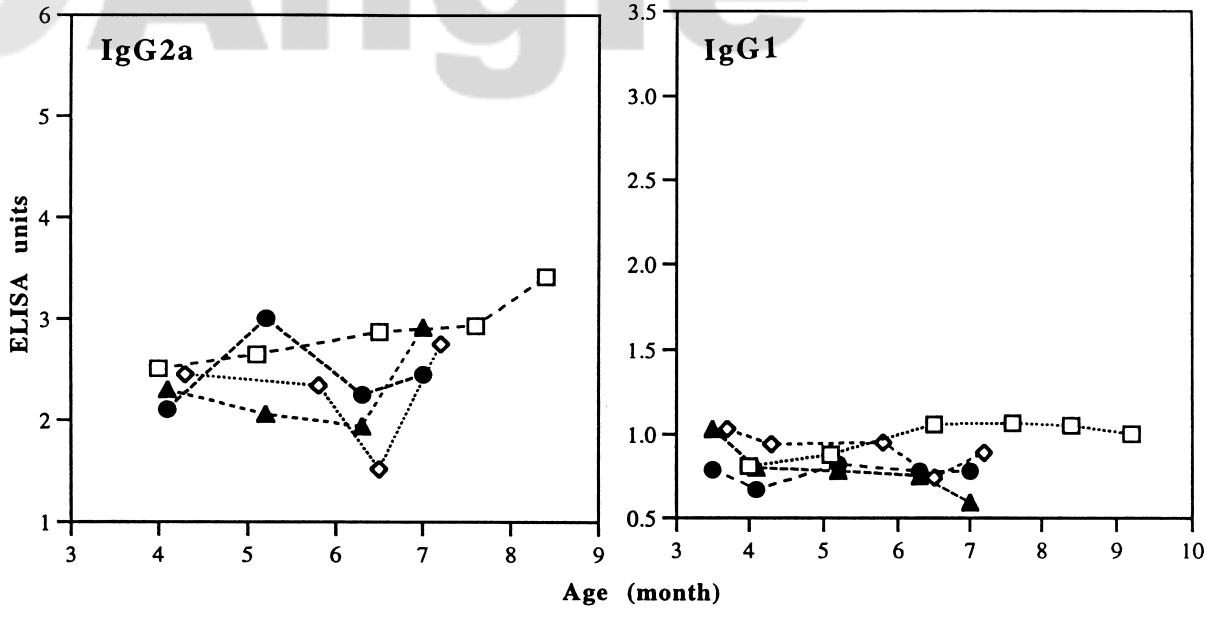

Figure 3. Total serum IgG1 and IgG2a levels in NZB/NZW Fl mice.
Mice were treated with different doses of vitamin D_3. Blood samples were obtained every two weeks up to 9 mo of age. Total serum IgG1 (A) and IgG2a (B) were measured by ELISA with mouse-IgG-subtype-specific antibodies. Serum of naive BALB/c mice served as a control to define ELISA unit. Results represent mean value of each group.□: untreated; ◇: 50 μL olive oil; ▲: 0.4 μg vitamin D_3/50 μL olive oil; ●: 2 μg vitamin D_3/50 μL olive oil.

higher than those of groups treated with olive oil alone and 0.4 μg vitamin D_3/olive oil, no other differences between these three groups was observed in terms of immune activities and symptoms. Therefore, we concluded that the amount of olive oil used in this study did not significantly modify the immune system of F1 mice. The speculation that low survival rate in olive oil-treated mice is attributable to force feeding process, being a kind of stress for animal, awaits to be sustentation by extensive study.

As mentioned earlier, vitamin D_3 could modulate cytokine gene expression in cultured murine and human T cells. Direct injection of 1α,25-dihydroxyvitamin D_3 could prevent the induction of murine experimental autoimmune encephalomyelitis[4] and thyroiditis[20], and it did not always affect the formation of autoreactive anti-dsDNA antibody. Although we found that 2 μg vitamin D_3 might prolong life span, it did not change the cytokine expression, serum level of IgG subtypes and autoreactive anti-dsDNA antibody. It seems that the biological activity of vitamin D_3 *in vivo* is different from the activity *in vitro*. The secosteroid vitamin D_3 is obtained either from ingestion or from conversion of 7-dehydrocholesterol in the epidermis. It is then transported by a vitamin D-binding protein to liver where it becomes 25-hydroxyvitamin D_3. This metabolite will be further hydroxylated by cytochrome p450 in the mitochondria in kidney[2,21]. The conversion of 25-hydroxyvitamin D_3 to calcitriol, with a 100-1000-fold increase in biologic activity and receptor binding, is tightly regulated by the mineral need of the individual[22]. Therefore, an alternative explanation of our results is that dietary vitamin D_3 did not converte into enough amount of active metabolites in the mice with balanced mineral metabolism. The question about how high-dose vitamin D_3 affects the life span awaits further study. Although we had not measured protein urea in this study, relative improvement in renal

function was suggested by reduced edema in mice receiving 2 μg vitamin D_3 as compared with that in control mice. Since the active form of vitamin D_3 is generated in the kidney and damages in renal system represent a major course of death in NZB/NZW F1 mice, we speculate that the protective effect of vitamin D_3 may be mediated through a mechanism not directly associated with immune reactions.

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攝食維生素D_3對紅斑性狼瘡小鼠體內T細胞相關活性的影響

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摘　要

本論文研究維他命D_3對於紅斑性狼瘡小鼠T輔助細胞活性以及疾病發展的影響。0.4或2μg維他命D_3溶在50μL橄欖油中，每隔一天灌食小鼠。然後，以RT-PCR方法測量小鼠體內包括：IL-2, -4, -5, -6, -10, -12及IFN-γ等基因的表現。血清中IgG1, IgG2a, anti-dsDNA免疫球蛋白則利用免疫吸附法定量。整體而言，紅斑性狼瘡小鼠發病過程中，T輔助細胞活性的時序與發病的嚴重程度有關。但是，維他命D_3餵食組中T輔助細胞活性相關細胞素基因的表現與控制組相近，IgG1, IgG2a與自體抗體量亦無明顯不同。儘管如此，餵食2μg維他命D_3的小鼠隻生命期確較其他組別來得長。顯然維他命D_3對於紅斑性狼瘡小鼠的保護效果似乎並非透過影響T輔助細胞活性相關細胞素，或是免疫球蛋白的種類來達成。

關鍵詞：維他命D_3，細胞激素，狼瘡。