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1,25-Dihydroxyvitamin D_3 Is a Positive Regulator for the Two Anti-Encephalitogenic Cytokines TGF-β1 and IL-4[1]

Margherita T. Cantorna, William D. Woodward,[2] Colleen E. Hayes, and Hector F. DeLuca[3]

Previously we demonstrated that 1,25-dihydroxyvitamin D_3 blocks the progression of relapsing encephalomyelitis. We now propose that 1,25-dihydroxyvitamin D_3 blocks these autoimmune symptoms by stimulating the differentiation and/or function of cells that inhibit the encephalitogenic process. To support this belief, we have found that 1,25-dihydroxyvitamin D_3 administration to mice increases IL-4 transcripts by 3- to 25-fold and TGF-β1 transcripts by 4- to 24-fold. Similarly, IL-4 and TGF-β1 transcripts were higher in the central nervous system of 1,25-dihydroxyvitamin D_3-treated mice compared with controls. The number of cells recoverable from the lymph nodes of 1,25-dihydroxyvitamin D_3-treated mice was only 50% that of controls. Overall, 1,25-dihydroxyvitamin D_3 treatment causes a net loss in the total number of lymphocytes while the number of IL-4 and TGF-β1 transcripts increased. The systemic and local increase in the expression of these two anti-inflammatory cytokines by 1,25-dihydroxyvitamin D_3 may be responsible for the ability of this drug to block encephalomyelitis. ***The Journal of Immunology*****, 1998, 160: 5314–5319.**

Multiple sclerosis (MS)[4] is a chronic autoimmune disease of unknown etiology affecting the central nervous system (CNS). Epidemiologic studies show that MS is more prevalent in temperate latitudes than at equatorial latitudes (1–3). The environmental factor that explains the link between geography and MS, however, has been elusive. In 1974, Goldberg first suggested that the amount of vitamin D available in the environment either from sunshine exposure or from the diet might affect the prevalence of MS (4). Ultraviolet irradiation of the skin is the major and ultimate source of vitamin D. Areas with low supplies of vitamin D correlate with geographic regions associated with a high risk for MS. Conversely, the prevalence of MS is lower where vitamin D is abundant, as in sunny climates, high altitudes, and areas with diets rich in fish oil (4–6).

We have recently provided strong experimental evidence that 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$), the functional metabolite of vitamin D_3, can greatly reduce or eliminate the incidence and severity of experimental autoimmune encephalomyelitis (EAE), an animal model of MS (7). EAE is mediated by $CD4^+$ T cells, and more specifically Th1 cells that recognize proteins in the CNS (8). Th1 cells secreting IFN-γ and TNF-α are associated with EAE in mice (9, 10) and with MS in humans (11). Neutralization of TNF in vivo decreases the severity of EAE in mice (12). Interestingly, neutralization of IFN-γ in vivo and the use of IFN-γ knockout mice suggest a beneficial role for IFN-γ in EAE (13, 14). A number of T cell products and the development of Th2 cells specific for CNS proteins are associated with the suppression of EAE in mice (15). In particular, TGF-β1 treatment of mice exhibiting signs of EAE has been shown to be beneficial (16). Conversely, neutralization of TGF-β1 in vivo increases the severity of EAE (17). Finally, based on work with EAE, TNF-α inhibitors and exogenous TGF-β1 have been developed as new immunotherapies for MS (18).

There are at least two possible mechanisms whereby vitamin D might prevent or decrease the severity of EAE. The functional metabolite or hormonal form of vitamin D, 1,25-$(OH)_2D_3$, has been shown to inhibit the in vitro proliferation of T cells and to decrease the in vitro production of two Th1 cytokines, IFN-γ and TNF-α (19–21). The first possibility is that 1,25-$(OH)_2D_3$ is negatively regulating encephalitogenic T cells and the cytokines they produce. A second possibility is that 1,25-$(OH)_2D_3$ is positively regulating anti-encephalitogenic cells and the cytokines they produce. These potential mechanisms are not mutually exclusive.

Experiments are described below that were designed to probe the immunobiologic mechanism(s) underlying the treatment and prevention of EAE by 1,25-$(OH)_2D_3$. We measured four cytokines pivotal for the outcome of EAE: IFN-γ, TNF-α, IL-4, and TGF-β1. Our results support the hypothesis that 1,25-$(OH)_2D_3$ acts primarily by increasing the anti-inflammatory cytokines IL-4 and TGF-β1.

Materials and Methods

Mice

The B10.PL mice were produced in our colony using breeding pairs obtained from The Jackson Laboratory (Bar Harbor, ME). During this time, the mice were maintained on Purina Chow that provides abundant amounts of vitamin D. At 6 to 7 wk of age, male and female mice were fed experimental purified diets described previously (7) that contained no vitamin D. Although the diets do not contain vitamin D, the mice were housed in rooms with fluorescent light that catalyzes vitamin D production in skin. Therefore, the mice were not vitamin D deficient. The amount of calcium in the diet was uniformly 0.25%. Mice were maintained for 1 wk on this experimental diet before use in experiments. Experiments used no less than

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[4] Abbreviations used in this paper: MS, multiple sclerosis; 1,25-$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; CNS, central nervous system; EAE, experimental autoimmune encephalomyelitis; MBP, myelin basic protein; LN, lymph node; G3PDH, glyceraldehyde-3-phosphate dehydrogenase.

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5 and up to 10 mice in each treatment group. The complete experiment with all time points were repeated at least two times but as many as seven times.

EAE induction

EAE was induced exactly as described (7). Briefly, mice were immunized s.c. with 400 μg of guinea pig myelin basic protein (MBP) in CFA. In addition, on the day of immunization and 2 days later the mice received an i.p. injection with 200 ng of pertussis toxin (List Biological Laboratories, Campbell, CA) in saline.

EAE severity was scored daily by two blinded individuals using the following scoring system: 0 = no symptoms; 1 = limp tail; 2 = paraparesis; 3 = hind limb paralysis; 4 = fore and hind limb paralysis; and 5 = moribund. Intermediate scores of $\pm$0.5 were used for mice with symptoms in between any two scores. Twenty-five to thirty percent of control B10.PL mice developed severe EAE and were sacrificed. These animals were scored as 5 on the day they were sacrificed. The future EAE scores do not include scores from sacrificed mice.

1,25-$(OH)_2D_3$ treatment

For the first experimental design, the 1,25-$(OH)_2D_3$ treatment was started the day before EAE induction. Females were fed 1,25-$(OH)_2D_3$ at 50 ng/day and the males were fed 1,25-$(OH)_2D_3$ at 200 ng/day. These doses of 1,25-$(OH)_2D_3$ were found previously to be the minimum needed to prevent EAE completely (Ref. 7, and M. T. Cantorna, J. Humpal-Winter, and H. F. DeLuca, unpublished observations). A second experimental design was to start 1,25-$(OH)_2D_3$ treatment when the tails of immunized mice first became limp (first signs of EAE, about day 7). One group of mice was treated with an i.p. injection of 1,25-$(OH)_2D_3$ (300 ng) in 0.1 ml of propylene glycol, and the other group of mice was injected with propylene glycol alone (controls). Both male and female mice injected with 1,25-$(OH)_2D_3$ were maintained on the experimental diet to which 1,25-$(OH)_2D_3$ was added to provide 50 ng per mouse daily. Control animals were provided the experimental diet throughout. A third experimental design was to allow mice to develop severe EAE (severity score of 2.5). Mice with EAE severity scores of 2.5 were randomly placed into two groups. One group of mice was treated with an i.p. injection of 1,25-$(OH)_2D_3$ (300 ng) in 0.1 ml of propylene glycol, and the other group of mice was injected with propylene glycol alone (controls). Some of the 1,25-$(OH)_2D_3$-injected and control-injected mice were used 24 h later for cytokine transcript analyses. The others were maintained on the experiment diet (controls) or on the same diet supplemented with 1,25-$(OH)_2D_3$ to provide 50 ng per mouse daily.

Cell cultures

Axillary, brachial, and inguinal lymph nodes (LN) were collected from control- and 1,25-$(OH)_2D_3$-treated mice at days 3, 7, 10, 14, and 21 postimmunization. These LNs were chosen because they were enlarged and drained the site of immunization. Collected LNs were disrupted manually using a 23-gauge needle and a pair of forceps. Total cell numbers in the LNs were determined by counting the number of lymphocytes from control- and 1,25-$(OH)_2D_3$-treated mice and dividing by the number of mice in the group. Flow cytometry of fluorescent-labeled cell populations (Thy-1, class II, CD4, and CD8) was done on LN cells from control- and 1,25-$(OH)_2D_3$-treated mice. For cytokine PCR analysis, LN cells were saved for total cellular RNA isolation. For cytokine protein determination, equal numbers of cells from control- and 1,25-$(OH)_2D_3$-treated mice were cultured in HL-1 serum-free medium (BioWhittaker, Walkerville, MD) supplemented with 2-ME (50 μM), glutamine (2 mM), penicillin (10 U/ml), and streptomycin (10 μg/ml). To stimulate cytokine production, lymphocytes were restimulated with 25 μg/ml of MBP for 24 to 72 h. Nonrestimulated LN cells did not produce cytokines. Supernatants were harvested and assayed for cytokine production using cytokine-specific ELISAs.

Isolation of spinal cord and brain samples

Groups of five to eight control and five to eight 1,25-$(OH)_2D_3$-treated mice were sacrificed and perfused with 10 to 15 ml of sterile saline. The treatments were started the day before immunization and both groups of mice were sacrificed when the controls reached EAE severity scores of $\geq$2.5. The spinal cords were either saved for histopathology or the spinal cords and brains were pooled and manually homogenized in saline using a Dounce homogenizer. The debris was allowed to settle and the cells were resuspended in a 70% Percoll (Pharmacia, Piscataway, NJ) solution. The cell/Percoll suspension was overlaid with a 30% Percoll solution and spun for 15 min at 500 $\times$ *g*. Lymphocytes were collected from the 30/70% interface (22, 23). The lymphocytes were washed twice and relayered onto Histopaque 1083 (Sigma, St. Louis, MO) to remove contaminating red cells and additional debris. The lymphocytes were again collected from the interface, washed twice, and saved for the isolation of total cellular RNA.

Histopathology

Spinal cords from unimmunized B10.PL mice or from B10.PL mice treated with 1,25-$(OH)_2D_3$ or controls were removed and fixed in 10% formalin. Paraffin-embedded tissue sections were prepared and stained in luxol fast blue and periodic acid Shiffs reagent by the University of Wisconsin Veterinary Science Department. Stained sections were viewed by two individuals who were blinded as to the source of the spinal cords. Inflammation was scored as follows: 0, no sign of inflammation; 1, mild inflammation; 2, discrete lesions with substantial inflammation; and 3, multiple lesions with extensive inflammation.

Cytokine ELISAs

ELISAs were used to measure IFN-γ, TNF-α, and TGF-β1 (24). The IFN-γ ELISA was performed exactly as described (25). The capture and detection Abs for the TNF-α ELISA were purchased from Genzyme (Cambridge, MA). Color development was with avidin-β-D-galactosidase and *p*-nitrophenyl-β-D-galactosidase. Murine TGF-β1 was detected using the human TGF-β1 kit exactly as described by Promega (Madison, WI). The ELISA detection limits were 1 ng/ml IFN-γ, 50 pg/ml TNF-α, and 500 pg/ml TGF-β1.

Transcript analysis by quantitative competitive PCR (QC-PCR)

Cells for mRNA analysis were dissolved in acid guanidinium thiocyanate, and total RNA was isolated by the phenol chloroform extraction method (26). Total cellular RNA was reverse transcribed using oligo(dT) primers, according to the manufacturer's protocols (Promega) and quantitated by competitive PCR. Primers and mimic DNA specific for glyceraldehyde-3-phosphate dehydrogenase (G3PDH), IL-4, IFN-γ, TNF-α, and TGF-β1 were obtained from Clontech Laboratories (Palo Alto, CA) (27, 28). Competitive cDNA mimics, which included the G3PDH, IL-4, TNF-α, IFN-γ, and TGF-β1 primer sequences adjoining a neutral DNA segment, were serially diluted and added to test cDNA aliquots (26, 27). The authentic product to mimic bp sizes were 983/600 for G3PDH, 306/544 for IL-4, 333/500 for IFN-γ, 354/500 for TNF-α, and 525/390 for TGF-β1. The mixture was amplified under predetermined optimal conditions and the products were resolved by 1.5% agarose gel electrophoresis and ethidium bromide stained. The cytokine bands were identified by size with respect to m.w. standards. The mimic DNA dilution that yielded a band with a fluorescence intensity that matched the cytokine band was used to calculate cytokine cDNA copy number. The G3PDH transcript quantitation served as a control for reverse transcription efficiency. Values are reported as cytokine cDNA copies per 1000 copies of G3PDH cDNA.

Statistics

Where possible, values reported were averages from multiple mice or experiments. Because of the variability in EAE induction, peak severity, and cytokine gene expression from one experiment to another, some values were reported as the values from one representative experiment of two to seven experiments. Again where possible statistical analyses were done using a statistics program for the Macintosh computer (STATVIEW STUDENT). The unpaired two group Student's *t* test (and confirmed using the Mann-Whitney *U* test) was done and values of $\rho < 0.05$ were considered statistically significant.

Results

Histopathology

When the 1,25-$(OH)_2D_3$ treatment was started at the same time as EAE induction, the spinal cord sections looked identical to unimmunized B10.PL spinal cords (data not shown). The absence of inflammatory infiltrates from spinal cord sections of 1,25-$(OH)_2D_3$-treated mice reflected the absence of EAE symptoms (7). When 1,25-$(OH)_2D_3$ was started at the first symptoms of EAE, the histology scores from control mice were significantly ($\rho = 0.0001$) higher than those from 1,25-$(OH)_2D_3$-treated mice (Table I). The differences between the control- and 1,25-$(OH)_2D_3$-treated animal histology scores at day 14 posttreatment were less striking and statistically insignificant (Table I). In all cases, the histopathology scores reflected the visual EAE severity scores in our mice (Table I).

Table I. *Histopathology of spinal cords from 1,25-$(OH)_2D_3$-treated and control mice*

Days Postimmunization	Days Posttreatment	Histopathology Score[a]		EAE Severity	
		Control	1,25-$(OH)_2D_3$	Control	1,25-$(OH)_2D_3$
14	7	2.4 ± 0.5[b] (n = 5)	0.3 ± 0.4 (n = 3)	4.5 ± 0.0[b] (n = 5)	0.8 ± 0.6 (n = 3)
21	14	1.6 ± 0.8 (n = 8)	1.0 ± 0.7 (n = 3)	1.8 ± 1.0 (n = 8)	0.5 ± 0.7 (n = 3)

[a] Inflammation along the CNS was scored blindly and given a value of 0, 1, 2, or 3 for the degree of inflammation. EAE was scored as previously described (7). Numbers are the mean ± SD for each group of mice (n).
[b] Control values are significantly higher than 1,25-$(OH)_2D_3$ values.

Total cell numbers in the LN

At various times postimmunization, the LNs draining the site of injection from four to eight mice were collected, pooled, and counted. The number of cells recoverable in the LNs of control mice started between 1 and 2 × 10^7 cells/mouse and doubled by 10 to 14 days after immunization (Fig. 1*A*). Sampling on days 14 and 21 was repeated three and four times, respectively. LNs of control mice yielded 2.6 ± 1.3 (10^7) cells/mouse on day 14 and 3.9 ± 0.2 (10^7) cells/mouse on day 21 postimmunization. The number of cells recoverable from 1,25-$(OH)_2D_3$-fed mice did not increase with time postimmunization (Fig. 1*A*). LNs of 1,25-$(OH)_2D_3$-fed mice yielded 1.1 ± 0.5 (10^7) cells/mouse on day 14 and 1.8 ± 0.7 (10^7) cells/mouse on day 21 postimmunization. The control cell yields were significantly (ρ = 0.008) higher than 1,25-$(OH)_2D_3$ cell yields by day 21 postimmunization. 1,25-$(OH)_2D_3$ treatment inhibited the in vivo expansion of LN cells both when 1,25-$(OH)_2D_3$ treatment was started at the time of immunization (Fig. 1*A*) and 1 wk after immunization (Fig. 1*B*). Although the total number of cells recoverable from the LN of 1,25-$(OH)_2D_3$-treated mice was fewer than from controls, the proportion of $CD4^+$, $CD8^+$, Thy-1+7, and class II^+ cells were not different in the control- and 1,25-$(OH)_2D_3$-treated animals (data not shown).

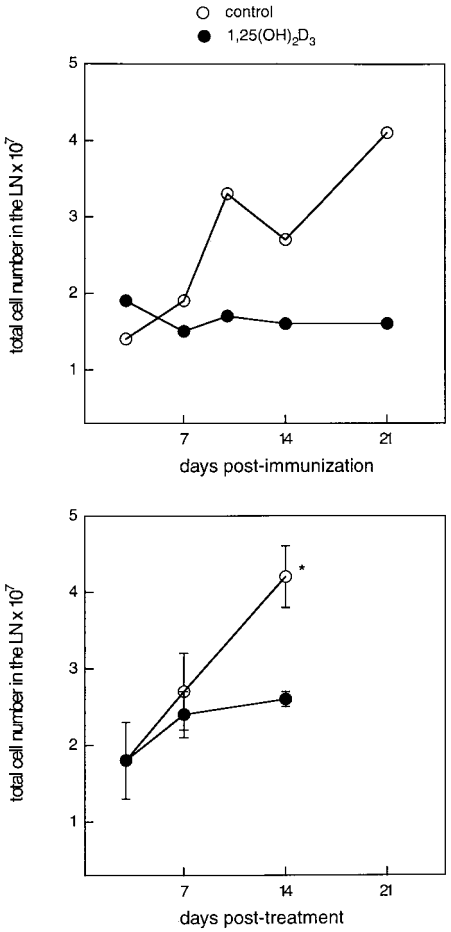

FIGURE 1. The total cells recoverable from the draining LN of control- and 1,25-$(OH)_2D_3$-treated mice. *A*, Control and 1,25-$(OH)_2D_3$ treatments were started the day before EAE induction. *B*, Control and 1,25-$(OH)_2D_3$ treatments were started at the first symptoms of EAE. The experiment in *B* was repeated three times with four to six mice per group. *, Control value is significantly ($\rho \leq 0.001$) higher than the 1,25-$(OH)_2D_3$ value.

IFN-γ and TNF-α

IFN-γ and TNF-α secretion was quantitated by assaying the amount of protein secreted in response to MBP in vitro. IFN-γ secretion was first detected 7 days postimmunization and peaked 10 days postimmunization (Fig. 2*A*). IFN-γ production from the LN cells of 1,25-$(OH)_2D_3$-treated mice was the same or in one case higher than IFN-γ secretion from the LN cells of control mice (Fig. 2*B*). The magnitude of IFN-γ secreted varied greatly from experiment to experiment but the amount of IFN-γ secreted in cells from 1,25-$(OH)_2D_3$-treated mice was in all but one case (Fig. 2*B*, 15 of 16 experiments) identical to that of controls. The number of IFN-γ transcripts was the same or lower (statistically not different) in CNS samples from 1,25-$(OH)_2D_3$-treated mice when compared with CNS samples from controls (Table II). Overall, the IFN-γ response of the LN cells reflected the IFN-γ response of cells from the CNS.

T cell-derived (MBP-specific) TNF-α secretion was first detected after 14 days of immunization, and TNF-α decreased thereafter (data not shown). On day 14 postimmunization, cells from control mice secreted 242 ± 15 pg/ml of TNF-α while cells from 1,25-$(OH)_2D_3$-treated mice secreted 194 ± 59 pg/ml of TNF-α. Although the differences were small, the amount of TNF-α secretion by LN cells from control mice was always higher (10 of 10 experiments) than that from 1,25-$(OH)_2D_3$-treated mice (data not shown). In the CNS, the number of TNF-α transcripts detectable from 1,25-$(OH)_2D_3$- and control-treated mice was low and not significantly different (Table II).

TGF-β1 and IL-4

Transcripts for IL-4 were first detected in the LN 21 days after immunization. Two weeks of 1,25-$(OH)_2D_3$ treatment increased IL-4 transcripts 25-fold over the controls (Fig. 3). Although the overall magnitude of the IL-4 response varied from experiment to experiment, this was a highly reproducible result. In six of six experiments, long-term 1,25-$(OH)_2D_3$-treatment increased IL-4 transcripts 3–25-fold higher then controls. IL-4 transcripts were detectable in samples from 1,25-$(OH)_2D_3$-treated but not control

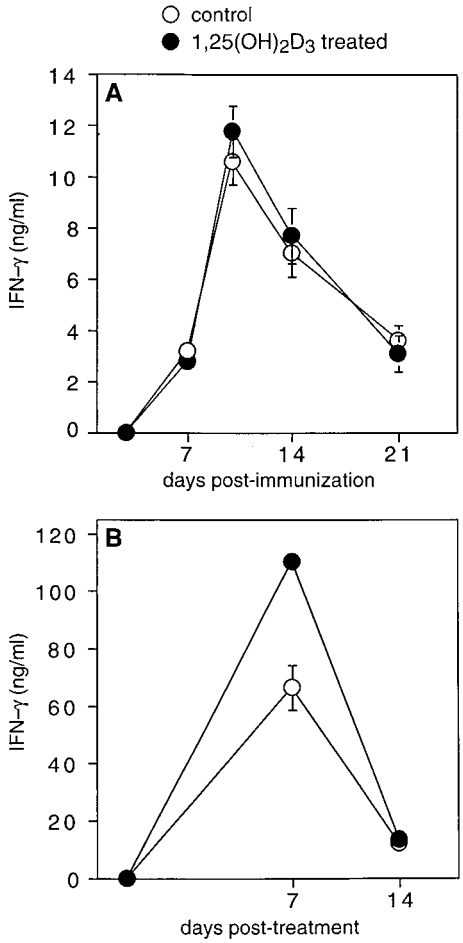

FIGURE 2. MBP-specific IFN-γ secretion in LN cells from control- or 1,25-$(OH)_2D_3$-treated mice. *A*, Control and 1,25-$(OH)_2D_3$ treatments were started the day before EAE induction. *B*, Control and 1,25-$(OH)_2D_3$ treatments were started at the time the first symptoms of EAE appeared. One representative experiment of three is presented. Each value represents the result from six to eight pooled mice. Values are reported as the mean ± SD of triplicate cultures.

CNS samples. This was a significant difference (Table II, $\rho = 0.0002$).

TGF-β1 transcripts were first detected in 1,25-$(OH)_2D_3$-treated mice at 14 days postimmunization (Fig. 4). Eightfold more TGF-β1 transcripts were detected in the LNs of 1,25-$(OH)_2D_3$-treated mice than controls 21 days after immunization (Fig. 4). Although the overall magnitude of the response varied from experiment to experiment, this was a highly reproducible result. In

Table II. *1,25-$(OH)_2D_3$ in vivo increases IL-4 and TGF-β1 mRNA in spinal cord and brain samples*[a]

	Control	1,25-$(OH)_2D_3$
IFN-γ/1000 G3PDH	85 ± 15	42 ± 11
TNF-α/1000 G3PDH	9 ± 3	20 ± 5
TGF-β1/1000 G3PDH	27 ± 8	171 ± 49[b]
IL-4/1000 G3PDH	0 ± 0	57 ± 0.3[b]

[a] QC-PCR of mRNA from the spinal cord and brain of control and 1,25-$(OH)_2D_3$-treated mice. Samples were taken when the EAE severity of control mice was ≥2.5. Values are the mean ± SEM of triplicate experiments. Each experiment used spinal cords and brains from six to eight pooled mice.

[b] Values from 1,25-$(OH)_2D_3$-treated mice are significantly higher than the controls; $p = 0.0002$.

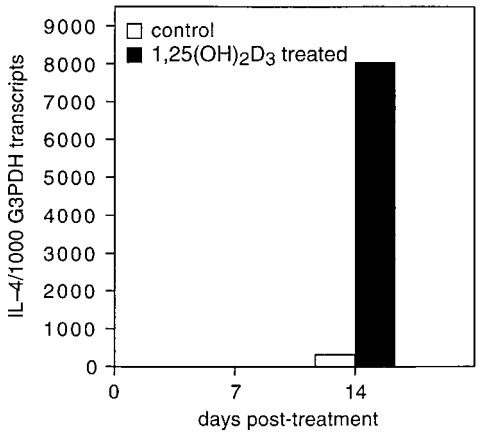

FIGURE 3. IL-4 transcript levels in the LN of control- or 1,25-$(OH)_2D_3$-treated mice. Control and 1,25-$(OH)_2D_3$ treatments were started when the first symptoms of EAE appeared. One representative experiment of three is presented. Each value represents the result from seven to nine pooled mice.

seven of seven experiments, TGF-β1 transcripts were 4- to 24-fold higher in LN of mice treated long-term with 1,25-$(OH)_2D_3$ compared with controls. Similarly, TGF-β1 transcripts were 5- to 6-fold higher (significant $\rho = 0.05$) in CNS samples from 1,25-$(OH)_2D_3$-treated mice than in controls (Table II). Because TGF-β1 is extensively posttranscriptionally regulated, we attempted to confirm our transcript measurements by quantitating TGF-β1 protein. MBP-specific TGF-β1 protein was undetectable from LN samples of 1,25-$(OH)_2D_3$-fed and control-fed mice at any time postimmunization (data not shown). LPS stimulation of resident peritoneal cells from 1,25-$(OH)_2D_3$-treated mice did make detectable TGF-β1 protein (630 ± 70 pg/ml), while peritoneal cells from control-treated mice did not (≤500 pg/ml).

Rapid in vivo effects of 1,25-$(OH)_2D_3$ treatment

1,25-$(OH)_2D_3$ treatment of mice with severe EAE caused a rapid decline in their symptoms (Fig. 5) compared with controls. Three of 10 controls but only 1 of 10 1,25-$(OH)_2D_3$-treated mice developed severe EAE (scored 5) and were sacrificed within 3 to 5 days of treatment. By day 6 post-1,25-$(OH)_2D_3$-treatment, the remaining 7 controls had more severe EAE ($\rho \leq 0.05$) than the 9 remaining 1,25-$(OH)_2D_3$-treated mice. The 7 control mice underwent a short-lived recovery at day 11 posttreatment (significant $\rho = 0.01$, then day 10 EAE scores), followed by a relapse. The 1,25-$(OH)_2D_3$-treated mice maintained a low level of symptoms as long as the diet was continuously supplemented with 1,25-$(OH)_2D_3$. Twenty-four hours after 1,25-$(OH)_2D_3$ treatment, the LN and serum were collected from 5 individual mice per treatment group. Serum calcium was raised slightly in the 1,25-$(OH)_2D_3$-treated mice, confirming the effectiveness of the treatment (Table III). IFN-γ and TNF-α transcripts were not significantly different following 1,25-$(OH)_2D_3$ treatment (Table III). IL-4 transcripts were fourfold higher (although statistically not different) in the 1,25-$(OH)_2D_3$-treated mice compared with controls. TGF-β1 transcripts were fivefold higher and significantly ($\rho \leq 0.04$) higher in mice receiving a single injection of 1,25-$(OH)_2D_3$ 24 h before sacrifice.

Discussion

Our results provide strong evidence that in vivo 1,25-$(OH)_2D_3$ treatment arrests the expansion of cells in the LNs of mice with EAE. In addition, histopathologic evaluation of spinal cords revealed less inflammation in 1,25-$(OH)_2D_3$-treated mice compared

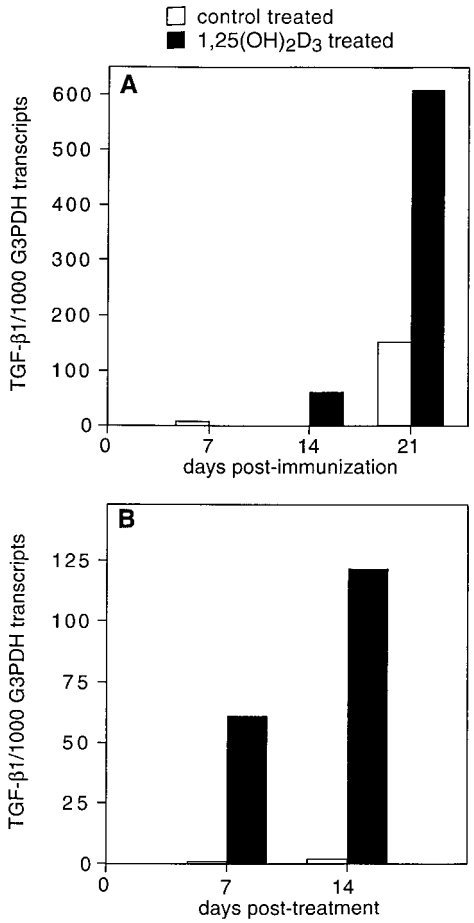

FIGURE 4. TGF-β1 transcripts in the LN control- or 1,25-$(OH)_2D_3$-treated mice. *A*, Control and 1,25-$(OH)_2D_3$ treatments were started the day before EAE induction. *B*, Control and 1,25-$(OH)_2D_3$ treatments were started at the times the first symptoms of EAE appeared. One representative experiment of three is presented. Each value represents the result from six to nine pooled mice.

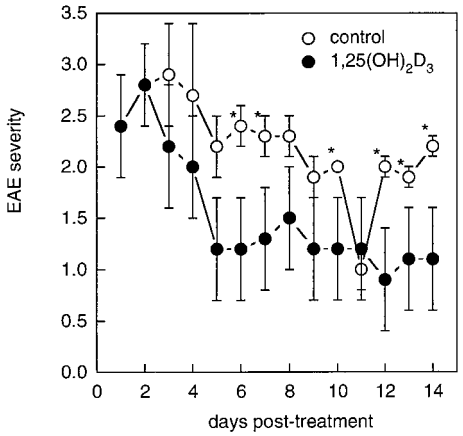

FIGURE 5. 1,25-$(OH)_2D_3$ treatment of EAE. 1,25-$(OH)_2D_3$ treatment of paralyzed mice decreased the severity and stopped the progression of EAE. Values are the mean EAE severity score of 10 mice per treatment group. One representative of two experiments is presented. *, Significantly different $\rho \leq 0.05$.

with controls. The various 1,25-$(OH)_2D_3$ treatments did not affect the amount of IFN-γ or TNF-α made on a per cell basis at any stage of EAE disease. However, control mice had as much as twice the number of mononuclear cells in the LNs than 1,25-$(OH)_2D_3$-treated mice, and the net effect for control mice would be a Th1 response that is twice that of the 1,25-$(OH)_2D_3$-treated mice. We conclude that 1,25-$(OH)_2D_3$ is not negatively regulating the Th1 cells that drive the encephalitogenic process, although this hormone may down-regulate the Th1 response through a modest effect on cellular numbers.

TGF-β1 is a potent modulator of cell growth (29). This cytokine inhibits the proliferation and differentiation of T and B cells (29), and antagonizes the effects of specific inflammatory cytokines, including TNF-α, IFN-γ, IL-1, IL-2, and IL-6 (30). More specifically, TGF-β1 is a critical regulator of autoimmune disease (30). Exogenous TGF-β1 can delay the onset and decrease the severity of EAE and arthritis in mice (16, 30, 31). Conversely, neutralization of TGF-β1 in vivo increases the severity of these two autoimmune diseases in the mouse (17, 30). In the present investigation, supplementation with 1,25-$(OH)_2D_3$ increased the number of transcripts of TGF-β1 mRNA in mononuclear cells both from sensitized LN and from affected central nervous tissue. This phenomenon was apparent in LN cells regardless of the state of disease at the time the hormone was first administered. Moreover, 1,25-$(OH)_2D_3$ was effective within only 24 h, an outcome that suggests a direct influence on cytokine production. Our current hypothesis, therefore, is that the actions of 1,25-$(OH)_2D_3$ in preventing and treating EAE may be in part a result of positive regulation of TGF-β1.

We did not detect an MBP-specific increase in TGF-β1 protein in vitro, but LPS stimulated TGF-β1 protein secretion in peritoneal exudate cells of mice given 1,25-$(OH)_2D_3$ supplements. It therefore seems likely that in our experiments TGF-β1 is coming from a macrophage and not from a T cell. LN cells are greater then 90% lymphocytes and have few macrophage cells relative to peritoneal cells. This is important in view of the finding that macrophage cells constitutively produce vitamin D receptor, while among T cells, activation is required for expression of the vitamin D receptor (32, 33). It seems likely that in EAE, 1,25-$(OH)_2D_3$ may be up-regulating the production of TGF-β1 by macrophage cells.

Long-term in vivo 1,25-$(OH)_2D_3$ treatment increased IL-4 transcripts in the LN and CNS compared with controls. Our data suggest that the effect of 1,25-$(OH)_2D_3$ on IL-4 may be indirect. The increase in IL-4 24 h after 1,25-$(OH)_2D_3$ injection was not significantly different from controls (Table III). We hypothesize that 1,25-$(OH)_2D_3$-driven TGF-β1 production by macrophage cells might make the T cell microenvironment more conducive to Th2-type cell differentiation. Experiments are underway to address this point.

Table III. *In vivo effect of 1,25-$(OH)_2D_3$ on cytokine transcript number*

	Mice[a]	
	Control	1,25-$(OH)_2D_3$
Serum Ca	8.4 ± 0.4	9.2 ± 0.4
TGF-β1/1000 G3PDH	38 ± 11	190 ± 51[b]
IL-4/1000 G3PDH	729 ± 280	2815 ± 1105
IFN-γ/1000 G3PDH	7.4 ± 1.5	7.3 ± 1.9
TNF-α/1000 G3PDH	17 ± 10	34 ± 11

[a] Control immunized mice with EAE severity scores of 2.5 were randomly split into two groups and 1,25-$(OH)_2D_3$ or control injected. Twenty-four hours later animals were sacrificed and the LN and serum of both groups of animals were saved for analyses. Values are the mean ± SEM of five mice.

[b] 1,25-$(OH)_2D_3$ treatment significantly increased the production of TGF-β1 compared to the controls.

We have previously shown that 1,25-$(OH)_2D_3$ can prevent and diminish the severity of EAE (7). The present results suggest that 1,25-$(OH)_2D_3$ acts by positively regulating the anti-encephalitogenic cytokines IL-4 and TGF-β1. We were unable to obtain results consistent with a direct effect of 1,25-$(OH)_2D_3$ on the encephalitogenic Th1 effector cell in terms of either IFN-γ or TNF-α expression. As a result of our 1,25-$(OH)_2D_3$ treatments, the numbers of cells recoverable from the LN and the number of inflammatory lesions discernible in histopathology sections were dramatically reduced. This could be due, in part, to increased production of TGF-β1, a cytokine known to inhibit T and B cell proliferation (16, 17). Based on the ability of TGF-β1 to ameliorate EAE (16), we hypothesize that 1,25-$(OH)_2D_3$-mediated up-regulation of TGF-β1 production might be the immunobiologic mechanism underlying the efficacy of 1,25-$(OH)_2D_3$ for halting the progression of EAE.

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