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1,25-Dihydroxycholecalciferol Prevents and Ameliorates Symptoms of Experimental Murine Inflammatory Bowel Disease[1]

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ABSTRACT   Anecdotal data suggest that the amount of vitamin D available in the environment either from sunshine exposure or diet may be an important factor affecting the development of inflammatory bowel disease (IBD) in humans. We tested the vitamin D hypothesis in an experimental animal model of IBD. Interleukin (IL)-10 knockout (KO) mice, which spontaneously develop symptoms resembling human IBD, were made vitamin D deficient, vitamin D sufficient or supplemented with active vitamin D (1,25-dihydroxycholecalciferol). Vitamin D–deficient IL-10 KO mice rapidly developed diarrhea and a wasting disease, which induced mortality. In contrast, vitamin D–sufficient IL-10 KO mice did not develop diarrhea, waste or die. Supplementation with 50 IU of cholecalciferol (5.0 μg/d) or 1,25-dihydroxycholecalciferol (0.005 μg/d) significantly ($P < 0.05$) ameliorated symptoms of IBD in IL-10 KO mice. 1,25-Dihydroxycholecalciferol treatment (0.2 μg/d) for as little as 2 wk blocked the progression and ameliorated ($P < 0.05$) symptoms in IL-10 KO mice with already established IBD.   J. Nutr. 130: 2648–2652, 2000.

KEY WORDS: • *vitamin D* • *inflammatory bowel disease* • *1,25-dihydroxycholecalciferol* • *mice*

Inflammatory bowel diseases (IBD)[3] are immune-mediated diseases of unknown etiology affecting the gastrointestinal (GI) tract. There are at least two distinct forms of IBD, ulcerative colitis and Crohn's disease. IBD are chronic recurring illnesses most commonly involving inflammation of the terminal ileum and colon, although these diseases can also affect many sites throughout the alimentary tract. Clearly, genetic factors predispose individuals to development of IBD (Podolosky 1991). In addition, the environment contributes to IBD development, and there is reason to believe that vitamin D may be an environmental factor affecting IBD. There is less vitamin D from sunlight exposure in areas in which IBD occurs most often because IBD is most prevalent in northern climates such as North America and Northern Europe (Podolosky 1991, Sonnenberg et al. 1991). A major source of vitamin D results from its manufacture via a photolysis reaction in the skin, and vitamin D availability from sunlight exposure is significantly lower in northern climates, particularly during the winter (Clemens et al. 1982, DeLuca 1993). Dietary intake of vitamin D is problematic because few foods are naturally rich in vitamin D. Weight loss occurs in 65–75% of patients diagnosed with Crohn's disease and 18–62% of patients with ulcerative colitis (Fleming 1995, Geerling et al. 1998). Vita-min deficiencies in general and vitamin D deficiency in particular have been shown to occur in IBD patients (Andreassen et al. 1998, Kuroki et al. 1993). To date, the possible association between vitamin D status and the incidence and severity of IBD in humans or animals has not been studied. The anecdotal information suggests that vitamin D status could be an environmental factor affecting the prevalence rate for IBD; this possible correlation warrants serious investigation.

The identification of vitamin D receptors in peripheral blood mononuclear cells sparked the early interest in vitamin D as an immune system regulator (Bhalla et al. 1983, Provvedini et al. 1983). In particular the CD4+ Th cells have vitamin D receptors and are therefore targets for vitamin D (Veldman et al. 2000). Hormonally active vitamin D [1,25-dihydroxycholecalciferol; $1,25(OH)_2D_3$] suppressed the development of at least two experimental autoimmune diseases (Cantorna et al. 1996 and 1998a). In vitro, $1,25(OH)_2D_3$ inhibited T-cell proliferation and decreased the production of interleukin (IL)-2, interferon (IFN)-γ and tumor necrosis factor (TNF)-α (Lemire and Adams 1992). In vivo, $1,25(OH)_2D_3$ injections were shown to inhibit the delayed type hypersensitivity reaction associated with the type-1 helper T (Th1) cell response (Lemire and Archer 1991, Lemire 1992). Vitamin D is a potent regulator of the immune system in general and T cells specifically.

For IBD, the immune-mediated attack is against the GI tract (Niessner and Volk 1995, Podolosky 1991). T cells, which preferentially produce the Th1 cytokines (IL-2, IFN-γ and TNF-α), have been shown to transfer Crohn's-like symptoms to naive mice (Aranda et al. 1997, Bregenholt and Claesson 1998), and the production of Th1 cytokines is asso-

ciated with IBD in humans as well (Niessner and Volk 1995). $1,25(OH)_2D_3$ treatment has been shown to suppress the development of other T-cell–mediated experimental autoimmune diseases (multiple sclerosis and arthritis; Cantorna et al. 1996 and 1998a). The hypothesis that vitamin D (through the production of 1,25-dihydroxycholecalciferol) would suppress the development and progression of IBD was tested.

Recently, a number of transgenic animals have been developed in which IBD symptoms occur spontaneously. One of the best animal models for Crohn's disease is the IL-10 knockout (KO) mouse (Kuhn et al. 1993, Mac Donald 1994). In conventional animal facilities, the IL-10 KO mice develop enterocolitis within 5–8 wk of life (Kuhn et al. 1993). Approximately 30% of the IL-10 KO mice die after the development of severe anemia and weight loss (Kuhn et al. 1993). The enterocolitis that develops in IL-10 KO mice is due to an uncontrolled immune response to conventional microflora because germfree IL-10 KO mice do not develop disease. In addition, mice raised in specific pathogen–free facilities develop milder disease, which does not result in the death of the mice (Kuhn et al. 1993). There are limitations involved in studying IL-10 KO mice as a model of IBD. If vitamin D is a regulator of IL-10 production, then the results in this animal model may not represent a "normal" immune response. However, patients with Crohn's disease show similar symptoms, have depressed IL-10 production and have been treated successfully with IL-10 (Narula et al. 1998).

MATERIALS AND METHODS

Mice. Age- and sex-matched C57BL/6 IL-10 KO and wildtype (WT) mice were produced in the Pennsylvania State University breeding colony; the breeding pairs were obtained from Jackson Laboratory (Bar Harbor, ME). The animal facilities at the Pennsylvania State University are specific pathogen free and therefore breeding IL-10 KO mice was successful. All of the procedures described were reviewed and approved by the Pennsylvania State University Institutional Animal Care and Use Committee on 1/25/99, IACUC# number: 98118-A0.

Diets From a single pool of breeding females fed commercial mouse diet (#5105 Ralston Purina; Richmond, IN), females in wk 2 of gestation were selected and distributed randomly into two groups. Feeding pregnant dams a vitamin D–deficient diet ensured that the weanlings would be vitamin D deficient by 5 wk of age (Cantorna et al. 1996). All mice were fed synthetic diets made in the laboratory [(Yang et al. 1993) as modified from Smith et al. (1987)]. The mice were vitamin D deficient, vitamin D sufficient or $1,25(OH)_2D_3$ supplemented. Mice were housed under yellow light to prevent the synthesis of vitamin D in skin. All of the mice were vitamin D deficient until weaning.

The 3-wk-old vitamin D–deficient mice were randomly assigned to various treatment groups as described below. Because $1,25(OH)_2D_3$ treatment of other experimental autoimmune diseases was most effective when dietary calcium was high (1 g/100 g diet), all mice were fed high calcium diets (Cantorna et al. 1999). Experimental diets were freshly prepared and replaced every 2–3 d during the experiment. To ensure that $1,25(OH)_2D_3$-treated mice ate all of the supplement provided, food cups containing 8 g of diet were replaced every other day (completely eaten) for the duration of each experiment (Cantorna et al. 1996 and 1998a). To monitor vitamin D toxicity, $1,25(OH)_2D_3$-supplemented mice were observed daily for signs of hypercalcemia, including overall health and weight loss.

Vitamin D treatments. In Experiment 1, the 3-wk-old vitamin D–deficient mice were either maintained vitamin D deficient or switched to the experimental diet which included 5.0 μg cholecalciferol/d (vitamin D sufficient). The severity of IBD development was compared in vitamin D–deficient and vitamin D–sufficient mice.

In Experiment 2, 3-wk-old vitamin D–deficient mice were divided into two groups. One group of mice was maintained on the vitamin D–deficient diet and the other group was supplemented with 0.005

μg/d $1,25(OH)_2D_3$. The vitamin D–deficient and $1,25(OH)_2D_3$-supplemented mice were killed 4 wk later at 9 wk of age.

In experiment 3, $1,25(OH)_2D_3$ treatment was started at the first signs of IBD symptoms (diarrhea, 7 wk of age). The 7-wk-old vitamin D–deficient mice were divided into two groups. One group was maintained vitamin D deficient and the other group was supplemented with 0.2 μg/d $1,25(OH)_2D_3$. The mice were treated for 2 wk and the 9-wk-old mice were killed.

Food restriction. Because of the dramatic weight loss and death of vitamin D–deficient IL-10 KO mice, a series of controlled feeding experiments were done. These experiments used three groups of mice. All of the mice for these experiments were maintained vitamin D deficient for the first 5 wk of life (the earliest signs of weight loss). At 5 wk, the vitamin D–deficient IL-10 KO mice were divided into two groups. Half of the mice were maintained vitamin D deficient and the other half were switched to a vitamin D–sufficient diet, which contained 5.0 μg/d cholecalciferol. In addition, a group of vitamin D–deficient WT mice were also switched to a diet that contained 5.0 μg/d cholecalciferol. The food eaten by vitamin D–deficient IL-10 KO mice was weighed daily, and the vitamin D–sufficient IL-10 KO and WT mice were fed a restricted diet that contained the amount of food eaten by vitamin D–deficient IL-10 KO mice in the previous 24 h to control for food intake.

Serum measurements. Mice were bled at 5 wk of age and at the end of the experiments to measure hemoglobin, calcium and RBC numbers. Serum was obtained every 2 wk and serum calcium measured (normal for mice is 2.00–2.75 mmol/L). Vitamin D deficiency was also monitored by serum calcium analysis (serum calcium <1.75 mmol/L). Calcium (587-A) and hemoglobin (525-A) colorometric kits were from Sigma Chemical (St. Louis, MO). RBC were counted using a hemocytometer.

IBD severity. Mortality associated with the development of diarrhea was recorded in IL-10 KO and WT mice. In addition, the small intestines (SI) were removed and weighed. The jejunum of IL-10 KO mice is visibly inflamed and has been used by others to monitor symptoms of IBD in mice (Kuhn et al. 1993). The jejunum of the SI was saved in 100 g/L formalin in PBS solution and sent to the Pennsylvania State Diagnostic Laboratory for sectioning and staining with hematoxylin and eosin. Four or more paraffin sections (4 μm) from each mouse were scored using the exact procedure described by Kuhn et al. (1993). The sections were scored without knowledge of their origin on a scale of 0–5 for inflammation as follows: 0, no inflammation;1, a few inflammatory cells; 2, mild inflammation; 3, abscess formation; 4, abscess formation with many inflammatory cells throughout; and 5, massive inflammation throughout the section.

Statistical analysis. Experiments were repeated as necessary; where possible, values were reported as the means from multiple experiments. A two-sample test for binomial proportions was used for statistical analysis of the percentage values shown in **Figure 1** as described (Rosner 1986). Body weights and weight gains were analyzed by repeated-measures ANOVA using simple contrasts to compare diet groups (main effects). Data were subjected to two-way ANOVA using diet and IL-10 genotype as the grouping factors. All post-hoc multiple comparisons were made using Fisher's protected Least Significant Difference test. Values were compared using a statistics program (Statview Student, Abacus Concepts, Berkeley, CA) for the Macintosh computer and differences of $P < 0.05$ were considered significant.

RESULTS

Mortality of vitamin D–deficient IL-10 KO mice. Figure 1 shows that vitamin D–deficient IL-10 KO mice begin to die at 7 wk of age and by 9 wk of age, 58% (15/26) of the vitamin D–deficient IL-10 KO mice were dead. After 9 wk of age, vitamin D–deficient IL-10 KO mice continued to waste and the death rate increased. In contrast, the vitamin D–sufficient IL-10 KO ($n = 10$) and the vitamin D–deficient WT ($n = 20$) mice appeared healthy, even at 13 wk of age.

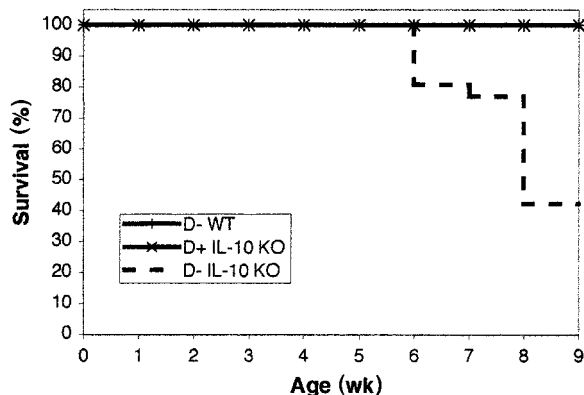The vitamin D–deficient IL-10 KO mice were growth re-

FIGURE 1 Vitamin D deficiency induces mortality of interleukin (IL)-10 knockout (KO) mice. Vitamin D–deficient IL-10 KO weanling mice were divided randomly into two groups. One group was maintained vitamin D deficient (−D, $n = 26$) and the other was fed the same diet which contained 5.0 μg cholecalciferol/d for the remainder of the experiment (+D, $n = 10$). Vitamin D–deficient wildtype (WT) (−D, $n = 20$) mice were also used in these experiments. Vitamin D–deficient IL-10 KO mice died after developing diarrhea. Vitamin D–deficient WT and vitamin D–sufficient IL-10 KO mice did not develop diarrhea or die.
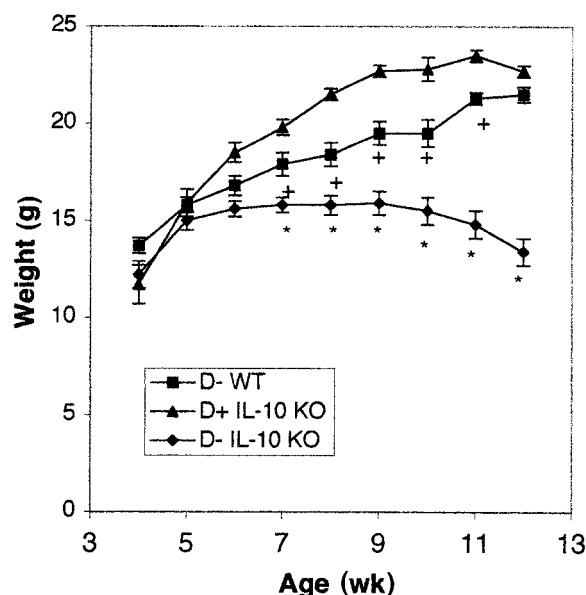
FIGURE 2 Growth curves for vitamin D–deficient and –sufficient interleukin (IL)-10 knockout (KO) mice and vitamin D–deficient wildtype (WT) mice. Vitamin D–deficient IL-10 KO weanling mice were divided randomly into two groups. One group was maintained vitamin D deficient (−D, $n = 14$ at the beginning of the experiment and $n = 5$ at the end) and the other was fed the same diet, which contained 5.0 μg cholecalciferol/d for the remainder of the experiment (+D, $n = 7$). Vitamin D–deficient WT (−D, $n = 9$) mice were also used in these experiments. Vitamin D–sufficient IL-10 KO mice grew rapidly compared with vitamin D–deficient IL-10 KO mice. The growth of vitamin D–deficient WT mice was retarded but constant over the 12-wk period (+the −D WT mice weighed significantly less than the +D IL-10 KO mice from 7 to 11 wk of age, $P < 0.05$); by 12 wk, the vitamin D–deficient WT mice did not differ from the vitamin D–sufficient mice in weight. Vitamin D–deficient IL-10 KO mice (*weighed significantly less then +D IL-10 KO mice, $P < 0.05$) stopped growing at 6 wk of age and began to lose weight and undergo a severe wasting disease, resulting eventually in death of the mice ($n = 5$ by 12 wk). Values are means ± SEM.

tarded compared with vitamin D–sufficient IL-10 KO and vitamin D–deficient WT mice (**Fig. 2**). The vitamin D–deficient WT mice grew more slowly than the vitamin D–sufficient IL-10 KO mice but by 12 wk of age, the vitamin D sufficient IL-10 KO and vitamin D deficient WT mice did not differ. By 6 wk of age and thereafter, the vitamin D–deficient IL-10 KO mice had stopped growing and were significantly smaller than the vitamin D–deficient WT mice (Fig. 2). At 9 wk of age, vitamin D–deficient IL-10 KO mice began to eat less and rapidly lost additional weight over the next 3 wk. Subsequent experiments were terminated at 9 wk to prevent unnecessary pain and suffering of the IL-10 KO mice. The vitamin D–deficient IL-10 KO mice died after a wasting disease, which was preceded by diarrhea.

IBD symptoms in vitamin D–deficient and 1,25(OH)$_2$D$_3$-supplemented IL-10 KO mice. Vitamin D–deficient WT and IL-10 KO mice weighed less than their 1,25(OH)$_2$D$_3$-supplemented counterparts at 9 wk of age (**Table 1**). The weights of the vitamin D–deficient IL-10 KO mice were lower than in previous experiments (Fig. 2) although data were consistent with the accelerated weight loss observed previously in vitamin D–deficient IL-10 KO mice. As expected, the serum calcium concentrations in 1,25(OH)$_2$D$_3$-supplemented mice were significantly ($P < 0.05$) higher than those of the vitamin D–deficient mice (Table 1). Hemoglobin levels and erythrocyte numbers were normal and not different in vitamin D–deficient, vitamin D–sufficient, and 1,25(OH)$_2$D$_3$- supplemented IL-10 KO and WT mice (data not shown).

WT mice that were vitamin D deficient and sufficient showed no signs of inflammation or abnormalities in the SI. Vitamin D–deficient IL-10 KO mice had significantly more inflammation in the SI than their 1,25(OH)$_2$D$_3$-supplemented or vitamin D–sufficient counterparts ($P < 0.05$, Table 1 and data not shown). Although the vitamin D–deficient IL-10 KO mice were the smallest in size, necropsy showed that they had extremely large SI. Future experiments will include SI weights as more quantitative measurements of inflammation in the SI.

Short-term 1,25(OH)$_2$D$_3$ treatment and IBD severity. There were no significant differences in the weight of any of the mice after 2 wk of 1,25(OH)$_2$D$_3$ treatment (data not shown). The SI of the vitamin D–deficient IL-10 KO mice,

however, were enlarged and weighed significantly more ($P < 0.05$) than the SI from 1,25(OH)$_2$D$_3$-supplemented IL-10 KO, vitamin D–deficient WT and 1,25(OH)$_2$D$_3$-supplemented WT mice (**Table 2**). In fact, the SI from vitamin

TABLE 1

Histopathology of vitamin D–sufficient and –deficient IL-10 KO and WT mice

Genotype	n	Vitamin D status[1]	Weight, g	Serum calcium, mmol/L	Histology score
IL-10 KO[2]	7	−D	12.4 ± 2.3*	1.74 ± 0.28*	3.0 ± 0.2*
IL-10 KO	7	1,25(OH)$_2$D$_3$	20.9 ± 0.8	3.00 ± 0.30	1.7 ± 0.4
WT	4	−D	16.7 ± 1.9*	1.67 ± 0.32*	0
WT	4	1,25(OH)$_2$D$_3$	21.1 ± 1.0	2.72 ± 0.25	0

[1] All of the mice were vitamin D deficient (−D) for the first 5 wk of life. At 5 wk, the mice were divided into two groups; half were supplemented with 0.005 μg/d 1,25(OH)$_2$D$_3$ for 4 wk. Values are means ± SEM; * significantly different from supplemented counterpart, $P < 0.05$.

[2] IL, interleukin; KO, knockout; −D, vitamin D deficient; 1,25(OH)$_2$D$_3$, 1,25-dihydroxycholecalciferol; WT, wildtype.

D–deficient IL-10 KO mice were 9.9% of the total body weight which is double the normal value (Table 2). Inflammation in the SI of IL-10 KO mice was reduced after as little as 2 wk of $1,25(OH)_2D_3$ treatment.

Food restriction vs. vitamin D deficiency and the symptoms of IBD. To rule out the possibility that weight loss and not vitamin D deficiency was associated with the increased symptoms of IBD observed, the food intake of vitamin D–sufficient IL-10 KO and WT mice was restricted (**Table 3**). Food restriction decreased the weight of vitamin D–sufficient IL-10 KO and WT mice, but the vitamin D–deficient IL-10 KO mice were still significantly lighter ($P < 0.05$, Table 3). The IL-10 KO mice were extremely ill by 9 wk in this series of experiments and had already undergone severe wasting. Food restriction did not change the symptoms of IBD in the vitamin D–sufficient mice. Food-restricted vitamin D–sufficient IL-10 KO mice did not develop overt enterocolitis or die, which occurred in vitamin D–deficient IL-10 KO mice. The relative SI weight of vitamin D–sufficient food-restricted IL-10 KO mice was not different than in previous experiments or compared with WT controls (Table 3). Histopathology confirmed the weight measurements in Table 3 (data not shown). The early symptoms of IBD in vitamin D–deficient IL-10 KO mice were associated with vitamin D deficiency and not with a reduction in energy or food intake.

DISCUSSION

Vitamin D deficiency exacerbates the symptoms of enterocolitis in IL-10 KO mice, and $1,25(OH)_2D_3$ treatment for as little as 2 wk ameliorated IBD symptoms in these mice. These findings provide strong evidence that vitamin D status may be an important factor in determining the incidence of IBD and furthermore establishes vitamin D as a physiologic regulator of IBD. This is the first experimental evidence to show a link between vitamin D status and IBD.

The time course of IBD development in vitamin D–deficient IL-10 KO mice is comparable to that of IBD that develops in IL-10 KO mice housed in conventional animal facilities (Kuhn et al. 1993). It is possible, although unlikely, that the microflora in the GI tract of IL-10 KO mice are disturbed during vitamin D deficiency such that disease-causing microbes expand and multiply to have an effect. Experiments to test this possibility could be done in vitamin D–de-

TABLE 2

$1,25(OH)_2D_3$ treatment decreases enterocolitis in IL-10 KO mice

| Genotype | n | Vitamin D status[1] | Small intestine | |
			g	$g/100~g$ body
IL-10 KO[2]	8	$-D$	1.67 ± 0.04*	9.9 ± 0.5*
IL-10 KO	8	$1,25(OH)_2D_3$	1.08 ± 0.05	5.4 ± 0.3
WT	12	$-D$	0.97 ± 0.02	5.8 ± 0.2
WT	8	$1,25(OH)_2D_3$	1.06 ± 0.04	5.3 ± 0.2

[1] All of the mice were vitamin D deficient ($-D$) for the first 7 wk of life. At 7 wk of age, the vitamin D–deficient IL-10 KO mice begin to show symptoms of enterocolitis (diarrhea and weight loss). The 7-wk-old IL-10 KO and WT mice were divided into two groups; half were supplemented with 0.2 μg/d $1,25(OH)_2D_3$ for 2 wk. Values are means $\pm$ SEM; * significantly greater than all other groups, $P < 0.05$.

[2] See Table 1 for abbreviations.

TABLE 3

Vitamin D deficiency and not a reduction in food intake causes inflammation in the small intestine in IL-10 KO and WT mice

| Genotype | n | Vitamin D status[1] | Weight, g | Small intestine | |
				g	$g/100~g$ body
IL-10 KO	7	$-D$	11.5 ± 1.2*	1.79 ± 0.02*	9.9 ± 0.4*
IL-10 KO	4	$+D$ restricted	16.1 ± 1.1	1.00 ± 0.02	6.2 ± 0.3
WT	4	$+D$ restricted	15.9 ± 1.1	0.85 ± 0.06	6.7 ± 0.2

[1] Five-wk-old vitamin D–deficient mice were divided into groups and either continued to consume diets that contained no added vitamin D ($-D$) or changed to a diet that contained 5.0 μg/d ($+D$) for 4 wk. The $+D$ mice were restricted in their food intake to the amount eaten by the vitamin D–deficient IL-10 KO mice in the previous 24 h. Values are means $\pm$ SEM; * significantly different from the other groups, $P < 0.05$.

[2] See Table 1 for abbreviations.

ficient germfree mice, although in the absence of any microflora, enterocolitis would probably not develop. It is more likely that the microflora do not change in response to vitamin D status but instead, the absence of vitamin D changes the immune response and the result in IL-10 KO mice is more severe IBD.

Accumulating evidence suggests that vitamin D is a regulator of CD4+ T cells, which cause autoimmune disease (Cantorna et al. 1996 and 1998c). One possible mechanism of vitamin D action is in the negative regulation of CD4+ T cells, which cause IBD. Vitamin D has been shown to inhibit directly the effector functions of CD4+ T cells both in vitro and in vivo (Cippitelli and Santoni 1998, Lemire 1992). The other possibility is that vitamin D is a positive regulator of T cells or other cells that inhibit the induction or function of IBD-causing T cells. Two possible vitamin D targets are transforming growth factor (TGF)-β1 and IL-4 secreting cells (Cantorna et al. 1998c). Increased production of TGF-β1 and IL-4 has been shown to occur in mice treated with $1,25(OH)_2D_3$ in vivo (Cantorna et al. 1998c). Furthermore, the production of TGF-β1 and IL-4 is associated with the inhibition of T-cell effector function and suppression of many autoimmune diseases (Groux et al.1997). Vitamin D regulation of the immune system is likely complex and includes multiple targets, which together explain the mechanism by which $1,25(OH)_2D_3$ suppresses the development of IBD.

Standard treatments of patients with IBD include short-term, high dose and long-term, low dose prednisone use (Andreassen et al. 1998, Podolosky 1991). Prednisone and other corticosteroid therapies result in decreased bone mineral density and many times result in higher risks for vertebral fracture (Andreassen et al. 1997 and 1998). Vitamin D supplementation of patients on corticosteroids has been shown to prevent steroid-induced bone loss (Buckley et al. 1996). The hormonally active form of vitamin D [$1,25(OH)_2D_3$] increases bone mineralization when given to experimental animals (Cantorna et al. 1998b) and humans (Ongphiphadhanakul et al. 2000). Thus, a further benefit of vitamin D and/or $1,25(OH)_2D_3$ supplementation may be the maintenance of bone mineral density.

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