

Dual effects of vitamin D-induced alteration of T_H1/T_H2 cytokine expression: Enhancing IgE production and decreasing airway eosinophilia in murine allergic airway disease

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Background: Vitamin D, a common food additive, has been shown to prevent the induction of experimental autoimmune diseases in mice. A possible immune deviation from T_H1 to T_H2 responses has been postulated. Although there is no doubt about the beneficial effects of vitamin D, its role in allergy has not been investigated.

Objective: To define the role of vitamin D in modulating the development of a T_H2-mediated disease, we used a murine model of pulmonary eosinophilic inflammation.

Methods: Five-week-old mice were primed on day 0 with ovalbumin intraperitoneally. Then they were nasally challenged with ovalbumin on days 7, 8, 9, and 10, and on day 11, samples were studied. Some mice received subcutaneous injections of vitamin D every second day as follows: days -3, -1, 1, 3, 5, 7, and 9. The control groups received PBS on the same days.

Results: Early treatment with vitamin D augmented allergen-induced T-cell proliferation along with T_H2 cytokine (IL-4 and IL-13) and IgE production. Surprisingly, the local inflammatory response in bronchoalveolar lavage fluid and lung tissue was significantly ameliorated with impaired recruitment of eosinophils and inferior levels of IL-5. These findings were attributed to late treatment with vitamin D after establishment of an early immune response.

Conclusion: We suggest that excess supplementation of vitamin D could influence the development of a sustained T_H2 response, leading to an increasing prevalence of allergy, whereas vitamin D might hold promising beneficial effects in airway eosinophilia. (*J Allergy Clin Immunol* 2003;112:585-92.)

Key words: Vitamin D, eosinophils, cytokines, IL-4, IL-5, IL-13, allergy, costimulation, inflammation, knockout, lung

1,25-Dihydroxycholecalciferol (1,25-[OH]₂-D₃), the active metabolite of vitamin D, is a key molecule in reg-

Abbreviations used

1,25-(OH)₂-D₃: 1,25-Dihydroxycholecalciferol
BAL: Bronchoalveolar lavage
EAE: Experimental autoimmune encephalomyelitis
OVA: Ovalbumin
VDR: Vitamin D receptor

ulating serum calcium levels, phosphate levels, and bone remodeling. The action of vitamin D is mediated through a specific intracellular receptor, the vitamin D receptor (VDR), which is a member of the steroid hormone receptor family. Recent studies have shown a linkage between VDR and the immune system, with the presence of VDR in several immune cells, such as monocytes, macrophages, and activated T and B cells.¹ Vitamin D has been shown to inhibit in vitro T-cell proliferation² and production of cytokines, such as IL-2, IL-12, and IFN-γ.³⁻⁵ In addition, vitamin D has been demonstrated to suppress Ig production,^{6,7} and more recently, inhibition of T_H1-mediated immune response has been suggested.⁸ Vitamin D has also been shown to reversibly block or prevent the in vivo induction of several autoimmune diseases, such as murine experimental autoimmune encephalomyelitis (EAE),⁹⁻¹² type I diabetes,^{13,14} and collagen-induced arthritis,¹⁵ and to prolong graft survival.¹⁶ Moreover, an immune T_H1/T_H2 shift has been postulated.^{17,18}

Allergic diseases, such as atopic dermatitis, allergic rhinitis, and asthma, are characterized by increased serum IgE levels and an inflammatory reaction, with increased numbers of eosinophils, mast cells, and CD4⁺ T cells and T cell-derived T_H2 cytokines, mainly IL-4 and IL-5. The incidence of atopic diseases is increasing in developed countries.¹⁹ This difference is not accounted for by a corresponding increase in the prevalence of allergic sensitization.^{20,21} The phenomenon is most likely the result of complex interactions between multiple genetic and environmental factors, including diet, associated with the westernized lifestyle.²¹⁻²³ One difference in the diet between developing and developed countries is the presence of vitamin D-fortified food products in addition to vitamin D supplements in children's diets to avoid rickets. The benefit of that is beyond doubt,

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although no studies have been performed to investigate the possible role of excess vitamin D on atopic status. To investigate the role of *in vivo* vitamin D supplementation in modulating the development of a T_H2-mediated disease, we used a murine model of pulmonary eosinophilic inflammation. Here we report that treatment in mice with the same dose range of vitamin D as the Scandinavian recommendation in children led to increased allergen-induced T-cell proliferation, IL-4 and IL-13 cytokine levels, and serum IgE production. However, the inflammatory pulmonary response was significantly suppressed, with impaired recruitment of eosinophils and reduced levels of IL-5 in the airways.

METHODS

Mice and diet

Female B10.RIII mice (weight, 17–21 g; age 5–7 weeks) were used. During the experiments, the mice were fed Beekay Standard Rat and Mouse Diet No.2 (BK002PS, B&K Universal AB) containing 1.574 IU of vitamin D per gram of diet and 0.63% calcium and were kept in a climate-controlled environment with 12-hour cycles of light-dark and sound. Therefore they were not vitamin D deficient. Other sets of experiments were performed with female IL-4-deficient mice of the B10.Q background (IL-4^{-/-}) with wild-type litter mates, both homozygotes (IL-4^{+/+}) and heterozygotes (IL-4^{+/-}), used as control animals to determine the role of IL-4.

Induction of airway inflammation and experimental design

Groups of mice were immunized by means of intraperitoneal injection of 50 µg of chicken egg albumin (ovalbumin [OVA]; Sigma Chemical Co) complexed with 5 mg of aluminum potassium sulphate (Sigma Chemical Co) on day 0. On day 7, the mice were challenged with 50 µg of OVA in 50 µL of PBS delivered to the airways as intranasal drops after achievement of slight anesthesia with isoflurane. The challenge was performed once daily for 4 consecutive days. On day 11, 24 hours after the last allergen exposure, the mice were assessed for lung allergic inflammatory response.²⁴

We were interested in investigating how vitamin D affects the prevalence, as well as the severity, of allergy. Thus 3 days before immunization (day -3), mice received a subcutaneous injection of Calcijex²⁵ (Abbott Labs, Chicago, Ill) containing 100 ng of calcitriol (1,25-[OH]₂-D₃) in PBS-Tween 20 buffer.⁶ That dose is approximately 2 µg/kg, and it is in the same dose range as the Scandinavian recommendation of 10 µg/d in children weighing 5 to 10 kg. Subsequently, the injections were also given every second day until the end of the experiment as follows: days -3, -1, 1, 3, 5, 7, and 9. Control groups received subcutaneous injections with PBS-Tween 20 buffer on the same days.

Next we were interested to study whether the effects of vitamin D differed when given at sensitization or at challenge. Thus 20 mice were immunized and challenged as above. One group received subcutaneous injections of 100 ng of calcitriol on days -1, 1, and 3 (group vitamin D/PBS). The second group received subcutaneous injections of 100 ng of vitamin D on days 5, 7, and 9 (group PBS/vitamin D). The third group received vitamin D during the entire protocol every second day, as mentioned above (group vitamin D/vitamin D). Finally, the fourth group did not receive any vitamin D treatment (group PBS/PBS). All mice (n = 5 mice per group) received PBS buffer subcutaneously on days when the rest were treated with vitamin D. Blood and samples were collected 24 hours after the last allergen exposure.

Cell proliferation

The spleens were dissected out on day 11. Single-cell suspensions from each mouse were prepared with Dulbecco modified Eagle medium with glutamax I (GIBCO BRL, Life Technologies) supplemented with 10% heat-inactivated FCS, 10 mmol/L HEPES, 50 mmol/L β-mercaptoethanol, 100 U/mL penicillin G, and 100 µg/mL streptomycin as culture medium. Cells were incubated (5 × 10⁶/mL) in triplicate at 37°C and 5% CO₂ in a humidified incubator. Medium, concanavalin A, or OVA (111 µmol/L) were separately added to the cultures. Fifty-four hours later, 5 µL of tritiated thymidine (100 µCi/mL) was added to each well before harvesting, and its incorporation was measured 18 hours later in a β-scintillation counter. Simultaneously, in separate parallel cultures 1,25-(OH)₂-D₃ (10⁻⁹ mol/L)²⁶ was added.

Determination of total and OVA-specific IgE levels

The mice were bled at the time of death. A sandwich ELISA protocol determined the levels of total and specific IgE, IgG, IgG1, and IgG2a in serum according to the manufacturer's instructions (BD Pharmingen).

Assessment of bronchoalveolar lavage fluid

Mice were deeply anesthetized and killed 24 hours after the last OVA exposure. The trachea was cannulated, bronchoalveolar lavage (BAL) fluid was collected twice with 0.5 mL of PBS, and the collected fluid was pooled. Total cell counts were determined by using an automated hemocytometer (Sysmex CDA-500, Toa Medical Electronics Co, Ltd), the fluid was centrifuged, and the supernatant was used to determine the airway cytokine content. The cells were applied to slides by using a cytospin apparatus, and eosinophils were detected by means of histochemical staining of cyanide-resistant eosinophil peroxidase activity with 3,3 diaminobenzidine tetrahydrochloride as a substrate, as described previously.²⁴ The slides were examined by means of light microscopy (40× magnification) in a blinded fashion, counting at least 500 cells per slide.

Lung histology

On day 11, the left lung was removed, embedded immediately in OCT compound (Tissue-Tek, Sakura Finetek Europe BV), snap-frozen, and stored at -70°C. Ten-micrometer cryosections were prepared, and eosinophils were detected by means of histochemical staining of cyanide-resistant eosinophil peroxidase activity. Forty areas in 6 fields were randomly selected in each section and examined by means of light microscopy for eosinophils (40×).

Cytokine ELISA

BAL fluid was obtained as previously described. The supernatants were assessed for cytokine content by using standard ELISA protocols with commercial IL-4, IL-5, and IFN-γ (BD Pharmingen) and IL-13 (R&D Systems Europe) ELISA kits. Standard curves were constructed with purified IL-4, IL-5, and recombinant IL-13. Detection levels were 25 pg/mL for IL-4, IL-5, and IL-13 and 12.5 pg/mL for IFN-γ.

Analysis of peripheral blood cells by means of flow cytometry

Blood from vitamin D-treated and control mice was obtained. After lysis of red blood cells, FcR was blocked with antibody against FcRII/III by using 2 µg/5 × 10⁵ cells. The following antibodies were then added: anti-CD3 FITC (145-2C11), anti-CD4 phycoerythrin (H129.19), anti-CD8 cytochrome (53-6.7), anti-CD19 FITC (ID3), anti-B220 cytochrome (RA3-6B2), and anti-CD40 li-

gand biotinylated (MRI; BD Pharmingen). Streptavidine labeled with cytochrome or phycoerythrin (BD Pharmingen) was then added to the biotinylated CD40 ligand. Analysis of a minimum of 10,000 total events was performed by using flow cytometry (FAC-Scan, Becton Dickinson).

Statistic analysis

Results were expressed as mean values $\pm$ SEM except for proliferation assays, in which SD was used. The Mann-Whitney *U* test was used to analyze the difference between the experimental groups. Significance was assumed at a *P* value of .05 or less.

RESULTS

Vitamin D treatment results in expansion of OVA-specific T cells

Because T lymphocytes contain VDR and the levels are increased when activated and treated with vitamin D,¹ we investigated the effect of vitamin D treatment on the peripheral T-cell response. We observed that in vivo treatment of mice with vitamin D results in a significant increase of OVA-specific T-cell proliferation (Fig 1, A). This activity was specific for OVA because nonrelevant control antigen (collagen type II peptide 707-721) did not result in proliferation (data not shown). In addition, the effect of vitamin D treatment on cell proliferation was entirely OVA specific because there was no difference between the groups in response to concanavalin A (Fig 1, A).

In separate cultures vitamin D was added in vitro to determine whether the in vivo and in vitro responses to vitamin D differed. We found that the proliferation of T cells from both vitamin D-treated and control mice was dramatically suppressed after adding vitamin D in vitro (Fig 1, B).

Bimodal effects of vitamin D treatment on OVA-specific T_H2 response

Next we were interested to investigate the effector function of OVA-specific T cells (Fig 1, C). Thus we checked the cytokines produced by these cells on restimulation with OVA in vitro. We found that OVA-specific IL-4 and IL-13 levels were significantly enhanced in vitamin D-treated mice compared with in control mice. On the contrary, IL-5 and IFN- γ production was significantly decreased in vitamin D-treated mice.

In vivo supplementation of vitamin D increased the levels of total and OVA-specific IgE but decreased the IgG response

Ig levels were measured to study the effect of vitamin D on antibody production. Total IgE levels in serum were significantly augmented in vitamin D-treated mice compared with in control mice (Fig 2). In accordance, a significant increase of OVA-specific IgE was also observed (Fig 2, A). However, IgG levels were significantly decreased, including the IgG1 isotype (Fig 2, B). IgG2a isotype levels were not significantly different between groups, although a tendency toward decrease was

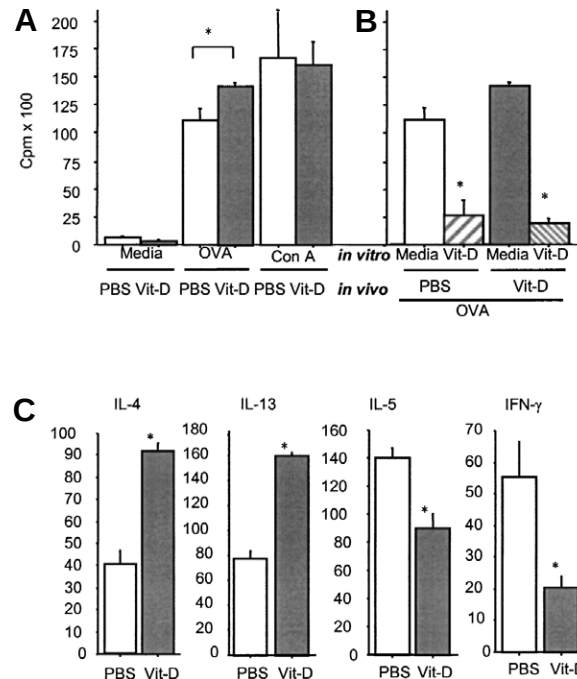


FIG 1. In vitro response in cultured splenocytes. **A**, No difference between control ($\square$) and vitamin D-treated ($\blacksquare$) mice after unspecific stimulation was seen. However, there was an increase in OVA-specific response in the vitamin D-treated group. **B**, Proliferation was suppressed by means of in vitro addition of vitamin D in both vitamin D-treated ($\blacksquare$) and control ($\square$) mice. **C**, OVA-specific cytokine production by spleen cells in vitamin D-treated ($\blacksquare$) and control ($\square$) mice is shown. IL-5 levels were significantly decreased in vitamin D-treated mice. Histograms show values expressed as means $\pm$ SEM (*n* = 6 mice per group). **P* < .05.

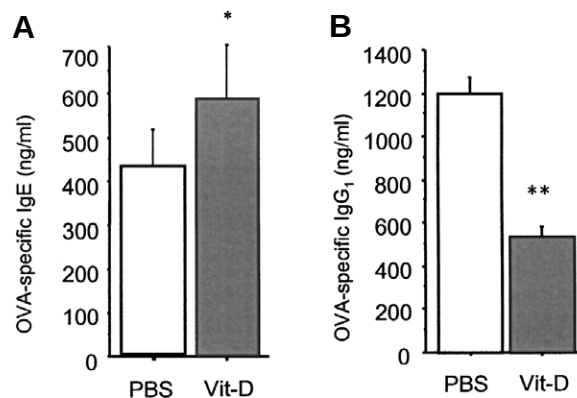


FIG 2. Serum levels of OVA-specific IgE (**A**) and IgG1 (**B**) in control ($\square$) and vitamin D-treated ($\blacksquare$) mice determined by means of ELISA. Histograms show values expressed as means $\pm$ SEM in picograms per milliliter. **P* < .05; ***P* < .01.

observed (320 ± 110 μ g/mL in vitamin D-treated mice vs 234 ± 42 μ g/mL in PBS-treated mice).

Moreover, we addressed the question of whether the effects of vitamin D treatment on increased IgE production is CD40 ligand dependent because other hormones, such as glucocorticoids, upregulate IgE through

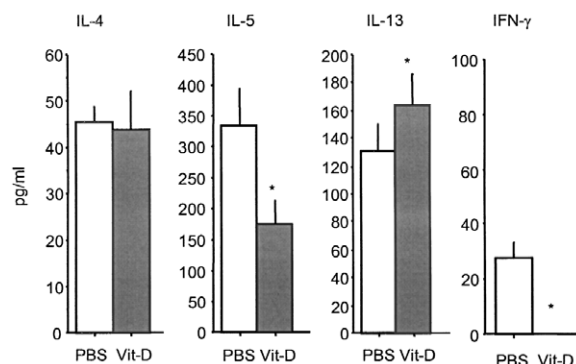


FIG 3. Cytokine levels in BAL fluid determined by means of ELISA in control (□) and vitamin D-treated (■) mice. IFN-γ levels were undetectable. Histograms show values expressed as means ± SEM in picograms per milliliter. * $P < .05$.

enhancement of CD40 ligand expression.²⁷ Using FACS analysis, we did not observe any significant difference in CD40 ligand expression on gated T cells between vitamin D-treated (2.3%) and control (2.1%) mice.

Production of T_H2 cytokines in BAL fluid was also differentially regulated by vitamin D

Because we observed that vitamin D treatment has different effects on systemic OVA-specific T_H2 response, we were interested to investigate whether it exerts similar effects locally in BAL fluid (Fig 3). We observed no difference in IL-4 protein levels between vitamin D-treated and control mice. Interestingly, IL-5 protein levels were significantly decreased in vitamin D-treated mice. However, the IL-13 protein in BAL fluid was significantly enhanced whereas IFN-γ protein levels were significantly decreased in vitamin D-treated mice.

Vitamin D treatment significantly reduced eosinophilia in airways and parenchyma

Because of the suppression of IL-5 production both in vitro and in vivo, we further investigated whether vitamin D treatment influenced the recruitment of eosinophils to the airways and the lung parenchyma. Vitamin D treatment did not change the total number of cells in BAL fluid ($5.98 \pm 0.67 \times 10^5$ cells/mL in vitamin D-treated mice vs $7.27 \pm 0.79 \times 10^5$ cells/mL in control mice). However, the percentage (42.3% vs 62.3%, $P < .05$) and the total number of eosinophils were significantly decreased in vitamin D-treated mice (Fig 4, A). Subsequently, we found that vitamin D-treated mice sensitized and challenged with OVA had significantly decreased numbers of eosinophils in the parenchyma (Fig 4, A-C).

Reduction of airway eosinophilia by vitamin D treatment was IL-4 independent

It has been reported that IL-4, in addition to being a switch factor for IgE, is involved in eosinophilia in the airways.²⁸ To study whether IL-4 is involved in vitamin D-mediated regulation of eosinophilia, we used IL-4 knockout mice to induce OVA-specific allergic

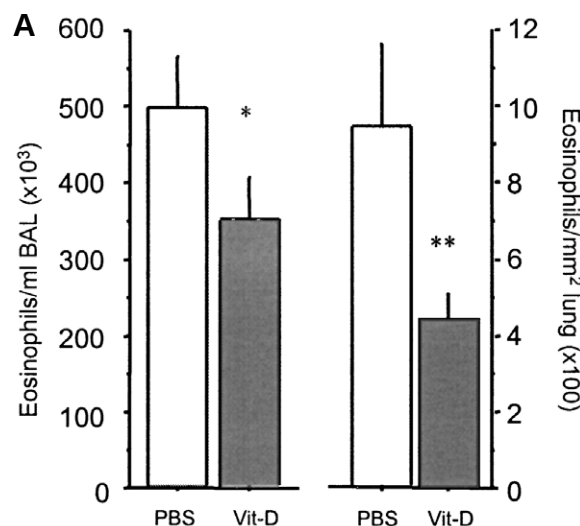


FIG 4. A, Eosinophils in BAL fluid and lung samples were decreased in vitamin D-treated (■) compared with control (□) mice. B, Eosinophils, with their characteristic brown color, are observed in a collapsed airway with a mucus plug from a control mouse. C, Few eosinophils are seen in a vitamin D-treated mouse. D, Percentages and total numbers of eosinophils in IL-4^{-/-} mice were decreased in vitamin D-treated mice. The number of affected airways with a presence of airway epithelial mucus cells was also diminished after treatment with vitamin D. Percentages and total numbers of eosinophils in airways of IL-4^{-/-} mice were decreased in vitamin D-treated mice. Histogram shows values expressed as means ± SEM. * $P < .05$; ** $P < .01$. (Figure continued)

eosinophilic inflammation and treated them with vitamin D as described earlier. Interestingly, although our data confirmed that eosinophilia is partially dependent on IL-4, we could observe less eosinophilia after vitamin D treatment (Fig 4, D).

Bimodal effect of vitamin D on allergy was time dependent

We subsequently investigated whether the modulating effect of vitamin D on parameters of allergy was time dependent. The group of mice receiving vitamin D around the time of sensitization (group vitamin D early = days -1, 1, and 3) and the group receiving vitamin D around the time of challenge (group vitamin D late = days 5, 7, and 9) had increased IgE levels compared with those of control mice. The levels of IgE in these groups were comparable with those of the group receiving vitamin D during the whole period (regular vitamin D-treated mice; Fig 5, A), although the time window for effect of vitamin D on downregulating IgG1 production seems to be early in association with sensitization (Fig 5, B).

Eosinophilia in BAL fluid in the vitamin D late group was significantly reduced similarly to that when vitamin D was given during the whole period (Fig 5, C). However, vitamin D treatment in the vitamin D early group did not affect eosinophilia. These results clearly demonstrate that the effect of vitamin D treatment on prevention of eosinophilia is attributed to a late-stage effect.

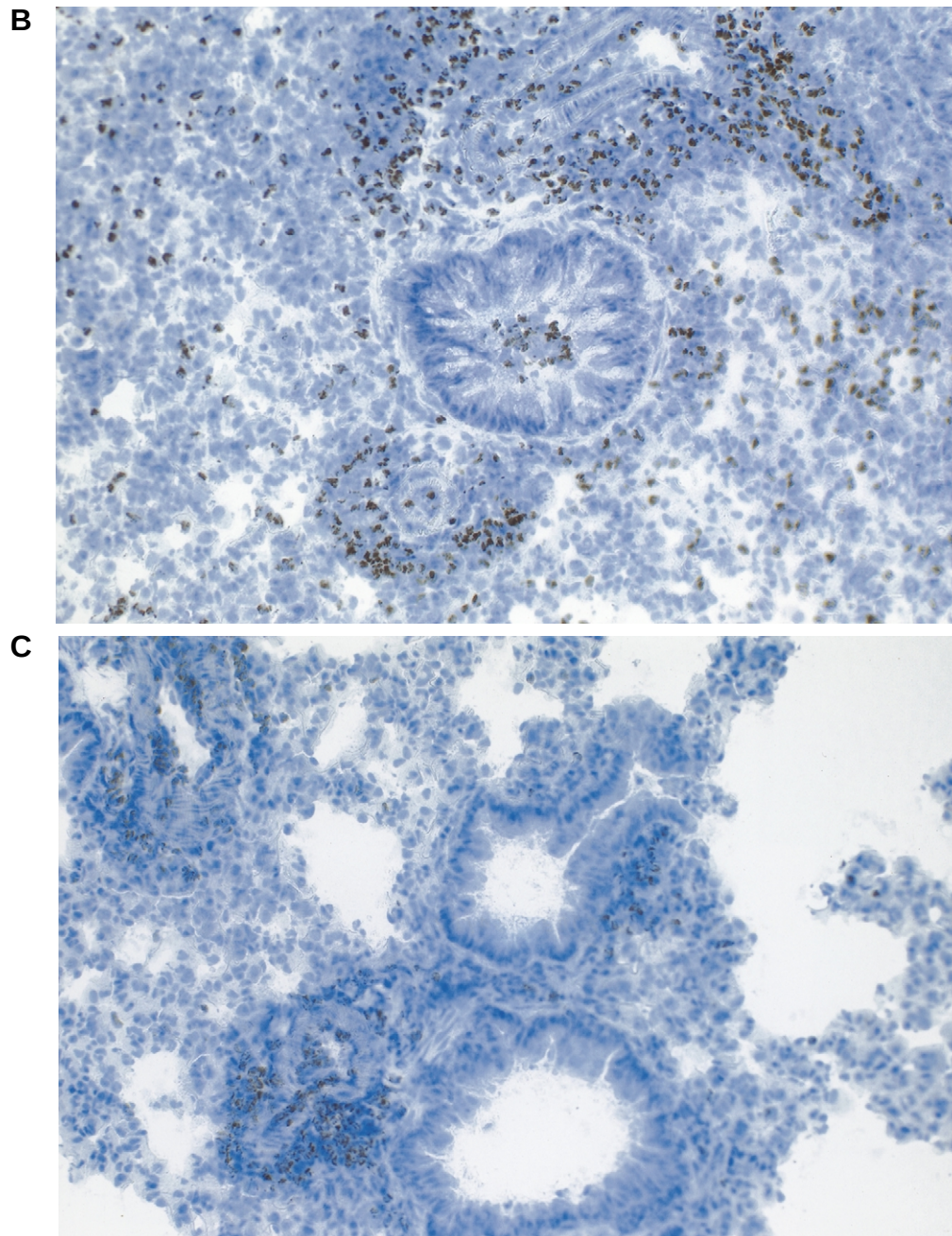


FIG 4. (continued)

DISCUSSION

1,25-(OH)₂-D₃, the active metabolite of vitamin D, has been reported to prevent or inhibit progression of different T_H1-mediated autoimmune diseases,^{13-16,29} probably by inducing a T_H1 to T_H2 shift,^{17,18} with inhibition of IL-12,³⁰ IL-2, and IFN-γ³⁻⁵ and upregulation of IL-4³¹ in mice. Previous studies have also demonstrated that doses of 50 to 100 ng of vitamin D are nontoxic and have several effects on the immune system in mice. Pakkala et al³² have shown that doses of 500 ng were lethal, with 250 ng being the highest

tolerated dose. Furthermore, less than 100 ng per day maintained serum calcium levels of less than 12 mg/dL³²⁻³⁴ and resulted in resolution of EAE,⁶ inflammatory bowel disease in mice,³⁵ and transplantation tolerance.³⁶ Because the small and safe range of vitamin D used to prevent EAE lesions in a black mouse strain, such as B10.P,¹¹ is well established, we considered 100 ng per mouse of B10.RIII strain every other day as an optimal, nontoxic, and nonhypercalcemic supplement of vitamin D administered by means of subcutaneous injection, avoiding any interference with absorption at the gastrointestinal tract.

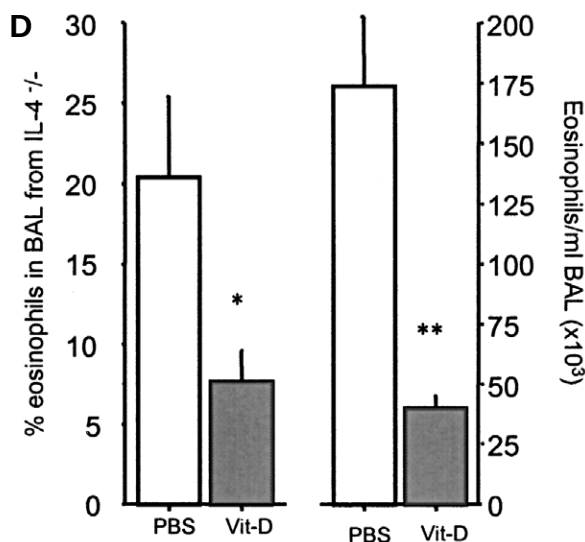


FIG 4. (continued)

Our main goal was to use this previous information in a murine model of allergic disease. This study conclusively demonstrates that vitamin D has bimodal effects in mice, both on the T_H2 immune response and on allergic inflammation. Clearly, in vivo treatment with vitamin D, in contrast to its in vitro effect, enhanced allergen-specific T-cell activation. This finding is also in accordance with the report that autoantigen (GAD65)-specific T-cell proliferation is enhanced as a result of vitamin D treatment.¹⁷ However, here we report that the T_H2 effector functions of the expanded T cells were differently affected by vitamin D. Although OVA-specific IL-4 and IL-13 production was enhanced on vitamin D treatment, secretion of IL-5, another T_H2 cytokine, was selectively inhibited. This indicates that vitamin D does not upregulate all cytokines involved in the T_H2 response after augmented priming of T_H2 cells but rather after original T-cell activation and upregulation of VDR.¹ This is further supported by the fact that the effect of vitamin D on T-cell activation and T_H2 cytokine regulation was directed toward OVA antigen but not toward irrelevant antigen (CII) or mitogen (concanavalin A). Indeed, it has been shown that IL-5 synthesis by human T_H cells is regulated selectively at the transcriptional level that differs from IL-2 and IL-4.³⁷ Subsequently, it has been reported that regulation of these cytokines is related to their different responsiveness to the transcription factor GATA-3.³⁸ The basic mechanism by which vitamin D regulates IL-5 versus IL-4 and IL-13 requires further investigation.

Moreover, we were interested to investigate whether vitamin D-dependent, OVA-specific IL-4 and IL-13 upregulation had an effect on the systemic production of IgE because IL-13 shares a common receptor with IL-4

(IL-4R) and cooperates in the regulation of IgE production.³⁹ We found that systemic IgE levels were highly enhanced as a result of vitamin D treatment, whereas IgG levels were downregulated. This decrease was mainly due to suppression of IgG1 levels, with no effect on IgG2a. Because IL-4 and IL-13 might be necessary for inducing the IgE isotype switch, the upregulation of vitamin D-dependent antigen-specific IL-4 and IL-13 production observed in our study could be closely related to the augmented levels of IgE. The upregulation of some costimulatory molecules, such as CD40 ligand, by other hormones than vitamin D has been described as a promoter of Ig switching.^{27,41} Therefore we investigated whether vitamin D had any effect on CD40 ligand expression of T cells, which could explain in part the upregulation of IgE levels. However, FACS analysis could not detect any difference in the expression of CD40 ligand between the vitamin D-treated and control groups. On the other hand, suppression of IgG levels has also been correlated with allergic inflammation. Our data are in part in agreement with earlier reports showing that vitamin D given in vivo inhibited T_H1 -induced IgG1⁷ or IgG2a⁸ in mice.

Subsequently, we were interested to explore whether the effect of vitamin D treatment differed between systemic and local inflammatory responses because this has been observed by others.^{41,42} Although IL-4 levels did not differ in BAL fluid between the vitamin D-treated and control groups, the increase in the IL-13 level was obvious. Nevertheless, the effect of vitamin D on IL-5 levels was once again prominently downregulatory. It is noteworthy that IL-5 has been postulated as the key cytokine involved in recruitment of eosinophils to the local inflammatory site in models of allergic asthma, in which the eosinophilic inflammation could be inhibited by an anti-IL-5 neutralizing antibody.^{43,44} It is well known that the expansion of IL-5-producing T_H2 cells leads to a recruitment of eosinophils,⁴⁵ and thus we investigated how eosinophilia in the airways and lung was affected by vitamin D treatment. We then observed that eosinophilia in both compartments was significantly reduced by vitamin D. This is once more supporting the role of vitamin D as a selective inhibitor of IL-5-mediated eosinophilic recruitment to local inflammatory sites. Because eosinophils are considered to be major effector cells in T_H2 -mediated diseases, such as bronchial asthma and atopic dermatitis, with the degree of eosinophilia tending to correlate with severity,⁴⁶⁻⁴⁸ vitamin D could be considered as a beneficial agent in these conditions. Indeed, it was also reported that a synthetic vitamin D analog (calcipotriene) could inhibit skin eosinophilia and epidermal proliferation in chronic proliferative dermatitis, a spontaneous mutation in C57BL/Ka mice.⁴⁹ It has also been postulated that IL-4 is involved in eosinophilia in airways in addition to being a switch factor for IgE.²⁸ To study this, we used IL-4 knockout mice. However, our data conclusively demonstrated that vitamin D-mediated inhibition of eosinophilia is IL-4 independent.

We subsequently investigated whether the modulating effect of vitamin D on parameters associated with allergy

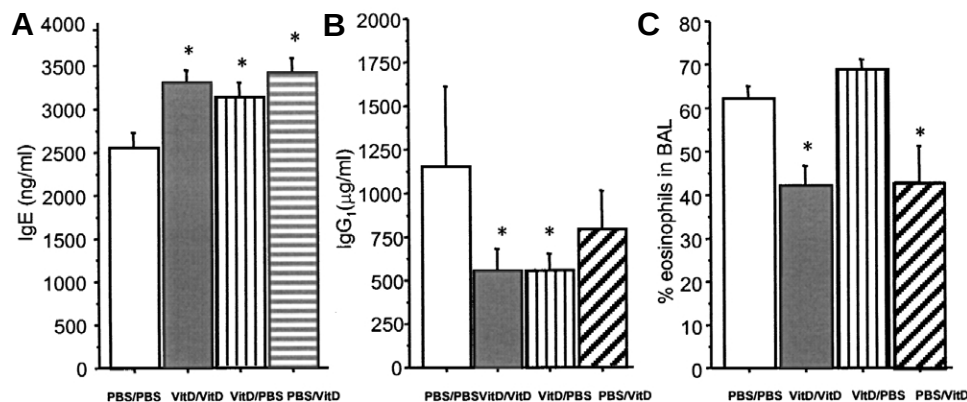


FIG 5. Time table for vitamin D's effects. **A**, IgE levels are increased in all groups treated with vitamin D. **B**, IgG1 is decreased in all groups receiving early vitamin D treatment at sensitization from day -1 to day 3. **C**, Vitamin D downregulates eosinophilia as a late-stage effect when given at challenge from day 5 to day 9. * $P < .05$.

was time dependent. Surprisingly, we observed that early treatment with vitamin D during immunization led to augmented IgE production. On the other hand, reduced levels of IL-5 with impaired recruitment of eosinophils in the airways were attributed to treatment with vitamin D after establishment of the immune response. Thus we suggest that excess supplementation with vitamin D could induce the development of profound T_H2 responses in mice, promoting allergic sensitization, whereas vitamin D might hold promising beneficial effects in established chronic inflammatory responses.

These results are of interest because they provide some evidence of a possible role of vitamin D in the development of allergic disease. Do these findings in mice have any relevance for the diet of newborn children? We think so because the mice received a daily vitamin D supplement of approximately 2 μg/kg, which is in the same dose range as the Scandinavian recommendation of 10 μg/d in children weighing 5 to 10 kg. Thus in addition to several other environmental trigger factors, dietary vitamin D should be considered as a hormone with the potential of skewing the immune system toward T_H2 -mediated immune reactions and allergic responses.

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REFERENCES

- Veldman CM, Cantorna MT, DeLuca HF. Expression of 1,25-dihydroxyvitamin D(3) receptor in the immune system. *Arch Biochem Biophys* 2000;374:334-8.
- Lemire JM, Adams JS, Kerami-Arab V, Bakke AC, Sakai R, Jordan SC. 1,25-Dihydroxyvitamin D3 suppresses human T helper/inducer lymphocyte activity in vitro. *J Immunol* 1985;134:3032-5.
- Iho S, Kura F, Sugiyama H, Takahashi T, Hoshino T. The role of monocytes in the suppression of PHA-induced proliferation and IL 2 production of human mononuclear cells by 1,25-dihydroxyvitamin D3. *Immunol Lett* 1985;11:331-6.
- Reichel H, Koeffler HP, Tobler A, Norman AW. 1 alpha, 25-Dihydroxyvitamin D3 inhibits gamma-interferon synthesis by normal human peripheral blood lymphocytes. *Proc Natl Acad Sci U S A* 1987;84:3385-9.
- Mathieu C, Bouillon R, Rutgeerts O, Vandeputte M, Waer M. Potential role of 1,25(OH)₂ vitamin D3 as a dose-reducing agent for cyclosporine and FK 506. *Transplant Proc* 1994;26:3130.
- Lemire JM, Adams JS, Sakai R, Jordan SC. 1 alpha, 25-dihydroxy-vitamin D3 suppresses proliferation and immunoglobulin production by normal human peripheral blood mononuclear cells. *J Clin Invest* 1984;74:657-61.
- Yang S, Smith C, DeLuca HF. 1 alpha, 25-Dihydroxyvitamin D3 and 19-nor-1 alpha, 25-dihydroxyvitamin D2 suppress immunoglobulin production and thymic lymphocyte proliferation in vivo. *Biochim Biophys Acta* 1993;1158:279-86.
- Lemire JM, Archer DC, Beck L, Spiegelberg HL. Immunosuppressive actions of 1,25-dihydroxyvitamin D3: preferential inhibition of Th1 functions. *J Nutr* 1995;125:1704S-8S.
- Lemire JM, Archer DC. 1,25-dihydroxyvitamin D3 prevents the in vivo induction of murine experimental autoimmune encephalomyelitis. *J Clin Invest* 1991;87:1103-7.
- Lemire JM, Adams JS. 1,25-Dihydroxyvitamin D3 inhibits the passive transfer of cellular immunity by a myelin basic protein-specific T cell clone. *J Bone Miner Res* 1992;7:171-7.
- Cantorna MT, Hayes CE, DeLuca HF. 1,25-Dihydroxyvitamin D3 reversibly blocks the progression of relapsing encephalomyelitis, a model of multiple sclerosis. *Proc Natl Acad Sci U S A* 1996;93:7861-4.
- Mattner F, Smeraldo S, Galbiati F, Muller M, Di Lucia P, Poliani PL, et al. Inhibition of Th1 development and treatment of chronic-relapsing experimental allergic encephalomyelitis by a non-hypercalcemic analogue of 1,25-dihydroxyvitamin D(3). *Eur J Immunol* 2000;30:498-508.
- Mathieu C, Laureys J, Sobis H, Vandeputte M, Waer M, Bouillon R. 1,25-Dihydroxyvitamin D3 prevents insulinitis in NOD mice. *Diabetes* 1992;41:1491-5.
- Casteels KM, Mathieu C, Waer M, Valckx D, Overbergh L, Laureys JM, et al. Prevention of type I diabetes in nonobese diabetic mice by late intervention with nonhypercalcemic analogs of 1,25-dihydroxyvitamin D3 in combination with a short induction course of cyclosporin A. *Endocrinology* 1998;139:95-102.
- Cantorna MT, Hayes CE, DeLuca HF. 1,25-Dihydroxycholecalciferol inhibits the progression of arthritis in murine models of human arthritis. *J Nutr* 1998;128:68-72.
- Cantorna MT, Hullett DA, Redaelli C, Brandt CR, Humpal-Winter J, Sollinger HW, et al. 1,25-Dihydroxyvitamin D3 prolongs graft survival without compromising host resistance to infection or bone mineral density. *Transplantation* 1998;66:828-31.
- Overbergh L, Decallonne B, Waer M, Rutgeerts O, Valckx D, Casteels KM, et al. 1alpha,25-dihydroxyvitamin D3 induces an autoantigen-specific T-helper 1/T-helper 2 immune shift in NOD mice immunized with GAD65 (p524-543). *Diabetes* 2000;49:1301-7.
- Cantorna MT, Woodward WD, Hayes CE, DeLuca HF. 1,25-dihydroxyvitamin D3 is a positive regulator for the two anti-encephalitogenic cytokines TGF-beta 1 and IL-4. *J Immunol* 1998;160:5314-9.

19. Holgate ST. The epidemic of allergy and asthma. *Nature* 1999;402:B2-4.
20. Priftanji A, Strachan D, Burr M, Sinamati J, Shkurti A, Grabocka E, et al. Asthma and allergy in Albania and the UK. *Lancet* 2001;358:1426-7.
21. Steerenberg PA, Van Amsterdam JG, Vandebriel RJ, Vos JG, Van Bree L, Van Loveren H. Environmental and lifestyle factors may act in concert to increase the prevalence of respiratory allergy including asthma. *Clin Exp Allergy* 1999;29:1303-8.
22. Matricardi PM. Prevalence of atopy and asthma in eastern versus western Europe: why the difference? *Ann Allergy Asthma Immunol* 2001;87:24-7.
23. Hijazi N, Abalkhail B, Seaton A. Diet and childhood asthma in a society in transition: a study in urban and rural Saudi Arabia. *Thorax* 2000;55:775-9.
24. Matheu V, Navikas V, Issazadeh S. Susceptibility of B10.RIII mouse strain to develop inflammatory allergic pulmonary disease. *Alergol Immunol Clin* 2001;16:282-90.
25. Smith EA, Frankenburg EP, Goldstein SA, Koshizuka K, Elstner E, Said J, et al. Effects of long-term administration of vitamin D3 analogs to mice. *J Endocrinol* 2000;165:163-72.
26. Jirapongsananuruk O, Melamed I, Leung DY. Additive immunosuppressive effects of 1,25-dihydroxyvitamin D3 and corticosteroids on TH1, but not TH2, responses. *J Allergy Clin Immunol* 2001;106:981-5.
27. Jabara HH, Brodeur SR, Geha RS. Glucocorticoids upregulate CD40 ligand expression and induce CD40L-dependent immunoglobulin isotype switching. *J Clin Invest* 2001;107:371-8.
28. Brusselle GG, Kips JC, Tavernier JH, van der Heyden JG, Cuvelier CA, Pauwels RA, et al. Attenuation of allergic airway inflammation in IL-4 deficient mice. *Clin Exp Allergy* 1994;24:73-80.
29. Casteels K, Waer M, Laureys J, Valckx D, Depovere J, Bouillon R, et al. Prevention of autoimmune destruction of syngenic islet grafts in spontaneously diabetic nonobese diabetic mice by a combination of a vitamin D3 analog and cyclosporine. *Transplantation* 1998;65:1225-32.
30. D'Ambrosio D, Cippitelli M, Cocciolo MG, Mazzeo D, Di Lucia P, Lang R, et al. Inhibition of IL-12 production by 1,25-dihydroxyvitamin D3. *J Clin Invest* 1998;101:252-62.
31. Cantorna MT, Humpal-Winter J, DeLuca HF. In vivo upregulation of interleukin-4 is one mechanism underlying the immunoregulatory effects of 1,25-dihydroxyvitamin D(3). *Arch Biochem Biophys* 2000;377:135-8.
32. Pakkala S, de Vos S, Elstner E, Rude RK, Uskokovic M, Binderup L, et al. Vitamin D3 analogs: effect on leukemic clonal growth and differentiation, and on serum calcium levels. *Leuk Res* 1995;19:65-72.
33. Zhou JY, Norman AW, Chen DL, Sun GW, Uskokovic M, Koeffler HP. 1,25-Dihydroxy-16-ene-23-yne-vitamin D3 prolongs survival time of leukemic mice. *Proc Natl Acad Sci U S A* 1990;87:3929-32.
34. Meehan TF, DeLuca HF. CD8(+) T cells are not necessary for 1 alpha,25-dihydroxyvitamin D(3) to suppress experimental autoimmune encephalomyelitis in mice. *Proc Natl Acad Sci U S A* 2002;99:5557-60.
35. Adorini L, Penna G, Casorati M, Davalli AM, Gregori S. Induction of transplantation tolerance by 1,25-dihydroxyvitamin D(3). *Transplant Proc* 2001;33:58-9.
36. Cantorna MT, Munsick C, Bemiss C, Mahon BD. 1,25-Dihydroxycholecalciferol prevents and ameliorates symptoms of experimental murine inflammatory bowel disease. *J Nutr* 2000;130:2648-52.
37. Mori A, Kaminuma O, Mikami T, Inoue S, Okumura Y, Akiyama K, et al. Transcriptional control of the IL-5 gene by human helper T cells: IL-5 synthesis is regulated independently from IL-2 or IL-4 synthesis. *J Allergy Clin Immunol* 1999;S429-36.
38. Zhang DH, Yang L, Ray A. Differential responsiveness of the IL-5 and IL-4 genes to transcription factor GATA-3. *J Immunol* 1998;161:3817-21.
39. Morawetz RA, Gabriele L, Rizzo LV, Noben-Trauth N, Kuhn R, Rajewsky K, et al. Interleukin (IL)-4-independent immunoglobulin class switch to immunoglobulin (Ig)E in the mouse. *J Exp Med* 1996;184:1651-61.
40. Ferlin WG, Severinson E, Strom L, Heath AW, Coffman RL, Ferrick DA, et al. CD40 signaling induces interleukin-4-independent IgE switching in vivo. *Eur J Immunol* 1996;26:2911-5.
41. Sullivan S, Broide DH. Compartmentalization of eosinophil granulocyte-macrophage colony-stimulating factor expression in patients with asthma. *J Allergy Clin Immunol* 1996;97:966-76.
42. Wang J, Palmer K, Lotvall J, Milan S, Lei XF, Matthaei KI, et al. Circulating, but not local lung, IL-5 is required for the development of antigen-induced airways eosinophilia. *J Clin Invest* 1998;102:1132-41.
43. Gulbenkian AR, Egan RW, Fernandez X, Jones H, Kreutner W, Kung T, et al. Interleukin-5 modulates eosinophil accumulation in allergic guinea pig lung. *Am Rev Respir Dis* 1992;146:263-6.
44. Mauser PJ, Pitman A, Witt A, Fernandez X, Zurcher J, Kung T, et al. Inhibitory effect of the TRFK-5 anti-IL-5 antibody in a guinea pig model of asthma. *Am Rev Respir Dis* 1993;148:1623-7.
45. Martin LB, Kita H, Leiferman KM, Gleich GH. Eosinophils in allergy: role in disease, degranulation, and cytokines. *Int Arch Allergy Immunol* 1996;109:207-15.
46. Bousquet J, Chanez P, Lacoste JY, Barneon G, Ghavanian N, Enander I, et al. Eosinophilic inflammation in asthma. *N Engl J Med* 1990;323:1033-9.
47. Louis R, Lau LC, Bron AO, Roldaan AC, Radermecker M, Djukanovic R. The relationship between airways inflammation and asthma severity. *Am J Respir Crit Care Med* 2000;161:9-16.
48. Obase Y, Shimoda T, Mitsuta K, Matsuo N, Matsuse H, Kohno S. Correlation between airway hyperresponsiveness and airway inflammation in a young adult population: eosinophil, ECP, and cytokine levels in induced sputum. *Ann Allergy Asthma Immunol* 2001;86:304-10.
49. Gijbels MJ, Elliott GR, HogenEsch H, Zurcher C, van den Hoven A, Bruijnzeel PL. Therapeutic interventions in mice with chronic proliferative dermatitis (cpdm/cpdm). *Exp Dermatol* 2000;9:351-8.