1,25-Dihydroxycholecalciferol Inhibits the Progression of Arthritis in Murine Models of Human Arthritis[1,2]

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ABSTRACT 1,25-Dihydroxycholecalciferol [1,25-$(OH)_2D_3$] has been shown to inhibit the progression of experimental autoimmune encephalomyelitis (EAE). Here we tested the possibility that 1,25-dihydroxycholecalciferol might be therapeutic for another autoimmune disease, arthritis. Two different animal models of arthritis were tested, namely, murine Lyme arthritis and collagen-induced arthritis. Infection of mice with *Borrelia burgdorferi* (the causative agent of human Lyme arthritis) produced acute arthritic lesions including footpad and ankle swelling. Supplementation with 1,25-dihydroxycholecalciferol of an adequate diet fed to mice infected with *B. burgdorferi* minimized or prevented these symptoms. Mice immunized with type II collagen also developed arthritis. The symptoms of this disease were also prevented by dietary supplementation with 1,25-dihydroxycholecalciferol. 1,25-Dihydroxycholecalciferol given to mice with early symptoms of collagen-induced arthritis prevented the progression to severe arthritis compared with untreated controls. These results suggest that 1,25-dihydroxycholecalciferol and/or its analogs may be a valuable treatment approach to this disease. J. Nutr. 128: 68–72, 1998.

KEY WORDS: • *autoimmune disease* • *calcium* • *vitamin D* • *mice* • *lymphocytes*

Rheumatoid arthritis (RA),[4] which affects ~1% of the human population, is the most common of the chronic inflammatory arthritides that also include Lyme arthritis, reactive arthritis, juvenile arthritis and others (McCarty 1989). Although the etiology of these diseases is largely unknown, they share certain pathologic features. The rheumatoid joint shows an inflammatory cell infiltrate comprised of neutrophils, macrophages, T and B lymphocytes and dendritic cells (Feldmann et al. 1996). An inflammatory reaction is thought to underlie the joint pathology seen in chronic arthritis, namely, thickening of the synovial lining with increased vascularization and ultimately, irreversible damage to cartilage and bone (McCarty 1989).

One useful animal model with strong parallels to human RA, collagen-induced arthritis (CIA) (Courtenay et al, 1980,Trentham et al. 1977), has facilitated a detailed analysis of the joint-damaging, inflammatory reaction. Immune reactivity to autologous type II collagen, a major cartilage component, is well documented in RA patients (Londei et al. 1989). Further, CIA in animals shows a strong association with particular major histocompatability complex haplotypes as does human RA (Nepom et al. 1989). A second useful animal model, acute Lyme arthritis in mice (Barthold et al. 1990), is induced with the same pathogenic *Borrelia burgdorferi* spirochete that causes chronic arthritis in about 10% of infected humans (Burgdorfer et al. 1982). Lyme arthritis exemplifies the reactive arthritides that can follow certain bacterial infections, for example with *Borrelia*, *Yersinia*, *Salmonella*, *Shigella*, *Campylobacter* and *Chylamydia* species (Schlaak et al. 1992). Lyme arthritis in mice (Schaible et al. 1991) and in humans shows a strong major histocompatability complex association (Steere et al. 1990). Thus, these two animal models, CIA and Lyme arthritis, reflect the inflammatory response, the antigenic specificity and the genetic susceptibility of known arthritic diseases in humans.

A relationship between vitamin D and the immune system was first suspected when the vitamin D receptor was detected in activated lymphocytes (Bhalla et al. 1983, Provvedini et al. 1983). Further, vitamin D deficiency (Yang et al. 1993a) and large doses of 1,25-$(OH)_2D_3$ or its analogs retarded T-cell–mediated immunity (Yang et al. 1993b). Finally, an autoimmune disease, experimental autoimmune encephalomyelitis (EAE), can be prevented or halted by 1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$] (Cantorna et al. 1996).

It appeared reasonable that an inflammatory disease such as rheumatoid arthritis might be sensitive to 1,25-$(OH)_2D_3$ because this disease clearly involves T-cell– and macrophage-mediated responses (Feldmann et al. 1996, Keane-Myers and Nickell 1995). Using the two animal models (*B. burgdorferi*–induced and collagen-induced) of rheumatoid arthritis, we demonstrate that 1,25-$(OH)_2D_3$ can prevent and halt the arthritic progression.

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[4] Abbreviations used: CIA, collagen-induced arthritis; 1,25-$(OH)_2D_3$, 1,25-dihydroxycholecalciferol; EAE, experimental autoimmune encephalomyelitis; IFN-γ, interferon-γ; IL-2, interleukin-2; RA, rheumatoid arthritis; TNF-α, tumor necrosis factor-α.

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MATERIALS AND METHODS

Experimental diets. Upon arrival from commercial vendors, all mice were fed Purina diet 5008 Formilab (Richmond, IN), containing vitamin D. For experiments, all of the mice were fed synthetic diets made in this laboratory (Yang et al. 1993a; modification of Smith et al. 1987). The experimental diet contained either 1 g calcium/100 g or 20 mg calcium/100 g as indicated. Mice were each provided 4 g of the experimental diet per day (which was totally consumed). Food cups containing 8 g diet were replaced every other day for the duration of each experiment. Groups of 8–12 mice were fed the experimental diet (control treatment) or the experimental diet plus various levels of 1,25-$(OH)_2D_3$ as indicated in each experimental design. Although the experimental diet has no added vitamin D, the mice were exposed to fluorescent light, which provides for synthesis of vitamin D in skin. In addition, vitamin D was provided initially in the Purina 5008 diet. Thus, the mice were not vitamin D deficient and must be considered as having adequate vitamin D. For mice with severe symptoms of arthritis, food was placed in small dishes on the bottom of the cage. At the end of the experiments, mice were killed by CO_2 asphyxiation, weighed and bled. All of the procedures described were reviewed and approved by the University of Wisconsin-Madison Research Animal Resources Center Committee Review Board on 09/09/94 and the protocol number is A-07–3000-A00755–4-08–94.

Serum calcium analyses. Calcium was determined by atomic absorption spectrometry (Spectrometer 3110, Perkin Elmer, Norwalk, CT) in 1 g/L $LaCl_3$ solution.

Lyme arthritis and 1,25-$(OH)_2D_3$ treatments. The C3H/He mice were purchased from Sprague Dawley (Indianapolis, IN). Spirochetes were grown in BSK-II medium and enumerated as described (Barbour 1984). Cloned *B. burgdorferi* strain N40, generously provided by S. W. Barthold (Yale University School of Medicine, New Haven, CT), was used in the Lyme arthritis experiment (Barthold et al. 1993). Mice were injected intradermally with 10^4 N40 strain spirochetes. At the end of the experiments, bladder samples were collected and cultured for 2 wk in BSK-II medium and examined microscopically to confirm infection. Lyme arthritis was induced in two groups of mice. The control mice received an experimental diet with 1 g calcium/100 g and without vitamin D, whereas the second group of mice received the same experimental diet supplemented with 20 ng 1,25-$(OH)_2D_3$/d. This dose was selected on the basis of previous experience with mice (Yang et al. 1993a and 1993b); in preliminary experiments, it was found to be effective without producing hypercalcemia. Dietary treatments were started 1 d before infection. Arthritic lesions were quantitated with an engineer's caliper using the hind ankle joints and footpads as described previously (Keane-Myers and Nickell 1995). All measurements and observations were made on ether-anesthetized mice by an observer who was unaware of treatments. Hind paw or ankle measurements for each mouse were averaged and group means used for statistical analyses. At the end of the experiment, mice were killed by CO_2 suffocation. Ankles and joints were removed, fixed in 10% formalin, embedded, sectioned and stained with hematoxylin and eosin.

Collagen arthritis. Bovine collagen type II was purchased from Elastin Products (Owensville, MO). Collagen was dissolved in 0.1 mol/L acetic acid at 4 g/L. The collagen solution was emulsified with an equal volume of Complete Freund's Adjuvent (Difco) containing *Mycobacterium tuberculosis* H37Ra. Male DBA/1LacJ mice were purchased from Jackson Laboratories (Bar Harbor, ME). Ether-anesthetized 8-wk-old mice were immunized subcutaneously with 100 μg collagen. In addition, 21 d later, the mice were given an intraperitoneal injection of 100 μg collagen in sterile saline. Arthritis symptoms developed within 4–5 wk of the initial collagen injection. Arthritis symptoms did not resolve but instead the mice had chronically swollen paws. Both front and hind paws were affected. Visual scores have been correlated with histologic severity of arthritis in mice (Trentham et al. 1977).

Mice were observed daily, and ankle and paw inflammation was scored every 2–3 d by an observer uninformed as to the treatments. The scoring system of Trentham et al. (1977) for collagen-induced arthritis in mice was used. Each of the four paws of each mouse was scored as follows: 0-no symptoms; 1-redness, definite swelling with some paw distortion; 2-difficulty using the paw with severe redness and swelling. The scores of the four paws and ankles of each mouse were averaged. The average scores of each mouse in a group were averaged and these values ± SEM were used for statistical analyses. The maximum arthritis severity score for an individual mouse was 8. At the end of the experiment, animals were killed by CO_2 suffocation. Ankles and joints were removed, fixed in 100 g/L formalin and examined histologically as described above.

1,25-$(OH)_2$D3 treatments for collagen arthritis. Mice were immunized subcutaneously with type II collagen (100 μg) and divided into two groups on d 20 after primary immunization (the day before the second collagen injection). Half of the mice were fed the experimental diet that contained 1 g calcium/100 g; the other half were fed the same diet except that it provided 20 ng/d 1,25-$(OH)_2D_3$. The above experiment was repeated and the dose of 1,25-$(OH)_2D_3$ was increased to 50 ng/d beginning on d 14 postimmunization. To increase the effectiveness of treatment and reduce the danger of hypercalcemia, we removed the calcium carbonate from the diet, yielding a 20 mg/100 g calcium diet.

In a separate experiment, 20 mice consumed the experimental diet without 1,25-$(OH)_2D_3$; mice were immunized with 100 μg collagen subcutaneously and monitored daily for the development of arthritis. On d 28 postimmunization, the mice began to show symptoms of arthritis. When each mouse had one or more paws scoring a 1 or higher, the mouse was randomly assigned to one of two groups. Half of the mice received an intraperitoneal injection of 300 ng 1,25-$(OH)_2D_3$ in saline and were given an experimental diet that provided 50 ng/d 1,25-$(OH)_2D_3$ and contained 20 mg/100 g calcium. The other half were controls, injected with saline and given the same experimental diet that contained no vitamin D.

Statistics. Values are mean ± SEM, n = 8–10 mice. The data were analyzed using the statistics program, Statview Student for the Macintosh. The unpaired two-group Student's t test was done and differences of $P < 0.05$ were considered significant. Changes over time were evaluated (see Fig. 1) by using the paired Student's t test; differences of $P < 0.05$ were considered significant.

RESULTS

1,25-$(OH)_2D_3$ decreases the severity of Lyme arthritis. C3H/He mice were injected intradermally with N40 *B. burgdorferi* spirochetes as described above and they developed acute arthritic symptoms within 7 d (**Fig. 1**). Infection by *B. burgdorferi* was confirmed by culture methods described above. The disease severity reached a maximum at 21 d after injection and spontaneously resolved thereafter (Barthold et al. 1991 and 1993, Cantorna and Hayes 1996). Resolution of the arthritic lesions was not due to clearance of the spirochete, because these mice are chronically infected (Barthold et al. 1991 and 1993).

Dietary treatments were begun 1 d before infection. The ankle measurements of control mice continued to increase throughout the 21-d study (statistically higher); the footpad measurements reached a maximum at 7 d (statistically different) and remained at this maximum throughout the study (Fig. 1). Additionally, the control mice showed clearly impaired use of their ankles and feet from 7 d postinfection onward. The 1,25-$(OH)_2D_3$–treated mice showed only a small and statistically insignificant increase in ankle measurements at 14 d postinfection, and their footpad measurements did not change (Fig. 1). The ankles and footpads from 1,25-$(OH)_2D_3$–treated mice were significantly smaller than the controls. No evidence of disuse was noted in this group. After mice consumed the experimental diets for 21 d, the serum calcium concentrations were 0.102 ± 0.006 (0.0026 mmol/L) and 0.112 ± 0.002 g/L (0.0028 mmol/L) in the controls and 1,25-$(OH)_2D_3$–treated mice, respectively. The body weights of the control mice were 24.0 ± 0.9 g on d 14 and 25.2 ± 1.5 g on d 21 postinfection. The body weights of the 1,25-$(OH)_2D_3$–treated mice were not different (23.1 ± 0.8 g on d 14 and 25.4 ± 1.7 g on d 21 postinfection).

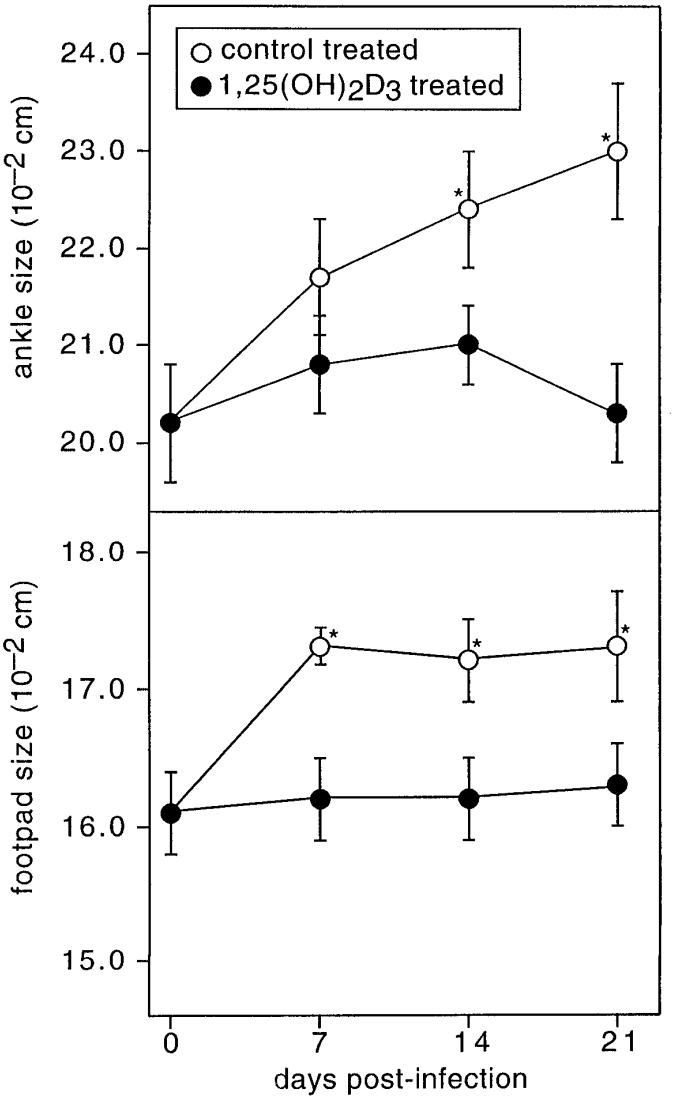

FIGURE FIGURE 1 1,25-Dihydroxycholecalciferol [1,25-$(OH)_2D_3$] supplementation lessens the severity of Lyme arthritis in C3H/He mice. The experimental diet for each mouse contained 20 ng/d 1,25-$(OH)_2D_3$, whereas the control diet contained no 1,25-$(OH)_2D_3$. The diets were started on the day before infection. Uninfected paw measurements (d 0) were made the day before infection. Values are means ± SEM of the averages of 8–10 individual mice. *Significantly different, $P \leq 0.05$.

1,25-(OH)$_2$D$_3$ completely prevents collagen-induced arthritis. Mice given 20 ng/d 1,25-$(OH)_2D_3$ had milder arthritic symptoms and a 50% lower incidence of arthritis (**Fig. 2**). After mice consumed these diets for 48 d, the serum calcium concentrations were 0.102 ± 0.006 (0.0026 mmol/L) and 0.112 ± 0.007 g/L (0.0028 mmol/L) for the controls and 1,25-$(OH)_2D_3$–treated mice, respectively, and were not significantly different from each other. The body weights of the controls were 25.1 ± 3.4 g and the 1,25-$(OH)_2D_3$–treated mice were 25.0 ± 2.5 g. The dose of 1,25-$(OH)_2D_3$ was increased to 50 ng/d in a subsequent experiment. At the end of the experiment, serum calcium concentrations were 0.078 ± 0.001 and 0.078 ± 0.001 g/L (0.0020 mmol/L) in the controls and 1,25-$(OH)_2D_3$–treated groups, respectively. At the end of the experiment, the body weights were also not different [control 27.8 ± 1.4 g and 1,25-$(OH)_2D_3$–supplemented 27.1 ± 0.7 g]. Treatment with 1,25-$(OH)_2D_3$ completely prevented the development of arthritis symptoms in DBA/1LacJ mice (**Fig. 3**). Histopathology sections from these mice confirmed our visual measurements and severity scores. Control mice had obvious signs of arthritis by d 30 postimmunization. By d 42, these arthritis scores reached 1–2. A score of 2 means that a mouse had at least one distorted paw and one or more paws that were not being used. The 1,25-$(OH)_2D_3$–treated mice all had scores of 0 throughout the experiment. A mouse with a score of 0 has no signs of arthritis and shows unrestricted movement. Thus, the arthritic lesions were prevented by 1,25-$(OH)_2D_3$ without an effect on serum calcium or body weight.

1,25-(OH)$_2$D$_3$ halts the progression of arthritis. Mice were treated individually as they developed arthritis symptoms. 1,25-$(OH)_2D_3$ treatment halted the progression of severe arthritis ($P < 0.05$) compared with the controls (**Fig. 4**). At the end of the experiment, the serum calcium concentrations for these two groups of mice were 0.078 ± 0.001 g/L (0.0020 mmol/L) in the control and 0.079 ± 0.001 g/L (0.0020 mmol/L) in the 1,25-$(OH)_2D_3$–treated groups. The weights of the mice were 27.8 ± 1.4 g for the controls and 27.3 ± 0.7 g for the 1,25-$(OH)_2D_3$–treated mice. Again, the improvement brought about by 1,25-$(OH)_2D_3$ took place in the absence of a change in serum calcium or body weight.

DISCUSSION

This study demonstrates that 1,25-$(OH)_2D_3$ supplementation dramatically decreased arthritic symptoms (inflamed and swollen ankles and paws) induced by collagen injections in DBA/1LacJ mice or by *B. burgdorferi* infection in C3H/He mice. Further, when given after arthritic lesions have resulted from the immunizations, 1,25-$(OH)_2D_3$ supplementation halted the progression of arthritis. The lesions produced by the immunization, the improvement due to 1,25-$(OH)_2D_3$, and the prevention of lesions were unmistakable. First, disuse of affected limbs was quite obvious as were swollen and red-

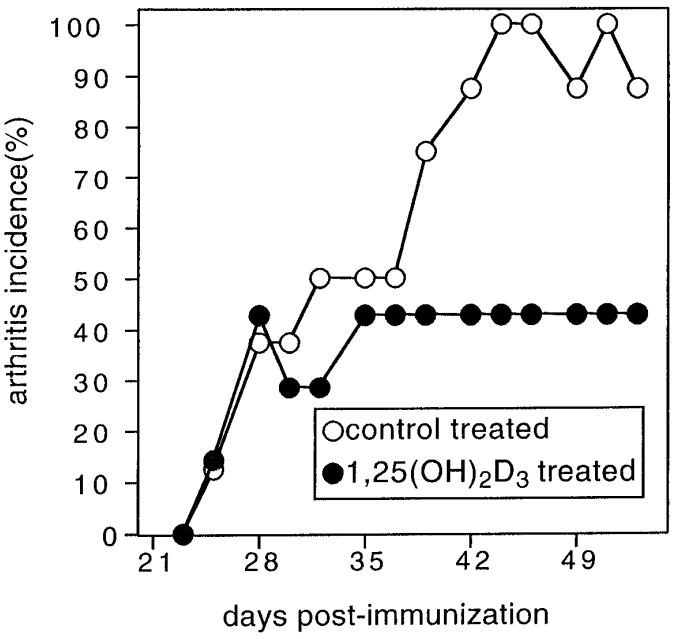

FIGURE 2 Low concentrations of 1,25-dihydroxycholecalciferol [20 ng daily 1,25-$(OH)_2D_3$] and high calcium (1 g/100 g) decrease the incidence of collagen arthritis by 50% in DBA/1LacJ mice. The experimental diets were started on d 20 postimmunization. The control diet contained no 1,25-$(OH)_2D_3$, and the 1,25-$(OH)_2D_3$–supplemented diet contained 20 ng/d 1,25-$(OH)_2D_3$. Calcium was 1 g/100 g.

dened paws and joints. Additionally, the investigator scoring the lesions was unaware of treatment groups. Finally, the lesions were confirmed by histologic examination of sections by another investigator unaware of the treatments. Cumulatively, the data suggest that 1,25-$(OH)_2D_3$ supplementation might be useful in ameliorating symptoms of arthritis.

The mechanisms of arthritic inflammation are not fully understood, but inflammatory T cells are likely involved. They are likely type 1 T-helper cells that secrete such cytokines as tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), and interleukin-2 (IL-2) (Al-Janadi et al. 1993). Further, the mechanisms whereby 1,25-$(OH)_2D_3$ can prevent or suppress the inflammatory process are not known. It is clearly not mediated by increasing serum calcium because in these experiments, 1,25-$(OH)_2D_3$ did not affect serum calcium, but it prevented the arthritic lesions.

The action of 1,25-$(OH)_2D_3$ is both rapid and prolonged. In established arthritis, 1,25-$(OH)_2D_3$ produced a rapid improvement (Fig. 4). This suggests a direct action on arthritogenic cells. On the other hand, 1,25-$(OH)_2D_3$ treatment of mice 14 d after immunization with collagen prevented the inflammatory response completely. This may have been due to an inhibition of expansion of the inflammatory cells or to stimulation of cells producing antiarthritogenic cytokines. Because of the many different in vitro observations of the actions of 1,25-$(OH)_2D_3$ and their conflicting nature on cytokine secretion, it is not possible to specify what events are occurring in vivo. For example, 1,25-$(OH)_2D_3$ has been reported to decrease in vitro production of IL-2, TNF-α and IFN-γ (Manolagas et al. 1986, Muller et al. 1993). Whether this also occurs in vivo is under investigation. Regardless of mechanism, these in vivo results suggest that 1,25-$(OH)_2D_3$ and its analogs may have considerable promise in the control of arthritic lesions that certainly warrants further investigation.

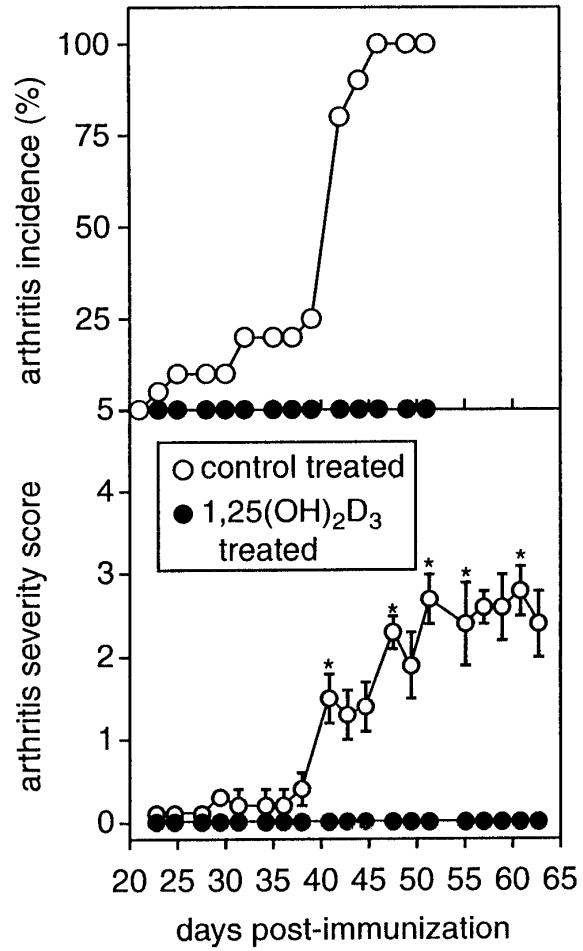

FIGURE 3 Low dietary calcium and 1,25-dihydroxycholecalciferol [1,25-$(OH)_2D_3$] supplementation within 14 d of immunization eliminate symptoms of collagen-induced arthritis in DBA/1LacJ mice. The experimental diet (20mg/100g calcium) provided 50 ng/d 1,25-$(OH)_2D_3$ or no vitamin D (control). The experimental diet was started on d 14 postimmunization, and the paws were scored as described in Materials and Methods. The 4-paw scores for each mouse were averaged and used to calculate the mean for each group. Values are means ± SEM, n = 10. *Significantly different, $P \leq 0.05$.

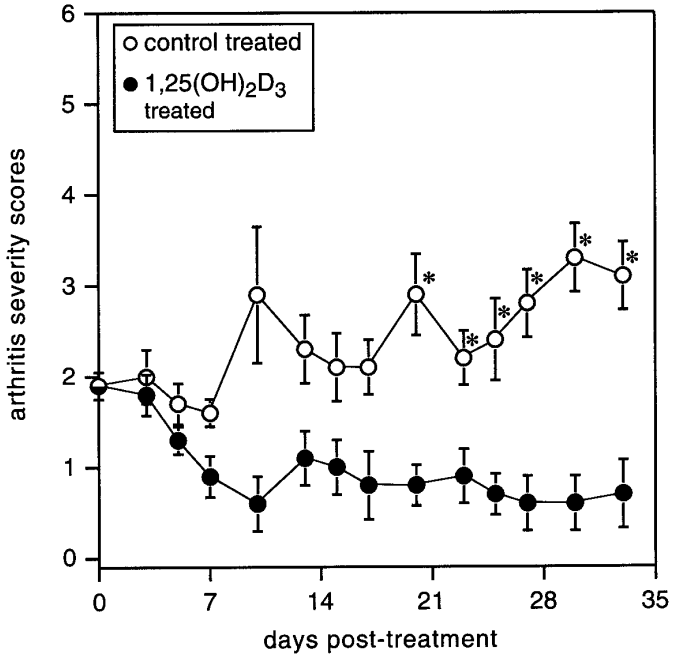

FIGURE 4 1,25-Dihydroxycholecalciferol [1,25-$(OH)_2D_3$] supplementation of DBA/1LacJ mice at the onset of arthritis halts the further progression of disease. As mice became arthritic, they were given an intraperitoneal injection of 300 ng of 1,25-$(OH)_2D_3$ or control injection (equivalent amount of saline). At the time of treatment, the diet was supplemented as described in Materials and Methods to provide 50 ng/d 1,25-$(OH)_2D_3$ and 20 mg/100 g calcium or no 1,25-$(OH)_2D_3$ and 20 mg/100 g calcium. The 4-paw scores for each mouse were averaged, and the average score of each mouse was used to calculate the average score for each group ± SEM, n = 8–10. *Significantly different, $P < 0.05$.

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