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Research Article

Recent studies have suggested that vitamin D may have other important biologic activities in addition to its well-characterized role in the maintenance of calcium homeostasis. Discovery of cytosolic receptors for vitamin D in human peripheral blood monocytes and lectin-stimulated lymphocytes prompted us to study the effects of 1,25-dihydroxyvitamin D3 (calcitriol), the most biologically active metabolite of vitamin D, upon phytohemagglutinin (PHA)-induced lymphocyte blast transformation. We have found that calcitriol is a potent inhibitor of PHA-induced lymphocyte proliferation, achieving 70% inhibition of tritiated thymidine incorporation after 72 h in culture. Furthermore, calcitriol suppressed interleukin-2 (IL-2) production by PHA-stimulated peripheral blood mononuclear cells in a concentration-dependent fashion. Lastly, the suppressive effect of calcitriol on cellular proliferation was partially reversed by the addition of saturating amounts of purified IL-2. We conclude that calcitriol is a potent inhibitor of PHA-induced lymphocyte blast transformation and that this effect is mediated, in part, through suppression of IL-2 production. Thus, calcitriol appears to possess immunoregulatory properties that have been unappreciated heretofore.

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Inhibition of T Lymphocyte Mitogenesis by 1,25-Dihydroxyvitamin D₃ (Calcitriol)

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Abstract. Recent studies have suggested that vitamin D may have other important biologic activities in addition to its well-characterized role in the maintenance of calcium homeostasis. Discovery of cytosolic receptors for vitamin D in human peripheral blood monocytes and lectin-stimulated lymphocytes prompted us to study the effects of 1,25-dihydroxyvitamin D₃ (calcitriol), the most biologically active metabolite of vitamin D, upon phytohemagglutinin (PHA)-induced lymphocyte blast transformation. We have found that calcitriol is a potent inhibitor of PHA-induced lymphocyte proliferation, achieving 70% inhibition of tritiated thymidine incorporation after 72 h in culture. Furthermore, calcitriol suppressed interleukin-2 (IL-2) production by PHA-stimulated peripheral blood mononuclear cells in a concentration-dependent fashion. Lastly, the suppressive effect of calcitriol on cellular proliferation was partially reversed by the addition of saturating amounts of purified IL-2. We conclude that calcitriol is a potent inhibitor of PHA-induced lymphocyte blast transformation and that this effect is mediated, in part, through suppression of IL-2 production. Thus, calcitriol appears to possess immunoregulatory properties that have been unappreciated heretofore.

Introduction

The role of vitamin D as a steroid hormone involved in calcium homeostasis has been well defined in recent years (1). The traditional target tissues for vitamin D action (intestine, bone, and kidney) possess specific high-affinity cytosolic recep-

tors for vitamin D and its metabolites (1). The discovery of receptors for vitamin D in cells and cell lines derived from tissues not clearly associated with calcium homeostasis (1) has prompted reevaluation of the scope of the biologic role of vitamin D.

The most biologically active form of vitamin D, 1,25-dihydroxyvitamin D₃ (calcitriol), has been demonstrated to induce differentiation of both human and murine myeloid leukemia cell lines and to prolong survival of mice injected with a murine leukemia cell line (2-4). In addition, calcitriol has been demonstrated to inhibit growth and induce differentiation of the U937 monoblast cell line (5) derived from a patient with histiocytic lymphoma (6). More recently, receptors for vitamin D have been found in monocytes and in lymphocytes activated in vitro by either mitogenic lectin or Epstein-Barr virus (7, 8).

The demonstration of vitamin D receptors in cells of lymphoid origin prompted us to examine the effects of calcitriol on lectin-induced lymphocyte blast transformation. Calcitriol was found to be a potent inhibitor of phytohemagglutinin (PHA)-induced cellular proliferation as measured by tritiated thymidine (³H]TdR) incorporation at concentrations as low as 10⁻¹¹ M. Since proliferation of T lymphocytes in response to either antigen or lectin has been shown to be mediated by interleukin-2 (IL-2) (9, 10), the effects of calcitriol on IL-2 production by PHA-stimulated lymphocytes was examined. Calcitriol inhibited IL-2 production with a concentration dependence that approximated that found in the cellular proliferation studies. The addition of saturating amounts of purified exogenous IL-2 reversed the inhibition of proliferation induced by calcitriol. Thus, calcitriol is a potent inhibitor of lectin-stimulated lymphocyte blast transformation, at least in part as a result of its suppression of IL-2 production.

Methods

Vitamin D compounds. Preservative free 1,25-dihydroxycholecalciferol (calcitriol) and 25-hydroxycholecalciferol (25-OH-D₃) were the generous

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1. *Abbreviations used in this paper:* 25-OH-D₃, 25-hydroxycholecalciferol; CTLL, cloned murine IL-2-dependent T lymphocyte line; IL-2, interleukin-2; PBM, peripheral blood mononuclear cells; PHA, phytohemagglutinin; TdR, thymidine.

gifts of Dr. Milan Uskokovic (Hoffman-La Roche, Inc., Nutley, NJ) and Dr. Rodney Carlson (Upjohn Co., Kalamazoo, MI), respectively. Vitamin D₃ was purchased from Sigma Chemical Co. (St. Louis, MO). All compounds were dissolved in 95% sterile ethanol and stored in glass vials at -20°C.

Cell cultures. Human peripheral blood mononuclear (PBM) cells isolated by Ficoll-Hypaque discontinuous gradient centrifugation were cultured at 1×10^6 cells/ml in RPMI 1640 medium (KC Biologicals, Lenexa, KS) supplemented with 10% heat-inactivated (56°C, 30 min) fetal bovine serum (Sterile Systems, Inc., Logan, UT) and 50 µg/ml gentamicin sulfate (U. S. Biochemical Corp., Cleveland, OH) at 37°C in a humidified atmosphere of 5% CO₂ in air. Cells for proliferation were stimulated with that concentration of PHA (Difco Laboratories, Detroit, MI) which was found to cause maximal stimulation for 72 h. Cell viability, as determined by acridine orange/ethidium bromide staining (11), was equivalent between control and treated cultures after 72 h in culture.

Cells to be assayed for IL-2 production were cultured at 5×10^6 cells/ml in RPMI 1640 with 10% FBS in the presence of PHA (1.5 µg/ml) (Wellcome Reagents Ltd., Beckenham, England) for 24 h. The cell-free supernatant was harvested by centrifugation (1000 g; 15 min) and stored at 4°C until assayed. The concentrations of these PHA preparations (Difco Laboratories and Wellcome Reagents, Inc.) used were found to give equivalent stimulation of PBM cells (10^6 /ml) after 72-h culture, as measured by [³H]TdR (New England Nuclear, Boston, MA) incorporation as described below.

Both sets of cultures were performed in the absence or presence of varied concentrations of calcitriol or 25-OH-D₃. Control cultures contained equal volumes of ethanol, which never exceeded 0.1% in control or treated cultures. Time course experiments were performed with addition of calcitriol to cultures at various time points after PHA stimulation, achieving a final concentration found to cause maximal effect (10^{-7} M).

Proliferation studies. After 72 h in culture, equal volumes (100 µl) of each sample were plated in quadruplicate into 96-well plates (Becton-Dickinson Labware, Oxnard, CA). Cells were pulsed with 0.5 µCi of [³H]TdR for 5 h at 37°C and harvested onto glass fiber filters with a Mash II Cell Harvester (Microbiological Associates, Walkersville, MD) and counted by liquid scintillation. Data are expressed as: percent inhibition = $1 - (\text{cpm calcitriol culture}/\text{cpm control culture}) \times 100$.

IL-2 biologic activity. IL-2 activity of cell-free supernatants was determined as previously described (12) by the IL-2 concentration-dependent stimulation of a cloned murine IL-2-dependent T lymphocyte line (CTLL-2, subclone 15 G). CTLL proliferation, as monitored by [³H]TdR (0.5 µCi) incorporation, was determined during the last 4 h of a 24-h culture period in the presence of serial twofold dilutions of a standard IL-2 preparation and the experimental sample. The dilutions that yielded 50% of the maximal CTLL [³H]TdR incorporation were determined by probit analysis; dilution values of the samples were divided by that of the standard to give the concentration of IL-2 in units per milliliter. The standard IL-2 preparation, which had been arbitrarily assigned a value of 1 unit/ml, routinely yielded 50% of the maximal [³H]TdR incorporation at a dilution of 1:25. CTLL proliferation was found to be unaffected by calcitriol over the range of concentrations used (10^{-5} – 10^{-12} M), so that any difference in CTLL proliferation between control and treated (containing calcitriol) supernatants was a function of IL-2 concentration. Stimulation by control supernatants from each donor (measured as units IL-2 per milliliter) were assigned a value of 100%, with treated supernatant activity calculated as a percentage of that value.

Production and preparation of affinity-purified IL-2. Jurkat subclone 6.8 cells (10) were routinely harvested from the exponential phase of cell growth (0.8 – 1.0×10^6 cells/ml), centrifuged (250 g; 10 min), placed into serum-free Dulbecco's Modified Eagle's medium (Gibco Laboratories, Grand Island, NY), and cultured at 4.0×10^6 cells/ml for 14–18 h in the presence of PHA (1.5 µg/ml) and phorbol myristate acetate (50 ng/ml; Consolidated Midlands Corp., Brewster, NY). The cell-free supernatant was harvested, filtered (0.45 µm), and stored at 4°C. IL-2 was purified using an immunoaffinity column constructed with an IgG2a monoclonal antibody to IL-2 (DMS-3), as previously described (13). IL-2 activity was assayed as described above.

Results

Effect of vitamin D compounds on PHA-stimulated lymphocyte proliferation. Calcitriol inhibited PHA-stimulated proliferation of PBM cells in a dose-dependent fashion (Fig. 1). Demonstrable inhibition was present at calcitriol concentrations as low as 10^{-12} M with maximal suppression (70–80%) usually apparent at 10^{-7} M. Furthermore, the immediate biologic precursor of calcitriol, 25-OH-D₃, was without significant inhibitory effect at concentrations below 10^{-6} M (Fig. 1). Vitamin D₃ lacked detectable antiproliferative effect at concentrations as high as 10^{-6} M (data not shown). As the relative inhibitory activity of these vitamin D metabolites reflects the much greater affinity of the vitamin D cytosolic receptor for calcitriol (14), it seems likely that this inhibitory effect is receptor-mediated.

In an attempt to define whether there was a critical point in the mitogenic stimulation of lymphocytes that was sensitive to calcitriol inhibition, time course studies were performed on

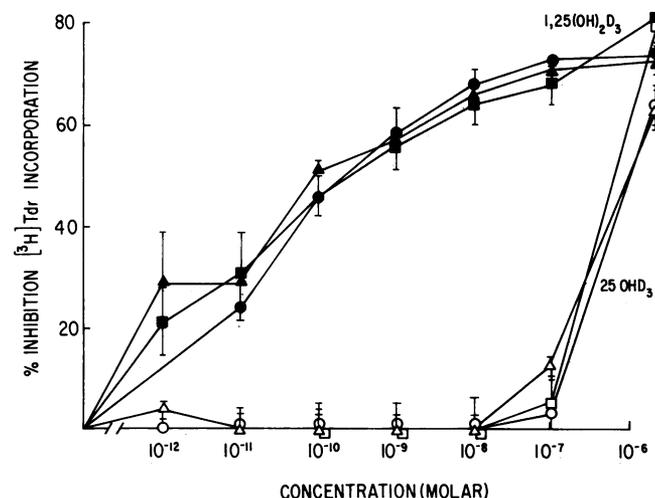


Figure 1. Inhibition of [³H]TdR incorporation by PHA-stimulated PBM cells by calcitriol. PBM cells from three donors were stimulated with PHA for 72 h with an ethanol control, calcitriol (■, ▲, ●), or 25-OH-D₃ (□, △, ○) and pulsed for 5 h with [³H]TdR. Error bars represent standard deviation of quadruplicate samples. Percentage inhibition was calculated as: percent inhibition = $1 - (\text{cpm treated culture}/\text{cpm control culture}) \times 100$.

PBM cells (Fig. 2). Although there was individual variability, if addition of calcitriol was delayed relative to PHA stimulation, less inhibition resulted. In all cases, significant reduction of calcitriol's inhibitory effect (measured 72 h after PHA stimulation) was apparent when it was added 24 h after culture with PHA. In some cases, the inhibition of proliferation was nearly obviated by the addition of calcitriol after 48 h.

Effect of calcitriol on IL-2 production. Since IL-2 is essential for the proliferation of lectin-stimulated T lymphocytes, studies of calcitriol's effect on IL-2 production were conducted. The production of IL-2 by PHA-stimulated lymphocytes was inhibited by calcitriol in a linear, dose-dependent fashion (Fig. 3). At a concentration of 10^{-9} M, calcitriol suppressed IL-2 production by 50%. Time course studies of IL-2 production revealed that if addition of calcitriol were delayed for 12 h following lectin stimulation, there was reduced inhibition (Fig. 4). These data show that calcitriol's effects on IL-2 production and T cell proliferation are mediated at an early stage of mitogenesis. Addition of calcitriol after substantial IL-2 production has taken place, results in the attenuation of its antiproliferative effect.

Lastly, to determine whether calcitriol had any inhibitory effect on PHA-stimulated lymphocyte proliferation independent of IL-2 production, we studied the effect of purified exogenous IL-2 on calcitriol-treated cells (Table I). Supplementation of PHA-stimulated PBM cells with saturating concentrations of IL-2 reversed to varying degrees (29–66%) the inhibition of proliferation induced by calcitriol (10^{-7} M). In all of the six donors studied, IL-2 addition did not restore proliferation to control levels.

Discussion

In this paper, we report that at physiologic levels, calcitriol, the most biologically active form of vitamin D, is a potent

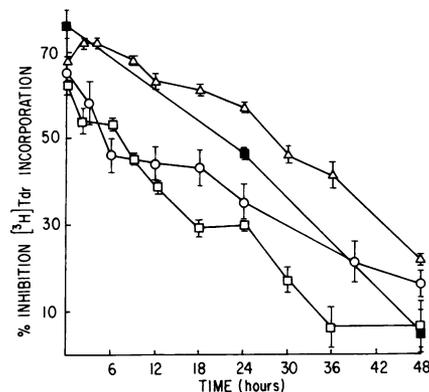


Figure 2. Time course of calcitriol-mediated inhibition of $[^3\text{H}]\text{Tdr}$ incorporation. PBM cells from four donors (Δ , \circ , \square , \blacksquare) were stimulated with PHA at time 0, and calcitriol (10^{-7}) was added to cultures at various time points. Inhibition of proliferation was measured as described in Fig. 1.

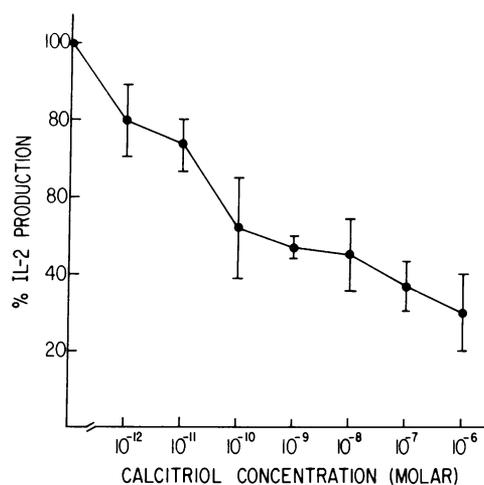


Figure 3. Effect of calcitriol on IL-2 production. Supernatants of PBM cells cultured in the absence or presence of calcitriol were measured 24 h after PHA stimulation. The effect of calcitriol on IL-2 was calculated as a percentage of control for each donor. Each point represents the mean and standard deviation with three donors.

inhibitor of PHA-induced lymphocyte proliferation. The degree of inhibition of proliferation by calcitriol was unaffected by PHA concentrations ranging from suboptimal levels up to twice those required to achieve maximal stimulation (data not shown). The immediate biologic precursor of calcitriol, 25-OH- D_3 , was without comparable effect unless used at 10,000-fold higher concentrations (10^{-6} M), and vitamin D_3 had no detectable effect. Since the cytoplasmic receptor for vitamin D has a much greater affinity for calcitriol than for 25-OH- D_3 (14), it seems likely that this suppression of lymphocyte proliferation is receptor-mediated. The absence of antiproliferative activity with vitamin D_3 provides further evidence of the specificity of calcitriol's effects on T lymphocyte mitogenesis. In addition, our finding that calcitriol decreased IL-2 production and that its inhibition of proliferation could be at least partially overcome by the addition of exogenous IL-2 suggested that the antiproliferative effects of calcitriol may, in part, result

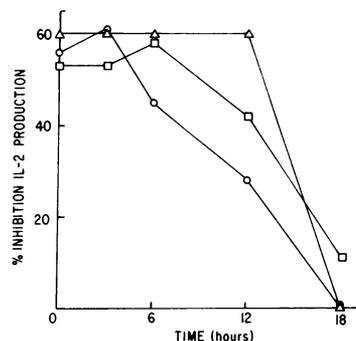


Figure 4. Time course study of effects of calcitriol on IL-2 production. PBM cells from three donors (\circ , Δ , \square) were stimulated with PHA at time 0, and calcitriol (10^{-7} M) was added to cultures at various time points. Supernatants were harvested at 24 h and IL-2 activity was measured. Inhibition was calculated as described in Fig. 1.

Table I. Ability of IL-2 to Overcome Inhibition of T Cell Mitogenesis by Calcitriol*

Donor (age [yr], sex)	PHA	PHA/calcitriol	PHA/IL-2	PHA/IL-2/calcitriol
	<i>cpm</i>			
1 (39, F)	103,700±6,400	38,100±600 (37%)	101,500±8,900 (98%)	90,700±3,600 (88%)
2 (38, F)	142,200±3,200	45,900±2,100 (32%)	135,800±2,800 (96%)	120,800±4,100 (85%)
3 (21, F)	91,200±5,700	19,700±1,900 (22%)	95,500±6,200 (105%)	63,100±5,700 (69%)
4 (43, M)	111,000±6,800	49,300±3,500 (44%)	136,700±5,100 (123%)	83,900±4,800 (75%)
5 (23, M)	102,500±3,600	27,400±2,200 (27%)	92,200±3,900 (90%)	57,500±1,900 (56%)
6 (24, M)	119,000±2,500	22,300±2,000 (19%)	111,000±7,500 (93%)	100,900±4,100 (85%)

* [³H]Tdr incorporation mediated by 72-h PHA-stimulated PBM cells (10⁶/ml) from six healthy donors. Cultures were conducted in the presence of either ethanol control, calcitriol (10⁻⁷ M), or IL-2 (3 U/ml), or both calcitriol and IL-2. Numbers in parentheses denote percentage of control (PHA alone) values.

from its suppression of IL-2 production. Studies using purified IL-2 also indicate that calcitriol may inhibit mitogenesis through other mechanisms in addition to inhibition of IL-2 production. In contrast, the effect of glucocorticoids on T cell mitogenesis can be overcome with exogenous IL-2 (9, 15). These studies indicate that the steroid hormone, calcitriol, may be involved in immunoregulation in addition to its well-recognized role in calcium homeostasis.

Calcitriol's effects on proliferation and IL-2 production appear to involve the early stages of mitogenesis, since there is a significant loss of its suppressive properties when added to cultures 12–18 h after lectin stimulation. Furthermore, it seems unlikely that calcitriol inhibits IL-2 production through a direct effect on the T cell. This is suggested by the finding that calcitriol must be added to cultures within 12 h of PHA stimulation to effect suppression of IL-2 production, whereas specific receptors for vitamin D are not detectable in T lymphocytes until 24 h after lectin stimulation (12). After induction of vitamin D receptors, calcitriol could potentially exert a direct antiproliferative effect on the T cell. One possible mechanism might be through down-regulation of IL-2 receptor expression (16). Such an effect could explain the failure of exogenous IL-2 to fully restore proliferation to that of controls.

Another possibility consistent with this data is that calcitriol's suppressive effects are mediated through monocytes, which normally express cytosolic receptors specific for vitamin D (7, 8). Calcitriol may, for example, decrease the capability of the monocyte to produce interleukin I in response to PHA stimulation, which would result in turn in decreased IL-2 production. In addition, calcitriol may induce the monocyte to release a factor with direct inhibitory effects on lymphocyte proliferation.

It has been recently reported that pulmonary alveolar macrophages from patients with sarcoidosis have 1-hydroxylase activity and can synthesize calcitriol from 25-OH-D₃ (17). In addition, peripheral blood lymphocytes from patients with sarcoidosis have been shown to have depressed proliferative responses to mitogens (18). Our demonstration of the immu-

nomodulatory effects of calcitriol on PHA-stimulated lymphocyte proliferation suggests a pathophysiologic mechanism which could link these two observations. Moreover, it may be that, under certain physiologic or other pathologic conditions, macrophages may be induced to synthesize calcitriol from 25-OH-D₃. Thus, calcitriol may represent a product of macrophages involved in the regulation of lymphocyte proliferation or monocyte-macrophage differentiation and function as has been reported in both human and murine myeloid cell lines (2–5).

The demonstration that calcitriol has immunoregulatory properties including effects on lymphocytes similar to those of glucocorticoids suggests that calcitriol, or other vitamin D metabolites, may have potential utility in mediating immunosuppression. The development of vitamin D analogs that exert less effect on calcium homeostasis but retain their inhibitory effects on lymphocyte proliferation might enable their use as immunosuppressive agents. Such agents would lack the morbidity of therapy (diabetes mellitus, osteopenia) common to pharmacologic doses of glucocorticoids.

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