Effects of long-term administration of vitamin D₃ analogs to mice

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Abstract

This study explores the effects of chronic administration of vitamin D₃ compounds on several biological functions in mice. Knowledge of long-term tolerability of vitamin D₃ analogs may be of interest in view of their potential clinical utility in the management of various pathologies such as malignancies, immunological disorders and bone diseases. Four unique vitamin D₃ analogs (code names, compounds V, EO, LH and LA) and 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃) were administered i.p. for 55 weeks to Balb/c mice. Each analog had previously been shown to have potent *in vitro* activities. After 55 weeks of administration, the mice had a profound decrease in their serum levels of interleukin-2 (IL-2). Likewise, several analogs depressed serum immunoglobulin G concentrations (compounds LH and LA), but levels of blood lymphocytes and splenic lymphocyte subsets (CD4, CD8 and CD19) were not remarkably depressed. The percent of committed myeloid hematopoietic stem cells was 4- to 5-fold elevated in the bone marrow of the mice that received analogs LH and V; nevertheless, their peripheral blood white and red cell counts and platelets were not significantly different in any of the groups. The mice that received 1,25(OH)₂D₃ had a decrease in bone quantity and quality with a

decrease in cross-sectional area and cortical thickness, and a 50% reduction in both stiffness and failure load compared with the control group. In contrast, the cohort that received a fluorinated analog (compound EO) developed bones with significantly larger cross-sectional area and cortical thickness as well as stronger mechanical properties compared with the control group. At the conclusion of the study, body weights were significantly decreased in all experimental mice. Their blood chemistries were normal. Extensive gross and microscopic autopsy analyses of the mice at the conclusion of the study were normal, including those of their kidneys. In conclusion, the vitamin D₃ analogs were fairly well tolerated. They did suppress immunity as measured by serum IL-2 and may provide a means to depress the immune response after organ transplantation and for autoimmune diseases. Use of these analogs prevented the detrimental effects of vitamin D₃ administration on mechanical and geometric properties of bone, while one analog (compound EO) actually enhanced bone properties. These results suggest that long-term clinical trials with the analogs are feasible.

Journal of Endocrinology (2000) **165**, 163-172

Introduction

Vitamin D₃ plays a central role in bone and calcium homeostasis by promoting the absorption of calcium from bone. The active hormonal form of vitamin D₃ is 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃; code name, compound C), the circulating level of which is tightly regulated (Haussler *et al.* 1997). 1,25(OH)₂D₃ also has diverse, non-classical biological effects on normal and malignant tissues *in vitro*. 1,25(OH)₂D₃ induces differentiation of normal cells including those from hematopoietic, digestive and integumentary systems (Reichel *et al.* 1989). 1,25(OH)₂D₃ can also inhibit proliferation and induced differentiation of various cancer cells, such as those from breast, ovary, skin, colon, brain and prostate tissue as well

as leukemia cells (Campbell & Koeffler 1997, Colston 1997, Gross *et al.* 1997, Van Leeuwen & Pols 1997). Previous studies have shown that both a genomic and non-genomic pathway are involved in these regulatory actions (Norman 1997). 1,25(OH)₂D₃ binds to nuclear vitamin D₃ receptors (VDR), which attaches to the VDR response elements in the promoter region of target genes (Mangelsdorf *et al.* 1995, Haussler *et al.* 1997). Meanwhile, 1,25(OH)₂D₃ exerts rapid effects in a variety of cells, perhaps by changing membrane voltage-gated channels, phospholipase C activity and the sodium/hydrogen antiport (Baran 1994).

The hypercalcemic effect of 1,25(OH)₂D₃ has prevented its broad application as a pharmacological agent. Recently, vitamin D₃ analogs have been developed that

have profound effects on a variety of cells but have less ability to cause hypercalcemia than does 1,25(OH)₂D₃. These analogs can inhibit proliferation and induce differentiation of cancer cells and decrease the development and progression of carcinoma *in vivo* without causing hypercalcemia (Zhou *et al.* 1989, 1990, Abe *et al.* 1991, Jung *et al.* 1994, Koshizuka *et al.* 1998, Kubota *et al.* 1998). These analogs may have utility in a variety of other disease states including psoriasis, immunological abnormalities and bone and endocrine disorders. Therefore, knowing the full scope of side-effects from chronic administration of vitamin D₃ analogs is important before embarking on long-term clinical trials. To our knowledge, this is the first study of the effects of prolonged administration of vitamin D₃ analogs *in vivo*; four vitamin D₃ analogs and 1,25(OH)₂D₃ were given to mice for 55 weeks. In general, the mice appeared healthy. They did, however, weigh less than their litter male controls, and had a dose-dependent hypercalcemia and a significant depression of serum interleukin-2 (IL-2) levels. Although 1,25(OH)₂D₃ decreased mechanical and structural integrity of bone, fluorine-containing analogs enhanced the strength and size of bone. Taken together, these findings suggest that vitamin D₃ can be chronically administered to individuals.

Materials and Methods

Eight-week-old female Balb/c mice from Jackson Laboratories (Bar Harbor, ME, USA) were maintained in pathogen-free conditions and fed a standard laboratory diet. Animals were weighed every 2 to 3 months. At the end of 55 weeks, the animals were killed by CO₂ asphyxiation in accordance with approved vivarian practices, and autopsies were performed.

Vitamin D₃ analogs

1,25(OH)₂D₃ (code name, compound C); 1,25(OH)₂-16ene-23yne-D₃ (compound V); 1,25(OH)₂-16ene-23yne-26,27-F₆-D₃ (compound EO); 1,25(OH)₂-16ene-23yne-19-nor-26,27-F₆-D₃ (compound LH); 1,25(OH)₂-16,22R,23-triene-D₃ (compound LA) (Fig. 1) were synthesized at Hoffmann-LaRoche, Nutley, NJ, USA. The compounds were dissolved in absolute ethanol at 10⁻³ mol/l and stored in aliquots at -20 °C. At the time of administration, analogs were diluted with PBS.

Treatment protocol and measurement of serum calcium

Mice were divided randomly into six groups of ten mice each and the cohorts received either no treatment (diluent control), or compound C, V, EO, LH or LA. Vitamin D₃ analogs were administered i.p. three times a week (Monday, Wednesday and Friday) at the following doses:

Figure 1 Structure, code names and Hoffmann-LaRoche Ro numbers of the vitamin D₃ analogs used in this study.

compound C, 0.0625 µg/mouse; compound V, 0.25 µg/mouse; compound EO, 0.0125 µg/mouse; compound LH, 0.0125 µg/mouse; compound LA, 0.0125 µg/mouse. The doses were chosen from our previous experience (Pakkala *et al.* 1995). Serum calcium levels were measured 1 day after administration of vitamin D₃ compounds; blood was collected from the orbital sinus at 6, 16, 20, 27, 31, 37, 43 and 55 weeks of treatment. Serum calcium levels were measured every 6 to 12 weeks by atomic absorption spectrophotometry (Perkin-Elmer 560, Perkin-Elmer, Norwalk, CT, USA).

Immunological tests and analysis of blood chemistries and the hematopoietic system

After 55 weeks of administration of vitamin D₃ analogs, blood was collected from the orbital sinus of each mouse for serum levels of IL-2 and immunoglobulin G (IgG). Serum IL-2 was determined with a Mouse IL-2 Quantikine ELISA Kit (R & D SYSTEMS, Minneapolis, MN, USA) and the optical density was read at 450 nm. Serum IgG was determined with a Mouse IgG RID kit

(The Binding Site Inc., San Diego, CA, USA). Blood was also collected from the orbital sinus for chemistries and hematopoietic cell concentrations after 1 year of injection of the vitamin D₃ compounds, using a Dupont Analyst Benchtop Chemistry System (Dade International, Newark, DE, USA) and a SeronoBaker 9000 Diff (Biochem Immuno-Systems, Allentown, PA, USA) respectively.

Flow cytometric analysis of murine lymphocytes

Lymphocyte populations in the spleen of mice were examined by two-color flow cytometric analysis. Cells obtained from vitamin D₃-treated and non-treated mice were stained with the following monoclonal antibodies: CD3, 4 and 8 for T-cells and CD19 for B-cells (PharMingen, San Diego, CA, USA), using two-color flow cytometric analysis with excitation by an argon ion laser (488 nm) (FACStar, Becton-Dickinson, San José, CA, USA). The ratio of T- to B-cells was defined as CD3/CD19.

Myeloid colony assay

Bone marrow cells were prepared from the femurs of mice by flushing with RPMI 1640 medium (GIBCO Laboratories, Grand Island, NY, USA) supplemented with 10% fetal calf serum (FCS) (GIBCO Laboratories), and single cell suspensions of spleen cells were prepared with the same medium. Mononuclear cells (MNCs) were isolated on Ficoll-Paque (Pharmacia Biotechnology, Uppsala, Sweden) and the colony-forming unit-granulocyte-macrophage (CFU-GM) assay was performed according to a modification of a method described previously (Koeffler *et al.* 1984). Briefly, the MNCs from bone marrow and spleen from three untreated mice (control group) and treated mice (two to three mice per group) were collected after 55 weeks of treatment with the various vitamin D₃ analogs. The MNCs (5×10^5) were plated in 35-mm culture dishes (Becton Dickinson, Franklin Lakes, NJ, USA) in 1 ml of a mixture containing Iscove's modified Dulbecco's medium (GIBCO Laboratories), 0.9% methylcellulose (FLUKA, Buchs, Switzerland), 30% FCS, 10^4 mol/l 2-mercaptoethanol, 2 mmol/l L-glutamine, 50 U penicillin, 50 µg streptomycin, 50 U recombinant murine IL-3 (Biosource, Camarillo, CA, USA) and 0.5 ng recombinant murine granulocyte-macrophage colony-stimulating factor (GM-CSF) (Biosource). Triplicate dishes for each vitamin D₃ concentration were cultured at 37 °C in 5% CO₂, and colonies containing more than 30 cells were scored on day 10 of culture.

Biomechanical characterization of bone

The mechanical integrity of whole bone specimens is reflective of the quality of the bone tissue, as well as its

amount and geometric organization. Analysis of both whole bone mechanical properties and geometry can therefore provide information about the characteristics of bone tissue itself. Rather than making an estimate of stress by dividing the load by cross-sectional area (CSA), we chose to measure structural properties and geometric properties separately. This allows us to test whether any alterations in whole bone properties can be explained by geometry. If in fact changes in whole bone properties are not easily explained by changes in cross-sectional geometry, then the inherent material properties of the bone must be altered. Our methods allow us to reach these conclusions without making the poorly validated assumptions of uniform cross-section with homogeneous, isotropic properties.

The geometry of the right femur from 40 mice was evaluated using a microComputed tomography (µCT) system developed at the University of Michigan. This system provides a complete three-dimensional digitization of structures at a resolution of approximately 30 µm in the x, y and z directions (Kuhn *et al.* 1990). The specimens were oriented so that the anterior-posterior, medial-lateral and longitudinal axes corresponded with the x-, y- and z-axes respectively. The mid-diaphyseal region, defined as the mid-50% of the length of the bone, was extracted from the total image and the data were thresholded to distinguish between 'bone' and 'non-bone' voxels. CSA, cortical thickness (CT), and moments of inertia (I_{xx} , I_{yy} and J) were determined for each cross-section, and were then averaged along the length of the mid-diaphysis.

Whole bone mechanical properties were determined by testing to failure in four-point bending on an MTS servohydraulic testing machine (Minneapolis, MN, USA) as previously described (Bonadio *et al.* 1993). Specimens were oriented with their anterior surface facing upward, and the mid-diaphyses were loaded at a constant displacement rate of 0.5 mm/s. Load-displacement data were acquired using Lab View software (National Instruments, Austin, TX, USA), and loads and displacements to yield and failure were directly measured. Stiffness was calculated as the slope of the linear portion of the load-displacement curve. Statistical significance was determined using a one-way ANOVA with Tukey's post-hoc test for group comparisons ($P < 0.05$ considered significant).

Analysis of organs

At completion of the study, tissues were fixed in 10% buffered formaldehyde after killing, embedded in paraffin blocks, sectioned, stained with hematoxylin and eosin and examined by light microscopy. The lung, heart, liver, kidney, spleen, adipose tissue, sternum and bone marrow were evaluated and compared with diluent-injected control mice.

Figure 2 Serum calcium levels of the Balb/c mice. Serum calcium levels were measured every 6 to 12 weeks by atomic absorption spectrophotometry. Data are expressed as the mean $\pm$ S.D. of five to ten mice.

Statistical analysis

The statistical significance of the differences was analyzed by a non-parametric Mann–Whitney U test.

Results

Calcium levels

An initial 6 week calcium study was performed in order to attempt to find the dose of each of the vitamin D₃ compounds that gave an upper range of normal serum calcium level (data not shown). Subsequently, the long-term trial was begun; the doses of compounds C, V, EO, LH and LA were 0.0625, 0.25, 0.0125, 0.0125 and 0.0125 μ g/mouse respectively.

Figure 2 shows the serum calcium levels of the mice during their 55 weeks of i.p. administration of the vitamin D₃ compounds. Over the first 6 weeks, serum calcium levels of all the groups were within the normal range (normal 8.5–10.5 mg/dl). However, at 16 weeks, all the mice receiving the vitamin D₃ compounds developed hypercalcemia. The serum calcium levels of the compound C cohort were extremely high (mean, 15 mg/dl). The dose of each was lowered: compounds C, V, EO, LH and LA were decreased to 0.03125, 0.1875, 0.00625, 0.00625 and 0.009375 μ g/mouse respectively. At weeks 20–37, most of the cohorts had serum calcium in the normal range. Near the end of the study, hypercalcemia redeveloped especially in those which received compounds LH and EO. Compound V, which was given at a dose of 0.1875 μ g/mouse, was the least calcemic analog.

Immune system

The immunosuppressive effect of 55 weeks of administration of vitamin D₃ analogs was evaluated by the

measurement of serum IL-2 (Fig. 3), IgG (Fig. 4), subpopulations of splenic T- and B-lymphocytes (Table 1), total peripheral blood lymphocyte counts and examination of the splenic architecture. Serum IL-2 levels of all the treated groups except those which received compound C, were markedly depressed being around 15 pg/ml, which is nearly 15% of the levels present in the control group.

Figure 3 Effect of long-term administration of vitamin D₃ analogs on the levels of serum IL-2. After 1 year of administration of vitamin D₃ analogs, blood was collected from orbital sinus and IL-2 levels were measured. Results represent mean $\pm$ S.D. of five mice. * and **, significantly different ($P < 0.01$) from control and compound C-treated groups respectively, as determined by a Mann–Whitney U test.

Figure 4 Serum IgG levels of experimental mice. After 1 year of injections as described in Materials and Methods, blood was collected and levels of IgG measured. Results represent mean $\pm$ S.D. of five mice per group.

Each of these treatment cohorts was statistically different from either the controls ($P < 0.01$) or those that received compound C ($P < 0.01$). The compound C group had a mean serum IL-2 level which was 60% of normal.

The level of serum IgG (mean 5.8 mg/ml) in the compound C-treated mice was elevated at 185% of the control group. Compound LH- and LA-treated cohorts had depressed serum IgG levels (mean 1.9 and 1.8 mg/ml respectively), which were about 60% of the control group. Analysis of T- and B-lymphocyte subsets in the spleen of the various cohorts at the end of the study were not statistically different (Table 1). The mean peripheral blood lymphocyte counts were slightly expanded in the compound C cohort ($3443/\text{mm}^3$) and somewhat contracted ($2205/\text{mm}^3$, $2597/\text{mm}^3$) in the mice that received compounds V and LA respectively compared with diluent-treated control mice ($3175/\text{mm}^3$). The splenic

morphology of each of the treatment groups was identical to the diluent-treated controls (data not shown).

Weights

During the study, the mice were weighed every 2 to 3 months; mean body weights of the experimental mice were less than the diluent-treated mice ($P < 0.05$). This decrease ranged from 20% in those that received compound V to 37% in those receiving compound EO (Fig. 5).

Myeloid committed stem cells

Effect of long-term exposure of the mice to the various vitamin D₃ analogs on their numbers of committed myeloid stem cells (CFU-GM) was examined at the end of the study. The bone marrow and spleen cells were harvested and MNCs were purified and plated in soft-gel cultures with a maximally stimulating concentration of the hematopoietic growth factor, murine GM-CSF. The number of CFU-GM colonies were enumerated after 12 days of culture, and results were expressed as a percent of myeloid colonies found in diluent-treated mice (Fig. 6). Compound C had no significant effect on the number of CFU-GM from the spleens compared with untreated mice (marrow cells were not tested). In contrast, compounds V, LH and LA enhanced the numbers of CFU-GM within both the spleens (169, 166 and 149%) and the bone marrows (371, 484 and 194%) respectively.

Blood hematopoietic and serum chemistry data

The blood hematopoietic data at the end of the study are presented in Table 2. The white blood cell (WBC) count was a little lower in the groups which received compounds V and LA. Groups that received either compound C or EO had slightly higher levels of red blood cells (RBC), hemoglobins, and hematocrits than did the control groups (Table 2). The platelet numbers were comparable in each group, except they were slightly elevated in the animals receiving compound C.

Table 1 B- and T-lymphocyte populations mean $\pm$ S.D. for three mice for each cohort in spleens after 55 weeks of infections of vitamin D₃ analogs

	Compound					
	Control	C	V	EO	LH	LA
CD3 (T cells, %)	30 $\pm$ 3.5	26 $\pm$ 0.4	27 $\pm$ 5.0	28 $\pm$ 2.7	32 $\pm$ 4.8	34 $\pm$ 10.0
CD4 (% of CD3 cells)	56 $\pm$ 3.8	60 $\pm$ 1.9	58 $\pm$ 2.1	62 $\pm$ 0.1	61 $\pm$ 6.8	61 $\pm$ 3.5
CD8 (% of CD3 cells)	31 $\pm$ 2.9	30 $\pm$ 0.5	28 $\pm$ 3.0	27 $\pm$ 1.0	28 $\pm$ 3.9	26 $\pm$ 3.2
CD19 (B cells, %)	60 $\pm$ 4.7	66 $\pm$ 1.5	63 $\pm$ 3.9	59 $\pm$ 6.6	55 $\pm$ 0.5	56 $\pm$ 10.8
CD4/CD8	1.76	2.04	2.05	2.38	2.17	2.35
CD3/CD19	0.49	0.40	0.43	0.48	0.58	0.60

CD3, T-lymphocytes; CD4, helper T-lymphocytes; CD8, cytotoxic T-lymphocytes; CD19, B-lymphocytes.

Figure 5 Body weight of mice receiving vitamin D₃ compounds. Animals were weighed every 2 to 3 months. Results represent mean $\pm$ S.D. of five to ten mice. * and **, significantly different from the control group at the $P < 0.05$ and $P < 0.01$ levels respectively, as determined by a Mann–Whitney U test.

The renal function studies (blood urea nitrogen (BUN) and creatinine) were normal in all the cohorts (Table 3). Likewise, the liver tests (alkaline phosphatase, glutamic oxaloacetic transaminase (GOT), glutamic pyruvic transaminase (GPT), bilirubin and total protein) and the blood electrolytes (Na^+ , K^+ , Cl^- and HCO_3^-) and blood sugar were also normal. The uric acid in all the treatment groups was statistically ($P < 0.015$) higher than the control group (Table 3), but the lactate dehydrogenase was normal in each group.

Figure 6 *In vivo* effect of long-term exposure to vitamin D₃ analogs on numbers of CFU-GM in murine bone marrow (dense columns) and spleen (light columns). Results are expressed as a mean percent $\pm$ S.D. of control CFU-GM (untreated mice). Each point represents a mean of CFU-GM from at least two mice with each experimental point having triplicate dishes. The control plates had 169 ± 25 and 148 ± 47 colonies per 5×10^5 MNCs from the spleen and bone marrow respectively.

Histology of various organs

At the end of the study, autopsies of all the mice were performed. No gross abnormalities were observed in any of the mice. Histology of the spleens showed normal lymphoid architecture. Bone marrows had normal cellularity with orderly hematopoietic maturation. Some of the mice treated with various analogs revealed slight lymphocytic infiltrates in the liver and focal mild steatosis. The hearts, lungs and kidneys were normal in all cohorts. In contrast, before beginning the study, during the interval when the dosage of the analogs was being determined, LH was administered at too high a dose ($0.05 \mu\text{g}$) and the mice developed severe hypercalcemia with resulting nephrocalcinosis. One mouse which received compound EO had one calcified subcutaneous node. All other organs appeared normal in all of the animals (data not shown).

Biomechanical characterization of bone

Results are summarized in Table 4, with statistically significant differences only between control and treatment groups noted. Other differences between the treatment groups also existed. In general, mechanical and geometrical properties of bones from mice treated with either compounds C or EO were significantly different from controls. Groups LA, LH or V were not significantly different from the control group, although a slight trend towards increased cross-sectional geometry was exhibited by the LH group.

Mechanical testing results showed severely diminished mechanical properties of bones from cohort C. This treatment resulted in a 50% reduction in both stiffness and

Table 2 Serum chemistry data mean $\pm$ S.D. for five mice after 55 weeks of injections of vitamin D₃ analogs

	Compound					
	Control	C	V	EO	LH	LA
ALP	142.0 $\pm$ 9.49	184.4 $\pm$ 26.4	150.8 $\pm$ 15.1	160.0 $\pm$ 11.4	113.6 $\pm$ 5.86	123.2 $\pm$ 14.1
GOT	151.4 $\pm$ 31.5	106.3 $\pm$ 16.3	154.8 $\pm$ 25.7	160.3 $\pm$ 32.6	111.6 $\pm$ 24.5	163.8 $\pm$ 35.2
GPT	178.2 $\pm$ 94.4	ND	113.4 $\pm$ 60.1	127.8 $\pm$ 68.3	68.8 $\pm$ 45.4	181.8 $\pm$ 75.2
BUN	19.1 $\pm$ 1.35	20.9 $\pm$ 3.06	24.1 $\pm$ 0.86	25.7 $\pm$ 2.41	19.0 $\pm$ 1.36	17.4 $\pm$ 0.87
Cr	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2
Glu	131.0 $\pm$ 14.3	93.4 $\pm$ 11.7	145.0 $\pm$ 8.80	94.8 $\pm$ 7.95	145.2 $\pm$ 23.0	116.6 $\pm$ 20.2
Chol	150.6 $\pm$ 13.1	146.4 $\pm$ 5.41	136.4 $\pm$ 11.9	152.6 $\pm$ 14.3	140.2 $\pm$ 21.9	118.6 $\pm$ 9.7
TG	113.0 $\pm$ 12.2	97.2 $\pm$ 21.7	107.8 $\pm$ 18.5	112.6 $\pm$ 35.4	93.8 $\pm$ 34.2	80.4 $\pm$ 13.0
UA	2.44 $\pm$ 0.22	4.12 $\pm$ 1.00	5.84 $\pm$ 1.53	6.12 $\pm$ 0.59	5.58 $\pm$ 0.72	4.13 $\pm$ 0.15
Bil	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
T-pro	5.92 $\pm$ 0.49	5.88 $\pm$ 0.45	5.32 $\pm$ 0.69	5.54 $\pm$ 0.38	5.52 $\pm$ 0.33	5.16 $\pm$ 0.46

ALP, alkaline phosphatase; T-pro, total protein; Cr, creatinine; Bil, bilirubin; UA, uric acid; Glu, glucose; Chol, cholesterol; TG, triglycerides.

Table 3 Hematopoietic blood values mean $\pm$ S.D. for five mice after 55 weeks of injections of vitamin D₃ analogs

	Compound					
	Control	C	V	EO	LH	LA
WBC ($10^3/\text{mm}^3$)	5.88 $\pm$ 1.94	6.26 $\pm$ 1.14	4.24 $\pm$ 1.76	5.78 $\pm$ 0.83	5.90 $\pm$ 1.47	4.90 $\pm$ 1.34
RBC ($10^6/\text{mm}^3$)	9.72 $\pm$ 1.02	11.4 $\pm$ 1.46	9.49 $\pm$ 0.96	11.1 $\pm$ 0.55	9.39 $\pm$ 0.66	9.42 $\pm$ 0.60
Hgb (g/dl)	15.1 $\pm$ 1.50	16.7 $\pm$ 1.47	14.6 $\pm$ 1.29	16.6 $\pm$ 0.97	14.6 $\pm$ 0.91	14.4 $\pm$ 0.72
Hct (%)	45.7 $\pm$ 4.76	50.9 $\pm$ 5.39	43.8 $\pm$ 4.15	51.1 $\pm$ 3.05	43.6 $\pm$ 3.05	43.3 $\pm$ 2.18
MCV	47.0 $\pm$ 0.13	44.9 $\pm$ 1.14	47.2 $\pm$ 2.43	46.0 $\pm$ 0.69	46.5 $\pm$ 0.65	46.0 $\pm$ 0.86
MCH	15.6 $\pm$ 0.11	14.7 $\pm$ 0.69	15.4 $\pm$ 0.43	14.9 $\pm$ 0.15	15.6 $\pm$ 0.30	15.3 $\pm$ 0.57
MCHC	33.1 $\pm$ 0.22	23.8 $\pm$ 0.82	33.3 $\pm$ 0.25	32.5 $\pm$ 0.25	33.6 $\pm$ 0.36	33.3 $\pm$ 0.68
Plt ($10^3/\text{mm}^3$)	1127 $\pm$ 98.9	1405 $\pm$ 92.9	1132 $\pm$ 253	1090 $\pm$ 78.3	1147 $\pm$ 193	1188 $\pm$ 76.1

Hgb, hemoglobin; Hct, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; Plt, platelet counts.

Table 4 Effects on mechanical and geometric properties of bone of vitamin D₃ analogs

Treatment	Compound					
	C (n=4) ^a	Control (n=7)	EO (n=5)	LA (n=8) ^a	LH (n=8)	V (n=8)
Yield load (N)	16.4 $\pm$ 3.9	30.2 $\pm$ 9.9	37.7 $\pm$ 11.4	34.4 $\pm$ 8.5	31.3 $\pm$ 6.6	30.1 $\pm$ 8.3
Failure load (n)	18.6 $\pm$ 2.6	37.4 $\pm$ 9.4	48.0 $\pm$ 14.3	40.3 $\pm$ 7.8	39.2 $\pm$ 8.8	37.4 $\pm$ 8.6
Stiffness (N/mm)	136.2 $\pm$ 37.0*	275.3 $\pm$ 72.2	394.5 $\pm$ 66.0*	321.7 $\pm$ 67.6	281.7 $\pm$ 58.1	291.4 $\pm$ 68.9
CSA (mm ²)	0.917 $\pm$ 0.035	1.042 $\pm$ 0.112	1.257 $\pm$ 0.087**	1.017 $\pm$ 0.111	1.103 $\pm$ 0.083	0.922 $\pm$ 0.078
CT (mm)	0.232 $\pm$ 0.007**	0.287 $\pm$ 0.020	0.336 $\pm$ 0.026*	0.281 $\pm$ 0.018	0.309 $\pm$ 0.039	0.254 $\pm$ 0.013
<i>I</i> _{xx} (mm ⁴)	0.300 $\pm$ 0.035	0.280 $\pm$ 0.054	0.332 $\pm$ 0.033	0.268 $\pm$ 0.063	0.308 $\pm$ 0.039	0.236 $\pm$ 0.045
<i>I</i> _{yy} (mm ⁴)	0.161 $\pm$ 0.017	0.137 $\pm$ 0.025	0.120 $\pm$ 0.016**	0.139 $\pm$ 0.030	0.153 $\pm$ 0.016	0.126 $\pm$ 0.019
J (mm ⁴)	0.460 $\pm$ 0.051	0.417 $\pm$ 0.079	0.532 $\pm$ 0.048	0.407 $\pm$ 0.091	0.460 $\pm$ 0.054	0.363 $\pm$ 0.063

* $P < 0.05$ vs control; ** $P < 0.01$ vs control.

^aOne specimen was excluded from analysis due to errors during testing.

failure load, and a 45% decrease in yield load when compared with the control group. Specimens from cohort C also showed a significant decrease in CT and a trend towards decreased CSA in comparison with specimens from the control group. These alterations were, however, accompanied by a slight increase in moments of inertia, which suggests a geometric redistribution of bone material farther from the neutral bending axis of the bone.

In contrast, specimens from cohort EO exhibited significant increases in both mechanical and geometric properties when compared with the control group. Mechanical testing results showed significant increase in stiffness and trends towards increased yield and failure loads of cohort EO. Mice treated with compound EO also had significantly larger CSA and CT, and a trend towards increased moments of inertia.

Discussion

This study sought to determine the toxicities of chronic (55 weeks) administration of vitamin D₃ analogs. We attempted to choose doses of these compounds that would result in levels of serum calcium at the high end of the normal range. Because of an initial pilot study as well as a prior short-term investigation, we chose doses that we thought would meet this criterion (Pakkala *et al.* 1995). At week 6 of vitamin D₃ administration, all the mice were normocalcemic; but at week 16, most had either a high or upper range of normal serum calcium level. The dose of each of the vitamin D₃ analogs was lowered. Analog V (0.1875 µg/mouse) was the least calcemic. A dose of compound C that was almost 6-fold lower than compound V was required to produce the same level of sera calcium. We do not know why analog V has the least potential for hypercalcemia, perhaps the compound is more rapidly metabolized resulting in a shorter *in vivo* half-life. Compounds LH and EO were the most calcemic; both of these analogs have six fluorines on the side-chain, which may slow their metabolism and thus their inactivation.

The vitamin D₃ analogs were tested for their effects on the immune system *in vivo*. A key element of the immune system is the helper T-cells (CD4-expressing cells). At least three different T-helper cell subsets have been described T_{H0}, T_{H1}, and T_{H2} cells. T_{H0} are the early precursors that differentiate into T_{H1} or T_{H2}. T_{H1} and T_{H2} cells are defined by the cytokines that they secrete. For instance T_{H1} cells produce IL-2 and interferon-γ (IFN-γ) and T_{H2} cells produce IL-4, -5, -6 and -13 (Mosmann & Coffman 1989, Manetti *et al.* 1993). The T_{H1} cells can help B-cells produce antibodies of the isotype IgG_{2a}, while T_{H2} cells can help these cells secrete IgG₁. Adding to the complexity of the function of T-lymphocyte subsets, each can cross-regulate the other. For example, IFN-γ produced by T_{H1} cells can down-modulate T_{H2} cells, and IL-4 and IL-10 produced by T_{H2} cells can inhibit T_{H1} cells (Mosmann 1991). We found that each of the vitamin D₃ analogs profoundly decreased levels of IL-2 in the sera. These effects may be mediated by direct inhibition of IL-2 production by T_{H2}-lymphocytes (Tsoukas *et al.* 1984, Rigby *et al.* 1985, Bhalla *et al.* 1986, Mangelsdorf *et al.* 1995). We previously showed *in vitro* that 1,25(OH)₂D₃ could inhibit in a dose- and time-dependent manner the production of lymphokines including IL-2, GM-CSF and IFN-γ produced by T-lymphocytes (Reichel *et al.* 1987, Tobler *et al.* 1988). This inhibition may occur through several different mechanisms including inhibition of AP-1 complexes (Matsui *et al.* 1986, Rigby *et al.* 1987). The decrease in serum IL-2 levels probably did not result from a decrease in CD4 T-lymphocytes in the experimental mice because the percent of these lymphocytes in the spleens of the mice receiving the analogs was normal and the total number of

circulating lymphocytes were similar to those in the control mice (Table 1).

The analogs were about 3-fold more potent than 1,25(OH)₂D₃ (compound C) in lowering the serum level of IL-2. Cyclosporin A is a potent inhibitor of T-lymphocytes, used to suppress the immune response in various circumstances such as organ transplantation (Emmel *et al.* 1989). Several prior studies have suggested that vitamin D₃ analogs may be more active than cyclosporin A in the suppression of production of cytokines *in vitro* (Koizumi *et al.* 1985, Gupta *et al.* 1989). Furthermore, a variety of animal models of autoimmunity have shown the beneficial effects of treatment using 1,25(OH)₂D₃ (Fournier *et al.* 1990, Lemire & Archer 1991, Lemire *et al.* 1992, Mathieu *et al.* 1992, Braisteau *et al.* 1993).

The long-term administration of vitamin D₃ analogs had a more subtle action on humoral immunity as measured by IgG. A previous study *in vitro* demonstrated that 1,25(OH)₂D₃ could decrease synthesis of IgG by B-lymphocytes *in vitro* (Iho *et al.* 1986). Other studies have shown that the B-cell suppressive effects could be mediated by inhibition of T-helper cells (Lemire *et al.* 1985). Paradoxically, we found that levels of serum IgG in the animals receiving 1,25(OH)₂D₃ were elevated (5.77 mg/ml IgG compared with 3.12 mg/ml in the controls). In contrast, levels were diminished in those animals receiving either analog LH or LA (1.9 and 1.8 mg/ml respectively). We are not sure why serum concentrations of IgG were elevated by 1,25(OH)₂D₃ and decreased by analogs LH and LA.

The peripheral blood levels of WBC, RBC and platelets were nearly normal. Surprisingly, the hematopoietic myeloid committed stem cells (CFU-GM) were elevated up to 5-fold in the bone marrows of some of the vitamin D₃ cohorts, especially those receiving analogs LH and V (Fig. 6). We have previously shown that 1,25(OH)₂D₃ could stimulate CFU-GM *in vitro* (Koeffler *et al.* 1984), and this enhancement occurred even when the CD34-positive stem cells were purified and directly exposed to 1,25(OH)₂D₃ (Lee *et al.* 1996). We do not at this time understand why the expansion of the myeloid committed stem cell pool was not reflected in increased levels of peripheral blood neutrophils in the mice receiving the vitamin D₃ analogs.

Chronic hypercalcemia can be associated with calcium deposition in both the kidneys (nephrocalcinosis) and subcutaneous fat. Indeed, animals which in our pilot experiment had severe hypercalcemia (LH group), also had prominent nephrocalcinosis. In contrast, none of the animals which received 1 year of vitamin D₃ analogs developed nephrocalcinosis; and only one mouse in the LH group had a subcutaneous calcium deposit. Furthermore, all the mice had normal serum BUN and creatinine levels, which also reflected the integrity of function of their kidneys. On the other hand, the mice were

underweight compared with the control mice (about 35% lower weight). The cohort V, with the least hypercalcemia, had the greatest mean weight; while the group LH, with the highest serum calcium, had the lowest weights. Hypercalcemia can produce nausea and lack of appetite as well as polydipsia because of dehydration. Therefore, we believe that the lack of weight gain probably reflects a toxicity of low-grade hypercalcemia. We can not, however, rule out the possibility that the vitamin D₃ compounds lowered levels of circulating growth hormone or somatomedins.

1,25(OH)₂D₃ and its metabolites are necessary for calcium homeostasis and therefore bone mineral deposition, primarily because they enhance absorption of calcium and phosphorus in the small intestine. 1,25(OH)₂D₃ has also been shown to directly affect bone cells. The vitamin D₃ metabolite stimulates matrix and alkaline phosphatase synthesis and production of bone specific proteins, specifically osteocalcin in osteoblasts (Lian *et al.* 1985). It also stimulates both differentiation and fusion of osteoclast progenitor cells and the activity of mature osteoclasts (Roodman *et al.* 1985). Treatment with vitamin D₃ analogs can therefore affect bone in multiple ways: (i) it can affect the amount of Ca²⁺ available for bone mineralization; (ii) it can affect the activity of cells that synthesize and resorb bone, therefore altering the quality or quantity of bone tissue, which can (iii) induce an adaptive response secondary to change in bone tissue properties. This type of adaptive response would most likely be a change in the amount and/or distribution of bone tissue that would allow the whole bone to better tolerate physiological loading.

Long-term administration of compound C appears to cause a severe decrease in quantity and quality of bone. Both CSA and CT were decreased compared with those in control animals; but surprisingly, I_{xx} was slightly increased (7%). This suggests that although these animals had less bone, they redistributed their bone mass away from the neutral bending axis. Despite this reorganization, and perhaps an attempt at optimization, it still resulted in drastically diminished mechanical properties. These two findings suggest an adaptive response to decreased bone tissue properties that were insufficient to return the whole bone properties to control values. Compound EO caused an opposite effect, as evidenced by the increase in both the amount of bone present and tissue properties of bone. The 43% increase in stiffness and the 25–28% increase in yield and failure loads can only be partially explained by the 19% increase in I_{xx}. Therefore, the animals treated with compound EO appear to have produced more bone that was of greater quality than that made by control animals. The slight increase in geometrical properties exhibited by cohort LH without concomitant increases in mechanical properties suggests that the bone produced by these animals may be inferior, although this alteration did not reach statistical significance.

In summary, our experiments showed that long-term administration of vitamin D₃ analogs is feasible. The mice did weigh less than the controls and monitoring of serum calcium with appropriate modification of dose was necessary. Their peripheral blood hematopoietic parameters and serum chemistries, as well as the microscopic examination of all of their major organs, were fairly normal. The immunosuppressive effects of the vitamin D₃ analogs are worthy of further exploration. The changes in bone suggest that selected vitamin D₃ analogs can enhance bone strength while compound C (1,25(OH)₂D₃) can have the opposite effect. Additional studies may help determine if the finding of this study may be of relevance for the treatment of osteoporosis.

Acknowledgements

We appreciate the excellent secretarial support of Kim Burgin and Marge Goldberg. This manuscript is dedicated to the memory of Marge Goldberg.

References

- Abe J, Nakano T, Nishi Y, Matsumoto T, Ogata E & Ikeda K 1991 A novel vitamin D₃ analog, 22-oxa-1,25-dihydroxyvitamin D₃, inhibits the growth of human breast cancer *in vitro* and *in vivo* without causing hypercalcemia. *Endocrinology* **129** 832–837.
- Baran DT 1994 Nongenomic actions of the steroid hormone 1α,25-dihydroxyvitamin D₃. *Journal of Cellular Biochemistry* **56** 303–306.
- Bhalla AK, Amento EP & Krane SK 1986 Differential effects of 1,25-dihydroxyvitamin D₃ on human lymphocytes and monocyte/macrophages: inhibition of interleukin-2 and augmentation of interleukin-1 production. *Cellular Immunology* **98** 311–322.
- Bonadio J, Jepsen KJ, Mansoura MK, Jaenisch R, Kuhn JL & Goldstein SA 1993 A murine skeletal adaptation that significantly increases cortical bone mechanical properties. Implications for human skeletal fragility. *Journal of Clinical Investigation* **92** 1697–1705.
- Braisteanu DD, Leenaerts P, Van Damme B & Bouillon R 1993 Partial prevention of active Heymann nephritis by 1α,25-dihydroxyvitamin D₃. *Clinical and Experimental Immunology* **94** 412–417.
- Campbell MJ & Koeffler HP 1997 Toward therapeutic intervention of cancer by vitamin D compounds. *Journal of the National Cancer Institute* **89** 182–185.
- Colston K 1997 Vitamin D and breast cancer: therapeutic potential of new vitamin D analogs. In *Vitamin D*, pp 1107–1124. Eds D Feldman, FH Glorieux & J Wesley Pike. San Diego, CA, USA: Academic Press.
- Emmel EA, Verweil CL, Durand DB, Higgins KM, Lacy E & Crabtree S 1989 Cyclosporin A specifically inhibits function of nuclear proteins in T cell activation. *Science* **246** 1617–1620.
- Fournier C, Gepner P, Sadouk M & Charriere J 1990 *in vivo* beneficial effect of cyclosporin A and 1,25-dihydroxyvitamin D₃ on the induction of experimental autoimmune thyroiditis. *Clinical Immunopathology* **54** 53–63.
- Gross C, Peehl DM & Feldman D 1997 Vitamin D and prostate cancer. In *Vitamin D*, pp 1125–1140. Eds D Feldman, FH Glorieux & J Wesley Pike. San Diego, CA, USA: Academic Press.

- Gupta S, Fass D, Shimizu M & Vayuvegula B 1989 Potentiation of immunosuppressive effects of cyclosporin A by 1 α ,25-dihydroxyvitamin D₃. *Cellular Immunology* **121** 290–297.
- Haussler MR, Haussler CA, Jurutka PW, Thompson PD, Hsieh JC, Remus LS, Selznick SH & Whitfield GK 1997 The vitamin D hormone and its nuclear receptor: molecular actions and disease states. *Journal of Endocrinology* **154** S57–S73.
- Iho S, Takahashi T, Kura F, Sugiyama H & Hoshino T 1986 The effect of 1,25-dihydroxyvitamin D₃ on *in vitro* immunoglobulin production in human B-cells. *Journal of Immunology* **136** 4427–4431.
- Jung SJ, Lee YY, Pakkala S, deVos S, Elstner E, Norman AW, Green J, Uskokovic M & Koeffler HP 1994 1,25(OH)₂-16-ene-vitamin D₃ is a potent anti-leukemic agent with low potential to cause hypercalcemia. *Leukemia Research* **18** 453–463.
- Koeffler HP, Amatrua T, Ikekawa N, Kobayashi T & DeLuca HF 1984 Induction of macrophage differentiation of human normal and leukemic myeloid stem cell by 1,25-dihydroxyvitamin D₃ and its fluorinated analogues. *Cancer Research* **44** 5624–5628.
- Koizumi T, Nakano T, Matsui T, Nakagawa T, Katakami Y & Fujita T 1985 Effect of 1,25-dihydroxyvitamin D₃ on cytokine-induced thymocyte proliferation. *Cellular Immunology* **96** 455–461.
- Koshizuka K, Koike M, Kubota T, Said J, Binderup L & Koeffler HP 1998 Novel vitamin D₃ analog (CB1093) when combined with paclitaxel and cisplatin inhibit growth of MCF-7 human breast cancer cells *in vivo*. *Cancer Research* **13** 421–428.
- Kubota T, Koshizuka K, Koike M, Uskokovic M, Miyoshi I & Koeffler HP 1998 19-Nor-26,27-bishomo-vitamin D₃ analogs: a unique class of potent inhibitors of proliferation of prostate, breast and hematopoietic cancer cells. *Cancer Research* **58** 3370–3375.
- Kuhn JDL, Goldstein SA, Feldkamp LA, Goulet RW & Jasion G 1990 Evaluation of a microcomputed tomography system to study trabecular bone structure. *Journal of Orthopedic Research* **8** 833–842.
- Lee YY, Kim ES, Seol JG, Kim BK, Binderup L, Elstner E, Park DJ & Koeffler HP 1996 Effect of a vitamin D₃ analog, EB1089, on hematopoietic stem cell lines. *Leukemia* **10** 1751–1757.
- Lemire JM & Archer DC 1991 1,25-dihydroxyvitamin D₃ prevents the *in vivo* induction of murine experimental autoimmune encephalomyelitis. *Journal of Clinical Investigation* **87** 1103–1107.
- Lemire JM, Adams JS, Kermaniarab V, Bakke AC, Sakai R & Jordan SC 1985 1,25-Dihydroxyvitamin D₃ suppresses human T-helper inducer lymphocyte activity *in vitro*. *Journal of Immunology* **134** 3032–3035.
- Lemire JM, Ince A & Takashima M 1992 1,25-Dihydroxyvitamin D₃ attenuates the expression of experimental murine lupus of MRU1 mice. *Autoimmunity* **12** 143–148.
- Lian JB, Coutts M & Canalis E 1985 Studies of hormonal regulation of osteocalcin synthesis in cultured fetal rat calvariae. *Journal of Biological Chemistry* **55** 1–6.
- Manetti R, Parronchi P, Guidizi MG, Piccinni M-P, Maggi E, Trinchieri G & Romagnani S 1993 Natural killer cell stimulatory factor (interleukin 12(IL-12)) induces T helper type 1 (Th₁)-specific immune responses and inhibits the development of IL-4 producing Th cells. *Journal of Experimental Medicine* **177** 1199–1204.
- Mangelsdorf DJ, Thummel C, Beato M, Herrlich P, Schutz G, Umesono K, Blumberg B, Kastner P, Mark M, Chambon P & Evans RM 1995. The nuclear receptor superfamily: the second decade. *Cell* **83** 835–839.
- Mathieu C, Waer M, Laureys J, Rutgeerts O & Bouillon R 1992 1,25-Dihydroxyvitamin D₃ prevents insulinitis in NOD mice. *Diabetes* **41** 1491–1495.
- Matsui T, Takahashi R, Nakao Y, Koizumi T, Katakami Y, Mihara Y, Sugiyama T & Fujita T 1986 1,25-Dihydroxyvitamin D₃-regulated expression of genes involved in human T-lymphocyte proliferation and differentiation. *Cancer Research* **46** 5827–5831.
- Mosmann TR 1991 Cytokine secretion patterns and crossregulation of T cell subsets. *Immunology Research* **10** 183–188.
- Mosmann TR & Coffman RL 1989 Th₁ and Th₂ cells: different patterns of lymphokine secretion lead to different functional properties. *Annual Review of Immunology* **7** 145–173.
- Norman AW 1997 Rapid biological responses mediated by 1 α ,25-dihydroxyvitamin D₃: a case study of transcalcachia (rapid hormonal stimulation of intestinal calcium transport). In *Vitamin D*, pp 233–256. Eds D Feldman, FH Glorieux & J Wesley Pike. San Diego, CA, USA: Academic Press.
- Pakkala S, de Vos S, Elstner E, Rude B, Uskokovic M, Binderup L & Koeffler HP 1995 Vitamin D₃ analogs: effect on leukemic clonal growth and differentiation, and serum calcium. *Leukemia Research* **19** 65–71.
- Reichel H, Koeffler HP, Tobler A & Norman AW 1987 1- α ,25-dihydroxyvitamin D₃ inhibits gamma-interferon synthesis by normal human peripheral blood lymphocytes. *Proceedings of the National Academy of Sciences of the USA* **84** 3385–3389.
- Reichel H, Koeffler HP & Norman AW 1989 The role of the vitamin D endocrine system in health and disease. *New England Journal of Medicine* **320** 980–991.
- Rigby WFC, Noelle RJ, Krause K & Fanger MW 1985 The effects of 1,25-dihydroxyvitamin D₃ on human T lymphocyte activation and proliferation: a cell cycle analysis. *Journal of Immunology* **135** 2279–2285.
- Rigby WFC, Denome S & Fanger MW 1987 Regulation of lymphokine production and human T lymphocyte activation by 1,25-dihydroxyvitamin D₃. *Journal of Clinical Investigation* **79** 1659–1664.
- Roodman GD, Ibbotson KJ, MacDonald BR, Kuehl TJ & Mundy GR 1985 1,25(OH)₂D₃ causes formation of multinucleated cells with osteoclast characteristics in cultures of primate marrow. *Proceedings of the National Academy of Sciences of the USA* **82** 8213–8217.
- Tobler A, Miller CW, Norman AW & Koeffler HP 1988 1,25-Dihydroxyvitamin D₃ modulates the expression of a lymphokine (granulocyte-macrophage colony-stimulating factor) post-transcriptionally. *Journal of Clinical Investigation* **81** 1819–1823.
- Tsoukas CD, Prowedini DM & Manolagas SC 1984 1,25-Dihydroxyvitamin D₃: a novel immunoregulatory hormone. *Science* **224** 1438–1441.
- Van Leeuwen JPTM & Pols HAP 1997 Vitamin D: anticancer and differentiation. In *Vitamin D*, pp 1089–1106. Eds D Feldman, FH Glorieux & J Wesley Pike. San Diego, CA, USA: Academic Press.
- Zhou JY, Norman AW, Lübbert M, Collins ED, Uskokovic MR & Koeffler HP 1989 Novel vitamin D analogs that modulate leukemic cell growth and differentiation with little effect on either intestinal calcium absorption or bone calcium mobilization. *Blood* **74** 82–93.
- Zhou JY, Norman AW, Chen DL, Sun GW, Uskokovic M & Koeffler HP 1990 1,25(OH)₂-16ene-23yne-vitaminD₃ prolongs survival time of leukemic mice. *Proceedings of the National Academy of Sciences of the USA* **87** 3929–3940.

Received 8 March 1999

Revised manuscript received 4 October 1999

Accepted 18 November 1999