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**Research Article**

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## 1,25-Dihydroxyvitamin D<sub>3</sub> Prevents the In Vivo Induction of Murine Experimental Autoimmune Encephalomyelitis

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### Abstract

The hormone, 1,25-dihydroxyvitamin D<sub>3</sub> (1,25-[OH]<sub>2</sub>-D<sub>3</sub>), inhibits lymphocyte activation in vitro. We studied the ability of the vitamin D metabolite to interfere in vivo with a primary T cell-mediated model of autoimmunity, murine experimental autoimmune encephalomyelitis (EAE). Within 2 wk of antigenic challenge, immunized animals will develop acute paralysis with central nervous tissue inflammation. If mice survive, a rise in antibody titer develops within a month. The administration of 0.1 μg 1,25-(OH)<sub>2</sub>-D<sub>3</sub> i.p. given every other day for 15 d, starting 3 d before immunization, significantly prevented the development of EAE. The rise in antibody titer to myelin basic protein was also abrogated. Histopathologic lesions of EAE were inhibited by treatment with the sterol. These results suggest a potent immunosuppressive role for 1,25-(OH)<sub>2</sub>-D<sub>3</sub> in vivo in the modulation of a cell-mediated model of autoimmunity. (*J. Clin. Invest.* 1991. 87:1103–1107.) **Key words:** anti-myelin basic protein antibodies • brain lesions • dosage • survival • severity

### Introduction

The active vitamin D metabolite, 1,25-dihydroxyvitamin D<sub>3</sub> (1,25-[OH]<sub>2</sub>-D<sub>3</sub>),<sup>1</sup> has been shown to exert immunosuppressive properties in vitro (1, 2). We (3, 4) and others (5–7) have previously demonstrated a significant inhibition of DNA synthesis, immunoglobulin, and IL-2 production by activated peripheral blood mononuclear cells in the presence of increasing concentrations of the active metabolite. Despite further substantiation of its immunoregulatory role in vitro (8, 9), no data had yet confirmed a potential role of the hormone in vivo. The murine model of autoimmunity, experimental autoimmune encephalomyelitis (EAE) can easily be induced in susceptible animals with central nervous tissue extracts (10). An acute para-

lytic disease will develop within 12–15 d. If the animal survives, a rise in antibody titer to myelin basic protein (MBP) will be present within 2–4 wk of immunization (11). We now report that 1,25-(OH)<sub>2</sub>-D<sub>3</sub> given in vivo can modulate the expression of EAE and prevent the antibody production response.

### Methods

**Animals.** Mice. Inbred SJL/J females, 6–8 wk old, were obtained from The Jackson Laboratory, Bar Harbor, ME, and fed a normal or low calcium chow (Ralston Purina Co., Richmond, IN).

**Active induction of EAE.** Animals were immunized in the hind footpads with 4 mg of rat spinal cord homogenate in CFA containing 4.5 mg/ml H37 Ra Mycobacteria per mouse. Immediately after, the animals received an intravenous injection of 1 μg of Pertussigen (a gift from Dr. John A. Munoz, NIH, Hamilton, MA).

**Disease activity assessment.** Animals were examined daily for signs of disease and graded on a scale of 0 to 5 of increasing severity: 0, no abnormality; 1, floppy tail; 2, moderate hind limb weakness; 3, quadriplegia; 4, paraplegia with moderate forelimb weakness; 5, quadriplegia or premonitory state.

**Antimyelin basic protein antibody response.** Sera were obtained from animals by retroorbital bleeding at 1 mo postimmunization and assayed for anti-MBP antibody by using an ELISA as previously described (12). Sera were analyzed at serum dilutions of 1/50, 1/100, 1/300, 1/1,000, and 1/2,000.

**Serum calcium determination.** Serum calcium was determined by microcolorimetric assay, modified from total serum calcium test set (Stanbio Laboratory, Inc., San Antonio, TX). Determinations can be performed with volume as low as 2 μl, in microtiter plates, using an ELISA reader. Calcium standards were provided by Sera Chem (Fisher Diagnostics, Orangeburg, NY).

**Histological examination.** Brains from mice killed by ether inhalation at the end of the observation period were fixed in 15% formalin in balanced salt solution. Coronal sections of the brains were then stained with hematoxylin and eosin and examined by light microscopy for inflammatory cells.

**Treatment with 1,25-(OH)<sub>2</sub>-D<sub>3</sub>.** Dilutions of 1,25-(OH)<sub>2</sub>-D<sub>3</sub> (Calcijex; Abbott Laboratories, Chicago, IL) were prepared in normal saline in a volume of 0.2 ml and injected intraperitoneally on an alternate day basis. An additional group received a constant infusion of the active metabolite through an osmotic pump (Alzet 2001; Alza Corp., Palo Alto, CA) implanted subcutaneously. Control animals received normal saline.

### Results

**Optimization of treatment with 1,25-(OH)<sub>2</sub>-D<sub>3</sub>.** Recipient mice were fed a normal or low calcium diet. Each group was then submitted to treatment with 1,25-(OH)<sub>2</sub>-D<sub>3</sub>, starting 3 d before immunization with central nervous tissue. Each group of five mice received no treatment, 0.05 μg, or 0.10 μg 1,25-(OH)<sub>2</sub>-D<sub>3</sub>.

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1. **Abbreviations used in this paper:** EAE, experimental autoimmune encephalomyelitis; MBP, myelin basic protein; 1,25-(OH)<sub>2</sub>-D<sub>3</sub>, 1,25-dihydroxyvitamin D<sub>3</sub>.

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Table 1. Optimization of Therapy with 1,25-(OH)<sub>2</sub>-D<sub>3</sub>

Groups	Diet	1,25-(OH) <sub>2</sub> -D <sub>3</sub>	Serum Ca mg/dl	Treatment d	EAE No. mice	Disease Activity
A	Normal	—	8.0±0.5	—	5/5	5
B	Low Ca	—	7.9±0.5	—	5/5	5
C	Normal	.05 µg	9.1±0.6	15	5/5	5
D	Low Ca	.10 µg	9.7±0.6	15	1/5	3
E	Low Ca	.048 µg*	9.0±0.6	7	0/5	0

Recipient mice were fed a normal or low calcium chow. 1,25-(OH)<sub>2</sub>-D<sub>3</sub>, 0.05 or 0.10 µg, was given i.p. every other day for 15 d, starting 3 d before immunization. Serum calcium was measured on day 2. Disease activity represents the mean activity score based on clinical assessment of mice developing EAE per group (five mice).

\* 1,25-(OH)<sub>2</sub>-D<sub>3</sub> administered by subcutaneous pump delivering 1 µl/h of a solution of 2 µg/ml for 0.048 µg/d.

i.p. on an alternate day basis for a 15-d period. Serum calcium was measured on day 1 before and day 2 after immunization. An additional group received an osmotic pump (Alzet 2001; Alza Corp.) implanted subcutaneously 2 d before immunization to deliver 1 µl/h of a solution of 2 µg/ml of the metabolite, a total daily dose of 0.048 µg. Table I illustrates, as predicted, that animals challenged with antigen but not exposed to 1,25-(OH)<sub>2</sub>-D<sub>3</sub> developed severe disease activity whether maintained on a normal (group A) or low calcium chow (group B). While animals exposed to 0.05 µg 1,25-(OH)<sub>2</sub>-D<sub>3</sub> given every other day developed hypercalcemia, no inhibition of disease activity was observed (group C). However, when the dosage was increased to 0.10 µg, prevention of EAE was seen (group D), despite a comparable calcium level (group D vs. C). The continuous infusion administration (group E) was as effective in inhibiting disease expression while allowing for a dosage reduction (0.048 µg per 24 h).

**Suppression of EAE by 1,25-(OH)<sub>2</sub>-D<sub>3</sub>.** To further substantiate a definite suppression of the active metabolite on disease activity, antigen-challenged recipients were treated or not with

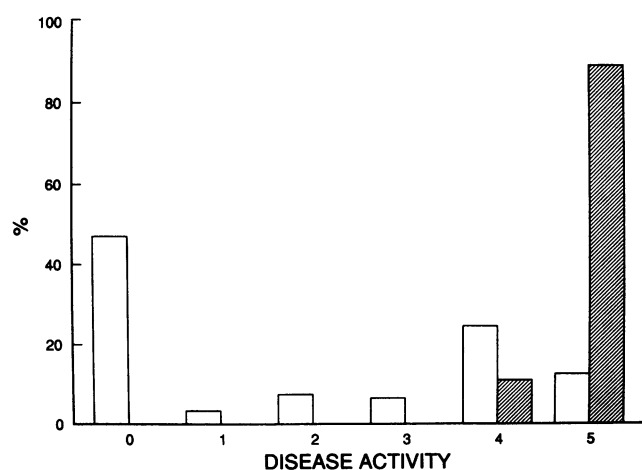


Figure 1. Percentage of mice expressing increasing disease score activity of EAE after antigenic challenge. Recipients were untreated (closed bars) or received 0.10 µg 1,25-(OH)<sub>2</sub>-D<sub>3</sub> (open bars) every other day for 15 d.

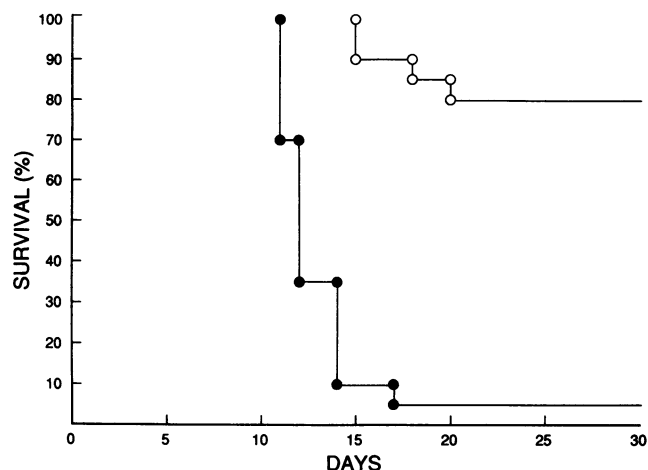


Figure 2. 1,25-(OH)<sub>2</sub>-D<sub>3</sub> prolongs survival of mice immunized with encephalitogenic doses of central nervous tissue. Naive (●) or 1,25-(OH)<sub>2</sub>-D<sub>3</sub>-treated (○) mice were challenged on day 0 with 4 mg rat spinal cord homogenate in CFA.

0.10 µg 1,25-(OH)<sub>2</sub>-D<sub>3</sub> i.p. on an alternate day basis for 15 d, starting 3 d before immunization. The study included 64 mice treated with the active metabolite while 52 mice not exposed to 1,25-(OH)<sub>2</sub>-D<sub>3</sub> served as controls. All animals were maintained on a low calcium diet. Control animals received an injection of normal saline with 0.1% ethanol, the vehicle of the hormone. As shown in Fig. 1, while none of the control group expressed a disease activity score < 4, the majority of the 1,25-(OH)<sub>2</sub>-D<sub>3</sub>-treated recipients had no or a milder disease activity. Moreover, treatment with the active vitamin D metabolite was associated with prolonged survival (Fig. 2). None of the treated animals have shown a relapse of the disease despite cessation of therapy. Recent experiments (not shown) have revealed similar outcomes despite discontinuation of treatment 5 d postimmunization.

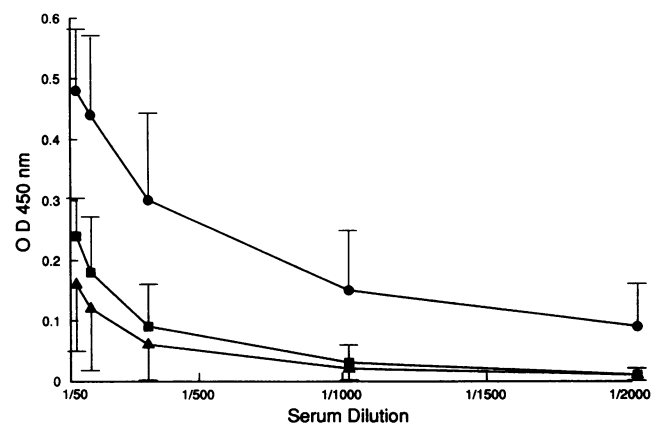
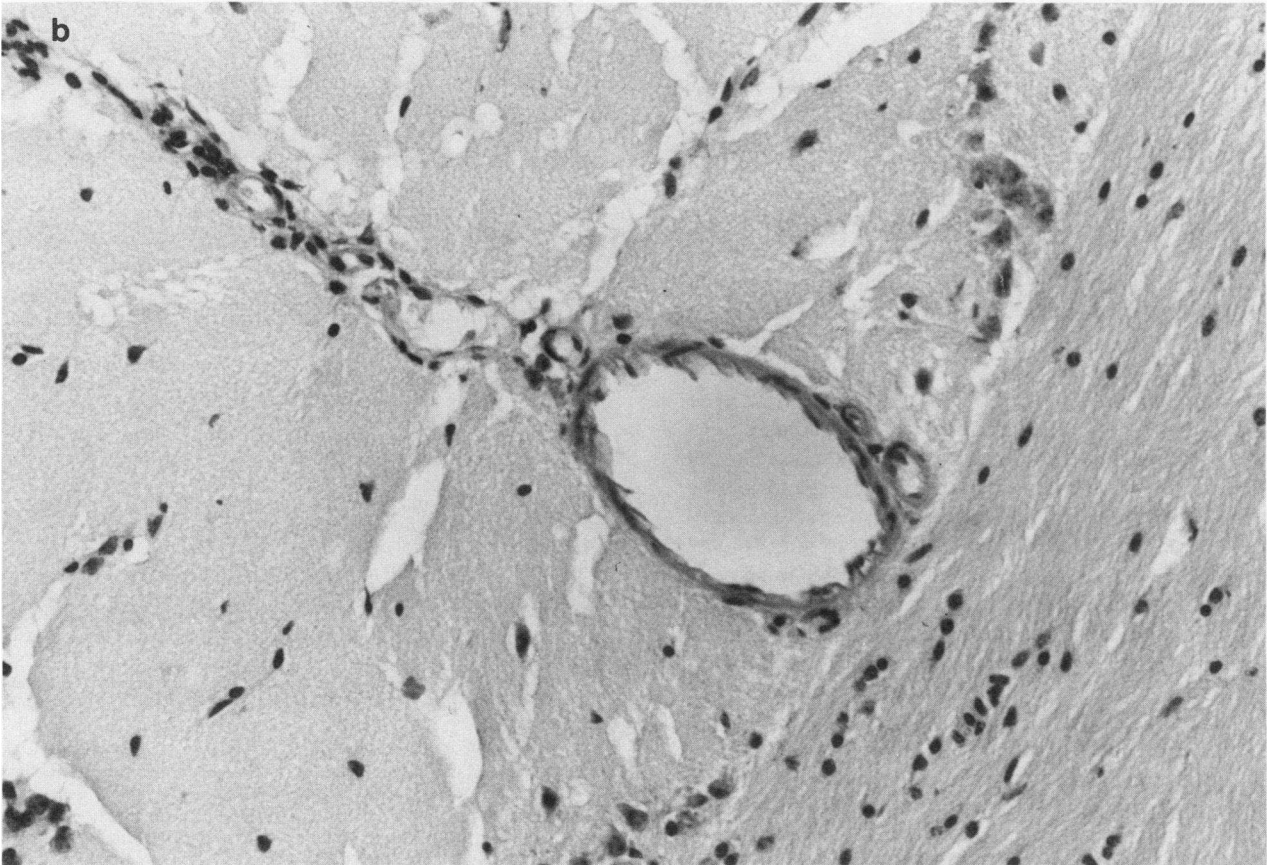
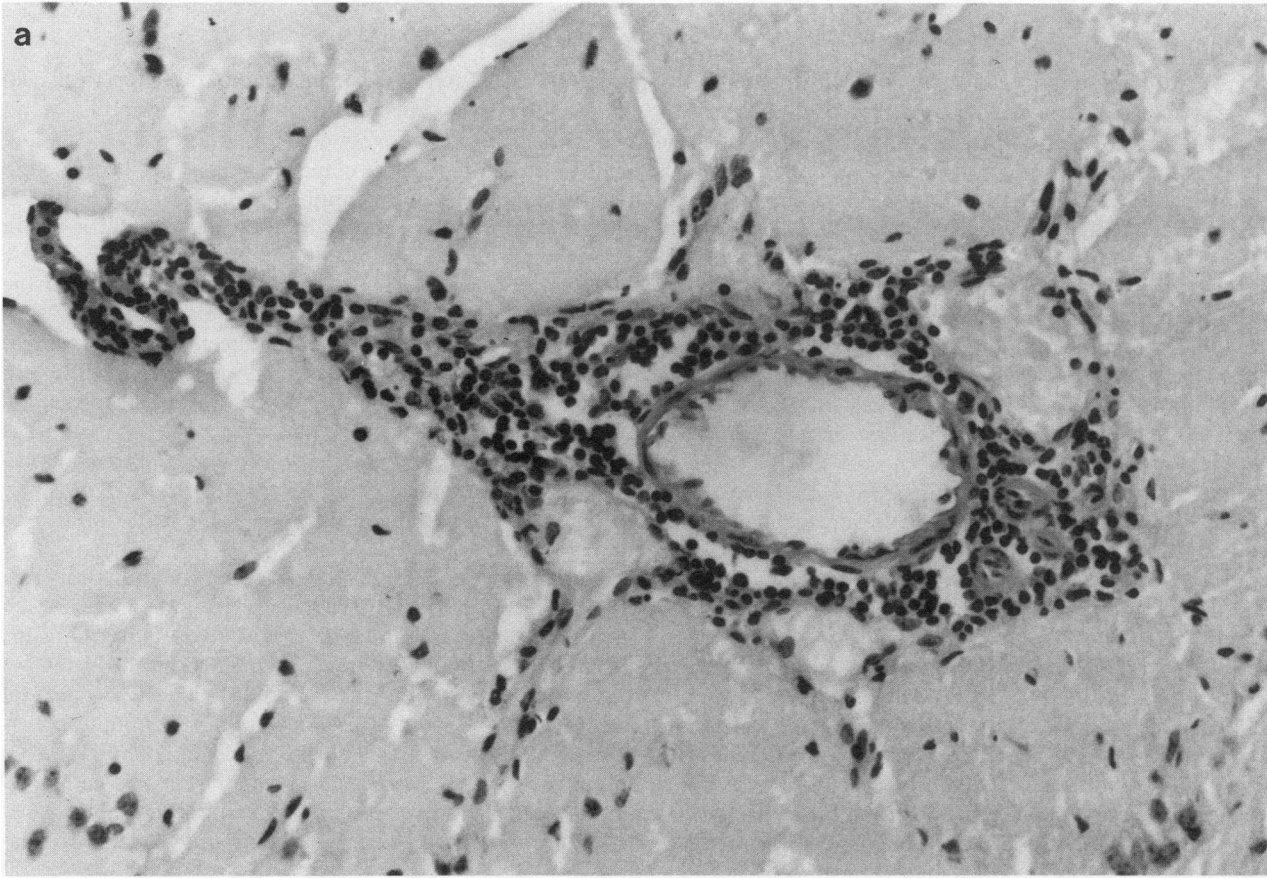


Figure 3. Inhibition of anti-MBP antibody production by 1,25-(OH)<sub>2</sub>-D<sub>3</sub>. Mice receiving 0.10 µg 1,25-(OH)<sub>2</sub>-D<sub>3</sub> every other day for 15 d (n = 64) (■, ▲) showed suppression of anti-MBP antibody production determined by ELISA compared with untreated mice (n = 12) (●) 30 d postimmunization. Disease expression (■) (n = 36) in 1,25-(OH)<sub>2</sub>-D<sub>3</sub>-treated recipients did not affect the inhibition of antibody production.



*Figure 4.* Inhibition of histologic lesions of EAE by 1,25-(OH)<sub>2</sub>-D<sub>3</sub>. Coronal sections of brains stained with hematoxylin and eosin ×200. Mice were challenged with central nervous system tissue 12 d previously and were untreated (*a*) or received 0.1 μg 1,25-(OH)<sub>2</sub>-D<sub>3</sub> every other day (*b*). Note the perivascular inflammatory reaction typical of EAE in (*a*).

*Suppression of anti-myelin basic protein antibody production by 1,25-(OH)<sub>2</sub>-D<sub>3</sub>.* Animals surviving the acute onset of EAE will first demonstrate antibodies to myelin basic protein on day 18 postimmunization (11). A pronounced humoral response is observed by day 30 and still present in animals sampled later than 200 d after antigenic challenge. Sera from control and 1,25-(OH)<sub>2</sub>-D<sub>3</sub>-treated animals were obtained on day 30 and tested for anti-myelin basic antibodies by ELISA. While sera from control animals (*n* = 12) exhibited elevated antibody titers, a dramatic inhibition of antibody production to MBP in the 1,25-(OH)<sub>2</sub>-D<sub>3</sub>-treated animals (*n* = 28) was seen. The antibody response was even abrogated in treated animals (*n* = 36) that expressed clinical disease activity (as depicted in Fig. 3).

*Inhibition of histologic lesions of EAE by 1,25-(OH)<sub>2</sub>-D<sub>3</sub>.* Control and 1,25-(OH)<sub>2</sub>-D<sub>3</sub>-treated animals were killed at day 15. The brains were carefully removed, fixed, and stained. Coronal sections were analyzed for lesions of EAE. Cellular infiltrates are usually present in the small veins of the meninges (13), as depicted in Fig. 4 a.

The lesions were characteristically present in immunized animals not exposed to 1,25-(OH)<sub>2</sub>-D<sub>3</sub>. The metabolite-treated animals challenged with the same encephalitogenic antigen that did not develop disease activity did not show any lesions of EAE (Fig. 4 b). The hormone-treated mice expressing a severe clinical disease demonstrated perivenous infiltrates if killed during the acute phase of EAE. However, these lesions were absent when the animals were killed after clinical recovery.

## Discussion

The sterol, 1,25-(OH)<sub>2</sub>-D<sub>3</sub>, has recently been shown to play a wider biological activity than in the regulation of calcium homeostasis. Cells of the immune lineage by their specific 1,25-(OH)<sub>2</sub>-D<sub>3</sub>-receptor expression upon various stages of activation are directly influenced by the hormone in vitro leading to inhibition of lymphocyte proliferation (3, 6, 8), antibody production (3, 14), IL-2 production and secretion (4, 5, 7), gamma-interferon and other lymphokines (15). This potent immunosuppressive effect, however, had yet to be substantiated in vivo.

The prodifferentiating effect of the vitamin D metabolite has prompted its use in vivo in mice with a prolongation of survival of animals injected with syngeneic leukemic cells (16, 17) and inhibition of human malignant melanoma and colonic cancer cell xenografts in immunosuppressed mice (18). A similar sterol dosage and injection regimen similar to our protocol (0.1 μg, 3 d/wk) was also used by Eisman et al. (18) but hypercalcemia was more pronounced in their animals (11.8 vs. 9.7 mg/dl). However, our observations allowed for a dissociation of the immunosuppressive effect of the sterol from the hypercalcemia produced since the disease was not prevented with lower metabolite concentration despite hypercalcemia.

The inhibitory effect of the hormone on active disease induction, antibody production, and development of histologic lesions was particularly remarkable. In experimental autoimmune encephalomyelitis, clones of T helper cells have been shown to exert multiple functions such as the passive transfer to disease and delayed-type hypersensitivity, the induction of antibody production in vivo and in vitro (12). With the preferential sensitivity of the T helper cell to the action of the hor-

mone (4, 9), 1,25-(OH)<sub>2</sub>-D<sub>3</sub> might inhibit in vivo the T cell help central to the development of this autoimmune disorder. Moreover, the significant recovery observed in the 1,25-(OH)<sub>2</sub>-D<sub>3</sub>-treated mice despite severe clinical disease could be related to their inability to develop an antibody response to the offending antigen. Anti-MBP antibodies do not appear to play a role in the induction of the disease but a potential pathophysiologic role has been suggested in the chronicity or relapses of the disease (19).

Of interest, 1,25-(OH)<sub>2</sub>-D<sub>3</sub> has not been shown to interfere with antibody production in vivo in another study (20). In experimental autoimmune thyroiditis, a state of tolerant T cells and competent B cells to the antigen thyroglobulin has been suggested (21). Antigen challenge would then trigger antibody production with no requirement for helper T cells and might explain the inability of 1,25-(OH)<sub>2</sub>-D<sub>3</sub> in that instance to inhibit antibody production.

Our observations demonstrated an immuno-inhibitory role of 1,25-(OH)<sub>2</sub>-D<sub>3</sub> in vivo on cellular and humoral responses in an animal model of autoimmunity. The exact mechanisms mediating the suppression in vivo remain to be elucidated. At this point, the hypercalcemia induced by the dosages of 1,25-(OH)<sub>2</sub>-D<sub>3</sub> required to produce optimal immunosuppression would restrict the use of the hormone for potential clinical immunosuppressive therapy. However, the development of vitamin D analogues with similar properties but with reduced hypercalcemic effects (22, 23) are currently under investigation.

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