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

Hector F. DeLuca *, Lori A. Plum

Department of Biochemistry, University of Wisconsin-Madison, 433 Babcock Drive, Madison, WI 53706-1544, USA

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

* Corresponding author. Fax: +1 608 262 7122.
E-mail addresses: deluca@biochem.wisc.edu, mings@biochem.wisc.edu (H.F. DeLuca).

[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

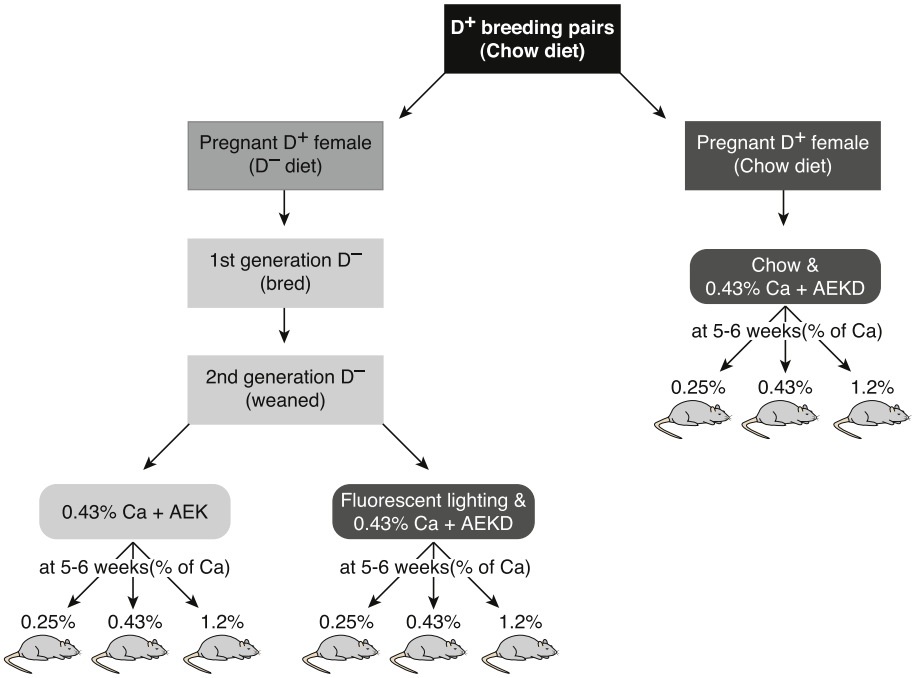

Fig. 1. Breeding scheme and experimental design for the mouse EAE experiments.

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.