Archives of Biochemistry and Biophysics 513 (2011) 140–143

Contents lists available at ScienceDirect

Archives of Biochemistry and Biophysics

journal homepage: www.elsevier.com/locate/yabbi

Vitamin D deficiency diminishes the severity and delays onset of experimental autoimmune encephalomyelitis

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ARTICLE INFO

Article history:
Received 26 May 2011
and in revised form 30 June 2011
Available online 19 July 2011

Keywords:
Multiple sclerosis
Autoimmune disease
Paralysis
Vitamin D function

ABSTRACT

Multiple sclerosis incidence is clearly inversely related to sun exposure. This observation led to the idea that vitamin D might be responsible for this relationship. Providing super-physiologic doses of the hormonal form of vitamin D, 1α,25-dihydroxyvitamin D_3, suppresses an animal model of multiple sclerosis, i.e. experimental autoimmune encephalomyelitis (EAE) but causes unwanted hypercalcemia. Further, dietary calcium is needed for this activity of 1α,25-dihydroxyvitamin D_3. B10PL mice were maintained on a vitamin D-deficient diet for two generations to produce frank vitamin D deficiency. These animals showed delayed onset and reduced severity of EAE compared to control animals on the same diet and given vitamin D_3 or provided a vitamin D-containing chow diet. Thus, vitamin D deficiency interferes with the development of this autoimmune disease rather than increasing susceptibility.

Introduction

There are many proven and suggested consequences of vitamin D deficiency. Among those suggested is increased susceptibility to autoimmune diseases such as multiple sclerosis (MS[1]), type I diabetes, rheumatoid arthritis and lupus [1,2]. Goldberg first noted an inverse relationship between MS incidence and latitude that in turn suggested an inverse relationship to sunlight exposure [3]. It seemed obvious that vitamin D production in skin might mediate this protective activity. The active form of vitamin D, i.e. 1α,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$) can prevent or markedly reduce the symptoms of experimental autoimmune encephalomyelitis (EAE) [4,5] at the expense of hypercalcemia. Calcium in the diet is necessary for maximal activity of 1,25-$(OH)_2D_3$ in reducing the symptoms and incidence of EAE [6]. Hypercalcemia itself is protective against EAE in female mice [7] and ultraviolet light (UV) itself is protective against EAE independent of vitamin D and without hypercalcemia [8]. A small clinical trial did not support the idea that a vitamin D compound is protective against MS [9], while another showed a non-significant trend toward improvement with vitamin D [10]. The question, therefore, of what role vitamin D may play in the development of MS is not settled. In the present study, we tested the idea that vitamin D deficiency might increase the symptoms, severity and onset of EAE in mice. We also decided to determine if dietary calcium might also be a precipitating factor. To our surprise, irrespective of dietary calcium level in the diet, vitamin D deficiency reduces the severity and delays the onset of EAE in mice.

Materials and methods

Animals and diet

B10PL(73NS)/sn breeding pairs were purchased from Jackson Laboratories (Bar Harbor, ME) for in-house breeding. Mice were maintained on a Purina Formulab diet (Richmond, IN) that contains 1% calcium and 3.3 IU/g vitamin D_3. A purified synthetic diet, devoid of vitamin D, was fed to all experimental groups and to the vitamin D-deficient breeders [11]. The diets contained 0.25%, 0.43%, or 1.2% Ca^{2+} provided from calcium carbonate. These diets were prepared in agar according to methods used previously [12].

The breeding scheme to generate the severely vitamin D-deficient mice is shown in Fig. 1. When the breeding pairs mated, two-thirds of the pups were used for vitamin D-deficient mouse production and one-third was used for mice provided with adequate vitamin D. The +D animals were maintained on the chow diet. For vitamin D-deficient animals, pregnant females were placed on the purified vitamin D-deficient diet containing 1.2% calcium and supplemented with oil containing the fat-soluble vitamins A, E, and K (AEK oil). To prevent vitamin D synthesis from occurring in the skin, mice were housed under incandescent lighting. At 18–21 days of age, the pups were weaned and raised on the indicated diet until 6 weeks old. At this time, the first generation vitamin D-deficient mice were bred to produce second generation −D mice. This was done to ensure that all the mice would be

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[1] *Abbreviations used:* EAE, experimental autoimmune encephalomyelitis; 1,25-$(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3; MS, multiple sclerosis.

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doi:10.1016/j.abb.2011.07.005

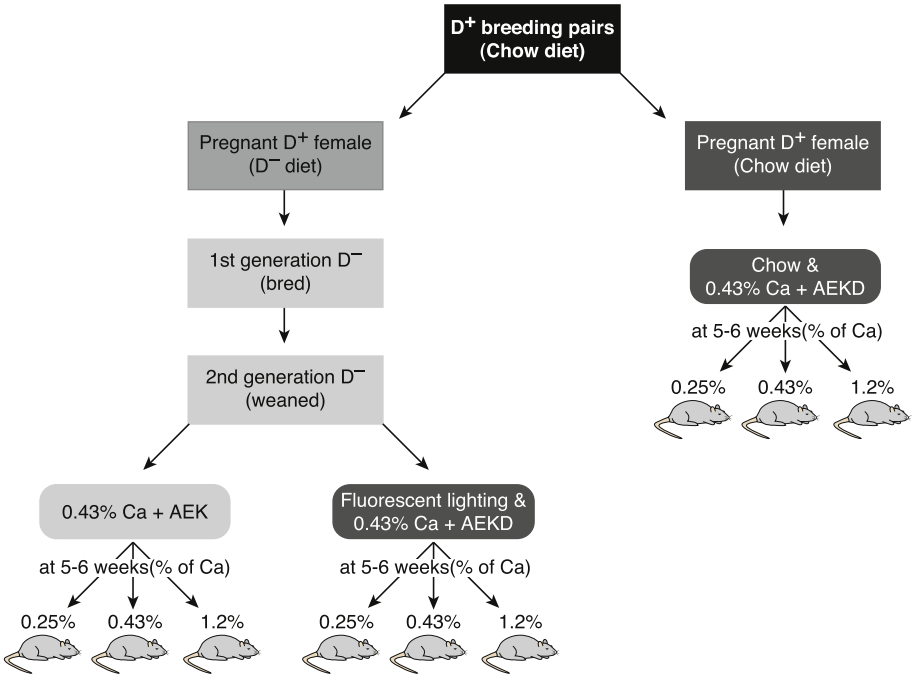

Fig. 1. Breeding scheme and experimental design for the mouse EAE experiments.

vitamin D-deficient. When the second generation −D mice were weaned, they were placed on 0.43% calcium diet with AEK oil (−D), while the other one-half were fed a diet containing 0.43% calcium and oil containing the fat-soluble vitamins A, E, D and K (AEDK oil (−D/+D)) [11]. Age-matched, chow-raised mice were also placed on 0.43% calcium diet with AEDK oil (+D) upon weaning. At 5–6 weeks of age, the mice were bled by tail nick to obtain serum for calcium determination. At this time, the mice were randomly assigned to remain on the 0.43% calcium diet or to be placed on the 0.25% or 1.2% calcium diets. Each group was composed of 6–11 mice with approximately one-half being male and one-half being female. All of the procedures were done in accordance with the Research Animal Resources Committee of the College of Agricultural & Life Sciences University of Wisconsin-Madison.

Induction of EAE

Mice were immunized at 7–8 weeks of age with guinea pig myelin basic protein (MBP). The MBP was extracted, lyophilized and stored at −80 °C [4]. Prior to immunization, MBP was diluted to 8 mg/ml in 0.1 M acetic acid. To make the 1:1 emulsion of MBP and complete Freund's adjuvant containing *mycobacterium tuberculosis* H37Ra (Difco), an Omni mixer homogenizer (Omni International, Warrenton, VA) was used. On day 0, the mice were immunized subcutaneously at four sites on the dorsal side with a total of 125 μl (500 μg MBP/mouse) of the emulsion and 100 μl of pertussis toxin (200 ng/mouse) (List Biological Labs, Campbell, CA) in sterile PBS was given intraperitoneally (IP). Another 100 μl of pertussis toxin was given IP on day 2 post-immunization.

The mice were scored daily for symptoms of EAE: 0, normal; 1, limp tail; 2, wobbly gait; 3, hind or fore limb paralysis; 4, hind and fore limb paralysis; 5, moribund. Animals with disease scores of 2 or greater were given the appropriate agar diet on the cage floor.

Serum calcium determinations

The experiments were ended on day 50 post-immunization. At this time, the mice were sacrificed by CO_2 asphyxiation. Weights were recorded and blood was collected by open-heart puncture for serum calcium determinations. The whole blood was clotted and centrifuged at 1100 × *g*. The serum was separated from the clot and stored at −20 °C until analysis. Serum calcium levels were determined from two measurements per sample by atomic absorption spectroscopy in 0.1% $LaCl_3$ (Perkin Elmer, Norwalk, CA).

Statistical analyses

Results are expressed as means ± SEM of one representative experiment due to the variability in the EAE development with different immunizations. Two separate experiments were conducted with similar results. SAS Version 8 (SAS Institute, Cary, NC) was used to determine the statistical significance. Mixed analysis was used to correct for covariance within the mouse cages. This method provides a conservative estimate of the *p* values compared to ANOVA analysis which would assume independence of the mice. A *p* value of <0.05 was considered significant.

Results

Vitamin D deficiency was confirmed in the second generation −D mice by low serum calcium and the occurrence of hypocalcemic tetany. At 5–6 weeks of age all mice were bled for serum calcium determinations. All of the −D mice that had serum calcium levels below 6.0 mg/dl were considered to be vitamin D-deficient [13] and were randomly assigned to the 0.25%, 1.2% or 0.43% calcium diet groups. Mice in the +D groups were also randomly assigned to one of the three groups. The serum calcium values obtained from the −D groups were significantly lower than the +D groups (Table 1).

Further evidence that the second generation −D mice were vitamin D-deficient was provided by a comparison of the percent mortality due to hypocalcemic tetany (not shown). The first generation −D mice had 0% mortality on 0.02%, 0.43%, and 1.2% calcium diets. However, the second generation −D mice had 100%, 30%, and 8% mortality on the 0.02%, 0.43%, and 1.2% calcium diets, respectively. After adjusting the calcium content of the low calcium diet to 0.25% calcium, we still observed 28% mortality in the −D group.

Table 1
The initial serum calcium values of mice at 5–6 weeks of age.[a]

Diet treatment group	Serum calcium (mg/dl)
−D 0.43% Ca	4.6 ± 0.2
−D/+D 0.43% Ca	7.7 ± 0.3[b]
+D 0.43% Ca	7.7 ± 0.6[b]

[a] All mice were bled by tail nick before they were placed on either 0.25% calcium, 1.2% calcium, or remained on 0.43% calcium diet treatments.
[b] Significantly higher, $p < 0.0001$ than −D group.

The average EAE disease score was lower in the −D mice compared to the +D mice (Fig. 2). The average disease scores for the −D groups were between 41–55% lower than the +D controls. There was also a lower average disease score for the −D/+D mice, ranging from 9–29% lower than the +D controls. The deficient animals given vitamin D had significantly increased disease scores, not equal to those maintained on vitamin D throughout. This might suggest that vitamin D plays a role in immune cell maturation, since the provision of vitamin D to −D animals had not yet achieved the disease incidence and onset demonstrated by the +D group.

The day of disease onset was determined when the mice had an EAE score of at least two for three days or more. The −D mice developed EAE about 11–17 days later than the +D mice (Fig. 3).

The terminal serum calcium values of the 0.25% and the 0.43% calcium −D groups were significantly lower than their corresponding +D groups (Table 2). The 1.2% calcium −D groups had lower calcium values, although not significant, than the +D and −D/+D groups (Table 2).

The terminal body weights were slightly lower for all −D groups compared to the +D and −D/+D groups with the exception of the +D/0.25% group (Table 2).

Discussion

There is no doubt that vitamin D plays a role in the immune system. First, activated T cells, antigen-presenting cells, and macrophages all contain significant amounts of VDR [14,15]. Secondly, vitamin D deficiency suppresses cell-mediated immunity [16], and thirdly, the hormonal form of vitamin D can at super-physiologic doses suppress the symptoms of EAE, lupus, rheumatoid arthritis and type I diabetes [5,6,13,17,18]. From these findings, it is expected that vitamin D may have profound effects on human autoimmune diseases.

The finding that MS is found in high incidence at high latitudes and is in low incidence at the equatorial regions triggered the idea

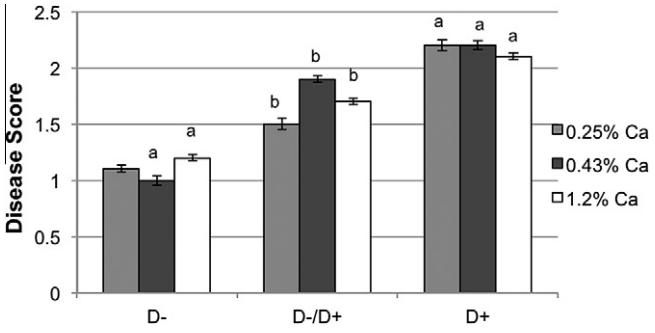

Fig. 2. The effect of vitamin D status and dietary calcium levels on EAE severity. The average disease score was calculated by dividing the total sum of the EAE scores by the number of days scored. The average disease score was significantly lower for each −D group, as compared to each +D group ($p \leqslant 0.0001$)[a]. The 0.25% and the corresponding 1.2% calcium −D/+D groups also had a lower average disease score than the +D controls ($p \leqslant 0.0180$)[b]. There were 8–10 animals in each of the −D and +D groups and 6–8 animals in the −D/+D group.

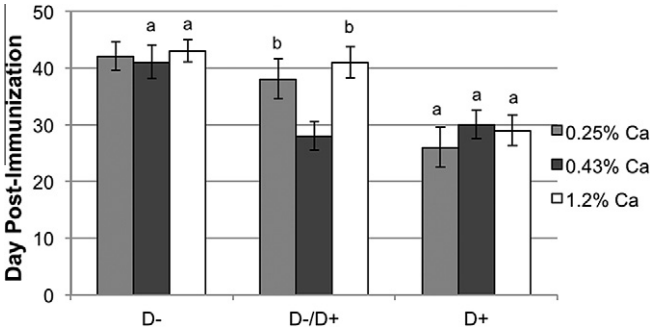

Fig. 3. Vitamin D deficiency delays the onset of EAE. The −D mice had a later onset of disease ($p = 0.0297$) than the +D controls[(a)]. The 0.25% and 1.2% calcium −D/+D groups also had a significant delay in disease onset ($p = 0.0156$) compared to the +D controls[(b)]. Animals that did not develop EAE by day 50 post-immunization were arbitrarily given an onset at day 50. The number of animals in each group is shown in the legend of Fig. 2.

Table 2
Terminal serum calcium values and body weights.

Groups	Terminal serum calcium (mg/dl)	Terminal body weights (g)
−D 0.25% Ca	8.5 ± 0.4[a,b]	23.37 ± 1.30
−D 0.43% Ca	7.9 ± 0.4[a,b]	21.86 ± 1.27
−D 1.2% Ca	8.6 ± 0.3	23.21 ± 1.06
−D/+D 0.25% Ca	9.6 ± 0.5[a,b]	25.64 ± 1.62
−D/+D 0.43% Ca	9.6 ± 0.4[a,b]	24.53 ± 1.43
−D/+D 1.2% Ca	9.9 ± 0.4	26.60 ± 1.21
+D 0.25% Ca	9.8 ± 0.5[a,b]	23.27 ± 1.63
+D 0.43% Ca	9.9 ± 0.5[a,b]	25.02 ± 1.63
+D 1.2% Ca	9.6 ± 0.5	26.73 ± 1.65

[a] Significantly lower serum calcium compared to the corresponding +D and −D/+D groups; $p = 0.05$.
[b] Serum calcium levels of the −D group compared to the corresponding −D/+D groups; $p = 0.0742$.

that vitamin D production might be protective against MS [3]. However, recent studies suggest that UV light itself might be protective independent of vitamin D [8]. Although this does not rule out a role for vitamin D as a protective agent in MS, it raises the question whether there is an involvement of vitamin D in this disease and if so, in what way.

In our studies of EAE, we noted a low incidence and severity of EAE in VDR null mice [unpublished results], although VDR was certainly required for the protective activity of super-physiologic doses of 1,25-$(OH)_2D_3$ [19].

In the case of type I diabetes, vitamin D deficiency certainly increased the severity and accelerated the onset of diabetes in NOD mice [13]. However, a study of true vitamin D deficiency in EAE has not been performed. Previously, we omitted vitamin D from the diet of mice in which EAE was induced [5]. In that case, the lack of vitamin D had little or no effect. However, precipitating vitamin D deficiency in mice is more difficult than omitting vitamin D from the diet. In the present study, we maintained mice for two generations on vitamin D-free diet and eliminated fluorescent lighting that emits significant UVB light. Severe hypocalcemia in these mice and significant tetany signaled clear vitamin D deficiency (Table 1). Further, in other studies mice raised for two generations under these conditions had undetectable levels of 1,25-$(OH)_2D_3$ in their blood by a cellular assay [20] that has a limit of detection of 5 pg/ml. These mice showed delayed onset and markedly reduced symptoms of EAE, as compared to the same animals on a chow diet and given vitamin D. It seems clear that vitamin D deficiency interfered with the autoimmunity exhibited by these mice. This is in

agreement with the finding of Yang et al. [16] and with the VDR null mutant findings [7]. During the preparation of this manuscript, another report appeared, supporting the idea that vitamin D deficiency protects against the development of this disease [21].

The results of this study are supportive of the idea that vitamin D may play a role in the development of functional components of the immune system. In the case of human MS, a high incidence of MS correlates with lower levels of 25-OH-D_3 in the blood [22,23]. However, the lower incidence also correlates with ultraviolet light exposure. Since UV light itself, independent of vitamin D, also suppresses EAE symptoms, another interpretation is clear, namely the UVB light increases blood levels of 25-OH-D_3, while at the same time directly suppresses EAE. The increase in 25-OH-D_3 is an indicator of UVB light but it is the UV light that actually suppresses the MS [8].

It is of some interest that vitamin D deficiency shortened the period of onset and increased the incidence of type 1 diabetes in the NOD mouse [13]. This model is complicated by the presence of large amounts of VDR in islet cells of the pancreas that are targeted for destruction in that disease. It is possible that the absence of 1,25-$(OH)_2D_3$ in the islet cells makes them more susceptible to destruction. In any case, these two different models of autoimmune disease differ in the impact of vitamin D deficiency.

The present result does not conflict with the result that excess 1,25-$(OH)_2D_3$ suppresses EAE and delays the onset, but it does add evidence that vitamin D deficiency found at higher and lower latitudes may not be the trigger of MS.

Acknowledgments

Ms. Jean Humpal-Winter's technical powers are gratefully acknowledged for this work. This work was funded by the Wisconsin Alumni Research Foundation.

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