1,25-Dihydroxyvitamin D₃ reversibly blocks the progression of relapsing encephalomyelitis, a model of multiple sclerosis

(vitamin D/vitamin D deficiency/myelin basic protein/autoimmunity)

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ABSTRACT Experimental autoimmune encephalomyelitis (EAE) is an autoimmune disease believed to be a model for the human disease multiple sclerosis (MS). Induced by immunizing B10.PL mice with myelin basic protein (MBP), EAE was completely prevented by the administration of 1,25-dihydroxyvitamin D₃ [1,25-$(OH)_2D_3$]. 1,25-$(OH)_2D_3$ could also prevent the progression of EAE when administered at the appearance of the first disability symptoms. Withdrawal of 1,25-$(OH)_2D_3$ resulted in a resumption of the progression of EAE. Thus, the block by 1,25-$(OH)_2D_3$ is reversible. A deficiency of vitamin D resulted in an increased susceptibility to EAE. Thus, 1,25-$(OH)_2D_3$ or its analogs are potentially important for treatment of MS.

Multiple sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system (CNS) that is certainly debilitating and can ultimately be fatal. This disease is characterized by a chronic course of relapses followed by periods of stability. Thus far, there is no clear method of treating this disorder.

One of the most useful models of this disease is murine experimental autoimmune encephalomyelitis (EAE). In this model, inflammatory T-helper type 1 (Th1) cells specific for myelin basic protein (MBP) or other CNS autoantigens penetrate the CNS and become activated by interacting with tissue antigen-presenting cells displaying autoantigenic epitopes (1, 2). An immune-mediated attack on the oligodendrocytes that synthesize MBP or other CNS proteins by the Th1 cells and the tissue macrophages (microglial cells) that become activated through Th1 cell cytokine action most probably mediates demyelination. Therefore, the EAE model presents an opportunity of determining the impact of various agents that might be useful in therapy or in prevention.

Our laboratory has been involved in studying the role of vitamin D beyond its calcemic actions (3). The first indication that vitamin D might modulate immune function was the discovery that peripheral blood cells have 1,25-dihydroxyvitamin D₃ [1,25-$(OH)_2D_3$] receptors (4, 5). Further, when added to mitogen-stimulated human peripheral blood lymphocytes *in vitro*, 1,25-$(OH)_2D_3$ inhibits their proliferation, their immunoglobulin synthesis, and their accumulation of transcripts for the interleukins 1, 2, and 6, tumor necrosis factors α and β, and interferon γ (6–9). Of considerable interest is that 1,25-$(OH)_2D_3$ induces promyelocytes to differentiate into monocytes (10) and thus there appears to be a possible complex role of 1,25-$(OH)_2D_3$ in the immune system. This is further reflected by *in vivo* experiments. Both excess of vitamin D and vitamin D deficiency suppress the delayed hypersensitivity response (11). Similarly, both vitamin D supplementation and vitamin D deficiency suppress immunoglobulin production (11). In contrast, selected antibody responses could be increased by topical 1,25-$(OH)_2D_3$ (12). In view of the complex

and divergent responses reported, it is unclear whether 1,25-$(OH)_2D_3$ can be used in a therapeutic mode.

Immunizing strain SJL/J mice having normal intakes of vitamin D with 5 mg of MBP has been used to induce EAE (13, 14). By placing these animals on a low calcium diet and supplying exogenous 1,25-$(OH)_2D_3$, survival of the mice could be prolonged but death could not be prevented (13, 14). The cause of death in these immunized SJL mice is uncertain, and it is unclear whether the low calcium diet or the supplemental 1,25-$(OH)_2D_3$ provided the benefit. Nevertheless, this model appeared to be of interest to study the action of 1,25-$(OH)_2D_3$ and its analogs in the autoimmune responses. Here we report a striking action of 1,25-$(OH)_2D_3$ as a reversible inhibitor of established EAE.

MATERIALS AND METHODS

Mice. The B10.PL mice were produced in our colony; the breeding pairs were obtained from The Jackson Laboratory. Experiments used weight-matched B10.PL male and female mice, which were 5 to 8 weeks of age and raised on mouse chow (Purina). In some experiments, mice were fed an experimental diet containing either no additional vitamin D or 20 ng per day per mouse of 1,25-$(OH)_2D_3$ (15, 16). In one experiment, only female mice were used (see Fig. 3).

EAE Disease Induction. MBP was isolated from guinea pig spinal cords following the procedure of Diebler *et al.* (17). MBP was lyophilized and stored at −20°C. For immunizations, MBP was dissolved in 0.1 M acetic acid at a concentration of 8 mg/ml. Mice anesthetized with ether were immunized subcutaneously with 0.1 ml of MBP (400 μg per mouse) emulsified in an equal volume of CFA (Difco) containing *Mycobacterium tuberculosis* H37 Ra. In addition, on the day of immunization and 2 days later, mice were injected i.p. with 200 ng of pertussis toxin (List Biological Laboratories, Campbell, CA) resuspended in sterile saline. This immunization protocol resulted in EAE induction in 100% of the mice. The EAE scoring system was: 0, normal; 1, limp tail; 2, paraparesis with a clumsy gait; 3, hind limb paralysis; 4, hind and fore limb paralysis; 5, moribund. The protocols used were approved by the Animal Care Committee of the University of Wisconsin-Madison (Protocol #A-07-3000-A00755-4-08-94).

RESULTS

By injecting low doses of MBP into B10.PL mice, we have achieved 100% incidence of EAE, reaching such severity that 27% of the animals had to be killed (Table 1). Supplying 1,25-$(OH)_2D_3$ to these animals 1 day before EAE induction

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Abbreviations: EAE, experimental autoimmune encephalomyelitis; MS, multiple sclerosis; MBP, myelin basic protein; 1,25-$(OH)_2D_3$, 1,25-dihydroxyvitamin D₃; Th1, T-helper type 1; CNS, central nervous system.
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Table 1. 1,25-(OH)$_2$D$_3$ prevents EAE in B10.PL mice

Diet	Day of onset	Peak severity	Incidence (no. paralyzed/ no. tested)	Terminal weight, g	Terminal serum Ca, mg/dl
No added vitamin D	18 ± 8	4.0	11/11	20.4 ± 3.1	8.1 ± 0.6
1,25-(OH)$_2$D$_3$ (20 ng per day)	None	0.0	0/9	19.1 ± 4.8	10.9 ± 0.8

Mice with normal vitamin D levels were transferred to a diet containing no additional vitamin D or the amounts of 1,25-(OH)$_2$D$_3$ shown above. The diet was changed on the day before the EAE induction. The mice were analyzed for disease onset, severity, weight, and serum Ca. Severity was scored as follows: 1, limp tail; 2, paraparesis; 3, hind limb paralysis; 4, fore and hind limb paralysis; 5, moribund. The 1,25-(OH)$_2$D$_3$ delayed the onset and reduced the severity of EAE.

completely prevented the appearance of any disability whatsoever (Table 1). It is important to note that in these experiments, unlike previous experiments (13, 14), the animals were fed a high calcium diet with the only variable being the administration of 1,25-(OH)$_2$D$_3$. Thus, the results clearly show that 1,25-(OH)$_2$D$_3$ itself blocks EAE.

We next investigated whether 1,25-(OH)$_2$D$_3$ could be used to prevent EAE progression. Two groups of 12 B10.PL mice were immunized with MBP in complete Freud's adjuvant as described. All the mice showed symptoms of EAE by day 10 postimmunization. When individual mice showed EAE symptoms of 1 or greater, they were given an i.p. injection containing 300 ng of 1,25-(OH)$_2$D$_3$ dissolved in ethanol or a mock injection with an equivalent amount of ethanol. At the time of treatment, the diet was also changed to an experimental diet containing no additional vitamin D (mock treatment) or 20 ng per day per mouse of 1,25-(OH)$_2$D$_3$ (Fig. 1). The results clearly show that 1,25-(OH)$_2$D$_3$ halted EAE progression and maintained the EAE disease severity below stage 2 for the duration of the observations (40 days postimmunization). On the other hand, the mock-treated mice all became paralyzed with peak EAE disease severity scores of 4. Serum calcium values were elevated 27% in the 1,25-(OH)$_2$D$_3$-treated mice as compared with the controls. Otherwise, the treatments were well-tolerated as shown by the normal appearance and weight of the mice [1,25-(OH)$_2$D$_3$-treated, 23.1 ± 4.0 g; mock-treated, 23.7 ± 3.4 g].

To test the reversibility of the 1,25-(OH)$_2$D$_3$ block, we continued the 1,25-(OH)$_2$D$_3$ supplementation study (Fig. 1), and on day 18, we removed 1,25-(OH)$_2$D$_3$ from the diet of 50% of the 1,25-(OH)$_2$D$_3$-treated mice (Fig. 2), creating three groups of mice. The 1,25-(OH)$_2$D$_3$-treated mice were fed the diet containing 1,25-(OH)$_2$D$_3$ at all times. The transiently

treated mice were given the 1,25-(OH)$_2$D$_3$ containing diet for 8–12 days, and were then fed the same diet but devoid of 1,25-(OH)$_2$D$_3$. Over the next 20 days, the control mice had severe stage 3 disease relapses and stage 1.5–2 disease remissions separated by 4–5 days. From day 18 to day 28, the 1,25-(OH)$_2$D$_3$-treated and transiently treated mice had mild stage 1.5–2 relapses and stage 0.5–1 remissions separated by 3 days. Ten days after 1,25-(OH)$_2$D$_3$ removal, the transiently treated mice resumed EAE disease progression, and reached a disease severity equal to the untreated controls by about day 32. Thus, 1,25-(OH)$_2$D$_3$ reversibly blocks EAE disease progression.

As a second approach to elucidate 1,25-(OH)$_2$D$_3$ immunoregulatory activity *in vivo*, we tested the susceptibility of vitamin D-deficient B10.PL mice to EAE. Pregnant mothers were placed on a vitamin D-deficient diet (15, 16). The female pups were taken at 3 weeks; half of each litter was maintained on the vitamin D-deficient diet and the other half was placed on the same diet but containing vitamin D (20 ng per day per mouse). At 6 weeks of age, serum Ca^{2+} and serum 1,25-(OH)$_2$D$_3$ analyses confirmed the vitamin D-deficient status of the mice [4.1 ± 0.4 mg/dl Ca^{2+}, <5–13 pg/ml 1,25-(OH)$_2$D$_3$ in vitamin D-deficient mice (−D) versus 8.0 ± 0.4 mg/dl Ca^{2+}, 38–65 pg/ml 1,25-(OH)$_2$D$_3$ in vitamin D-fed mice (+D)]. The +D and −D female mice were then immunized with MBP. Vitamin D deficiency decreased the mean day of EAE onset when compared with +D littermate controls (13 days in −D mice versus 23 days in +D mice; Fig. 3). Other measures of EAE severity (incidence and maximum severity) were unchanged by vitamin D deficiency (data not shown). Thus, in the absence of vitamin D, encephalitogenic cells developed more quickly, resulting in the earlier onset of EAE symptoms compared with +D controls. Histological examination of the
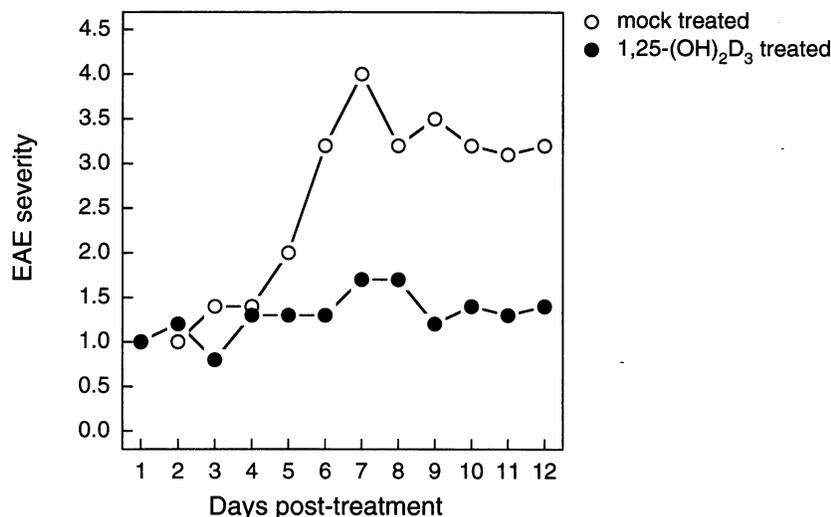

FIG. 1.   1,25-(OH)$_2$D$_3$ prevents the progression of EAE in B10.PL mice. When individual mice showed EAE symptoms of 1 or greater, they were given an intraperitoneal injection containing 300 ng of 1,25-(OH)$_2$D$_3$ dissolved in ethanol (●) or mock injected with an equivalent amount of ethanol (○). At the time of treatment, the diet was also changed to the experimental diet containing no additional vitamin D or to the same diet containing 20 ng per day per mouse of 1,25-(OH)$_2$D$_3$.
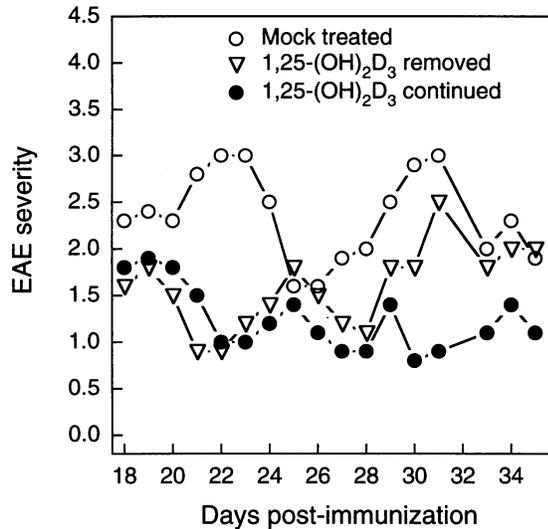
FIG. 2. Removal of 1,25-(OH)$_2$-D$_3$ results in increased EAE symptoms in B10.PL mice. The mice as described in Fig. 1 were split into three new groups. The first group of mice was mock-treated and maintained on a diet devoid of vitamin D (○). The second group of mice was treated with 1,25-(OH)$_2$D$_3$ for 8–12 days and then placed on a diet devoid of vitamin D (▽). The third group of mice were treated with 1,25-(OH)$_2$D$_3$ and maintained on a diet containing 20 ng per day per mouse for the remainder of the study (●).

CNS of a mouse that had previously undergone several relapses by Ian D. Duncan (University of Wisconsin School of Veterinary Medicine, Madison) showed axonal and myelin sheath damage in the superficial tracts of the thoracolumbar spinal cord (data not shown).

DISCUSSION

The findings that 1,25-(OH)$_2$D$_3$ reversibly blocks EAE progression and that vitamin D deficiency accelerates EAE onset provide strong evidence that vitamin D status may be an important factor in determining the incidence of MS and that it is a physiologically important immune system modulator. In view of these results, it seems possible that 1,25-(OH)$_2$D$_3$ has at least two mechanisms of action. The first is a fast action of 1,25-(OH)$_2$D$_3$ directly on encephalitogenic cells to inhibit their encephalitogenic function. The experiment that suggests this is
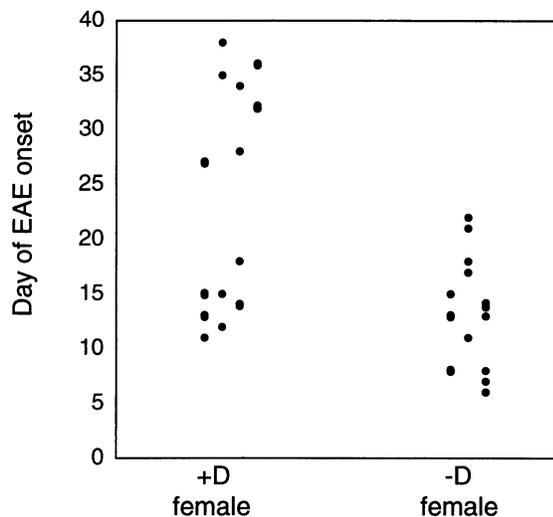
the rapid action of 1,25-(OH)$_2$D$_3$ in blocking EAE disease progression (Fig. 1). The possible cellular targets for this proposed action are the MBP-specific Th1 cells and the activated macrophage.

A second possible mechanism is the stimulation of differentiation of cells that inhibit encephalitogenic cell function or the blockage of differentiation of encephalitogenic cells, which was suggested by the 10-day delay between 1,25-(OH)$_2$D$_3$ removal and the resumption of EAE disease progression (Fig. 2). One interpretation of this result is that encephalitogenic cells were not deleted during 1,25-(OH)$_2$D$_3$ treatment, but were inhibited by an agent with a longer half-life than 1,25-(OH)$_2$D$_3$. A T cell producing antiinflammatory cytokines would be one such agent; 1,25-(OH)$_2$D$_3$ could stimulate T cell precursors to differentiate into antiinflammatory T cells. Another interpretation is that 1,25-(OH)$_2$D$_3$ blocks the differentiation of cells required for encephalitogenesis, and cell differentiation after drug removal took 10 days.

There are currently three main strategies for the treatment of MS. Antigen-nonspecific immunosuppressive drugs or treatments constitute the major therapy used (18). Examples are adrenocorticotrophic hormone, corticosteroid, prednisone, methylprednisone, 2-chlorodeoxyadenosine (Cladribine), mitoxantrone, sulfasalazine, methotrexate, total lymphoid irradiation, and possibly interferon-beta, although its mechanism of action remains undefined. Some immunosuppressants have been tried without success, i.e., azathioprine, cylclophosphamide, and cyclosporin. The limitations of this approach are risk of infection during nonspecific immunosuppression and the toxic side effects of some of the cytotoxic drugs.

Antigen-specific immunosuppressive drugs and treatments are in development and have shown some promise (18). Examples are feeding CNS antigens like myelin to tolerize the encephalitogenic T cells, injecting pathogenic T cells (T cell vaccination) or synthetic T cell receptor peptides to induce immune-mediated elimination of the pathogenic T cells, injecting tolerogenic peptides that are related to encephalitogenic peptides of CNS antigens like myelin, and giving intravenous immunoglobulin. The limitation of these approaches is that autoantigenic epitopes and T cell receptor peptide sequences may differ between MS patients, and within a single MS patient, as the autoimmune reaction spreads to additional epitopes within one protein and to additional proteins (19).

Cytokine-specific therapies are in development (18). Examples are neutralizing antibodies against tumor necrosis factors, soluble tumor necrosis factor-receptors, soluble interleukin-1 antagonists, and others. The limitations of these approaches are the problem of delivering the neutralizing agent in sufficient quantity to the CNS tissue site where it is required and the immunological side effects of long-term cytokine neutralizing activity.

Our results suggest that 1,25-(OH)$_2$D$_3$ and its analogs may be novel and effective treatments for MS patients. A possible limitation of this treatment is the hypercalcemia induced by 1,25-(OH)$_2$D$_3$. This may be solved by using one or more noncalcemic 1,25-(OH)$_2$D$_3$ analogs (20). The advantages of 1,25-(OH)$_2$D$_3$ as an immunomodulator and as a treatment for MS might include selective immunosuppression of autoimmune cells, ease of delivery, and the positive regulation of other immune cells that might decrease the risk of infections.

FIG. 3. Vitamin D deficiency decreases the number of days for the onset of EAE symptoms. Each symbol (●) represents an individual mouse.

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