Rag-1-dependent cells are necessary for 1,25-dihydroxyvitamin D_3 prevention of experimental autoimmune encephalomyelitis

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Abstract

Multiple sclerosis (MS) is a demyelinating disease involving genetic and environmental risk factors. Geographic, genetic, and biological evidence suggests that one environmental risk factor may be lack of vitamin D. Here, we investigated how 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$) inhibits experimental autoimmune encephalomyelitis (EAE), an MS model. The experiments used adoptive transfer of TCR-transgenic (TCR1) cells specific for myelin basic protein (MBP) peptide into unprimed recipients. When unprimed TCR1 splenocytes were transferred, and the recipients were immunized with peptide, the mock-treated mice developed EAE, but the 1,25-$(OH)_2D_3$-treated recipients remained disease-free. Both groups had TCR1 T cells that proliferated in response to MBP Ac1–11 and produced IFN-γ but not IL-4 in the lymph node. In the central nervous system (CNS), the mock-treated mice had activated TCR1 T cells that produced IFN-γ but not IL-4, while the 1,25-$(OH)_2D_3$-treated mice had TCR1 T cells with a non-activated phenotype that did not produce IFN-γ or IL-4. When activated TCR1 T cells producing IFN-γ were transferred into unprimed mice, the mock-treated and the 1,25-$(OH)_2D_3$-treated recipients developed EAE. Likewise, the 1,25-$(OH)_2D_3$ did not inhibit Th1 cell IFN-γ production or promote Th2 cell genesis or IL-4 production in vitro. Finally, the 1,25-$(OH)_2D_3$ inhibited EAE in MBP-specific TCR-transgenic mice that were *Rag-1*^{+}, but not in animals that were *Rag-1*-null. Together, these data refute the hypothesis that the hormone inhibits Th1 cell genesis or function directly or through an action on antigen-presenting cells, or promotes Th2 cell genesis or function. Instead, the evidence supports a model wherein the 1,25-$(OH)_2D_3$ acts through a *Rag-1*-dependent cell to limit the occurrence of activated, autoreactive T cells in the CNS. © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

Multiple sclerosis (MS) is a demyelinating disease of the central nervous system (CNS) with a puzzling etiology. It appears that inheriting genetic risk factors and exposure to unidentified environmental risk factors are both required to cause this disease (Ebers and Dyment, 1998). The fact that monozygotic twins show a high discordance rate for MS indicates that the disease might be preventable in genetically susceptible individuals if the environmental risk factors were known and could be avoided. It is our hypothesis, based on geographic, climatologic, and biolog-

ical evidence, that one avoidable environmental risk factor for MS might be lack of vitamin D (Hayes et al., 1997) [Hayes, 2000].

MS prevalence shows a striking geographic distribution that strongly suggests an environmental risk factor. The disease prevalence increases with increasing latitude in both hemispheres from a low of 1–2 cases per 10^5 population near the equator to a high of > 200 cases per 10^5 population at latitudes > 50° (Ebers and Sadovnick, 1993). Importantly, immigrants displayed the MS risk of their new homeland, reinforcing the conclusion that the latitude gradient of MS risk reflects environmental rather than genetic variables (Ebers and Sadovnick, 1994). For example, Irish immigrants to Hobart, Australia (42.8°S) had an MS prevalence about 5-fold higher than Irish immigrants to Queensland, Australia (25.1°S), regardless of age at migration (Hammond et al., 2000). Acheson et al. (1960)

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first proposed that sunlight might reduce the MS risk, since sunlight showed a striking inverse correlation with MS prevalence. A recent study confirmed that individuals with the highest sunlight exposures had the lowest odds ratio, 0.24, for mortality from MS (Freedman et al., 2000). This strong inverse correlation between high sunlight exposure and low MS risk indicates that sunlight might be a protective environmental factor in MS.

The vitamin D endocrine system is exquisitely responsive to sunlight and may mediate a protective effect of sunlight in MS. Sunlight is required for previtamin D_3 synthesis in skin (Velluz and Amiard, 1949). Previtamin D_3 is the precursor of 25-hydroxyvitamin D_3 (25-$(OH)D_3$), the stored form of vitamin D, and 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$), the biologically active hormone (Holick et al., 1971). We first proposed that sunlight might be protective in MS through the immunoregulatory actions of 1,25-$(OH)_2D_3$ (Hayes et al., 1997). This hypothesis is consistent with the geographic and climatologic evidence above. Further, some nutritional evidence supports the hypothesis, since MS prevalence is lower where diets are rich in fish oil, which is a natural vitamin D source (Swank et al., 1952). Also, vitamin D deficiency is prevalent in MS patients (Cosman et al., 1998; Nieves et al., 1994). Finally, the theory is consistent with studies showing seasonal fluctuations in MS severity (Auer et al., 2000). The peak of brain lesion frequency in MS patients occurred about 2 months after the nadir of serum 25-$(OH)D_3$ levels measured in controls, while the nadir of MS severity occurred about 2 months after the peak of serum 25-$(OH)D_3$ levels (Embry et al., 2000). The temporal correlation between MS severity and serum 25-$(OH)D_3$ levels and the nutritional reports point to a possible cause and effect relationship between lack of vitamin D and increased MS severity.

We have provided clear evidence that the biologically active hormone, 1,25-$(OH)_2D_3$, inhibits experimental autoimmune encephalomyelitis (EAE), a model of MS. Immunizing mice with spinal cord homogenate induced a progressively paralytic autoimmune disease, EAE, with strong similarities to MS (Olitsky and Yager, 1949). In this model, 1,25-$(OH)_2D_3$ treatment completely blocked EAE induction and progression of established EAE (Cantorna et al., 1996). Further, we showed that mice with severe acute EAE recovered from paralysis within a few days of 1,25-$(OH)_2D_3$ treatment (Nashold et al., 2000). Together, these experiments indicate that 1,25-$(OH)_2D_3$ is a profoundly important EAE inhibitor.

The mechanism by which 1,25-$(OH)_2D_3$ inhibits EAE induction is not known. The hormone mediates its activities primarily through a nuclear vitamin D hormone receptor (VDR). The VDR regulates the transcription of particular genes, mainly through a vitamin D responsive element in the promoter region (Haussler et al., 1997). Myeloid lineage cells and activated T lymphocytes possess this VDR (Bhalla et al., 1983; Provvedini et al., 1983), sug-

gesting that they may be targets of the hormone's mechanism of action as regards immune system regulation.

Several investigators have shown that 1,25-$(OH)_2D_3$ is a potent and specific inhibitor of activated Th1 cells in vitro (Lemire et al., 1995; Muller and Bendtzen, 1996; Provvedini et al., 1983; Reichel et al., 1987; Rigby, 1988; Rigby et al., 1987). Others have shown that 1,25-$(OH)_2D_3$ decreased IL-12 production in vitro (D'Ambrosio et al., 1998; Lemire, 2000), and inhibited dendritic cell (DC) maturation, activation, and antigen-presenting cell function in vitro (Griffin et al., 2000; Penna and Adorini, 2000; Piemonti et al., 2000). Since DC producing IL-12 are strong stimulators of Th1 differentiation (Trinchieri and Scott, 1999), and Th1 cells are encephalitogenic in EAE (Zamvil et al., 1986), one possible mechanism for 1,25-$(OH)_2D_3$ inhibition of EAE induction would be for the hormone to inhibit DC functions, thereby diminishing Th1 cell differentiation and/or function. Despite its appeal, this hypothesis has never been tested in vivo.

Here we tested the hypothesis that 1,25-$(OH)_2D_3$ might inhibit Th1 cell differentiation and/or Th1 cell function in vivo. Mice with TCR-transgenic T cells specific for myelin basic protein (MBP) residues #1–11, here termed TCR1 mice (Goverman et al., 1993), were used. The effects of 1,25-$(OH)_2D_3$ treatment on TCR1 T cell differentiation and function were examined in adoptive transfer experiments in vivo and cell culture experiments in vitro. The 1,25-$(OH)_2D_3$ treatment clearly inhibited EAE induction in unprimed B10.PL mice that received TCR1 cells and MBP Ac1–11 peptide, but it did not inhibit Th1 cell development or function, or promote Th2 cell development or function, or stimulate TCR1 T cell deletion. In the 1,25-$(OH)_2D_3$-treated mice, there were activated, MBP-responsive, TCR1 T cells producing cytokines in the peripheral lymphoid organs. However, in the CNS of these mice, there were only TCR1 T cells with a non-activated phenotype that did not produce cytokines. Finally, the 1,25-$(OH)_2D_3$ treatment inhibited MBP Ac1–11-induced EAE in TCR-transgenic mice that were *Rag-1*^{+}, but did not inhibit spontaneous EAE in TCR-transgenic mice that were *Rag-1*-deficient. This result rules out a direct effect of the hormone on antigen-presenting cell function and signals a requirement for *Rag-1*-dependent cells in the EAE inhibition mechanism. This new evidence suggests that the 1,25-$(OH)_2D_3$ treatment may inhibit EAE by enhancing a *Rag-1*-dependent cell mechanism that limits the occurrence of activated, autoreactive T cells in the CNS.

2. Materials and methods

2.1. Chemicals, antibodies, and cytokines

The 1,25-$(OH)_2D_3$ was purchased from Tetrionix (Madison, WI). It was dissolved in 100% ethanol at 1

mg/ml and stored frozen. MBP was isolated from guinea pig spinal cords as described (Diebler et al., 1972). The MBP acetylated #1–11 peptide (MBP Ac1–11) was synthesized by BioSynthesis (Lewisville, TX) and had the sequence acetyl-ASQKRPSQRSK (Zamvil et al., 1986).

Hybridomas secreting rat antibodies to mouse IFN-γ (clones XMG1.2 and R4.6A2) and to mouse IL-4 (clone 11B11) were purchased from the American Type Culture Collection (Manassas, VA) and produced as ascites in pristane-primed, athymic BALB/c mice, kindly donated by Dr. E. Balish (University of Wisconsin-Madison). Biotinylated anti-mouse IL-4 (clone BVD6-24G2), FITC-conjugated anti-mouse $V_{\alpha}2$ TCR (clone B20.1), PE-Cy5-conjugated anti-mouse CD44 (clone IM7), PE-conjugated anti-mouse CD45RB (clone 16A), and FITC-anti-mouse CD4 (clone GK1.5) were purchased from BD PharMingen (San Diego, CA). R-phycoerythrin labeled anti-mouse CD62L (Mel-14) was purchased from Caltag Laboratories (Burlingame, CA). Polyclonal rabbit antiserum to mouse IFN-γ was produced following the manufacturer's protocol for TDM Emulsion (RIBI ImmunoChem Research, Hamilton, MT). Biotin-labeled goat antibody to rabbit IgG was purchased from Kirkegaard & Perry Laboratories (Gaithersburg, MD). The rIL-4 and rIL-12 were purchased from R&D Systems (Minneapolis, MN); rIFN-γ was purchased from Genentech (South San Francisco, CA). Purified human rIL-2 was a generous gift from Dr. N. Street (University of Texas Southwestern Medical Center at Dallas).

2.2. Mice

The B10.PL(73NS)/Sn breeding pairs were obtained from The Jackson Laboratory (Bar Harbor, ME). MBP TCR1 transgenic mice (B10.PL-H2^u-H2-T18^a[73NS]/Sn-TgN [TCRα]/BlJg and B10.PL-H2^u-H2-T18^a[73NS]/Sn-TgN [TCRβ]/C14Jg) genetically engineered to express the MBP Ac1–11-specific TCR $V_{\alpha}2.3$ and $V_{\beta}8.2$ chains were produced and genotyped as described (Goverman et al., 1993). MBP $Rag-1^{-/-}$ TCR2 transgenic mice genetically engineered to express the MBP Ac1–11-specific $V_{\alpha}4$ and $V_{\beta}8.2$ TCR genes on a $Rag-1$-deficient B10.PL background (Lafaille et al., 1994) were generously donated by Dr. Susumu Tonegawa (Massachusetts Institute of Technology). All mice were bred at the University of Wisconsin-Madison Department of Biochemistry animal facility under pathogen-free conditions. Mice were maintained at 23 °C with 40–60% humidity and 12-h light–dark cycles. Experimental protocols were approved by the Institutional Animal Care and Use Committee. Unless stated otherwise, the mice were fed laboratory chow #5008 (Formulab, Richmond, IN), containing 3.3 IU cholecalciferol/g diet. Male and female mice age 4–8 weeks were used; they were age- and sex-matched within experiments. Animals showing spontaneous EAE signs were not used for experiments.

2.3. Synthetic diet, EAE disease induction, and 1,25-$(OH)_2D_3$ treatment

For the experiments, mice were fed a synthetic diet devoid of vitamin D, or supplemented to provide 50 ng/day (females) or 200 ng/day (males) of 1,25-$(OH)_2D_3$ (Cantorna et al., 1998). This intake was based on an average diet consumption of 4.5 g/day per mouse. The synthetic diet was formulated exactly as the complete diet described elsewhere (Smith and Hayes, 1987), except that vitamin D was omitted.

Two EAE model systems were used. In the first system, 10^7 TCR1 splenocytes ($\sim 10^6$ T cells) from 4–6-week-old unprimed B10.PL TCR1 mice were injected i.p. into unprimed B10.PL mice. One day after the transfer, the recipients were changed to a synthetic diet with or without 1,25-$(OH)_2D_3$. Three days after the diet change (4 days after transfer), the recipients were anesthetized with ether, and immunized subcutaneously with 0.1 ml of MBP Ac1–11 peptide emulsified in an equal volume of CFA containing Mycobacterium tuberculosis H37Ra (Difco, Detroit, MI). The mice were also injected i.p. with 100 ng of pertussis toxin (List Biological Laboratories, Campbell, CA). Synthetic diet feeding was continued and EAE severity was scored daily. In the second system, unprimed B10.PL mice were changed to a synthetic diet with or without 1,25-$(OH)_2D_3$. Three days after the diet change, graded numbers of in vitro activated TCR1 Th1 cells were injected i.p. Synthetic diet feeding was continued and EAE severity was scored daily. Animals were weighed when they were euthanized.

2.4. Cell culture

Splenocytes from individual 4–6-week-old unprimed B10.PL TCR1 mice were used for the in vitro Th differentiation studies (Hsieh et al., 1992; Seder et al., 1992). These splenocytes were cultured (10^6 cells/ml; 5 ml/well in six-well plates) in HL-1 serum-free medium (BioWhittaker, Walkersville, MD) supplemented with 2-ME (50 μM), L-glutamine (2 μM), penicillin (10 U/ml), and streptomycin (10 μg/ml). MBP Ac1–11 peptide (3 μM) was added to some wells. Some wells also received rIL-12 (10 ng/ml) and rIFN-γ (20 ng/ml), or rIL-4 (20 ng/ml) and neutralizing antibody to IFN-γ (10 μg/ml; XMG1.2 ascites). The 1,25-$(OH)_2D_3$ stock (1 mg/ml ethanol) was diluted to 100 nM in complete medium. This working hormone stock (0.04% ethanol vol/vol) was subsequently diluted into culture wells to the desired final concentration. The control cultures received an equal volume of 0.04% ethanol in medium. On day 3 of culture, the cells were split into two wells, and fresh medium with human rIL-2 (20 U/ml) was added. On day 7 of culture, the cells were collected, washed twice with Hank's balanced salt solution, counted, and re-stimulated (5×10^5 T cells/ml) with fresh, irradiated (3000 rad) B10.PL splenocytes (5×10^6

cells/ml) and peptide (3 μM) for 24 h. The triplicate re-stimulation cultures received hormone in 0.04% ethanol (vol/vol in medium) or 0.04% ethanol in medium as a control. Culture supernatants were collected and stored at −70 °C until analyzed by ELISA.

To produce differentiated Th1 cells for adoptive transfer, splenocytes from B10.PL TCR1 mice were cultured with rIL-12 plus rIFN-γ as described above. The Th1 cells were collected on day 7, washed, counted, and used for the adoptive transfer.

2.5. Cell proliferation

The T cell responses to MBP Ac1–11 were measured by a cell proliferation assay. Brachial, inguinal, and axillary lymph nodes were collected from individual mice and dissociated into single cell suspensions. The cells (0.5×10^6/well) were cultured in triplicate in supplemented HL-1 medium with and without graded amounts of MBP Ac1–11. The cultures were pulsed with a 1-μCi aliquot of [^{3}H]-thymidine in HL-1 medium for the last 18 h of a 72-h culture. The cells were collected onto glass fiber filters using a PhD Cell Harvester (Cambridge Technologies). The filters were washed, dried, and counted in a Beckman LS6500 liquid scintillation counter. The mean and S.D. of [^{3}H]-thymidine cpm incorporated were calculated for each individual mouse. The background [^{3}H]-thymidine cpm incorporated in the absence of antigen was subtracted. The mean and S.E.M. were calculated for groups of 3–5 mice.

2.6. Cytokine analysis

TCR1 splenocyte culture supernatants were analyzed for IFN-γ and IL-4 proteins by ELISA (Mosmann and Fong, 1989). Capture mAb were R4.6A2 (IFN-γ) and 11B11 (IL-4). Detection antibodies were polyclonal rabbit antiserum to murine IFN-γ and biotinylated BVD6-24G2 (IL-4). Rabbit IgG was detected with biotinylated goat antibody to rabbit IgG. ELISA reactions were developed with streptavidin-alkaline phosphatase (Gibco BRL, Life Technologies, Gaithersburg, MD) and Sigma 104 phosphatase substrate in alkaline buffer (Sigma Diagnostics, St. Louis, MO). Optical density was read at 405 nm using a Vmax Microplate Reader (Molecular Devices, Menlo Park, CA). The ELISA detection limits were 16 pg/ml of IFN-γ and 8 pg/ml of IL-4, as determined with recombinant cytokine standards.

For IFN-γ and IL-4 transcripts analysis, the animals were euthanized, thoroughly perfused with PBS, and the lymph node and CNS cells were collected. The RNA was extracted from the cells without restimulating them in vitro. The RNA was reverse-transcribed, and analyzed for IFN-γ and IL-4 transcripts by quantitative competitive PCR exactly as described (Cantorna et al., 1998).

2.7. VDR transcript analysis

The VDR transcript analysis was done on mRNA obtained from highly polarized Th1 and Th2 cells. To obtain these cells, TCR1 splenocytes were cultured with MBP Ac1–11 in the presence of exogenous IL-12 and IFN-γ or IL-4 and neutralizing antibodies to IFN-γ as described above. The cells harvested after 7 days of culture were purified by centrifugation on Percoll and examined for the $V_{\beta}8.2$ TCR chains by flow cytometry. The recovered cells were consistently > 95% $V_{\beta}8.2$ positive. The highly polarized Th1 and Th2 cell phenotypes were verified by their IFN-γ and IL-4 synthesis rates in the restimulation culture described above.

The mRNA was isolated from Th1 and Th2 cells and from the lymph node cells using Tri Reagent (Molecular Research Center, Cincinnati, OH), and reverse transcribed using the Reverse Transcription System (Promega, Madison, WI) according to the manufacturer's directions. The primers for VDR cDNA amplification were 5′TAGCTCC-CTGTACTTACGTC3′ and 5′CCGCATCACCAAG-GACAACC3′ (Liu et al., 1996); they were purchased from GIBCO-BRL (Grand Island, NY). Primers for glyceralde-hyde-3-phosphate dehydrogenase (G3PDH) cDNA amplification were 5′TGAAGGGTGTGAACGGATTTGGC3′ and 5′CATGTAGGCCATGAGGTCCACCAC3′ (Clontech, Palo Alto, CA). A quantitative competitive PCR for G3PDH was done on serially diluted cDNA samples. The products were electrophoresed in agarose gels, stained with ethidium bromide, and photographed. The sample concentrations were adjusted to give equivalent G3PDH PCR product band intensities at equal dilutions. The Th1 and Th2 cell cDNA samples were then serially diluted and subjected to the VDR-specific PCR. PCR products were electrophoresed, stained, and photographed.

2.8. Spinal cord cell isolation and flow cytometric analysis

Unprimed splenocytes from young TCR1 mice were injected i.p. into unprimed B10.PL mice, the recipients were changed to a synthetic diet with or without 1,25-$(OH)_2D_3$, and subsequently immunized i.p. with MBP Ac1–11 (200 μg/mouse) and 100 ng pertussis as above. Eleven days later, mice were euthanized and thoroughly perfused with phosphate-buffered saline. The spinal cords were removed by insufflation and minced. The debris was allowed to settle, and the single cell suspensions from mice within a group were pooled and centrifuged. The cell pellet was resuspended in 37% Percoll (Amersham Pharmacia Biotech) and centrifuged at 2,118 *g* for 15 min as described (Brabb et al., 2000). The cells were washed a second time in 37% Percoll, then resuspended in 30% Percoll and layered over 70% Percoll. After centrifugation, the cells collected from the Percoll gradient interface were

washed and resuspended in ice-cold PBS with 5% FCS and 0.1% sodium azide (staining buffer). All subsequent incubations were for 30 min at ice temperature, separated by two washes with staining buffer. Cells were stained with FITC-conjugated antibodies to TCR $V_{\alpha}2.3$, PE-Cy5-conjugated antibodies to CD44, and PE-conjugated antibodies to CD45RB. The stained samples were analyzed on a FACScalibur™ using CELLQuest™ software (Becton-Dickinson).

2.9. Data analysis

For in vivo experiments, individual mice were analyzed and the mean and S.D. were calculated for each group. The numbers of mice per group are given in the table and figure legends. The group means were compared and the significance of differences was determined using the Wilcoxon test as described (Schefler, 1979); $p < 0.05$ was considered significant.

The in vitro T cell differentiation experiments were done at least three times with triplicate cultures for each group. Each culture well was analyzed in duplicate and the average was recorded. The calculated means and S.D. for the triplicate cultures were compared using the two-tailed Student's t-test assuming unequal variances (Microsoft Excel 98, Microsoft, Redmond, WA); $p < 0.05$ was considered significant.

3. Results

3.1. 1,25-(OH)₂D₃ inhibition of EAE induction

EAE is a Th1 cell-mediated autoimmune disease, and previous reports suggested that the mechanism for 1,25-(OH)₂D₃-mediated inhibition of EAE induction might involve regulation of T cell differentiation or function. To investigate possible hormonal control of T cell differentiation, a TCR1 cell transfer system was established. This system allowed the transfer of unprimed or MBP-primed TCR1 cells, thereby increasing the MBP-specific T cell frequency to a point where TCR1 T cell differentiation could be measured, while retaining the other T cell populations found in non-transgenic mice at normal levels.

The first study tested whether the 1,25-(OH)₂D₃ would inhibit EAE when unprimed TCR1 cells were transferred, consistent with the actions we reported for hormone treatment in unprimed B10.PL mice immunized with MBP (Cantorna et al., 1996). Ten million unprimed TCR1 splenocytes were injected into unprimed B10.PL mice. One day later, the mice were changed to a synthetic diet with or without 1,25-(OH)₂D₃, and the synthetic diet feeding was continued for the duration of the experiment. After 3 days of synthetic diet feeding, the recipient mice were immunized with MBP Ac1–11 peptide and pertussis, and EAE severity was recorded daily thereafter. As ex-

pected, the mock-treated animals had a 93% (13/14) disease incidence and most animals (12/14) reached a peak severity of at least 2.0 or greater (Fig. 1). In sharp contrast, just 18% of the hormone-treated animals had mild disease symptoms and most animals showed no disease whatsoever. When the hormone was removed from the diet of the 1,25-(OH)₂D₃-treated mice on day 17, no EAE symptoms were seen through day 44 when the experiment was terminated. The control mice continued to exhibit relapsing–remitting EAE symptoms during this time. Thus, the 1,25-(OH)₂D₃ inhibited the transferred TCR1 cells from inducing EAE when the recipients were primed with MBP Ac1–11.

3.2. VDR expression in Th1 and Th2 cells

The mechanism for 1,25-(OH)₂D₃-mediated inhibition of EAE induction probably involves the binding of the hormone to the VDR, which is present in activated T lymphocytes (Bhalla et al., 1983; Provvedini et al., 1983). As a first step towards understanding 1,25-(OH)₂D₃-mediated inhibition of EAE induction, we examined VDR expression in Th1 and Th2 cells. TCR1 splenocytes were used to produce the Th1 and Th2 cells, and the RNA from these cells was analyzed for VDR transcripts. To produce Th1 cells, the TCR1 splenocytes were cultured with MBP Ac1–11 and exogenous IL-12 and IFN-γ. When washed and restimulated with MBP Ac1–11 and fresh, irradiated B10.PL splenocytes, the polarized Th1 cells produced IFN-γ (407 ± 30 ng/ml per 5×10^5 T cells in 24 h), but no IL-4. Similarly, to produce Th2 cells, the splenocytes were cultured with MBP AC1–11, exogenous IL-4, and
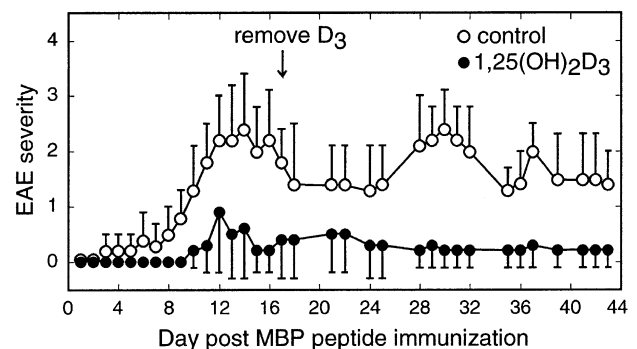

Fig. 1. The 1,25-(OH)₂D₃-treated B10.PL recipients of TCR1 splenocytes did not develop EAE when they were primed with MBP Ac1–11. (○) Mock-treated; (●) 1,25-(OH)₂D₃-treated. The TCR1 splenocytes (10^7 cells) were injected i.p. into naive B10.PL mice. One day later, the recipients were changed to a synthetic diet devoid of vitamin D, or supplemented with 1,25-(OH)₂D₃ as described in Materials and Methods. Three days after the diet change, the recipients were immunized with MBP Ac1–11. Synthetic diet feeding was continued and EAE severity was scored daily after peptide immunization. The experiment was performed three times. The values shown represent the composite mean and S.D. from these three experiments. Up to day 14 there were 20 mock-treated mice and 22 hormone-treated mice. Thereafter, there were 10 mice per group.

neutralizing antibodies to IFN-γ. The washed and restimulated Th2 cells produced IL-4 (439 ± 18 ng/ml per 5×10^5 T cells in 24 h), but no IFN-γ. The Th1 and Th2 cells recovered for VDR transcript analysis were $> 95\%$ $V_\beta 8.2$ positive by flow cytometric analysis (data not shown). The mRNA from these cells was isolated and reverse transcribed. A quantitative, competitive PCR was done for G3PDH, and the sample cDNA concentrations were adjusted to equalize the G3PDH cDNA content. The samples were then serially diluted and subjected to a VDR-specific PCR (Fig. 2). Both the Th1 and Th2 cells expressed good levels of the VDR mRNA suggesting they may be targets of $1,25\text{-}(OH)_2D_3$ action.

3.3. $1,25\text{-}(OH)_2D_3$ action on differentiated Th1 and Th2 cells

To learn whether the hormone might inhibit EAE by blocking the encephalitogenic funtions of Th1 cells in vivo, a Th1 cell adoptive transfer model of EAE was used. Polarized TCR1 Th1 cells were produced as described above. Graded Th1 cell numbers were injected into unprimed B10.PL mice that had been fed a synthetic diet with or without $1,25\text{-}(OH)_2D_3$ continuously, beginning 3 days prior to the Th1 cell transfer. EAE symptoms were scored daily after the transfer. The maximum EAE severity was proportional to the number of Th1 cells transferred; 0.2×10^6 Th1 cells did not induce disease, 1×10^6 Th1 cells yielded stage 1–2 EAE, and 2.5×10^6 Th1 cells yielded stage 2–3 EAE. The $1,25\text{-}(OH)_2D_3$-treated mice showed a slight delay in EAE onset compared to the mock-treated mice at the intermediate Th1 cell dose (Fig. 3; $p = 0.048$ at day 9). Nevertheless, the mock-treated and hormone-treated mice did not differ significantly in EAE incidence and peak severity at the intermediate (Fig. 3) and high (data not shown) Th1 cell doses, indicating that $1,25\text{-}(OH)_2D_3$ treatment did not inhibit the encephalitogenic function of the fully differentiated Th1 cells.

Fig. 3. The $1,25\text{-}(OH)_2D_3$-treatment does not prevent adoptive transfer of Th1 cell-mediated EAE disease. (○) Mock-treated; (●) $1,25\text{-}(OH)_2D_3$-treated. Naive B10.PL mice were continuously fed a synthetic diet devoid of vitamin D, or supplemented with $1,25\text{-}(OH)_2D_3$ beginning 3 days before the Th1 cell transfer. The Th1 cells were obtained by culturing the TCR1 splenocytes under Th1-driving conditions; 1×10^6 Th1 cells were injected i.p. into each recipient and clinical EAE disease was scored daily thereafter. The values represent the mean and S.D. of five individual male and female mice per group in one experiment of two.
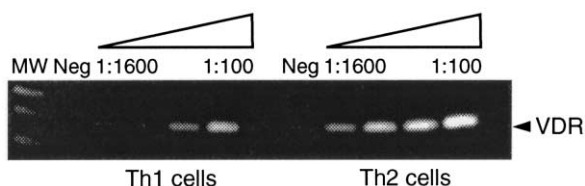

Fig. 2. The nuclear vitamin D receptor transcripts are present in Th1 cells and Th2 cells. Splenocytes from naive TCR1 mice were stimulated with MBP Ac1–11 peptide with exogenous cytokines to produce highly polarized Th1 and Th2 cells as described in Materials and methods. The mRNA was isolated from these cells, reverse transcribed, and a PCR for G3PDH was done. The Th1 and Th2 cDNA concentrations were adjusted to give equivalent amounts of G3PDH PCR product. The Th1 and Th2 cDNA samples were then serially diluted, subjected to the VDR-specific PCR as described in Materials and methods, electrophoresed, stained, and photographed. The arrowhead shows the position of the expected 300 bp VDR PCR product.
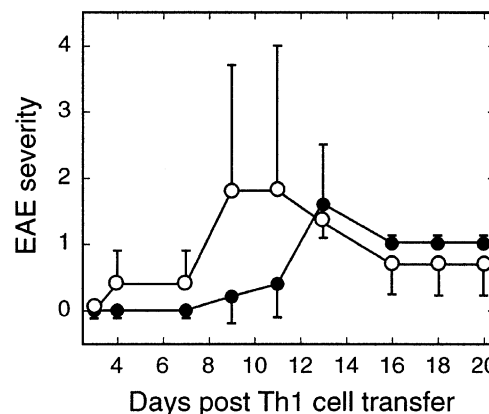
As a second method to test hormone inhibition of differentiated Th1 cell function, the effects of graded hormone doses on TCR1 Th1 cell IFN-γ synthesis was analyzed in vitro. Highly polarized Th1 cells were produced as described above, and restimulated overnight at a constant T cell density with MBP Ac1–11 and fresh, irradiated B10.PL splenocytes. In the absence of hormone, the Th1 cells (5×10^5) produced > 400 ng/ml of IFN-γ in 24 h. The hormone had no consistent, significant, or dose-dependent effect on IFN-γ synthesis (Fig. 4). Similarly, the hormone had no significant effect on IL-4 synthesis by polarized Th2 cells when they were restimulated overnight with MBP Ac1–11 and fresh, irradiated B10.PL splenocytes (Fig. 4). These results rule out a direct action of the hormone on differentiated Th1 cell IFN-γ synthesis and on differentiated Th2 cell IL-4 synthesis.

3.4. $1,25\text{-}(OH)_2D_3$ influence on Th1 and Th2 cell differentiation

Since the $1,25\text{-}(OH)_2D_3$ did not inhibit differentiated Th1 cell function, we considered the possibility that the hormone treatment may have inhibited Th1 cell development. The experiment shown in Fig. 1 was repeated, and on day 12, lymph node cells were recovered from the $1,25\text{-}(OH)_2D_3$-treated and mock-treated recipient mice and tested for a proliferative response to MBP Ac1–11 peptide in vitro. The $[^3H]$-thymidine incorporated into cells cultured without antigen was low and did not differ significantly between the groups (control group 2230 ± 133 cpm; hormone-treated group 2979 ± 635 cpm). This background of $[^3H]$-thymidine incorporation was subtracted. As ex-
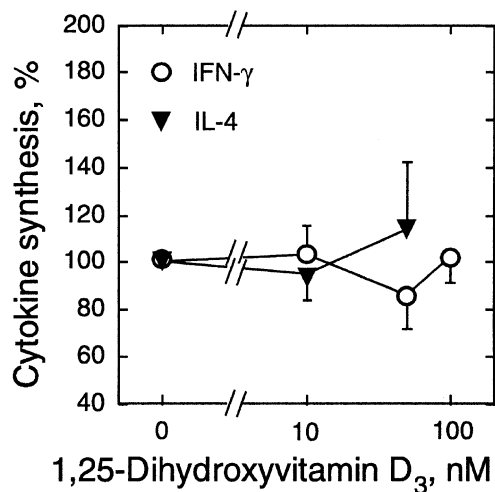
Fig. 4. The 1,25-$(OH)_2D_3$ does not inhibit differentiated Th1 and Th2 cell cytokine synthesis in vitro. (○) IFN-γ synthesis; (▼) IL-4 synthesis. Highly polarized Th1 and Th2 cells were produced as described in Materials and methods. The T cells were harvested at day 7 and restimulated in triplicate at 5×10^5 T cells/ml with fresh irradiated splenic APC and peptide, in the presence or absence of graded 1,25-$(OH)_2D_3$ concentrations. The 24-h restimulation culture supernatants were collected and IL-4 and IFN-γ proteins were quantified by ELISA. The data shown are the mean $\pm$ S.D. as a percentage of the control without added 1,25-$(OH)_2D_3$ for four (IL-4) or five (IFN-γ) separate determinations. The horizontal axis is a log scale.

pected, the cells recovered from mock-treated mice showed an antigen-dependent proliferative response to MBP Ac1–11 (Fig. 5A). Surprisingly, the cells recovered from the 1,25-$(OH)_2D_3$-treated mice also showed an antigen-dependent proliferative response to MBP Ac1–11, indicating

that priming had occurred. This result was unexpected because the hormone-treated mice had little or no EAE disease on day 12 (Fig. 1). On day 21, when mock-treated animals entered remission, the cells recovered from both groups of mice showed a very low proliferative response to MBP Ac1–11 (data not shown). However, memory T cells were present in both groups of mice on day 21, as judged by their strong and equivalent antigen-dependent proliferative responses in the presence of MBP Ac1–11 and exogenous IL-2 (Fig. 5B). These results rule out inhibition of TCR1 Th1 cell priming and TCR1 T cell deletion in the periphery as mechanisms for hormone-mediated inhibition of EAE induction. Further, the findings indicate that Th cell priming for an MBP Ac1–11 response occurred in the hormone-treated mice, despite the absence of EAE symptoms in these mice.

As a second method to detect Th1 cell priming for an MBP Ac1–11 response, we looked for IFN-γ transcripts. The experiment shown in Fig. 1 was repeated, and on various days after MBP Ac1–11 priming, the lymph node cells were collected and examined for IFN-γ transcripts by quantitative competitive PCR. The results showed very similar IFN-γ responses in the lymph nodes from the two groups of mice (Table 1). These data are entirely in agreement with our previous study showing equivalent LN cell IFN-γ protein secretion in mock-treated and hormone-treated B10.PL mice that were primed to induce EAE (Cantorna et al., 1998). These data are also consistent with the T cell proliferation data (Fig. 5A). Together, the present and previous experiments support the conclusion that Th1 cell priming for an MBP Ac1–11 response occurred in the lymph nodes of the hormone-treated mice.
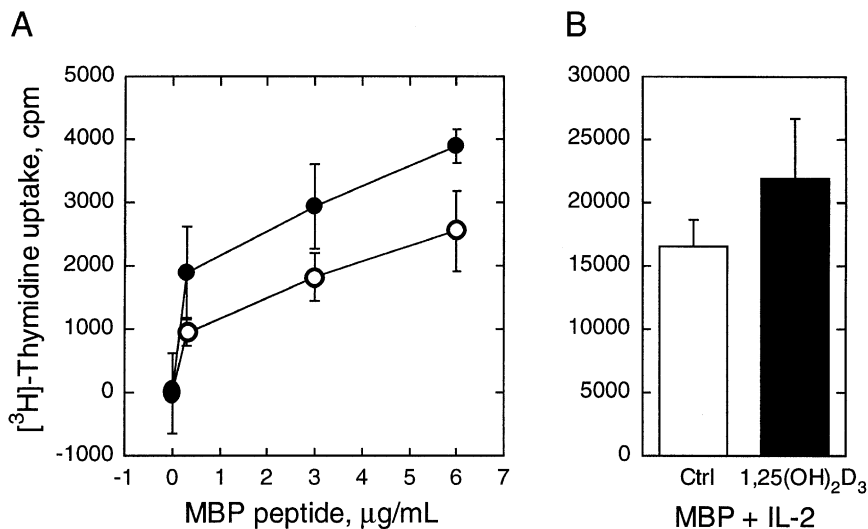

Fig. 5. Lymph node cells from 1,25-$(OH)_2D_3$-treated mice proliferate in response to MBP Ac1–11 in vitro. (○) Cells from mock-treated mice; (●) Cells from 1,25-$(OH)_2D_3$-treated mice. The experiment was done as described in the Fig. 1 legend. Panel A: 12 days after immunization with MBP Ac1–11, when the mock-treated mice showed peak EAE severity, lymph node cells were harvested and cultured in triplicate with graded amounts of MBP Ac1–11. Panel B: lymph node cells were harvested and cultured in triplicate with MBP Ac1–11 plus recombinant human IL-2. The proliferative response was quantified by [^{3}H]-thymidine uptake as described in Materials and methods. The background cpm in the absence of antigen was subtracted. The results shown are the mean $\pm$ S.E.M. for three to four individual mice per group from one experiment of three.

Table 1
Analysis of IFN-γ and IL-4 transcripts in the lymph node and spinal cord of TCR1 T cell recipients

Tissue	Day post-EAE induction	IFN-γ[a] transcripts		IL-4[a] transcripts	
		Control	1,25-$(OH)_2D_3$	Control	1,25-$(OH)_2D_3$
Lymph node	4	12 ± 7	4 ± 4[b]	< 1	< 1
	7	6 ± 3	10 ± 8	< 1	< 1
	14	14 ± 7	18 ± 16	50 ± 58	5 ± 8
	17	< 1	< 1	< 1	< 1
Spinal cord	4	< 1	< 1	10 ± 15	< 1
	7	< 1	< 1	< 1	< 1
	14	5 ± 4	< 1[b]	2 ± 3	< 1
	17	< 1	< 1	< 1	< 1

[a] The experiment was done as described in the Fig. 1 legend. EAE severity at day 14 was 2.3 ± 1.1 in the mock-treated group and 0.6 ± 0.0 in the $1,25(OH)_2D_3$-treated group. Mean and S.D. for 7–10 mice per group, except day 17 when there were four mice per group. Values given are transcript copies/1000 G3PDH copies.

[b] $p = 0.04$ by the Wilcoxon test; all other differences were not significant.

Some investigators have found that Th2 cells inhibit EAE induction and the progression of established EAE (Chen et al., 1994; Kuchroo et al., 1995), leading to the concept that induction of Th2 cells might be one mechanism of blocking autoimmune disease (Segal and Shevach, 1998). Thus, to explain the disease-free status of the $1,25$-$(OH)_2D_3$-treated mice, despite the presence of MBP-specific Th1 cells in these mice, one could propose that priming for a Th2 cell response to MBP may have occurred. To investigate this point, the experiment shown in Fig. 1 was repeated, and IL-4 transcripts were quantified at various times as described above for IFN-γ transcripts (Table 1). The results showed little or no IL-4 transcript accumulation in the lymph nodes from the $1,25$-$(OH)_2D_3$-treated mice, and a variable number of IL-4 transcripts only on day 14 in the mock-treated controls. Thus, if $1,25$-$(OH)_2D_3$ stimulated Th2 cell development as a mechanism of EAE inhibition, we were unable to detect the Th2 cells.

We also conducted experiments to investigate possible $1,25$-$(OH)_2D_3$-stimulated Th2 cell development in vitro. Unprimed TCR1 splenocytes were stained for the expression of the transgenic $V_\alpha 2.3$ and for CD62L (Mel-14), which is strongly positive on unprimed T cells. The $V_\alpha 2$-positive splenocytes had a high CD62L density (data not shown). When the unprimed TCR1 splenocytes were cultured without MBP Ac1–11, they did not proliferate spontaneously, suggesting that they had not activated in vivo. The unprimed TCR1 splenocytes were cultured with APC and MBP Ac1–11, in the presence or absence of polarizing cytokines (rIL-12 plus rIFN-γ or rIL-4 plus neutralizing antibodies to IFN-γ), with and without added $1,25$-$(OH)_2D_3$. The cultured cells were collected on day 7, counted, resuspended at a constant cell density, and tested

for cytokine synthesis in an overnight restimulation culture with fresh, irradiated B10.PL splenocytes, but without added hormone.

Without polarizing cytokines, a small IFN-γ response and no IL-4 response were obtained, and the hormone had no substantial effect on either response (Fig. 6). With polarizing cytokines, the rIL-12 and rIFN-γ addition promoted Th1 cell differentiation, whereas IL-4 addition plus neutralizing mAb to IFN-γ promoted Th2 cell differentiation as expected. Judging by cytokine synthesis in the polarizing cultures, $1,25$-$(OH)_2D_3$ reduced Th1 development in vitro by about 50–60%, and Th2 development by about 30–40% (Fig. 6). Thus, there was no evidence for $1,25$-$(OH)_2D_3$-stimulated Th2 cell development.
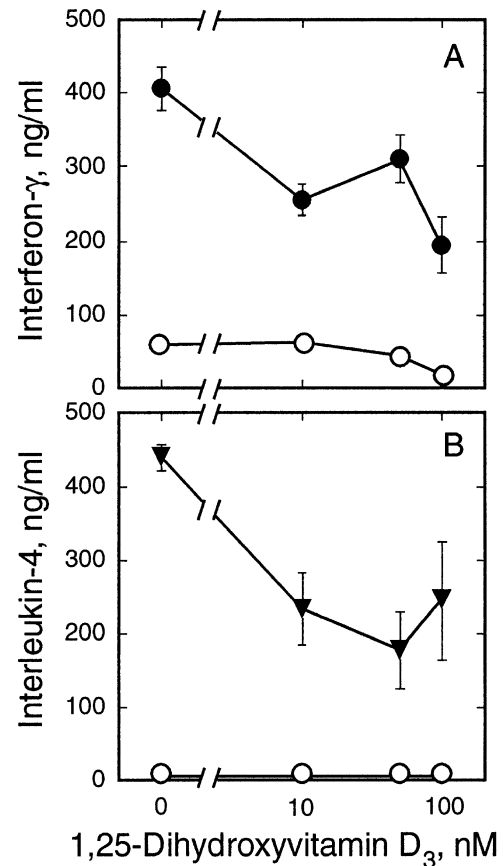

Fig. 6. The $1,25$-$(OH)_2D_3$ partially inhibited Th1 and Th2 cell development in vitro. ($\bigcirc$) No exogenous cytokines added; ($\bullet$) recombinant IL-12 and IFN-γ added; ($\blacktriangledown$) recombinant IL-4 and neutralizing mAb to IFN-γ added. Splenocytes from naive TCR1 mice were stimulated with MBP Ac1–11 peptide in the presence or absence of graded $1,25$-$(OH)_2D_3$ concentrations, with and without exogenous cytokines as indicated by the symbols. Cells harvested at day 7 were restimulated at 5×10^5 T cells/ml with fresh irradiated splenic antigen-presenting cells and MBP#1011 peptide. The 24-h restimulation culture supernatants were collected and IL-4 and IFN-γ proteins were quantified. The data shown are the mean $\pm$ S.D. of triplicate cultures from one representative experiment of six for Th1 cells and three for Th2 cells. The horizontal axis is a log scale.

3.5. 1,25-(OH)$_2$D$_3$ influence on activated T cells in the CNS

The results presented above indicated that the TCR1 cells transferred into hormone-treated recipients were not deleted, and in the lymph node, some differentiated into MBP-specific Th1 cells. Nevertheless, these hormone-treated recipients remained essentially disease-free. To explain this apparent paradox, we theorized that MBP-specific Th1 cells displaying a fully activated, cytokine-producing phenotype might not be present in the CNS, although they were present in the peripheral lymph nodes. Accordingly, we sought to detect activated Th1 cells in the CNS of mock-treated and hormone-treated mice. The experiment

shown in Fig. 1 was repeated, and on various days after MBP Ac1–11 priming, spinal cord cells were obtained from perfused animals, and the RNA extracted from the cells was analyzed for cytokine transcripts. IFN-γ and IL-4 transcripts were detected in the spinal cord samples from the mock-treated mice (Table 1). However, IFN-γ and IL-4 transcripts were completely lacking in the spinal cord samples from 1,25-(OH)$_2$D$_3$-treated mice. Thus, judging by cytokine transcript analysis, activated Th1 and Th2 cells were not detectable in the CNS of hormone-treated, disease-free mice.

To analyze whether TCR1 T cells were present but not activated or absent from the CNS of the 1,25-(OH)$_2$D$_3$-treated mice, immunofluorescence analysis was applied.

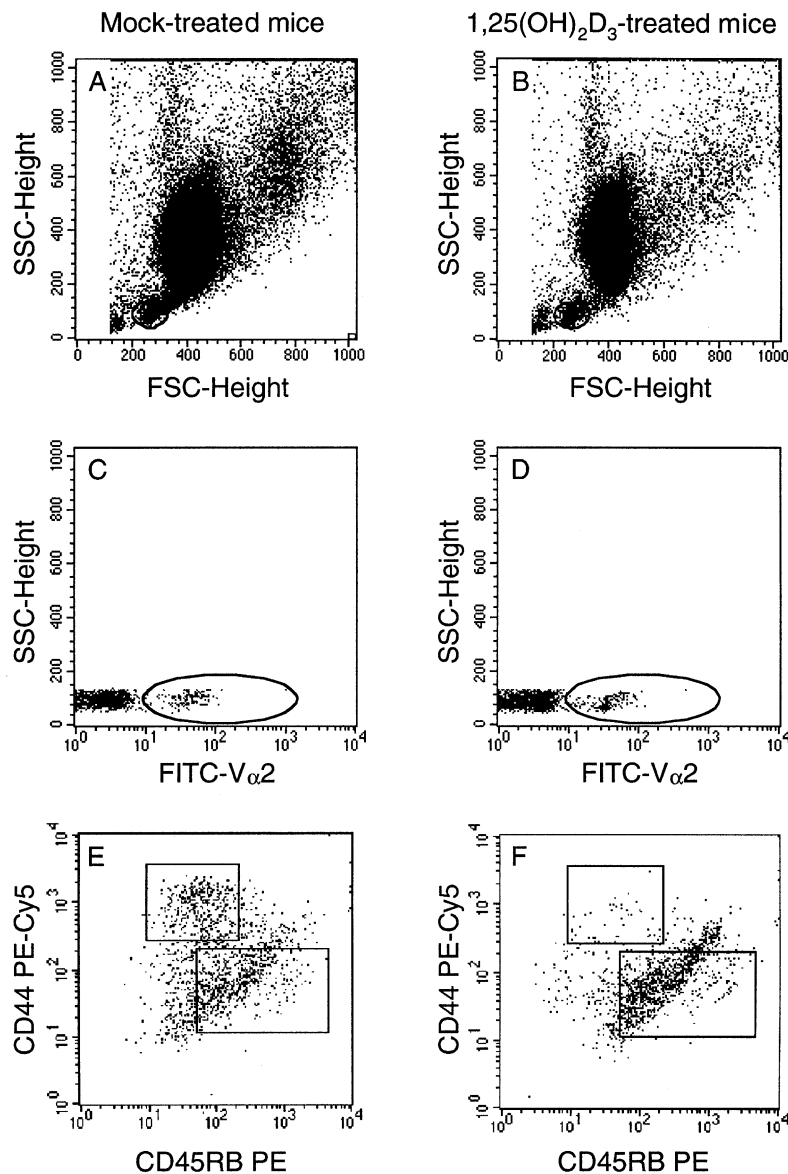Fig. 7. Spinal cord TCR1 T cells from 1,25-(OH)$_2$D$_3$-treated mice have an unprimed phenotype. Panels A, C, and E represent cells from mock-treated mice. Panels B, D, and F represent cells from 1,25-(OH)$_2$D$_3$-treated mice. The experiment was done as described in the Fig. 1 legend. On day 11, mice were euthanized and perfused with buffer. The spinal cord cells from each group ($n = 6$) were collected and pooled, separated on a Percoll gradient, washed, and immunostained as described in Materials and methods. The immunostained cells were analyzed by flow cytometry. Fluorescence is plotted on a log scale. The data shown represent one experiment of two.

Table 2
Analysis of activated and unprimed TCR-$V_\alpha 2^+$ lymphocytes in the spinal cord of TCR1 T cell recipients

The experiment was done as described in the Fig. 1 legend. The spinal cord cell analysis was done on day 11, when EAE severity was 2.2 ± 0.6 for the mock-treated mice ($n = 6$) and 0 ± 0 for the 1,25-$(OH)_2D_3$-treated mice ($n = 6$). Spinal cord cells were pooled for the analysis.

	Mock-treated		1,25-$(OH)_2D_3$-treated	
	%	10^3/mouse	%	10^3/mouse
Spinal cord cell recovery		2067		1217
Lymphocytes in the spinal cord	1.9	39	3.7	45
TCR $V_\alpha 2^+$ lymphocytes in the lymphocyte gate	10.8	4.2	9.8	4.3
Activated TCR $V_\alpha 2^+$ lymphocytes (CD44^{high}CD45RB^{low})	29.8	1.3	2.6	0.2
Unprimed TCR $V_\alpha 2^+$ lymphocytes (CD44^{high}CD45RB^{low})	43.0	1.8	71.7	3.2

The experiment shown in Fig. 1 was repeated, and when the mock-treated mice reached stages 2 to 3 EAE, spinal cord cells were isolated from six mice per group, pooled, stained, and analyzed as described (Brabb et al., 2000). Forward and side scatter gates were selected to encompass the live lymphocytes (Fig. 7A and B). The $V_\alpha 2^+$ T lymphocytes were gated (Fig. 7C and D), and analyzed for CD44 and CD45RB (Fig. 7E and F). Within the $V_\alpha 2^+$ T lymphocyte gate are the $V_\alpha 2 V_\beta 8.2$ T cells specific for MBP Ac1–11. There may also be a few $V_\alpha 2^+$ T lymphocytes that derive from the recipient mouse and may not be specific for MBP Ac1–11 (if they do not use $V_\beta 8.2$), and a few $V_\alpha 2^+$ T lymphocytes that derive from the TCR1 donor mouse and may have dual specificity due to incomplete allelic exclusion imposed by the $V_\alpha 2.3$ transgene.

Each mock-treated mouse and 1,25-$(OH)_2D_3$-treated mouse had about 4000 $V_\alpha 2^+$ lymphocytes in the CNS (Table 2). In the mock-treated mouse, ~ 1300 had an activated/memory phenotype (CD44^{hi}CD45RB^{lo}), while ~ 1800 had an unprimed phenotype (CD44^{lo}CD45RB^{hi}). In the 1,25-$(OH)_2D_3$-treated mouse, only ~ 200 had an activated/memory phenotype, while ~ 3200 had an unprimed phenotype. The finding that hormone-treated mice had mostly TCR1 T cells of an unprimed phenotype in the CNS is consistent with the cytokine transcript data and with the disease-free status of these animals.

3.6. 1,25-$(OH)_2D_3$ failure to inhibit EAE in Rag$^{-/-}$TCR2 mice

At least two models might explain the observation that hormone-treated mice had mostly unprimed TCR1 T cells in the CNS. First, the 1,25-$(OH)_2D_3$-treatment might have inhibited antigen-presenting cell function in the CNS, consistent with the reports that the hormone inhibited DC

maturation, activation, and antigen-presenting cell function in vitro (Griffin et al., 2000; Penna and Adorini, 2000; Piemonti et al., 2000). Second, the 1,25-$(OH)_2D_3$-treatment might have enhanced the function of regulatory cells that suppress the activation of self-reactive T cells, thereby preventing autoimmune disease (Sakaguchi, 2000; Shevach, 2000, 2001). To distinguish these models, we investigated whether the hormone could prevent EAE in *Rag-1$^{+/-}$*TCR2 and *Rag-1$^{-/-}$*TCR2 mice (Lafaille et al., 1994). Both strains have T cells that are specific for MBP Ac1–11. But, the *Rag-1$^{+/-}$*TCR2 mice have other *Rag-1*-dependent T and B cells, whereas the *Rag-1$^{-/-}$*TCR2 mice lack all other *Rag-1*-dependent T and B cells. If the hormone directly inhibits antigen-presenting cell function, then it would be expected to inhibit EAE in the *Rag-1$^{+/-}$*TCR2 and *Rag-1$^{-/-}$*TCR2 mice.

The *Rag-1$^{+/-}$*TCR2 and *Rag-1$^{-/-}$*TCR2 mice were fed a synthetic diet with or without 1,25-$(OH)_2D_3$ (50 ng/day for females; 100 ng/day for males) as soon as they were weaned and genotyped at age 3 weeks. At this age, we found no evidence of activated TCR2 T cells. Because EAE does not occur spontaneously in the *Rag-1$^{+/-}$*TCR2 mice (Lafaille et al., 1994), these mice were immunized with MBP Ac1–11. The mock-treated *Rag-1$^{+/-}$*TCR2 mice first showed EAE signs at day 5 post-immunization, and developed such severe disease by days 12–13 that they were euthanized (Fig. 8). In contrast, the 1,25-$(OH)_2D_3$-treated *Rag-1$^{+/-}$*TCR2 mice first showed EAE signs at days 8–9 post-immunization, and their EAE disease was less severe than the mock-treated mice. Thus, although the hormone did not inhibit EAE in these *Rag-1$^{+/-}$*TCR2 mice as effectively as it did in the B10.PL recipients of *Rag-1$^{+/-}$*TCR1 splenocytes (Fig. 1), the cells required for hormone-mediated inhibition were clearly present in the *Rag-1$^{+/-}$*TCR2 mice.

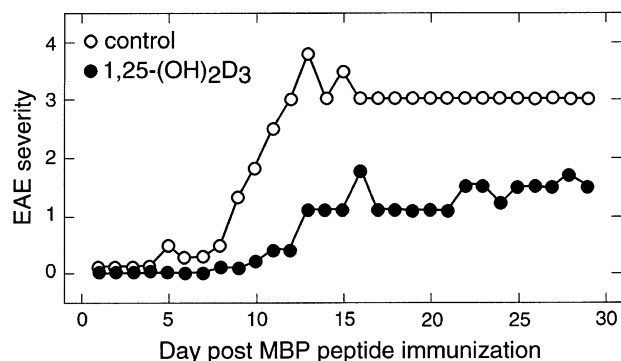EAE develops spontaneously in the *Rag-1$^{-/-}$*TCR2 mice (Lafaille et al., 1994). Signs of spontaneous EAE

Fig. 8. The 1,25-$(OH)_2D_3$ treatment inhibited peptide-induced EAE in the *Rag-1$^{+/-}$* TCR2 mice. The *Rag-1$^{+/-}$* TCR2 mice (three per group) were fed a synthetic diet with or without 1,25-$(OH)_2D_3$ (50 ng/day for females; 100 ng/day for males). One week after the diet change, the mice were immunized with MBP Ac1–11. The EAE disease severity was scored daily.

occurred at age 4–5 weeks in 100% of the mock-treated *Rag-1*$^{-/-}$ TCR2 mice (10/10) and the 1,25-(OH)$_2$D$_3$-treated *Rag-1*$^{-/-}$ TCR2 mice (6/6). The EAE was equally severe in the two groups of mice and required that they be euthanized at age 8–9 weeks. The complete failure of 1,25-(OH)$_2$D$_3$ treatment to delay the onset or reduce the severity or the incidence of EAE in *Rag-1*$^{-/-}$ TCR2 mice is not consistent with direct hormone inhibition of antigen-presenting cell function. Instead, these data indicate that one important cell type required for 1,25-(OH)$_2$D$_3$-mediated inhibition is absent in the *Rag-1*$^{-/-}$ TCR2 mice. Thus, hormone-mediated inhibition of EAE requires a *Rag-1*-dependent cell.

4. Discussion

Our goal was to investigate how 1,25-(OH)$_2$D$_3$ inhibits EAE induction, when animals that have MBP peptide-specific T cells are immunized with the peptide. The new evidence provided here clearly shows that 1,25-(OH)$_2$D$_3$ treatment did not directly inhibit Th1 cell encephalitogenic functions such as IFN-γ production, nor did it promote the deletion of the MBP peptide-specific T cells, or stimulate Th2 cell differentiation or IL-4 synthesis. Priming of the MBP peptide-specific T cells for a proliferative response with IFN-γ production occurred in the peripheral lymph nodes of the hormone-treated animals, indicating that the 1,25-(OH)$_2$D$_3$ treatment did not inhibit Th1 cell differentiation in these sites through an action on antigen-presenting cells or T cells. Significantly, the disease status of the mice correlated best with the activation status of the V$_\alpha$2$^+$ T cells that were detected in the CNS. In the mock-treated mice with EAE, these cells had an activated, cytokine-producing phenotype. In the 1,25-(OH)$_2$D$_3$-treated mice, they had an unprimed phenotype and did not produce cytokines. Thus, the hormone appeared to limit Th1 cell activation in the CNS. Significantly, the 1,25-(OH)$_2$D$_3$ treatment prevented EAE in *Rag-1*$^{+/-}$ TCR2 mice, but not in *Rag-1*$^{-/-}$ TCR2 mice. These data suggest a model wherein the hormone potentiates a natural *Rag-1*-cell-dependent mechanism that suppresses MBP-specific Th1 cell activation in the CNS.

Our data do not support the view that 1,25-(OH)$_2$D$_3$ is a potent and specific inhibitor of Th1 cells (Lemire et al., 1995; Muller and Bendtzen, 1996; Provvedini et al., 1983; Reichel et al., 1987; Rigby, 1988; Rigby et al., 1987). Many in vitro studies have shown that 1,25-(OH)$_2$D$_3$ addition to mitogen-stimulated cultures of human peripheral blood cells inhibited IFN-γ protein and mRNA production (Lemire, 2000). We replicated these in vitro studies here, showing that 1,25-(OH)$_2$D$_3$ addition to TCR1 splenocyte cultures decreased the IFN-γ response. However, this in vitro result could not be corroborated in vivo. The 1,25-(OH)$_2$D$_3$ failed to inhibit activated Th1 cell encephalitogenic function in the Th1 cell transfer model of

EAE, and it also failed to inhibit Th1 cell development in the MBP peptide induction model of EAE. Based on the in vivo results, we conclude that 1,25-(OH)$_2$D$_3$-mediated inhibition of Th1 cells is not the mechanism for hormone inhibition of EAE induction.

Our data also do not support the emerging view that 1,25-(OH)$_2$D$_3$ inhibits antigen presentation for T cell activation (Griffin et al., 2000; Penna and Adorini, 2000; Piemonti et al., 2000). Griffin et al. (2000) reported that 1,25-(OH)$_2$D$_3$ addition to mouse DC cultures reduced MHC class II, CD40, CD80 (B7-1), and CD86 (B7-2) expression in vitro, thereby impeding the capacity of the DC to stimulate allogeneic T cell activation. Penna and Adorini (2000) reported similar findings with human DC cultures, and also reported inhibitory effects of the hormone on DC differentiation and maturation in vitro. Finally, Piemonti et al. (2000) added 1,25-(OH)$_2$D$_3$ to human DC cultures and found enhancement of endocytic activity with little effect on costimulatory molecules, but a decrease in stimulation of allogeneic T cell activation. Based entirely on in vitro evidence, the investigators hypothesized that the immunosuppressive effects of the hormone may be attributable to decreased antigen-presenting cell function. We also tested this model in vivo. The model predicts that 1,25-(OH)$_2$D$_3$ treatment would inhibit EAE in the *Rag-1*$^{-/-}$ TCR2 mice. The finding that it did not, and that it also did not prevent Th1 cell activation in the lymph nodes of TCR1 cell recipients that were primed with MBP peptide, indicates that there was no functionally significant decrease in antigen-presenting cell function in vivo.

Induction of Th2 cells rather than Th1 cells has been suggested as one mechanism of blunting autoimmune disease (Segal and Shevach, 1998). This immune deviation concept is based on the findings that Th2 cells protected against EAE induction and the progression of established EAE (Chen et al., 1994; Kuchroo et al., 1995 and others). We previously found some IL-4 transcripts in a pooled sample from 1,25-(OH)$_2$D$_3$-treated animals without EAE, but none in a pooled sample from mock-treated animals with EAE (Cantorna et al., 1998); these results led us to postulate that 1,25-(OH)$_2$D$_3$ may have stimulated Th2 cell differentiation or IL-4 synthesis. However, in those experiments, the IL-4 transcript abundance was very low and highly variable. To increase the MBP peptide-specific T cell frequency so individual mice could be analyzed with greater precision, we transferred TCR1 splenocytes into B10.PL mice and repeated the IL-4 transcript analysis. In the current system, no IL-4 transcripts were detected at any time in any tissue in the hormone-treated mice, whereas these transcripts were detected occasionally and with high variability in the mock-treated recipients. In addition, an in vitro T cell differentiation system was used, and there was no direct effect of 1,25-(OH)$_2$D$_3$ on Th2 cell differentiation or IL-4 production in vitro. Thus, our current results do not support the postulate that 1,25-(OH)$_2$D$_3$ inhibits

EAE by stimulating Th2 cell differentiation or IL-4 synthesis. Our results showing that Th2 cells are not required for hormone-mediated EAE inhibition are consistent with evidence that Th2 cells were inefficient suppressors of EAE (Khoruts et al., 1995) and actually caused EAE in *Rag-1*-deficient mice (Lafaille et al., 1997), that EAE remissions occurred in IL-4-deficient mice (Liblau et al., 1997), and that IL-4 was not detectable during spontaneous EAE remissions in normal mice (Di Rosa et al., 1998). Therefore, IL-4-independent mechanisms must exist to inhibit EAE, and $1,25\text{-}(OH)_2D_3$ most likely operates through one of these IL-4-independent mechanisms.

Development of EAE requires the presence of activated, neural antigen-reactive T cells in the CNS, and several mechanisms have been documented that prevent such injurious autoimmune responses. One mechanism is active suppression. There is good genetic and biological evidence that activation of neural antigen-reactive T cells can be specifically suppressed. Antigen-specific suppression of EAE has been achieved by administering MBP i.v. in soluble form (Alvord et al., 1965; Levine et al., 1972; Swierkosz and Swanborg, 1975), or intracutaneously in IFA (Alvord et al., 1965; Rauch et al., 1968; Swierkosz and Swanborg, 1977), or i.v. coupled to cells or liposomes (McKenna et al., 1983; Strejan et al., 1981), or as an oral dose (Bitar and Whitacre, 1988; Higgins and Weiner, 1988). In most instances, adoptive transfer of T lymphocytes transferred antigen-specific suppression of EAE to unprimed animals, implicating regulatory T lymphocytes in this tolerance mechanism. Antigen-specific suppression of EAE has also been documented in the TCR1 and TCR2 mice. Van de Keere and Tonegawa (1998) and Olivares-Villagomez et al. (1998) reported that the MBP-specific TCR2 mice do not spontaneously develop EAE unless they are made genetically deficient in the *Rag-1* gene. By crossing the MBP-specific TCR transgene onto other null strains, and performing adoptive transfer experiments, these investigators determined that *Rag-1*-dependent $TCR\alpha\beta$ $CD4^+$ T cells suppress EAE induction but not Th1 cell encephalitogenic functions in the TCR2 mice. Our results showing that early $1,25\text{-}(OH)_2D_3$ treatment prevented EAE in the $Rag\text{-}1^{+/-}$ TCR2 but not $Rag\text{-}1^{-/-}$ TCR2 mice is consistent with a model wherein the $1,25\text{-}(OH)_2D_3$ treatment may have enhanced the development and/or function of the *Rag-1*-dependent $TCR\alpha\beta$ $CD4^+$ T cells that suppress EAE induction.

There is good evidence from TCR-transgenic mouse models that suppression of neural antigen-reactive T cell activation occurs in the CNS but not in the peripheral lymph nodes, suggesting that this tolerance mechanism operates in situ (Brabb et al., 2000). T cells from the CNS of unprimed TCR1 mice with no EAE signs had an unprimed phenotype and did not proliferate in response to MBP peptide in vitro, whereas cells from the cervical lymph nodes of these animals had an activated/memory phenotype and proliferated in response to MBP peptide in vitro (Brabb et al., 2000). Furthermore, the CNS cells from disease-free TCR1 mice suppressed the proliferative responses of TCR1 lymph node cells to MBP peptide. Our results were strikingly similar to these results. The experimental system we used was different, since we transferred TCR1 splenocytes into unprimed B10.PL mice and immunized the recipients with MBP peptide, whereas they studied spontaneous EAE in TCR1 mice. We found that mock-treated mice with EAE had many activated, IFN-γ-producing, TCR1 T cells in the CNS and in the peripheral lymph nodes, whereas the disease-free, $1,25\text{-}(OH)_2D_3$-treated mice had activated TCR1 T cells in the peripheral lymph nodes, but had only unprimed TCR1 T cells in the CNS. Additional experiments will be required to determine whether neural antigen-specific suppressor cells are present in the CNS of the disease-free, $1,25\text{-}(OH)_2D_3$-treated mice, and these experiments are in progress. However, based on the new evidence presented here, we suggest that the $1,25\text{-}(OH)_2D_3$ may inhibit EAE induction by augmenting the development and/or function of *Rag-1*-dependent cells (such as $TCR\alpha\beta$ $CD4^+$ T cells) that inhibit activation of neural antigen-reactive T cells in the CNS. If this hypothesis is correct, and $1,25\text{-}(OH)_2D_3$ is required for the optimal function of regulatory cells that maintain self-tolerance in the periphery, then lack of sufficient vitamin D to support $1,25\text{-}(OH)_2D_3$ biosynthesis could be an environmental risk factor for a large number of autoimmune disorders.

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