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1,25-DIHYDROXYVITAMIN D_3 INHIBITS SELECTIVELY THE MITOGENIC STIMULATION OF MOUSE MEDULLARY THYMOCYTES

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Mouse thymocytes were separated into cortical and medullary subpopulations by differential agglutination with peanut agglutinin. A high-affinity receptor for 1,25-dihydroxyvitamin D3 is present in medullary immunocompetent mouse thymocytes and is absent from cortical immature cells. 1,25-dihydroxyvitamin D_3, at physiological concentrations, inhibits the mitogenic response of the medullary cells to phytohemagglutinin and interleukin-2, but has no effect on the cortical subpopulation. Other less active metabolites of vitamin D had little or no effect on medullary cell stimulation.

1,25-dihydroxyvitamin D ($1,25(OH)_2D$) is the most active natural metabolite of vitamin D. Like the true steroidal hormone, $1,25(OH)_2D$ acts in the nucleus of target cells (1,2). This requires a multistep process involving translocation of hormone receptors from the cytosol to the nucleus and induction of specific messenger RNA and protein synthesis. This hormone is a potent differentiating agent for human promyelocytic leukemia and murine leukemia lines (3-5), inducing their maturation towards macrophages. $1,25(OH)_2D_3$ also induces the differentiation of monocytes into bone resorbing osteoclasts (6). These effects are probably mediated by interaction of the hormone with a specific, high affinity receptor.

Chemical inducers of differentiation in some cell types, such as dimethyl sulfoxide and butyric acid (7,8), inhibit mitogen-induced lymphocyte proliferation (9,10). In this study we measured the effect of the hormone on murine thymocytes stimulation by mitogens. We also measured the content of receptors for $1,25(OH)_2D_3$.

Abbreviations: $1,25(OH)_2D_3$ - 1,25-dihydroxyvitamin D_3; PNA - peanut agglutinin; PNA-positive cells - thymocytes bearing receptors for PNA; PNA-negative cells - thymocytes lacking receptors for PNA; PHA - phytohemagglutinin; TPA - 12-O-tetradecanoyl-phorbol-13-acetate; IL-1 - Interleukin-1; IL-2 - Interleukin-2.

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The major site of T lymphocyte maturation is the thymus which contains a major population of immature cortical cells and a minor population of immunocompetent mature medullary lymphocytes. We have found that freshly isolated medullary thymocytes, separated by peanut agglutinin (PNA) differential agglutination technique (PNA-negative cells), contained a high affinity, saturable receptor for $1,25(OH)_2D_3$, whereas PNA-positive cortical cells lack the receptor for the hormone. In parallel, we have observed that the mitogenic response of the mature PNA-negative cells was inhibited by low concentrations (10^{-10}M) of $1,25(OH)_2D_3$, whereas mitogenic stimulation of the immature PNA-positive cells was not affected by the hormone.

MATERIALS AND METHODS

Materials. Purified phytohemagglutinin (PHA) was obtained from Wellcome Research Laboratories, Beckenham, England. Bovine serum albumin, Peanut agglutinin and 12-O-tetradecanoyl-phorbol-13-acetate (TPA) were from Sigma Chemical Co., St. Louis, Mo. Escherichia coli 055:B5 Lipopolysaccharide was from Difco Laboratories, Detroit, Mich. $25(OH)D_3$, $24,25(OH)_2D_3$ and $1,25(OH)_2D_3$ were obtained from Hoffman LaRoche, Nutely, NJ (gift from Dr. M. Uskokovic). Aprotinin was from Bayer, Leverkusen, Germany. Bio-Gel HTP hydroxylapatite was from Bio-Rad Laboratories, Richmond, CA. $1,25[23,24(n)^3H](OH)_2D_3$ was from New England Nuclear, Boston, MA (160 Ci/mmol). Thymidine Methyl-^{3}H was from Nuclear Research Center - Negev, Beer-Sheva, Israel (2 Ci/mmol).

Isolation of cells. Thymocytes were removed from 4-6 weeks old BALB/c mice, minced and washed with phosphate buffered saline. Separation into cortical and medullary subpopulations was performed by differential peanut-agglutinin agglutination technique (10,11). Thymocytes used in cell cultures were separated by sedimentation through fetal calf serum. In the separation of cells for receptor assays fetal calf serum was replaced by bovine serum albumin solution to avoid contamination by serum vitamin D binding protein. PNA-treated cells were sedimented through a 3% bovine serum albumin solution in phosphate-buffered saline and the agglutinated PNA-positive cells were further purified by sedimentation through 5% bovine serum albumin solution.

Cell culture and stimulation conditions. The cells were suspended ($2x10^6$/ml) in Roswell Park Memorial Istitute Medium 1640 (Grand Island Biological Company, Grand Island, N.Y.) containing 5% heat-inactivated human AB serum and cultured in 0.2 ml aliquots in flat-bottomed Cooke microtiter plates (Nunc Denmark) at 37°C in a 95% air 5% CO_2 humidified atmosphere. The various additions included PHA (2 μg/ml), TPA (50 ng/ml) and various dilutions of soluble growth factor preparations. Cultures were incubated for 72 hr and [^{3}H]thymidine (1 μCi/well) was added 16 hr before harvesting on a microtiter Dynatech Automash Cell Harvester. All cultures were done in triplicates. Soluble growth factors used in the stimulation studies were prepared as previously reported (10,12). IL-1-containing preparations were prepared from human peripheral blood mononuclear cells incubated with lipopolysaccharide for 24 hr. IL-2-containing preparations were supernatants obtained from neuraminidase and galactose oxidase treated human peripheral blood mononuclear cells or form the human leukemia cell line , Jurkat stimulated with PHA and TPA. These IL-2-containing supernatants contain also IL-1 or TPA and were used only in cortical thymocytes stimulation experiments.

Binding of [^{3}H]1,25(OH)$_2$D$_3$ to soluble extract from cells. All procedures were performed with solutions maintained at 0-4°C. Freshly separated cells were washed 3 times with phosphate-buffered saline and 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 Units/ml approtinin). The suspension was sonicated (three 5 seconds pulses) with Heat-Systems-Vetrasonics Cell Disruptor model W-375 set at gain 3. The sonicate was centrifuged at 100,000 g for 60 minutes. The supernatant was saved and will be referred to as "cytosol". Freshly prepared "cytosols" were incubated with varying concentrations of [^{3}H]1,25(OH)$_2$D$_3$ (0.06-1 nM). Low affinity non-specific binding was assessed by coincubation with 1 μM 1,25(OH)$_2$D$_3$. Incubation conditions and hydroxylapatite batch separation procedure were as previously described (13). Protein was determined by the method of Lowry et al (14).

RESULTS

Thymocytes were separated into the cortical and medullary subpopulation by selective agglutination with peanut agglutinin. The medullary immunocompenent cells can be stimulated by incubation with the plant lectin PHA and the macrophage-derived interleukin-1 (IL-1). IL-1 can be replaced by the tumor promoter TPA (10). Under these conditions, the lymphocyte-derived interleukin-2 (IL-2) is produced, interacts with specific receptors and stimulates the cells to proliferate. PHA-treated medullary cells can also be stimulated directly by exogenous IL-2. The immature cortical cells are unable to produce IL-2 (15) and can be stimulated by the combined treatment with PHA and exogenous IL-2. The effect of 1,25(OH)$_2$D$_3$ on the stimulation of the two thymocyte subpopulations was investigated. 1,25(OH)$_2$D$_3$ inhibited the mitogenic response of the medullary PNA-negative cells to PHA and TPA or IL-1, but had no effect on the response of cortical PNA-positive cells to PHA and IL-2 (Fig. 1a). The inhibition was dose dependent and saturable. The half maximal effect was achieved at 2×10^{-10}M 1,25(OH)$_2$D$_3$ which is at the physiological plasma concentration range. The extent of inhibition never exceeded 50 percent even at a concentration of 10^{-7}M 1,25(OH)$_2$D$_3$ (Fig.1). The inhibition of the mitogenic response was not associated with cell damage or cell death as revealed by the trypan blue exclusion test. The addition of 1,25(OH)$_2$D$_3$ during the 16 hours of the thymidine incorporation assay had no effect on the incorporation of the label into DNA. We have studied the effect of two additional metabolites of vitamin D, 25(OH)D$_3$ and 24,25(OH)$_2$D$_3$, on the stimulation of PNA-negative cells by PHA and TPA. No significant dose dependent inhibition at a concentration range of 10^{-10}-10^{-7}M was observed with either of these metabolites (Fig. 1b).

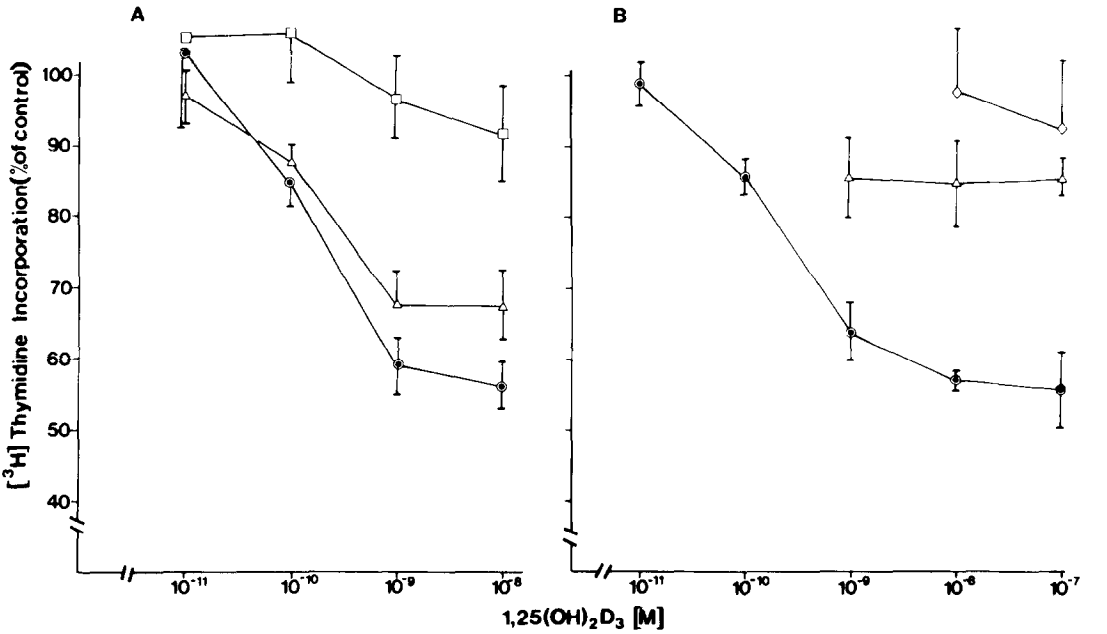

Fig. 1: a) Effect of $1,25(OH)_2D_3$ on the mitogenic stimulation of the two major thymocyte subpopulations. PNA-negative cells were cultured with PHA and TPA (θ), or PHA and IL-1 containing preparation (△). PNA-positive cells were cultured with PHA and IL-2 containing preparations from neuraminidase and galactose oxidase-treated human peripheral blood mononuclear cells or from the human leukemia cell line Jurkat (□). $1,25(OH)_2D_3$ was added to the cultures together with the mitogens. Figure represent the mean + S.E. of five separate experiments. The average values for (^3H)thymidine incorporation in control cultures were: PNA-negative cells; PHA + TPA, 274,300 cpm; PHA + IL-1, 118,400 cpm; PNA-positive cells; PHA + IL-2, 34,000 cpm.

b) Effect of different vitamin D metabolites on the mitogenic response of PNA-negative cells. $1,25(OH)_2D_3$ (θ), $25(OH)D_3$ (◇) or $24,25(OH)_2D_3$ (△) were added to PHA + TPA stimulated PNA-negative cells. (^3H)thymidine incorporation into control cultures was 304,000 cpm. Figure represent the mean ± S.D. of triplicate cultures of a representative experiment.

The different effect of $1,25(OH)_2D_3$ on the two thymocyte subpopulations can be attributed to an inherent property of the cells, or alternatively, to their mode of activation, namely by IL-1 or IL-2. We compared, therefore, the effect of the hormone on the stimulation of the two cell types by exogenous IL-2. The IL-2 preparations were a purified human IL-2 and a Gibbon IL-2 preparation devoid of IL-1 activity (16). The results in table 1 demonstrate that $1,25(OH)_2D_3$ inhibited to a similar extent medullary PNA-negative cells stimulated by either IL-1 or IL-2, whereas, the stimulation of the cortical PNA-positive cells by IL-2 was not affected by the hormone. Preliminary results have shown the same pattern of inhibition using a human recombinant IL-2. It is important to note that this inhibitory

Table 1. EFFECT OF $1,25(OH)_2D_3$ ON MITOGENIC STIMULATION OF THYMOCYTES INDUCED BY IL-2

	Thymocyte Subpopulations	Additions	[^{3}H]Thymidine incorporation (cpm)		
			None	$1,25(OH)_2D_3$ (10^{-9}M)	Inhibition[a] (%)
Exp.1.	PNA-negative	None	2736	2572	–
		PHA	25618	14696	43
		PHA+IL-1	116514	66706	43
		PHA+IL-2[b]	50465	25786	55
	PNA-positive	None	1225	1310	–
		PHA+IL-2[b]	19085	19339	0
Exp.2.	PNA-negative	PHA	16057	10141	37
		PHA+TPA	124198	52350	61
		PHA+IL-2[c]	81134	45822	45

[a]Percent inhibition of IL-1 and IL-2 responses was calculated after substraction of the respective PHA responses in the presence or absence of $1,25(OH)_2D_3$;

[b]Conditioned medium of the gibbon T cell line MLA-144 (100 µl/well);

[c]Purified human T cell growth factor (Electro-Nucleonics) (6 U/ml).

effect can be overcome by adding higher concentrations of recombinant or purified human IL-2 (40 U/ml).

The potent and specific inhibitory effects of $1,25(OH)_2D_3$ on the mitogenic stimulation of medullary thymocytes indicates the involvement of a high affinity specific receptor to $1,25(OH)_2D_3$ in this process. We measured the binding of [^{3}H]$1,25(OH)_2D_3$ to cytosol preparations from both PNA-negative and PNA-positive cells. The binding isotherm was obtained by incubating [^{3}H]$1,25(OH)_2D_3$, $(0.05\text{-}1)\times10^{-9}$M, with cytosol preparations in the presence or absence of 1×10^{-6}M unlabeled hormone. The binding in the presence of unlabeled hormone was considered to be non-specific. Specific binding of the hormone to cytosol preparations from both thymocyte subpopulations is presented in fig. 2. Saturable binding, as judged by the Scatchard analysis, was observed only for the medullary PNA-negative cells

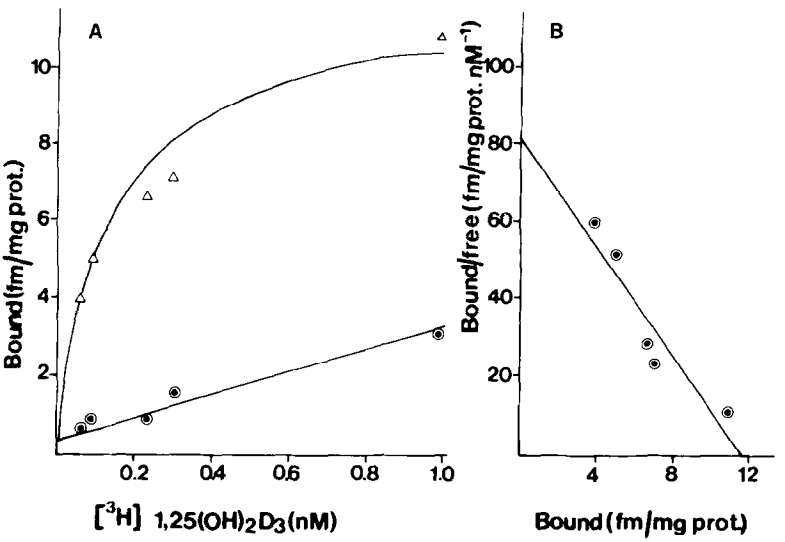

Fig. 2: Binding of [^{3}H]1,25(OH)$_2$D$_3$ to PNA-negative and PNA-positive thymocytes: Cytosols were prepared from PNA-positive ($4x10^8$ cells/ml) and PNA-negative ($1.4x10^8$ cells/ml) thymocytes. Non-specific binding was 38% of the total binding for PNA-positive cells and 24% of the total binding for PNA-negative cells. Specific binding isotherms for PNA-negative (Δ) and PNA-positive (θ) cells are shown in Figure 2A. Scatchard plot (2B) drawn from the data points for PNA-negative cells had a correlation coefficient of 0.926.

with an apparent dissociation constant of $(1.3 \pm 0.3)x\ 10^{-10}$M and number of sites of 11.6 ± 4.3 fmole/mg protein. The dissociation constant is very similar to the concentration of 1,25(OH)$_2$D$_3$ eliciting half of the maximal inhibition of stimulation.

DISCUSSION

We have demonstrated the presence of biologically active, high affinity, specific receptors for 1,25(OH)$_2$D$_3$ in mature medullary mouse thymocytes and their absence in cortical immature cells. It has been recently shown that human peripheral blood lymphocytes acquire receptors for 1,25(OH)$_2$D$_3$ upon mitogenic stimulation (6). Since both thymocyte subpopulations proliferate extensively in vivo (17), it appears that the acquirement of receptors for 1,25(OH)$_2$D$_3$ is not characteristic for proliferating lymphocytes per se, but rather reflects their differentiation stage. As noted above, 1,25(OH)$_2$D$_3$ caused only partial inhibition of thymocyte stimulation (approximately 50 percent) even at concentration of 10^{-7}M, which is thousand fold higher than the dissociation constant. This might indicate the presence of a subpopulation in the medullary cells which

is resistant to the hormone. The stimulation of the medullary cells was inhibited to a similar extent upon stimulation by PHA and IL-1 or by PHA and IL-2. This finding may indicate that the response to IL-2 is a target for $1,25(OH)_2D_3$ inhibition. Since the stimulation of the medullary thymocytes by IL-1 and PHA is dependent on the production of IL-2, an additional effect of $1,25(OH)_2D_3$ on IL-2 production cannot be ruled out. The presence of receptor for $1,25(OH)_2D_3$ in lymphocytes at a specific stage of differentiation raises the possibility for a role for the hormone in T cell maturation.

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