Defective Binding and Function of 1,25-Dihydroxyvitamin D_3 Receptors in Peripheral Mononuclear Cells of Patients with End-organ Resistance to 1,25-Dihydroxyvitamin D

Ruth Koren, Amiram Ravid, Uri A. Liberman, Zeev Hochberg, Yosef Weisman, and Abraham Novogrodsky
Metabolic Unit and Rogoff-Wellcome Medical Research Institute, Beilinson Medical Center, Petah-Tikva, Israel; Department of Pediatrics, Rambam Medical Center, Haifa, Israel; and Bone Disease Unit, Tel-Aviv Medical Center, Tel-Aviv, Israel

Abstract

Lectin-induced DNA synthesis by peripheral mononuclear cells from 17 normal donors was inhibited (40–60%) by 1,25-dihydroxyvitamin D_3 ($1,25[OH]_2D_3$) at physiological concentrations (10^{-10}–10^{-9} M). The lymphocytes acquire specific receptors for $1,25(OH)_2D_3$ upon activation by the lectins. This process precedes the inhibitory effect of $1,25(OH)_2D_3$. We studied lymphocytes from six patients from four different kindreds with the syndrome of hereditary end-organ resistance to $1,25(OH)_2D$ (the so-called vitamin D-dependent rickets type II). In five patients (three kindreds) peripheral blood mononuclear cells did not acquire receptors for $1,25(OH)_2D_3$ upon phytohemagglutinin-induced activation. Moreover, in contrast to normal lymphocytes, the mitogenic stimulation of these patients' lymphocytes by phytohemagglutinin and concanavalin A was not inhibited by $1,25(OH)_2D_3$. Activated lymphocytes of the sixth patient from a fourth kindred exhibited normal binding of $[^3H]1,25(OH)_2D_3$ but the hormone failed to inhibit the mitogenic stimulation. A similar pattern of the vitamin D effector system was previously observed in fibroblasts cultured from skin biopsies of the same group of patients. The conclusions from these findings are: (*a*) the inhibition of mitogenic stimulation by $1,25(OH)_2D_3$ is mediated by specific functional receptors to the hormone; and (*b*) the receptors for $1,25(OH)_2D_3$ in mononuclear cells are probably controlled genetically by the same mechanisms as the effector system in well-characterized target organs of the hormone, such as intestine and kidney.

Introduction

1,25-dihydroxyvitamin D ($1,25[OH]_2D$)[1] is the most biologically active natural metabolite of vitamin D. The target of $1,25(OH)_2D$, like that of other steroidal hormones, is the nucleus of the responding cells. $1,25(OH)_2D$ manifests its effect by a multistep process resulting in the induction of specific messenger RNA and protein synthesis (1, 2). Recent developments in the field of vitamin D endocrinology indicate that receptors for $1,25(OH)_2D$ are present not only in the classical target organs of vitamin D, the intestine, bone, and kidney, but also in other organs including cellular components of the immune system (3, 4).

Normal human resting T and B lymphocytes do not contain receptors for $1,25(OH)_2D_3$. However, they acquire such receptors upon lectin-induced stimulation or transformation with Epstein-Barr virus (5, 6). The receptors can be detected 24 h after lectin treatment (6). [^{3}H]Thymidine incorporation into DNA of stimulated peripheral blood T lymphocytes is inhibited by $1,25(OH)_2D_3$ at physiological concentrations (7). We have demonstrated the presence of high affinity receptors for $1,25(OH)_2D_3$ in medullary mouse thymocytes and their absence from cortical thymocytes. Concomitantly, we have found that $1,25(OH)_2D_3$ inhibits the mitogenic stimulation of the medullary cells but has no effect on the stimulation of the cortical cell subpopulation (8). These data suggest that the inhibitory effect of $1,25(OH)_2D_3$ on lymphocyte response is mediated by specific receptors.

Study of lymphocytes from patients with the syndrome of end-organ resistance to $1,25(OH)_2D$ (the so-called vitamin D-dependent rickets type II[DDII]) provides an unusual opportunity to challenge this assumption. DDII is a hereditary autosomal recessive syndrome characterized by early onset rickets, hypocalcemia, secondary hyperparathyroidism, and elevated serum levels of $1,25(OH)_2D$ before or during treatment with high doses of various vitamin D metabolites. In more than 50% of these patients total alopecia is an additional characteristic feature.

It was demonstrated recently that fibroblasts cultured from human skin biopsies have receptors for $1,25(OH)_2D_3$, the characteristic features of which are similar if not identical to those of known target cells for the hormone (9). Using fibroblasts cultured from skin biopsies of these patients, a spectrum of defects in the intracellular receptor mechanism has been demonstrated (10, 11). However, fibroblast culture is a costly and time-consuming procedure. If patients with DDII have a universal defect in $1,25(OH)_2D_3$ receptors in multiple organs, such a defect should be manifested in their lymphocytes as well. In this report we demonstrated that peripheral blood lymphocytes from a group of patients with DDII do not acquire receptors for $1,25(OH)_2D_3$ upon mitogen stimulation. Moreover, the stimulation of these lymphocytes, in contrast to normal lymphocytes, was refractory to inhibition by $1,25(OH)_2D_3$. These data strongly support the conclusion that inhibition of stimulation by $1,25(OH)_2D_3$ is mediated by specific receptors for the hormone and that peripheral lymphocytes may serve as a rapid diagnostic tool for DDII.

Methods

Subjects. This study included six patients aged 3–22 yr of four kindreds: Two affected siblings in kindreds 1 and 3, and one affected patient in

Address correspondence to Dr. Liberman, Metabolic Disease Unit, Beilinson Medical Center, Petah-Tikva, 49100 Israel.

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. *Abbreviations used in this paper:* Con A, concanavalin A; $1,25(OH)_2D$, 1,25-dihydroxyvitamin D; $1,25(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; DDII, vitamin D-dependent rickets type II; PHA, phytohemagglutinin.

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kindreds 2 and 4. The patients had typical clinical and biochemical features of DDII, including alopecia. Detailed clinical descriptions of the patients were published previously (11, 12). No measurable cytosol receptors or nuclear uptake of [^{3}H]1,25(OH)$_2$D$_3$ could be observed in studies with fibroblasts cultured from skin biopsies of kindreds 1–3 (11). Normal capacity and affinity of cytosol receptors and nuclear uptake was demonstrated for patient No. 4 (10). Normal donors were young adults except for one 10-yr-old girl (normal 2).

Materials. Purified phytohemagglutinin (PHA) was obtained from Wellcome Research Laboratories, Beckenham, Kent, England; concanavalin A (Con A), twice chrystallized, from Miles-Yeda, Rehovot, Israel; Ficoll-Hypaque from Pharmacia Fine Chemicals, Uppsala, Sweden; 1,25(OH)$_2$D$_3$ from Hoffmann-LaRoche Inc., Nutley, NJ (a gift from Dr. M. Uskokovic); aprotinin from Bayer, Leverkusen-Bayerwerk, Germany; and Bio-Gel HTP hydroxylapatite was from Bio-Rad Laboratories, Richmond, CA. 1,25[23,24(n)^{3}H](OH)$_2$D$_3$ was from New England Nuclear, Boston, MA (160 Ci/mmol); [*methyl*-^{3}H]thymidine was from Nuclear Research Center Negev, Beer-Sheva, Israel, (2 Ci/mmol); and tissue culture media were obtained from Biological Industries, Beth-Haemek, Israel.

Lymphocyte culture. Human peripheral blood mononuclear cells were obtained from 20-ml blood samples by Ficoll-Hypaque density gradient centrifugation as previously described (13). Mononuclear cells (1×10^6/ml) were suspended in RPMI 1640 medium containing 5% heat-inactivated human AB serum supplemented with penicillin (100 U/ml) and streptomycin (100 μg/ml). Cells were incubated at 37°C in a humidified 5% CO_2–95% air atmosphere in 0.2-ml aliquots in flat-bottomed Cooke microtiter plates (Nunc, Roskilde, Denmark). Con A (10 μg/ml), PHA (1 μg/ml), and 1,25(OH)$_2$D$_3$ (10^{-10}–10^{-6} M) were added at zero time. Cultures were incubated for 68 h. [^{3}H]Thymidine (1 μCi/well) was added 4 h before harvesting. All cultures were carried out in triplicates.

Binding studies. Binding of [^{3}H]1,25(OH)$_2$D$_3$ to receptors in mononuclear cells was assayed as previously described (8, 10). Peripheral blood mononuclear cells (1×10^6/ml) were incubated with PHA (1 μg/ml) under the same culture conditions described above in 9-cm tissue culture dishes (Nunc), 15 ml/dish. Stimulated cells were harvested after 3 d and washed 3 times with ice cold phosphate-buffered saline. All further procedures were performed at 0–4°C. Cells were suspended in a high KCl buffer (10 mM Tris–HCl, pH 7.4; 300 mM KCl; 10 mM sodium molybdate; 1.5 mM EDTA; 1.0 mM dithiothreitol; and 500 kallikrein inactivator U/ml approtinin). The suspension was sonicated (three 5-s pulses) with Heat-System-Ultrasonics Cell Disruptor model M-375 (Ultrasonic Corp. of America, Hicksville, NY) set at gain 3. The sonicate was centrifuged at 100,000 *g* for 60 min. The supernatant was saved and will be referred to as "cytosol." Freshly prepared cytosols were incubated with 1 nM [^{3}H]1,25(OH)$_2$D$_3$ for 17 h at 0°C. Bound [^{3}H]1,25(OH)$_2$D$_3$ was separated from free hormone by the hydroxylapatite batch separation procedure.

Nonspecific binding was assessed under conditions where 1,000-fold excess of cold hormone was present in the binding assay. Protein was determined by the method of Lowry et al. (14). Protein concentrations in the cytosols were between 1.2 and 2 mg/ml. All binding assays were performed in duplicates.

Statistical analysis. Data of [^{3}H]thymidine incorporation are presented as mean±SD. Statistical analysis was carried out by means of unpaired *t* test.

Results

The effect of 1,25(OH)$_2$D$_3$ at different concentrations on Con A-mediated stimulation of mononuclear cells from two normal volunteers is depicted in Table I. Inhibition of [^{3}H]thymidine incorporation into stimulated lymphocytes is observed already at 10^{-10} M 1,25(OH)$_2$D$_3$, and reaches a peak at 10^{-9} M with no additional change at concentrations of up to 10^{-6} M. Half of the maximal inhibition is achieved at a concentration of 1–2 $\times$ 10^{-10} M 1,25(OH)$_2$D$_3$. [^{3}H]Thymidine incorporation into

*Table I. Effect of 1,25(OH)$_2$D$_3$ on Con A-induced Stimulation of Mononuclear Cells from Normal Subjects and a Patient with DDII**

	[^{3}H]Thymidine incorporation		
[1,25(OH)$_2$D$_3$]	Normal 4a	Normal 4b	Patient 4
	cpm	*cpm*	*cpm*
None	42,400±7,500	66,900±5,000	78,300±12,400
10^{-10} M	27,100±7,500	61,400±600	76,600±3,900
3×10^{-10} M	19,800±5,600	51,000±9,500	74,100±6,400
10^{-9} M	10,800±1,300	47,500±2,300	75,700±2,300
10^{-8} M	13,200±1,900	47,500±900	95,700±14,600
10^{-7} M	10,300±1,900	45,200±600	84,900±20,100
10^{-6} M	12,600±3,000	46,200±5,400	83,200±3,300

* Mononuclear cells were incubated with Con A (10 μg/ml) and different concentrations of 1,25(OH)$_2$D$_3$ for 72 h as described in Methods. Cells were pulsed with [^{3}H]thymidine in the last 4 h of the incubation. Data are presented as the mean±SD of triplicate cultures. Data obtained in the presence of 10^{-9}–10^{-6} M 1,25(OH)$_2$D$_3$ were pooled and compared with the control by means of unpaired *t* test. The inhibition was significant ($P < 0.001$) for the normal donors. The slight enhancement observed for patient 4 is of no statistical significance.

lymphocytes obtained from 17 healthy subjects and incubated with Con A and 1 nM 1,25(OH)$_2$D$_3$ for 72 h was 60±11.5% (SE) of the control value. For every normal donor the inhibition was highly significant ($P < 0.01$). In preliminary experiments, performed under the same experimental conditions, we established that the decrease in thymidine incorporation was accompanied by a decrease in cell number. In a typical experiment cell number decreased by ~40%. When the cultures were pulsed with [^{3}H]thymidine at 48 h, no inhibitory effect by 1,25(OH)$_2$D$_3$ was observed. It should be noted that receptors for 1,25(OH)$_2$D can be detected on T lymphocytes 24 h after lectin treatment (6), and thus receptor appearance precedes the expression of inhibition.

The effect of 1,25(OH)$_2$D$_3$ on Con A-induced stimulation of mononuclear cells from children with DDII is depicted in Fig. 1 and Table I. Four separate experiments were carried out, each was performed on cells from children of different kindred, with normal donors serving as controls. The [^{3}H]thymidine incorporation into Con A-stimulated lymphocytes from the six patients was essentially refractory to inhibition by 1,25(OH)$_2$D$_3$. The deviations from control values never reached statistical significance. In contrast, 1,25(OH)$_2$D$_3$ inhibited the mitogenic response of lymphocytes from six normal subjects that served as controls in the same experiments. The inhibition was dose-dependent and ranged between 43 and 52%. The differences between the hormone effect on lymphocytes from patients and respective normal donors were highly significant ($P < 0.001$) (Fig. 1 and Table I).

We have studied the binding of 1,25(OH)$_2$D$_3$ to mononuclear cells stimulated by PHA. The cell preparations used in the binding experiments were the same as in the stimulation-inhibition experiments shown in Fig. 1 and Table I. In the binding experiments we used PHA- rather than Con A-stimulated cells, since PHA was more effective in generation of receptors for 1,25(OH)$_2$D$_3$, probably due to its higher potency as a mitogen. As shown in Table II, PHA stimulation of lymphocytes from normal donors is significantly ($P < 0.01$) inhibited by 1 nM 1,25(OH)$_2$D$_3$, similar to the inhibition of Con A stimulation

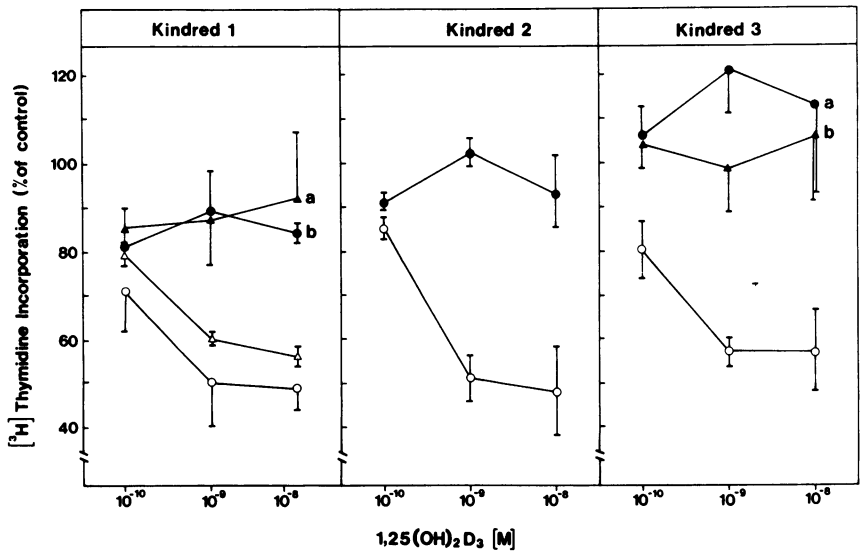

Figure 1. Effect of $1,25(OH)_2D_3$ on Con A-induced stimulation of mononuclear cells from normal subjects and patients with DDII. Each experiment dealt with a different kindred. Kindred 1: patient 1a (▲), patient 1b (●), normal 1a (○), and normal 1b (△). Kindred 2: patient 2 (●) and normal 2 (○). Kindred 3: patient 3a (●), patient 3b (▲), and normal 3 (○). Experimental details are as described in legend to Table I and in Methods. Data are presented as the mean of triplicate cultures. Data in the presence of 10^{-9} and 10^{-8} M $1,25(OH)_2D_3$ were pooled and compared with the control. The inhibition of thymidine incorporation for all normal donors was statistically significant ($P < 0.001$). The values for DDII patients were not significantly lower than the control values ($P > 0.05$). The differences between patients and respective controls were statistically significant ($P < 0.001$) for all kindreds.

described above. On the other hand, lymphocytes from patients with DDII are resistant to the inhibitory effect of the hormone. Occasionally, we observed an enhancing effect of $1,25(OH)_2D_3$ on PHA-induced thymidine incorporation into lymphocytes from these patients. However, this effect was not dose-dependent (data not shown) and was statistically significant only in two out of the six patients. The binding of $[^3H]1,25(OH)_2D_3$ to cytosols of PHA-stimulated mononuclear cells from patients with DDII is practically undetectable in patients 1a, 2, 3a, and 3b, and normal in patient 4. The lack of binding of $1,25(OH)_2D_3$ to lymphocytes from some of the patients could not be attributed to a reduction in the mitogenic response, since the rate of thymidine incorporation for cell preparations from patients is similar to the rate for normal donors (Table II). It should be noted that although the extent of inhibition by $1,25(OH)_2D_3$ is remarkably similar among different individuals (41–47%), the

*Table II. Effect of $1,25(OH)_2D_3$ on Thymidine Incorporation into PHA-stimulated Mononuclear Cells from Normal Donors and DDII Patients, and Binding of $[^3H]1,25(OH)_2D_3$ to Cytosol Preparations from the Same Cells**

Donor	[³H]Thymidine incorporation		[³H]1,25(OH)₂D₃ binding (specific§)
	PHA	PHA + 1,25(OH)₂D₃	
	cpm	*cpm*	*fmol/mg protein*
Experiment 1			
Patient 1a	110,200±10,500	155,800±3,000 (141)‡	NS‖
Normal 1a	83,800±9,400	49,600±7,100 (59)	84.3
Experiment 2			
Patient 2	100,300±11,000	111,000±3,100 (111)	NS
Normal 2	130,600±5,400	70,500±5,900 (54)	39.5
Experiment 3			
Patient 3a	110,200±8,500	108,600±12,500 (99)	NS
Patient 3b	105,700±6,000	113,700±13,300 (108)	NS
Normal 3	82,500±8,300	43,900±1,300 (53)	119.6
Experiment 4			
Patient 4	134,900±11,300	144,800±11,200 (107)	63.8
Normal 4b	53,500±4,000	14,800±2,400 (28)	30.8

* The mononuclear cells in the experiments are those used in the corresponding experiments described in Fig. 1 and Table I. The concentration of $1,25(OH)_2D_3$ was 10 nM. Data are presented as the mean±SD of triplicate cultures. The inhibition of thymidine incorporation into lymphocytes from normal donors is statistically significant ($P < 0.01$). The enhancement of thymidine incorporation into lymphocytes from DDII patients was not statistically significant, except for patient 1a ($P < 0.01$). ‡ Numbers in parentheses are the values of thymidine incorporation in the presence of $1,25(OH)_2D_3$, expressed as percents of the control values. § Specific binding was obtained by substracting nonspecific binding from total binding. ‖ NS, nonsignificant specific binding. NS was defined whenever the total binding was less than the nonspecific binding, or when the specific binding was <50% of the nonspecific binding. Nonspecific binding was always ~100 cpm and never exceeded 10% of the total binding in normal donors.

number of binding sites for [^{3}H]1,25(OH)$_2$D$_3$ in parallel cultures varies considerably (30.8–119.6 fmol/mg protein). This variability may be the result of the presence of monocytes and other non-T cell types.

Discussion

Recent studies suggest that the inhibition of lymphocyte proliferation by 1,25(OH)$_2$D$_3$ is mediated by a specific high-affinity receptor. This assumption is based mainly on indirect evidence: the inhibitory dose of 1,25(OH)$_2$D$_3$ which causes half maximal effect is similar to the dissociation constant observed in parallel binding studies ($\sim 2 \times 10^{-10}$ M); the relative efficacy of vitamin D metabolites as inhibitors is similar to their known affinity for the receptor binding site (7) and, finally, a subpopulation of mouse thymocytes that does not have a high affinity receptor is insensitive to inhibition (8).

Skin fibroblasts obtained from the patients included in this study with clinical evidence of end-organ resistance to 1,25(OH)$_2$D showed a receptor pattern similar to the one observed by us in their stimulated mononuclear cells (Table II): normal for patient 4 and undetectable for the others. Neither of these patients responded clinically or biochemically to prolonged treatment with high doses of vitamin D or its 1α-hydroxylated metabolites. The induction of 25-hydroxyvitamin D 24-hydroxylase by 1,25(OH)$_2$D$_3$ in cultured skin fibroblasts may serve as a biochemical marker for the effect of the hormone in vitro. No induction of the enzyme was observed in cultured skin fibroblasts from all four kindreds (reference 11 and unpublished observation). We show here that 1,25(OH)$_2$D$_3$ fails to inhibit the proliferation of mitogen-stimulated lymphocytes from the same patients that have defective effector systems for the hormone, including the patient with normal binding.

This finding strongly supports the conclusion that the receptor for 1,25(OH)$_2$D$_3$ mediates the inhibitory effect of the hormone on lymphocyte stimulation and that this effect requires the presence of an intact coupling between receptor binding and subsequent events. The expression of this receptor in mononuclear cells is probably genetically controlled by the same mechanism as the expression of receptors in skin fibroblasts and classical target organs like intestine and kidney. The susceptibility of mononuclear cells to inhibition by 1,25(OH)$_2$D$_3$ can readily be used to diagnose patients with total or partial end-organ resistance to 1,25(OH)$_2$D. Peripheral blood mononuclear cells provide an easily accessible and useful cellular source which may serve as a model for the study of the mechanism of 1,25(OH)$_2$D$_3$ action under physiological and pathological conditions.

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