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1,25-DIHYDROXYVITAMIN D_3 RECEPTORS IN A SUBSET OF MITOTICALLY ACTIVE LYMPHOCYTES FROM RAT THYMUS

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Summary: Lymphocytes from the rat thymus gland were separated on Percoll gradient in two subsets. The first subset was enriched in large mitotically active cells and the second in small mitotically inert cells. The former cell preparation possessed a 3.3 S macromolecule with high affinity for 1,25-Dihydroxyvitamin D_3 ($Kd=3.3x10^{-10}M$) which bound to DNA cellulose and was eluted from this resin with 0.26 M KCl. In contrast, the small-cell-enriched subset of thymic cells was negative for 1,25-Dihydroxyvitamin D_3 specific binding. These findings support evidence from studies in human lymphocytes that there exists an association between mitotic activity and 1,25-Dihydroxyvitamin D_3 receptor expression in this class of leukocytes.

It has been earlier shown that specific receptors for 1,25-Dihydroxy- vitamin D_3 [$1,25(OH)_2D_3$] are absent from normal human lymphocytes but occur in several established lines of malignant lymphocytes and are expressed in normal lymphocytes upon activation with mitogenic lectins and Epstein Barr virus (1,2). These findings have suggested that there may be an association between the mitotic activity of lymphoid cells and $1,25(OH)_2D_3$ receptors. To probe further into this intriguing association we have examined lymphoid cells of the rat thymus gland. It is known that two major subpopulations of lymphoid cells coexist in this gland: a) large size cells located in the subcapsular and medullary regions that are mitotically active and through a series of rapid divisions give rise to b) small mitotically inert cells which are located almost exclusively in the thymic cortex (3,4). After partial separation of these two subpopulations on Percoll gradient we searched for the presence of $1,25(OH)_2D_3$ receptors in each cell preparation.

MATERIALS AND METHODS

Reagents and chemicals: Ficoll-Hypaque and Percoll were purchased from Pharmacia Chem. Co. (Piscataway, NJ 08854). Radioactive 1,25-Dihydroxy-[26,27 methyl-

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^{3}H] cholecalciferol (specific activity: 158 Ci/mmol) and [methyl-^{3}H] Thymidine (specific activity: 5 Ci/mmol) were purchased for Amersham (Arlington Heights, IL 60005). Crystalline 1,25-dihydroxy-cholecalciferol was generously donated by Dr. Milan Uskokovic of Hoffmann-LaRoche.

Thymus cell preparation: Thymus glands were removed from 6-week old male Fisher rats. Cells were released from the stroma by gently pressing the glands through a stainless steel mesh and were resuspended in RPMI 1640 medium supplemented with 10% heat-inactivated Fetal Calf Serum. The resuspended cells were purified by Ficoll-Hypaque centrifugation (5), washed twice in medium and incubated in 65 mm plastic culture dishes for 30 min. at 37°C to remove adherent thymic monocytes (6); this step was necessary in order to eliminate the possibility that binding could be due to contaminating monocytes which possess $1,25(OH)_2D_3$ receptors (1,2). The non adherent cells were harvested, washed twice in medium and subsequently fractionated in two subpopulations by a continuous gradient of Percoll (7). The upper fraction of the gradient consisted of debris and cells with very low viability and was discarded to further exclude the possibility of monocyte contamination. The remaining cells of the gradient were distributed in two bands. Each band was harvested separately and washed in fresh medium. Aliquots were stained for the presence of non specific esterase (8) and analyzed for their size and light-scatter properties with a Cytofluorograph 50 H cell sorter (Ortho Diagnostic, Westwood, MA 02090). (9). Cell viability was determined by trypan blue exclusion.

Incorporation of [^{3}H] Thymidine: Freshly isolated cells were plated in flat bottom 96-wells plates in medium supplemented with 10% heat-inactivated Fetal Calf Serum ($0.2x10^6$ cells/0.2 ml). [^{3}H] Thymidine (5 μ Ci/ml) was added and incubations were carried for 19 hours. At the end of the incubation cells were harvested with a Titertek multiple cells harvester on glass fiber filter paper, dried overnight in oven at 60 C and counted in scintillation liquid. Statistical analysis of data was done using Student's paired t test.

$1,25(OH)_2D_3$ binding studies: Thymic cells from the upper and lower band of Percoll were incubated intact with [^{3}H] $1,25(OH)_2D_3$ ($2x10^{-9}$M) for one hour at 37°C in the presence or absence of 200-fold excess of unlabelled $1,25(OH)_2D_3$. Cells were then cooled at 4°C for 15 min, washed with isotonic buffer and were disrupted by sonication. Cytosol was prepared by ultracentrifugation (105,000xg) of the sonicate and was either layered onto sucrose density gradients (4-24% in 0.3 M KCl-buffer) or precipitated by ammonium sulfate, re-solubilized and applied to a DNA-cellulose column. The column was then eluted with a 90-ml linear gradient of KCl (0-0.6 M KCl). Fractions were collected and aliquots (1 ml) counted. Salt concentration was monitored by conductivity measurements. For saturation analysis of specific $1,25(OH)_2D_3$ binding, cells were incubated as described above with increasing concentrations of [^{3}H] $1,25(OH)_2D_3$ ($3x10^{-11}$ to $1x10^{-9}$M) alone or in the presence of 200-fold excess of unlabelled $1,25(OH)_2D_3$. After incubation, cells were washed and solubilized with Omnisol (WestChem, San Diego) and the internalized radioactivity was counted. The specific binding (total minus non specific) was plotted by the method of Scatchard. The above methodology has been described in greater detail in previous publications (1,10,11).

RESULTS

The cell viability following Ficoll-Hypaque density centrifugation was greater than 98%. Using this mixed thymic cell preparation we performed Percoll density gradient centrifugation. Excluding cell debris from the top of the gradient, the remaining cells were separated in two bands. Light scatter propertv analysis in a total of nine experiments indicated that, in average, the upper

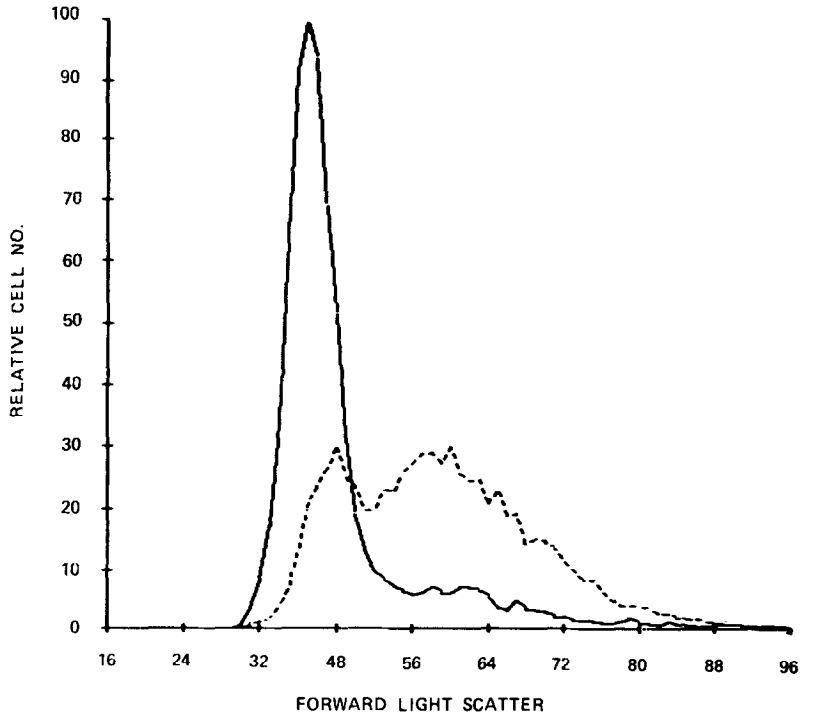

Figure 1: Cytofluorographic analysis of enriched subsets of rat thymus lymphocytes. Cells, purified on Ficoll-Hypaque, were fractionated by Percoll density gradients sedimentation in two subpopulations. Continuous line indicates light scatter of small- cell-enriched subpopulation (lower fraction of Percoll). Dotted line indicates light scatter of large-cell-enriched subpopulation (upper fraction of Percoll). Eight units on abscissa $\approx 15\ \mu^3$.

band was composed of 52% large cells (diameter $\geq$6μm) and 48% small cells (diameter $\leq$6μm) and the lower band was composed of 89% small and 11% large cells. Representative results of the enriched populations characteristics are shown in Figure 1. The cell viability was 98% and 94% for the upper and lower band respectively. In neither population we could detect cells stained for the presence of non specific esterase; this documented that our preparations were free from contaminating monocytes. The mitotic activity of each enriched cell subpopulation was established using [^{3}H] Thymidine incorporation. The mean values of radioactivity ($\pm$ S.D.) from nine experiments, in each one using triplicate determinations, were: upper fraction 94,381 ($\pm$ 16,830) cpm/10^6 viable cells; lower fraction 35,293 ($\pm$ 1945) cpm/10^6 viable cells ($p<0.001$). Figure 2 depicts sucrose density gradient sedimentation analysis of [^{3}H]1,25(OH)$_2$D$_3$ binding in the two cell subpopulations. A [^{3}H] 1,25(OH)$_2$D$_3$ binding macromolecule was detected in the large- cell-enriched preparation (upper band). The peak of the

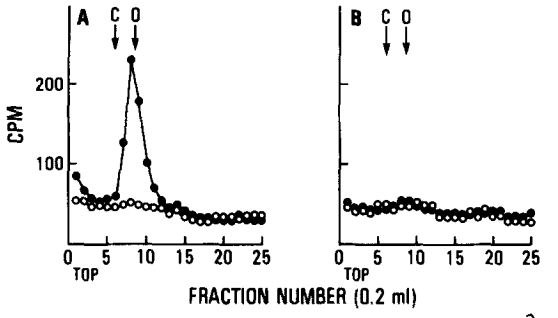

Figure 2: Sucrose density gradient (4-24%) analysis of [^{3}H] 1,25(OH)$_2$D$_3$ binding in rat thymus lymphocytes. 20×10^6 cells from the large-cell-enriched preparation (Panel A) and 60×10^6 cells from the small-cell-enriched preparation (Panel B) were incubated intact with [^{3}H] 1,25(OH)$_2$D$_3$ (2×10^{-9} M) alone (●---●) or in the presence of 200-fold M excess of unlabelled 1,25(OH)$_2$D$_3$ (o---o). Arrows indicate sedimentation markers: C=chymotrypsinogen (S=2.54); O=ovalbumin (S=3.7).

bound radioactivity sedimented at 3.3 S, between chymotrypsinogen and ovalbumin, and it was completely displaceable by 200-fold M excess of unlabelled hormone (Figure 2a). In contrast (Figure 2b) we found no specifically bound radioactivity in the small-cell-enriched preparation (lower band). Similar results were obtained in three separate experiments. The binding of [^{3}H] 1,25(OH)$_2$D$_3$ in the large-cell-enriched subpopulation was further examined by saturation analysis. Figure 3a depicts the Scatchard plot of specific binding of [^{3}H] 1,25(OH)$_2$D$_3$ (i.e., total minus non specific) derived from the saturation analysis. This plot yielded a straight line, indicating a single class of bind-

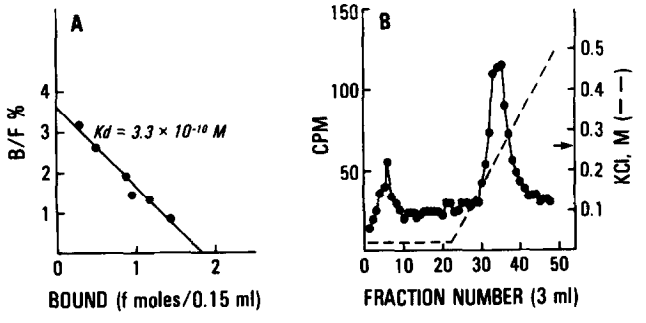

Figure 3: a) Scatchard plot of [^{3}H] 1,25(OH)$_2$D$_3$ specific binding in rat thymus lymphoid cells. Cells from the upper fraction of Percoll (4×10^6/0.15 ml) were incubated intact in triplicates with increasing concentrations of [^{3}H] 1,25(OH)$_2$D$_3$ alone or in the presence of 200-fold M excess of unlabelled 1,25(OH)$_2$D$_3$. Specific binding (total minus non specific) has been plotted by the method of Scatchard. b) DNA-cellulose chromatography of [^{3}H] 1,25(OH)$_2$D$_3$ binding. Cells from the upper fraction of Percoll (130×10^6/ml) were incubated intact with [^{3}H] 1,25(OH)$_2$D$_3$ (2×10^{-9} M). Radioactivity (●---●) and salt concentration (----) are depicted. ARROW indicates concentration of KCl which corresponds with the peak of the eluted radioactivity.

ing sites. We have calculated an apparent K_d of 3.3 x 10^{-10}M with a maximum concentration of binding sites of $0.5x10^{-15}$ moles /10^6 cells. Finally, Figure 3b illustrates DNA-cellulose chromatography of the [^{3}H] 1,25(OH)$_2$D$_3$-binding macromolecule complex. The complex bound to DNA and it could be eluted from this affinity resin with 0.26 M KCl.

DISCUSSION

We have examined in this study the possibility that the presence of 1,25(OH)$_2$D$_3$ receptors in lymphocytes is related to their mitotic activity. To do so we prepared two subsets of lymphocytes from rat thymus glands. The first subset was enriched in large size cells (52%) and the second in small size cells (89%). The former preparation exhibited ≈ 3-fold higher thymidine incorporation than the latter. Other workers, by using homogeneous preparations of the large and small size thymic cells have established that only the large cells are mitotically active (12). The reason for employing enriched rather than homogeneous preparations from either subset was that the latter do not produce sufficient numbers of cells for 1,25(OH)$_2$D$_3$ receptor experiments.

We have found that the large-cell-enriched subset possessed a 1,25(OH)$_2$D$_3$ binding macromolecule. The sedimentation characteristics of this macromolecule, its affinity for 1,25(OH)$_2$D$_3$ and the behavior of the 1,25(OH)$_2$D$_3$-macromolecule complexes on DNA-cellulose are similar to those established for the 1,25(OH)$_2$D$_3$ receptor in classical target tissues for this hormone (13,14). In contrast to the large-cell-enriched subset, we found that the small-cell-enriched subset did not possess such binding. Reinhard et al. have detected 1,25(OH)$_2$D$_3$ receptor-like activity in homogenates of the calf thymus but did not search for the particular cells that possess this activity (15). Our results indicate that 1,25(OH)$_2$D$_3$ receptors occur only in one of the two major subsets of thymic lymphoid cells, namely the large, mitotically active lymphoblasts. This evidence strengthens further the likelihood that there exists an association between the expression of 1,25(OH)$_2$D$_3$ receptors and the mitotic stage of lymphoid cells. It should be noted however that large cells were present in similar numbers in both fractions of Percoll. In view of this and the fact that cells harvested in the

lower fraction of Percoll (i.e. small-cell-enriched fraction) were negative for $1,25(OH)_2D_3$ receptors in these experiments, it is likely that the expression of $1,25(OH)_2D_3$ receptors is limited further to a specific subset of mitotically active cells. Several subpopulations of mitotically active thymic lymphocytes with distinct phenotypic markers have been identified and are believed to represent cells at different stages of the maturation pathway (16). Hence, further studies will be required to identify the particular subset of mitotically active thymic cells that express the $1,25(OH)_2D_3$ receptor.

The presence of the receptor in a subset of thymic cells implies that these cells are target for hormonal action. In preliminary studies we have indeed found that $1,25(OH)_2D_3$ prolongs the survival of thymic cells in short term culture. At this stage the biological significance of the association between mitosis and $1,25(OH)_2D_3$ receptor expression, and the role of the hormone on thymic cells is unknown. A series of recent studies have shown that $1,25(OH)_2D_3$ promotes the differentiation of hematopoietic cells, including normal monocytes and leukemia cells (1,17,18). In view of this and the present evidence indicating that $1,25(OH)_2D_3$ receptors are expressed only at certain stages of the differentiation pathway of lymphoyctes, it is likely that $1,25(OH)_2D_3$ plays a role also in the differentiation of this class of leukocytes. The thymus gland, where T lymphocyte precursors proliferate and co-exist with their more differentiated progeny, should serve as a useful model for exploring this possibility.

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