Rapid Publication

1α,25-Dihydroxyvitamin D_3 Suppresses Proliferation and Immunoglobulin Production by Normal Human Peripheral Blood Mononuclear Cells

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Abstract. Activated B and T lymphocytes from normal human subjects are known to have the specific high-affinity receptor for 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2$-D_3). In an attempt to determine a functional role for the sterol in such cells, we studied the effect of 1,25-$(OH)_2$-D_3 on DNA synthesis and Ig production by normal human peripheral blood mononuclear (PBM) cells activated in vitro by the polyclonal lymphocyte activators pokeweed mitogen and phytohemagglutinin, and the specific antigen dermatophyton O. A dose-dependent inhibition of [^{3}H]thymidine incorporation was observed in cells incubated with 1,25-$(OH)_2$-D_3 in concentrations ranging from 10^{-10} to 10^{-7} M. Production of IgG and IgM, determined by enzyme-linked immunosorbent assay, was similarly inhibited by increasing concentrations of 1,25-$(OH)_2$-D_3. Half-maximal inhibition of DNA and Ig synthesis was found at 10^{-10} to 10^{-9} M 1,25-$(OH)_2$-D_3. This suppressive effect was specific for 1,25-$(OH)_2$-D_3; of the other vitamin D metabolites examined, only 10^{-7} M 24R,25 dihydroxyvitamin D_3 (24,25-$(OH)_2$-D_3) had a similar inhibitory effect. 1,25-$(OH)_2$-D_3 was not cytotoxic and did not affect unactivated PBMs. These data demonstrate that 1,25-$(OH)_2$-D_3 is a potent inhibitor of human PBM Ig production in vitro and suggest that this action is mediated through the hormone's antiproliferative effect on Ig-producing B cells and/or helper T cells.

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Introduction

The active metabolite of vitamin D_3, 1α,25-dihydroxy-vitamin D_3 (1,25-$(OH)_2$-D_3), is well recognized as an important modulator of calcium homeostasis in man (1). The biological effects of the sterol in target tissues, like intestinal epithelium where it promotes transcellular calcium transport, are initiated by interaction of the hormone with its specific high-affinity intracellular receptor (2–4). In recent years, receptors for 1,25-$(OH)_2$-D_3 have been identified in a variety of cells not previously recognized as a target for the hormone; among them are both malignant (5, 6) and nonmalignant cells (7, 8) of hematopoietic origin. For example, 1,25-$(OH)_2$-D_3 has a potent antiproliferative effect on promyelocytic leukemia cells (5) and has been shown to induce differentiation of both leukemia cells (5, 9) and myelocytic precursors from normal human bone marrow (7) along the monocyte-macrophage pathway. The recent identification of receptors for 1,25-$(OH)_2$-D_3 in activated normal human B and T lymphocytes (8, 10) suggests that these cells may be potential targets for the action of the sterol. In this report, we describe an inhibitory role of 1,25-$(OH)_2$-D_3 on DNA synthesis and immunoglobulin production by normal activated human peripheral blood mononuclear cells (PBMs)[1] in vitro.

1. *Abbreviations used in this paper:* FBS, fetal bovine serum; PBM, peripheral blood mononuclear cell; PHA, phytohemagglutinin; PWM, pokeweed mitogen.

Methods

Culture conditions. PBMs were obtained from four normal adult volunteers by centrifugation of heparinized venous blood on Ficoll-Hypaque (Histopaque; Sigma Chemical Co., St. Louis, MO). The isolated fraction contained >95% mononuclear cells. Cells were cultured in medium RPMI-1640 supplemented with penicillin (50 U/ml), streptomycin (50 μg/ml), L-glutamine (0.29 mg/ml), and 10% heat-inactivated fetal bovine serum (FBS) in microtiter plates (all from Flow Laboratories, McLean, VA) at a concentration of 1×10^6 cells/ml. A control group of unstimulated PBMs was cultured under identical conditions.

At the initiation of the culture period, pokeweed mitogen (PWM) (Sigma Chemical Co.), phytohemagglutinin-P (PHA-P) (Difco Laboratories, Detroit, MI), or dermatophyton O, extracted from Candida albicans (Hollister-Stier Laboratories, Spokane, WA), was added to a second group of cell cultures at a final concentration of 2.5 μg/ml or at a dilution of 1/1,000 or 1/32, respectively. Authentic, crystalline 25-OH-vitamin D_3 (25-OH-D_3) was generously provided by Dr. John Babcock (Upjohn Co., Kalamazoo, MI). 1,25-$(OH)_2$-D_3 and 24R,25 dihydroxyvitamin D_3 (24,25-$(OH)_2$-D_3) were provided by Dr. Milan Uskokovic (Hoffmann-La Roche Inc., Nutley, NJ). Vitamin D sterols were solubilized in absolute ethanol and added to culture medium which contained 10% FBS as a 0.025% solution in concentrations ranging from 10^{-10} to 10^{-7} M. 10% FBS contained 2.0×10^{-11} M 1,25-$(OH)_2$-D_3. Control PBMs were cultured in 0.025% ethanol concentration. The cells were then incubated at 37°C in an atmosphere of 5% CO_2/95% air.

Measurement of DNA synthesis. PBM DNA synthesis was evaluated on the fifth day of cell culture. 12 h before harvesting, 1.5 μCi of [^{3}H]thymidine (6.7 Ci/mM, New England Nuclear, Boston, MA) was added to each well. Cells were lysed and harvested in a semiautomatic cell harvester (Skatron, Inc., Sterling, VA), and cell debris was maintained on glass-fiber filters which were dried and counted in liquid scintillation fluid (Scintillar, Mallinckrodt Inc., Paris, KY) and counted in a Beta counter (S-8000; Beckman Instruments Co., Fullerton, CA).

Measurement of immunoglobulin synthesis. Ig production by PBMs was determined by an enzyme-linked immunosorbent assay system developed in our laboratory. After 12 d in culture, IgG and IgM were measured from culture supernatants on flat-bottomed microtiter plates (Linbro; Flow Laboratories) which were coated with 200 μl of a 2.5-μg/ml solution of goat anti-human IgG ($F(ab')_2$ fragments) or rabbit anti-human IgM in a 0.05 M carbonate buffer containing 2.93 g/liter of sodium bicarbonate and 1.59 g/liter of sodium carbonate, at 4°C overnight. After a washing with phosphate-buffered saline, 50 μl of the culture supernatants was incubated at 37°C in assay plates for 1 h. The plates were then washed three times with 0.05% solution of Tween-20 in 10 mM Tris-HCl, pH 8.0. Horseradish peroxidase conjugated to protein A (Sigma Chemical Co.) or anti-human IgM (μ-chain specific; Cappel Laboratories Inc., Cochranville, PA) was added to the plates at a dilution of 1/6,000 or 1/12,000 to detect IgG or IgM, respectively. The dilutions were made in Tris-buffered saline containing 0.05% Tween-20, 1% bovine serum albumin, and 0.01% sodium azide. After incubation and washing, O-phenylenediamine (OPD; New England Nuclear) in a 0.1 M solution containing 10.505 g/liter of citric acid and 8.90 g/liter disodium hydrogen phosphate, pH 6.3, was added to each well. Samples were analyzed on an automated spectrophotometer (Flow Laboratories) with each supernatant assayed in triplicate. The values were related to standard concentrations of IgG and IgM (Calbiochem-Behring Corp., American Hoechst Corp., La Jolla, CA). The results were expressed in absolute values (nanograms per milliliter).

After 12 d of culture, the cell pellets were resuspended in RPMI-1640 and the cells were counted in the presence of trypan blue to assess cell viability. Total cellular protein determinations were performed according to the method of Bradford (11), and DNA content was determined according to the method of Kissane and Robbins (12) as modified by Benya and Shaffer (13).

Statistical analysis of the data was performed by paired *t* test.

Results

The data shown in Fig. 1 typify the effects of the various vitamin D metabolites on Ig production by stimulated PBMs from normal human subjects. Suppression of production of IgG and IgM by 1,25-$(OH)_2$-D_3 in cultures activated with either PWM or dermatophyton O was similar, with maximal inhibition evident at 10^{-8} M to 10^{-7} M 1,25-$(OH)_2$-D_3. Inhibition of Ig synthesis occurred only in the presence of high concentrations of 24,25-$(OH)_2$-D_3 (10^{-7} M), whereas incubation of cells with 25-OH-D_3 had no identifiable effect on Ig production (Table I). The

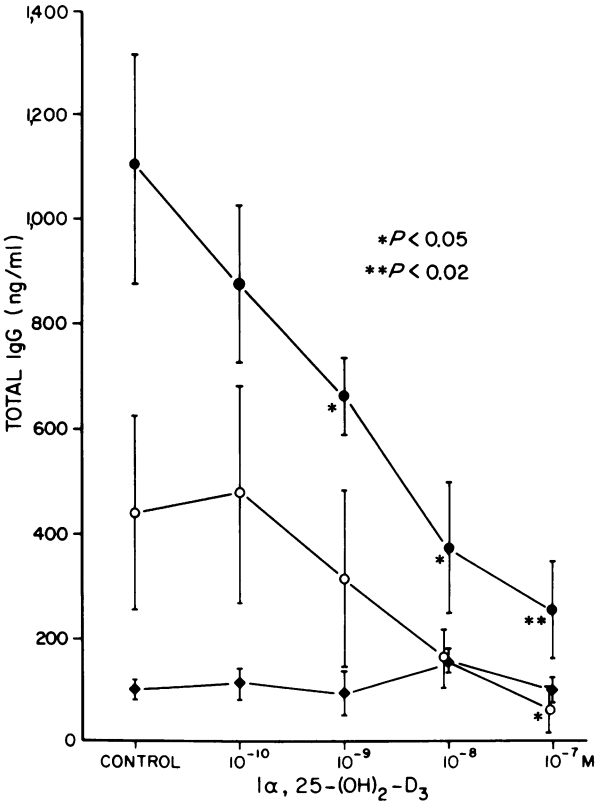

Figure 1. The effect of 1,25-$(OH)_2$-D_3 on IgG production in vitro by normal human PBMs unstimulated (♦) and stimulated with either PWM (●) or dermatophyton-O (○). The results are expressed in nanograms per milliliter. Control cultures with 10% FBS contain 2×10^{-11} M 1,25-$(OH)_2$-D_3. Each point represents the mean value of four normal human subjects assayed in triplicate.

Table I. Effect of 1,25-(OH)$_2$-D$_3$, 25-OH-D$_3$, and 24,25-(OH)$_2$-D$_3$ on IgG Production by Activated Human PBMs

	Vitamin D metabolites						
		1,25-(OH)$_2$-D$_3$		25-OH-D$_3$		24,25-(OH)$_2$-D$_3$	
Activators	None	10^{-8} M	10^{-7} M	10^{-8} M	10^{-7} M	10^{-8} M	10^{-7} M
		ng/ml	*ng/ml*	*ng/ml*	*ng/ml*	*ng/ml*	*ng/ml*
None	100±20	160±24	100±26	120±22	110±20	120±24	100±27
PWM	1,106±225	373±140*	253±90‡	880±162	900±186	840±154	496±102*
D-O	440±191	166±76	66±58*	460±186	480±190	386±169	406±174

Values are total IgG production. Each value represents the mean of four normal human subjects assayed in triplicate, D-O, dermatophyton O.
* $P < 0.05$. ‡ $P < 0.02$.

addition of various concentrations of 1,25-(OH)$_2$-D$_3$ had no effect on unstimulated PBMs. When Ig production was evaluated, cell viability was identical in all cultures regardless of the activator, vitamin D metabolite, or metabolite concentration used.

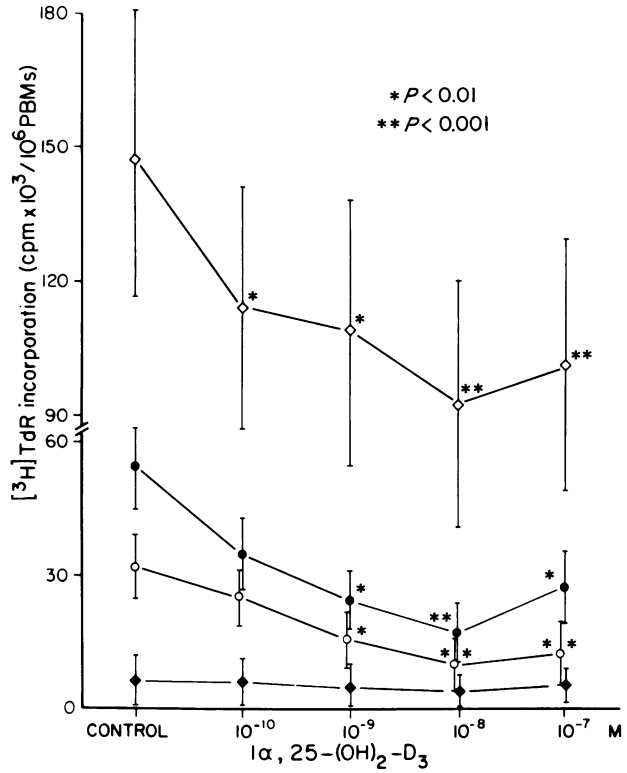

Figure 2. The effect of 1,25-(OH)$_2$-D$_3$ on DNA synthesis in vitro by normal human PBMs unstimulated (♦) and stimulated with either PHA (◇), PWM (●), or dermatophyton-O (○). The results are expressed as [^{3}H]thymidine ([^{3}H]TdR) incorporation (cpm × $10^3/10^6$ PBMs). Control cultures with 10% FBS contain 2×10^{-11} M 1,25-(OH)$_2$-D$_3$. Each point represents the mean value of four normal human subjects assayed in triplicate.

The dose-related effect of 1,25-(OH)$_2$-D$_3$ on Ig production was paralleled by a reduction in DNA synthesis (Fig. 2). The addition of 1,25-(OH)$_2$-D$_3$ resulted in a dose-dependent inhibition of DNA synthesis and total DNA content at concentrations ranging from 10^{-10} to 10^{-7} M. Half-maximal inhibition of [^{3}H]thymidine incorporation occurred in the presence of 10^{-11} to 10^{-10} M 1,25-(OH)$_2$-D$_3$ in cells activated by PHA and PWM, and 10^{-10} to 10^{-9} M 1,25-(OH)$_2$-D$_3$ in cultures activated by dermatophyton O. When 24,25-(OH)$_2$-D$_3$ was added to cell cultures activated by PWM, PHA, and dermatophyton O, pronounced inhibition was observed at 10^{-7} M, but no significant effect was seen at lower concentrations (Table II). The PWM-stimulated PBMs cultured in the presence of 25-OH-D$_3$ showed minimal inhibition of DNA synthesis.

Discussion

The data presented here demonstrate an inhibitory effect of 1,25-(OH)$_2$-D$_3$ on DNA synthesis and Ig production by activated PBMs. Ig synthesis was inhibited by the active vitamin D metabolite in a dose-dependent fashion, with an effect seen at concentrations as low as 10^{-10} M 1,25-(OH)$_2$-D$_3$ in the culture medium. Incubation of cultures with 25-OH-D$_3$ and 24,25-(OH)$_2$-D$_3$ indicated that this suppressive effect was specific for 1,25-(OH)$_2$-D$_3$; the half-maximal effect of 1,25-(OH)$_2$-D$_3$ was present at concentrations of hormone 100 to 1,000 times less than concentrations of 24,25-(OH)$_2$-D$_3$ and 25-OH-D$_3$, respectively. These results are consistent with a 1,25-(OH)$_2$-D$_3$ receptor-mediated phenomenon, with the suppressive effects of the various metabolites reflecting the relative affinity with which the sterols are bound to the specific receptor for 1,25-(OH)$_2$-D$_3$ (4). Moreover, the exquisite sensitivity of cultured PBMs to the effects of 1,25-(OH)$_2$-D$_3$ is particularly striking when one considers that <10% of the total 1,25-(OH)$_2$-D$_3$ present in the 10% FBS-containing incubation medium is unbound and available for interaction with its intracellular receptor (14). Because 10^{-9} M

Table II. Effect of 1,25-(OH)$_2$-D$_3$, 25-OH-D$_3$, and 24,25-(OH)$_2$-D$_3$ on DNA Synthesis by Activated Human PBMs

	Vitamin D metabolites						
		1,25-(OH)$_2$-D$_3$		25-OH-D$_3$		24,25-(OH)$_2$-D$_3$	
Activators	None	10^{-8} M	10^{-7} M	10^{-8} M	10^{-7} M	10^{-8} M	10^{-7} M
		cpm $\times 10^3/10^6$ PBMs	*cpm $\times 10^3/10^6$ PBMs*	*cpm $\times 10^3/10^6$ PBMs*	*cpm $\times 10^3/10^6$ PBMs*	*cpm $\times 10^3/10^6$ PBMs*	*cpm $\times 10^3/10^6$ PBMs*
None	6.5±4.9	6.0±3.9	5.9±2.6	7.0±3.2	6.8±2.8	6.9±3.0	6.6±2.6
PHA	137.2±26.8	92.1±22.6‡	99.4±23.1‡	126.7±23.6	129.5±24.3	129.2±32.8	104.3±23.3*
PWM	45.3±7.8	14.4±5.1‡	22.9±5.6*	40.4±7.0	28.6±6.2	33.4±6.4	14.7±5.3‡
D-O	26.7±6.9	8.6±5.0‡	10.7±5.2‡	35.6±7.0	30.4±6.9	37.3±7.5	12.3±5.0*

DNA synthesis is reflected by [^{3}H]thymidine incorporation (in counts per minute $\times 10^3/10^6$ PBMs). Each value represents the mean of four normal human subjects assayed in triplicate, D-O, dermatophyton O. * $P < 0.01$. ‡ $P < 0.001$.

1,25-(OH)$_2$-D$_3$ is a receptor-saturating concentration of hormone in a serum-free environment, it is also not surprising that the maximal inhibition of DNA and Ig synthesis was observed in cultures exposed to 10^{-8} to 10^{-7} M 1,25-(OH)$_2$-D$_3$. The absence of an inhibitory effect in resting (unstimulated) PBM cultures is probably explained by the lack of receptors for 1,25-(OH)$_2$-D$_3$ in these cells (15, 10).

Whereas 25-OH-D$_3$ had no inhibitory effect on Ig production, 24,25-(OH)$_2$-D$_3$ was associated with a significant inhibition of Ig synthesis, but only at a concentration of 10^{-7} M. This discrepancy in the effect of these two vitamin D metabolites, despite their similar affinities for the 1,25-(OH)$_2$-D$_3$ receptor, may be explained by a greater affinity of vitamin D binding protein for 25-OH-D$_3$ than for 24,25-(OH)$_2$-D$_3$ (4, 16).

The 1,25-(OH)$_2$-D$_3$ suppression of antigen-induced Ig production is usually more unpredictable, since identifiable responses vary with the immunization status and exposure of individuals tested (17). However, in addition to the effect of 1,25-(OH)$_2$-D$_3$ in PBMs activated with dermatophyton O, normal controls did show antigen-inducible responses to diptheria or tetanus toxoid, which could be inhibited with 1,25-(OH)$_2$-D$_3$.

The current experimental results are best explained by a 1,25-(OH)$_2$-D$_3$-induced antiproliferative effect on a population of mononuclear cells, which can modulate Ig synthesis. Since both activated T and B cells are known to have receptors for 1,25-(OH)$_2$-D$_3$, the primary site of hormone action may be on one or both of these cells. Studies are underway to define more clearly the mechanisms of 1,25-(OH)$_2$-D$_3$-induced inhibition of Ig synthesis.

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References

1. Coburn, J. W., and E. Slatopolsky. 1981. Vitamin D, parathyroid hormone and renal osteodystrophy. *In* The Kidney. B. M. Brenner and F. C. Rector, editors. W.B. Saunders Co., Philadelphia. Second ed. 2:2213–2305.

2. Wecksler, W. R., and A. W. Norman. 1980. Biochemical properties of 1,25-dihydroxyvitamin D receptors. *J. Steroid Biochem.* 13:977–989.

3. Norman, A. W., and W. R. Wecksler. 1978. Vitamin D receptors and biologic responses. *In* Receptors and Hormone Action. B. W. O'Malley and L. Birnbaumer, editors. Academic Press, New York. First ed. 2:533–571.

4. Franceschi, R. T., R. U. Simpson, and H. F. DeLuca. 1981. Binding proteins for vitamin D metabolites: serum carriers and intracellular receptors. *Arch. Biochem. Biophys.* 210:1–13.

5. Haussler, M. R., D. J. Mangelsdorf, H. P. Koeffler, C. A. Donaldson, and J. W. Pike. 1983. 1,25-dihydroxyvitamin D-induced differentiation of human promyelocytic leukemia (HL-60) cells: a receptor mediated phenomenon. *Arthritis Rheum.* 6:23. (Abstr.)

6. Frampton, R. J., S. A. Omond, and J. A. Eisman. 1983. Inhibition of human cancer cell growth by 1,25-dihydroxyvitamin D$_3$ metabolites. *Cancer Res.* 43:4443–4447.

7. McCarthy, D. M., J. F. San Miguel, H. C. Freake, P. M. Green, H. Zola, D. Catovsky, and J. M. Goldman. 1983. 1,25-dihydroxyvitamin D$_3$ inhibits proliferation of human promyelocytic leukaemia (HL60) cells and induces monocyte-macrophage differentiation in HL60 and normal human bone marrow cells. *Leuk. Res.* 7:51–55.

8. Bhalla, A. K., E. P. Amento, T. L. Clemens, M. F. Holick, and S. M. Krane. 1983. Specific high affinity receptors for 1,25-dihydroxyvitamin D$_3$ in human peripheral blood mononuclear cells: presence in

monocytes and induction in T lymphocytes following activation. *J. Clin. Endocrinol. Metab.* 57:1308–1310.

9. Abe, E., C. Miyara, H. Sakagami, M. Takeda, K. Konno, T. Yamazaki, S. Yoshiki, and T. Suda. 1981. Differentiation of mouse myeloid and leukemia cells induced by 1,25-dihydroxyvitamin D_3. *Proc. Natl. Acad. Sci. USA.* 78:4990–4995.

10. Provvedini, D. M., C. D. Tsoukas, L. J. Deftos, and S. C. Manolagas. 1983. 1,25-dihydroxyvitamin D_3 receptors in human leukocytes. *Science (Wash. DC).* 221:1181–1183.

11. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248–254.

12. Kissane, J. M., and C. Robbins. 1958. The fluorometric measurement of deoxyribonucleic acid in animal tissues with special reference to the central nervous system. *J. Biol. Chem.* 233:184–188.

13. Benya, P. D., and J. D. Shaffer. 1982. Dedifferentiated chondrocytes reexpress the differentiated collagen phenotype when cultured in agarose gels. *Cell.* 30:215–224.

14. Bouillon, R., F. A. Van Assche, H. Van Baelen, W. Heyns, and P. De Moor. 1981. Influence of the vitamin D-binding protein on the serum concentration of 1,25-dihydroxyvitamin D_3. *J. Clin. Invest.* 67:589–596.

15. Bar-Shavit, Z., S. L. Teitelbaum, P. Reitsman, A. Hall, I. E. Pegg, J. Trial, and A. J. Kahn. 1983. Induction of monocytic differentiation and bone resorption by 1,25-dihydroxyvitamin D_3. *Proc. Natl. Acad. Sci. USA.* 80:5907–5911.

16. Bouillon, R., H. Van Baelen, and P. De Moor. 1980. Comparative study of the affinity of the serum vitamin D-binding protein. *J. Steroid Biochem.* 13:1029–1034.

17. Pahrva, S. G., R. A. Good, and M. K. Hoffmann. 1981. The role of mitogens and antigens in the generation of antibody-producing human B-lymphocytes. *J. Clin. Immunol.* 1:101–105.