1,25-Dihydroxyvitamin D_3 Receptors in Human Leukocytes

Abstract. *A 1,25-dihydroxyvitamin D_3 receptor macromolecule was detected in peripheral mononuclear leukocytes from normal humans. This macromolecule was found to be present in monocytes but absent from normal resting peripheral B and T lymphocytes. However, it was present in established lines of malignant B, T, and non-B, non-T human lymphocytes, as well as in T and B lymphocytes obtained from normal humans and activated in vitro.*

Because of evidence that 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$] might play a role in leukemic cell differentiation (*1*), we examined normal human leukocytes for the presence of receptors for this hormone (*2*). We found that peripheral mononuclear leukocytes from normal humans have a receptor macromolecule which binds ^{3}H-labeled 1,25$(OH)_2D_3$ with specificity and high affinity [dissociation constant, $1.6 \times 10^{-10} M$] and sediments at 3.3*S* in sucrose density gradients. By separating leukocytes from six normal subjects into monocyte-enriched and lymphocyte-enriched populations, we have determined that this receptor occurs in monocytes but is apparently absent from normal B and T lymphocytes. In contrast to normal lymphocytes, we found that established lines of malignant human B, T, and non-B, non-T lymphocytes have this receptor. Further, we have demonstrated that T and B lymphocytes obtained from normal humans and activated by mitogenic lectins and Epstein-Barr virus, respectively, also express the 1,25$(OH)_2D_3$ receptor. That the receptor may have a role in normal leukocyte differentiation was suggested by the occurrence of morphological and biochemical changes in 1,25$(OH)_2D_3$-treated human monocytes.

1,25-Dihydroxyvitamin D_3 can induce murine and human myeloid leukemia cells in vitro to differentiate into macrophages or granulocytes, or both, and administration of 1,25$(OH)_2D_3$ into mice inoculated with leukemia cells prolongs their survival (*1*). The effect of 1,25$(OH)_2D_3$ on skeletal resorption (*3*) may also be related to a possible role of 1,25$(OH)_2D_3$ on blood-cell differentiation. Resorption is mediated by bone osteoclasts, and osteoclasts are believed to derive primarily from marrow cells and blood monocytes that reach the bone surface through the bloodstream (*4*).

Mononuclear cell concentrates from fresh blood samples obtained from normal human volunteers were isolated by means of Ficoll density gradients (*5*). The average cellular composition of the final concentrate from 13 blood samples, determined by the absence or presence of nonspecific esterase and fluoride inhibition (*6*) as well as by forward versus 90° light-scatter analysis on a cytofluorograph (*7*), was 84 percent lymphocytes, 15 percent monocytes, 1 percent granulocytes, and a few red cells. Binding of [^{3}H]1,25$(OH)_2D_3$ to intracellular receptors was established by incubating intact cells with the ligand (*8*). We found that the mononuclear leukocytes contained a macromolecule that bound [^{3}H]1,25$(OH)_2D_3$ and sedimented at 3.3*S* on a sucrose gradient (*9*). The radioactive ligand was completely displaceable by 200-fold excess of unlabeled 1,25$(OH)_2D_3$ but only partially displaceable by the same molar concentration of 25-OH-D_3 (Fig. 1a). A Scatchard plot of the specific [^{3}H]1,25$(OH)_2D_3$ binding formed a straight line, indicating the presence of a single class of binding sites. The dissociation constant of [^{3}H]1,25$(OH)_2D_3$ for this binder was $1.6 \times 10^{-10} M$ (Fig. 1b). The specificity, sedimentation characteristics, and the affinity of [^{3}H]1,25$(OH)_2D_3$ to this binding macromolecule are similar to those of known 1,25$(OH)_2D_3$ receptors (*2*).

In order to determine whether the [^{3}H]1,25$(OH)_2D_3$ binding macromolecule occurred in both or only in one of the two major cell populations of the mononuclear concentrates, we fractionated the Ficoll-isolated cells from six normal individuals by subsequent Percoll density gradient centrifugation (*10*) into lymphocyte-enriched and monocyte-enriched cells. The monocyte-enriched cell population contained 40 percent monocytes and 60 percent lymphocytes. The lymphocyte-enriched population was practically pure, containing more than 97 percent lymphocytes. To define further the composition of these cell populations, we performed immunofluorescence studies with monoclonal antibodies specific to surface antigens that are expressed either in T lymphocytes (T101), B lymphocytes (B1), or monocytes (OKM 1) (*11*). The antibody to OKM 1 also recognizes a subpopulation of lymphocytes. A fluorescein-conjugated rabbit antibody to mouse immunoglobulin was used as second antibody and fluorescent cells were analyzed on the cytofluorograph. On average, the monocytes in the monocyte-enriched fraction were 73 percent positive for OKM 1, 4 percent positive for B1, and negative for T101; the lymphocytes in the same fraction were 50 percent positive for T101, 6 percent positive for B1, and 31 percent positive for OKM 1. The lymphocytes in the lymphocyte-enriched fraction were 85 percent positive for T101, 5 percent positive for B1, and 22 percent positive for OKM 1. In blood samples from all six individuals we found a 3.3*S* [^{3}H]1,25$(OH)_2D_3$-binding macromolecule in the monocyte-enriched fractions (Fig. 2a). In contrast, the lymphocyte-enriched fractions from all the individuals were negative for [^{3}H]1,25$(OH)_2D_3$ binding (Fig. 2b).

Because of the evidence that normal B and T lymphocytes lack 1,25$(OH)_2D_3$ receptors and the reports that 1,25$(OH)_2D_3$ was active on leukemia cells, we searched for 1,25$(OH)_2D_3$ receptors in established malignant human cell lines (*12*) of B lymphocytes (Raji cells), T lymphocytes

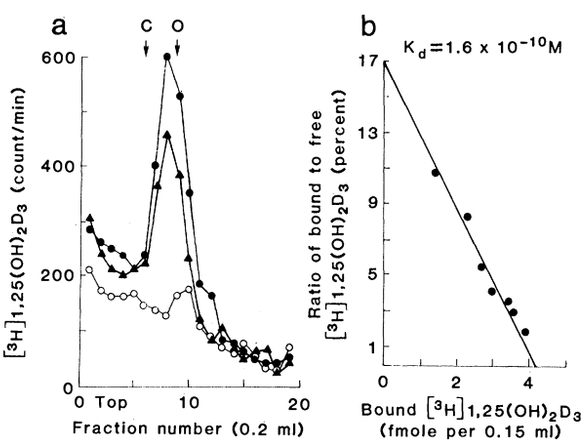

Fig. 1. (a) Sucrose density sedimentation and (b) Scatchard analysis of specific [^{3}H]-1,25$(OH)_2D_3$ binding in normal human mononuclear leukocytes. For the sucrose density sedimentation, intact cells (30×10^6 per 0.3 ml) were incubated with [^{3}H]1,25$(OH)_2D_3$ ($1 \times 10^{-9} M$) (●) alone (*9*) or in the presence of (▲) $2 \times 10^{-7} M$ unlabeled 25-OH-D_3 or (○) $2 \times 10^{-7} M$ unlabeled 1,25$(OH)_2D_3$ for 1 hour at 37°C. Cells were then washed with iced isotonic buffer resuspended in hypertonic buffer and sonicated. The sonicate was centrifuged to give a supernatant (cytosol), and 0.3-ml portions of this were layered onto 4 to 24 percent sucrose gradients and centrifuged for 20 hours at 144,000*g*. Chymotrypsinogen (*C*) ($S = 2.5$) and ovalbumin (*O*) ($S = 3.7$) were used as sedimentation coefficient standards. Saturation analysis of [^{3}H]1,25$(OH)_2D_3$ binding in intact cells (5×10^6 per 0.15 ml) was done by the method described earlier (*8*); specific binding of [^{3}H]1,25$(OH)_2D_3$, was plotted for Scatchard analysis.

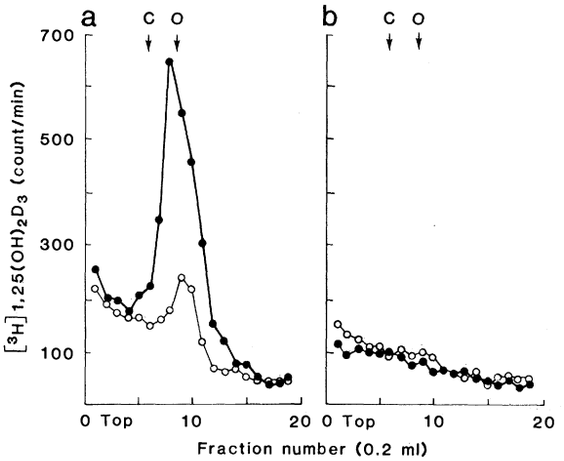

Fig. 2. Sucrose gradient sedimentation analysis of [^{3}H]-1,25(OH)$_2$D$_3$ binding in (a) monocyte-rich and (b) lymphocyte-rich leukocytes from normal humans. Intact cells (15×10^6 monocyte-rich cells per 0.3 ml or 30×10^6 lymphocyte-rich cells per 0.3 ml) were incubated with (●) [^{3}H]1,25(OH)$_2$D$_3$ alone ($2 \times 10^{-9}M$) or in the presence of (○) unlabeled 1,25(OH)$_2$D$_3$ ($2 \times 10^{-7}M$). Sucrose gradient analysis of [^{3}H]1,25-(OH)$_2$D$_3$ binding was performed as described for Fig. 1. Results of a representative experiment (from a total of six) are shown.

(HSB-2), and non-B, non-T cells (K562). All three malignant lines, in contrast to the normal lymphocytes, contained a [^{3}H]1,25(OH)$_2$D$_3$ binding macromolecule that sedimented on sucrose density gradients at 3.3*S*, identical to the value for the macromolecule of the normal monocytes. The [^{3}H]1,25(OH)$_2$D$_3$-macromolecule complex of all three malignant cell lines bound to DNA-cellulose and it could be eluted from this affinity resin with a KCl gradient of 0 to 0.6*M*; the peak radioactivity of the eluted material occurred at about 0.24*M* KCl. This elution profile is similar to that of the 1,25(OH)$_2$D$_3$ receptor of normal target cells and of other malignant cell lines (*13*). In addition, T lymphocytes from normal humans were activated in vitro by culturing the Percoll-isolated lymphocyte fraction for 4 days in the presence of the mitogenic lectins phytohemagglutinin P (1 percent) or concanavalin A (20 μg/ml) (*14*). The lymphocytes that were cultured in the presence of either of the lectins, unlike control lymphocytes from the same individual, expressed a 3.3*S* macromolecule that had a dissociation constant for [^{3}H]1,25(OH)$_2$D$_3$ of $3.8 \times 10^{-10}M$ (Fig. 3). The expression of this receptor was associated with a transformation of 41 percent of the normal lymphocytes to lymphoblasts. Similarly, B lymphocytes isolated after removal of T lymphocytes by the sheep red blood cell technique and subsequently infected with Epstein-Barr virus for 3 weeks (*15*) expressed the 3.3*S* 1,25(OH)$_2$D$_3$ binding protein (dissociation constant, $4 \times 10^{-10}M$), in contrast to noninfected B lymphocytes from the same normal donor. Finally, in order to obtain information regarding a biological function of 1,25(OH)$_2$D$_3$ on normal human leukocytes, as is implied by the presence of the specific receptor, we cultured monocytes from normal donors in the presence of 1,25(OH)$_2$D$_3$ ($10^{-8}M$) for a 4-week period. We observed morphological changes in the 1,25(OH)$_2$D$_3$-treated monocytes that are consistent with differentiation of monocytes toward macrophages. In addition, 1,25(OH)$_2$D$_3$-treated monocytes exhibited a progressive increase in β-acetylglucosaminidase activity in comparison with untreated cells from the same donor; the difference was detectable by day 9, and the enzyme activity of treated cells was four times that of untreated cells by the end of the culture. This increase reflects maturation of monocytes to macrophages (*16*).

We have presented evidence to suggest that human monocytes have specific 1,25(OH)$_2$D$_3$ receptors and are targets for 1,25(OH)$_2$D$_3$ actions that are presumably mediated by the receptor protein. In view of the evidence for the differentiating effect of 1,25(OH)$_2$D$_3$ on monocytes, the blood cells that are the origin of the bone-resorbing osteoclasts (*4*), we speculate that part of the potent influence of 1,25(OH)$_2$D$_3$ on skeletal homeostasis might be exerted in vivo by regulating monocyte-to-osteoclast differentiation. We have also unraveled a striking difference in the expression of 1,25(OH)$_2$D$_3$ receptors between normal resting versus malignant or activated B and T lymphocytes. These findings point to an association between the expression of the 1,25(OH)$_2$D$_3$ receptor protein and the mitotic activity of lymphocytes. This association has been further strengthened by our preliminary results indicating the expression of 1,25(OH)$_2$D$_3$ receptor in mitotically active rat thymoblasts, but not in mitotically inert thymocytes. Receptors for 1,25(OH)$_2$D$_3$ in mitotically active lymphocytes perhaps serve to mediate an antiproliferative prodifferentiation effect of the hormone or a role of 1,25(OH)$_2$D$_3$ in immune phenomena. We believe that the discovery of 1,25(OH)$_2$D$_3$ receptors in an easily accessible tissue such as leukocytes will greatly facilitate the study of this receptor in humans.

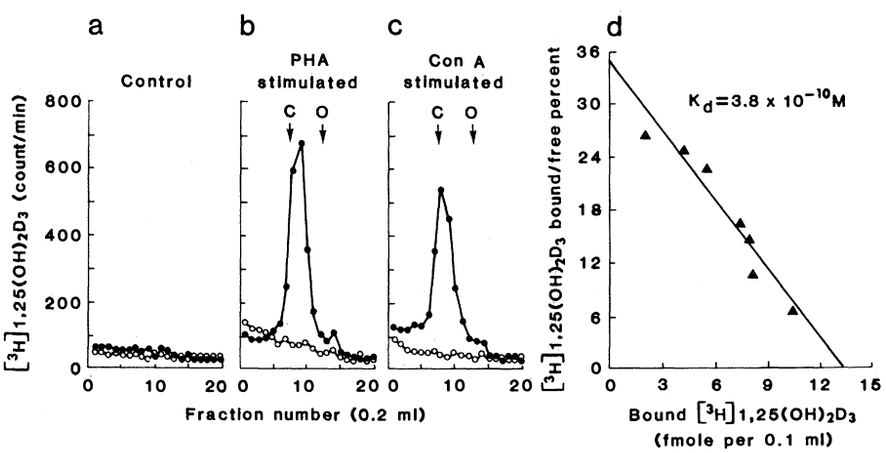

Fig. 3. Peripheral blood lymphocytes were cultured for 4 days in RPMI 1640 plus 10 percent heat-inactivated fetal calf serum (a) alone or in the presence of (b) phytohemagglutinin P (*PHA*) (1 percent) or (c) concanavalin A (*Con A*) (20 μg/ml); (d) Scatchard plot. At the end of the culture, cells were harvested and sucrose gradient analysis as well as Scatchard analysis of the specific [^{3}H]1,25(OH)$_2$D$_3$ binding were performed, as described for Fig. 1, with 7×10^6 intact cells per 0.3 ml and 1×10^6 intact cells per 0.1 ml, respectively.

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9. In preliminary experiments with cells isolated from blood, receptor binding was masked by the overwhelming presence of a protein which, unlike true receptor, bound [^{3}H]1,25(OH)$_2$D$_3$ in a nonsaturable and nonspecific fashion. This protein sedimented at 4.2*S*, identical to the serum D binding protein; the presence of this protein appeared to be the result of heavy cell contamination by serum. This interference was overcome by performing all of the incubations of isolated cells with [^{3}H]1,25(OH)$_2$D$_3$ in the presence of vitamin D$_3$ (1×10^{-5} *M*); vitamin D$_3$ has high affinity for serum D binding protein, but it does not bind to the 1,25(OH)$_2$D$_3$ receptor. This technical maneuver enabled us to saturate the 4.2*S* protein without interfering with the [^{3}H]-1,25(OH)$_2$D$_3$ binding to intracellular receptor.
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Altered Activity Patterns During Development Reduce Neural Tuning

Abstract. *Neonatal mice were reared in an acoustic environment that repetitively entrained activity in a large proportion of primary auditory afferents during the period when the frequency tuning of auditory neurons normally develops. The tuning curves obtained from these mice were significantly broader than those of normally reared mice of the same age. This suggests that the normal frequency tuning of neurons was prevented or delayed by synchronizing the pattern of activity imposed on the auditory pathway.*

Neural activity is critically involved in the normal maturation of response properties in the mammalian visual system (*1*) and a number of experiments indicate that coactivation of convergent inputs plays an important role in this process (*2*). We have investigated the role played by activity during the development of the mouse auditory pathway, using the hypothesis that coactive neural inputs are more likely to form lasting connections with a postsynaptic neuron. If this is the case, precise convergence onto postsynaptic neurons could be affected by selecting inputs with temporally correlated spike trains from an initially larger array of terminals. This idea has evolved from a theory originally put forward by Hebb (*3*) to explain the neural basis of associative learning.

Our studies were conducted on mice since their postnatal development is well defined and rapid (*4–7*). The experimental procedure was to raise young mice in an environment of repetitive clicks. Since this stimulus entrains the activity in a large proportion of auditory nerve fibers, it should have produced relatively synchronized firing patterns among these axons during development (*8*). We reasoned that in normal animals close temporal correlations in activity might provide the cues necessary to fine-tune a central auditory neuron because inputs of similar best frequency are likely to be active together. We predicted that in the absence of cues which might arise from these similar firing patterns, the ontogenetic fine-tuning of central auditory neurons would be relatively ineffective. Neurons which would have become more selective to frequency during development would consequently show abnormally broad tuning curves.

All mice were of the C57BL/6J strain. At 8 days of age litters were culled to seven animals and placed in standard laboratory cages along with their mother. The entire cage was then placed in an anechoic box within an acoustic isolation room where the mice were exposed to clicks presented at a rate of 20 per second from an overhead speaker. Each click had a peak-to-peak sound pressure level (SPL) of 88 dB (with reference to 0.0002 dyne/cm^2) and a frequency power spectrum that was essentially flat from 0 to 15 kHz (Fig. 1A). Mouse pups remained in this rearing environment until 19 to 24 days after birth, an age at which neural response properties resemble those of adults in normally reared mice (*6*). Litters to be used as normal controls were also culled to seven animals and maintained in the auditory environment provided by our animal facility.

Frequency tuning curves were taken from single units in the central nucleus of the inferior colliculus in normal and click-reared (CR) mice immediately upon removal from the click-rearing box. The animals were anesthetized with sodium pentabarbitol (Nembutal, 55 mg/kg, injected intraperitoneally), the inferior colliculus was surgically exposed, then covered with a 2 percent agar solution. Glass micropipettes filled with 2*M* NaCl and 1 percent fast green were used to record single units and compound potentials. The position of recording was marked subsequently by iontophoresing fast green. During the recording session all animals were tranquilized with chlorprothixene (Taractan, 7 mg/kg, injected intraperitoneally) (*9*), and their orally measured temperature was maintained within 1°C of one another. All auditory stimuli were presented closed field through a calibrated earphone (Stax SR-44; B & K 1/4-inch condenser microphone) fitted to the contralateral ear. The entire preparation was enclosed within an acoustic isolation chamber (IAC). Only units which responded to clicks were isolated and analyzed for frequency tuning with pulsed (1.5 per second) tone pips of 150 msec-duration (10-msec rise-fall time). Compound action potentials were recorded from the cochlear nucleus and were averaged (Tracor-Northern 1550) to detect threshold responses to pure tones (*10*).

The majority of neurons in the central nucleus of the inferior colliculus responded to a fixed range of frequencies each at characteristic intensity, and one frequency, the best frequency, generally drove the neuron when presented at lowest intensity or threshold. Q values are conventionally used to indicate the relative degree of tuning or stimulus selectivity of such auditory neurons (*11*). Larger bandwidths correspond to smaller Q values and, therefore, indicate less selectively tuned neurons.

We have obtained frequency tuning curves from 408 single units in 67 normal and CR mice. Values of Q were calculated for units in both groups. The mean Q values calculated at 20 dB above threshold (Q_{20}) for units of similar best frequency are shown in Fig. 1C along with the 95 percent confidence intervals. Units from CR animals had significantly lower Q_{20} values for characteristic frequencies in the range 10 to 15 kHz [analysis of variance, $P < 0.0005$)] and significant decreases in tuning ($P > 0.05$) were also observed in the 4 to 4.9, 8 to 8.9, and 15 to 15.9 ranges. Moreover