1,25-Dihydroxyvitamin D_3-induced Differentiation in a Human Promyelocytic Leukemia Cell Line (HL-60): Receptor-mediated Maturation to Macrophage-like Cells

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ABSTRACT The human-derived promyelocytic leukemia cell line, HL-60, is known to differentiate into mature myeloid cells in the presence of 1,25-dihydroxyvitamin D_3 (1,25[OH]$_2D_3$). We investigated differentiation by monitoring 1,25(OH)$_2D_3$-exposed HL-60 cells for phagocytic activity, ability to reduce nitroblue tetrazolium, binding of the chemotaxin *N*-formyl-methionyl-leucyl-[^{3}H]phenylalanine, development of nonspecific acid esterase activity, and morphological maturation of Wright-Giemsa-stained cells. 1,25(OH)$_2D_3$ concentrations as low as 10^{-10} M caused significant development of phagocytosis, nitroblue tetrazolium reduction, and the emergence of differentiated myeloid cells that had morphological characteristics of both metamyelocytes and monocytes. These cells were conclusively identified as monocytes/macrophages based upon their adherence to the plastic flasks and their content of the macrophage-characteristic nonspecific acid esterase enzyme. The estimated ED_{50} for 1,25(OH)$_2D_3$-induced differentiation based upon nitroblue tetrazolium reduction and *N*-formyl-methionyl-leucyl-[^{3}H]phenylalanine binding was 5.7×10^{-9} M. HL-60 cells exhibited a complex growth response with various levels of 1,25(OH)$_2D_3$: $\leq 10^{-10}$ M had no detectable effect, 10^{-9} M stimulated growth, and $\geq 10^{-8}$ M sharply inhibited proliferation. We also detected and quantitated the specific receptor for 1,25(OH)$_2D_3$ in HL-60 and HL-60 Blast, a sub-clone resistant to the growth and differentiation effects of 1,25(OH)$_2D_3$. The receptor in both lines was characterized as a DNA-binding protein that migrated at 3.3S on high-salt sucrose gradients. Unequivocal identification was provided by selective dissociation of the 1,25(OH)$_2D_3$-receptor complex with the mercurial reagent, *p*-chloromercuribenzenesulfonic acid, and by a shift in its sedimentation position upon complexing with anti-receptor monoclonal antibody. On the basis of labeling of whole cells with 1,25(OH)$_2$[^{3}H]D_3 in culture, we found that HL-60 contains ~4,000 1,25(OH)$_2D_3$ receptor molecules per cell, while the nonresponsive HL-60 Blast is endowed with ~8% of that number. The concentration of 1,25(OH)$_2D_3$ (5×10^{-9} M) in complete culture medium, which facilitates the saturation of receptors in HL-60 cells, is virtually identical to the ED_{50} for the sterol's induction of differentiation. This correspondence, plus the resistance of the relatively receptor-poor HL-60 Blast, indicates that 1,25(OH)$_2D_3$-induced differentiation of HL-60 cells to monocytes/macrophages is occurring via receptor-mediated events.

The induced differentiation of the human promyelocytic leukemia, HL-60, by a variety of compounds such as phorbol esters, DMSO, retinoic acid (RA),[1] and 1α,25-dihydroxyvitamin D_3 (1,25[OH]$_2D_3$), is of great current interest (1–4). The most potent inducer of differentiation in HL-60 cells is

[1] *Abbreviations used in this paper:* FMLP, *N*-formyl-methionyl-leucyl-phenylalanine; KET, 1mM EDTA, 10 mM Tris-HCl, pH 7.4, and various concentrations of KCl; NBT, nitroblue tetrazolium; PBS, phosphate-buffered saline; PCMBS, *p*-chloromercuribenzene sulfonic acid; RA, retinoic acid; 1,25(OH)$_2D_3$, 1,25-dihydroxyvitamin D_3.

THE JOURNAL OF CELL BIOLOGY · VOLUME 98 FEBRUARY 1984 391–398
 · 0021-9525/84/98/0391/08 $1.00

the active metabolite of vitamin D_3, $1,25(OH)_2D_3$, which is effective at nanomolar concentrations and is reported to elicit differentiation along the granulocyte pathway (4). The potential physiologic significance of $1,25(OH)_2D_3$-mediated differentiation of myeloid cells to granulocytes is unknown. Furthermore, $1,25(OH)_2D_3$ similarly causes the differentiation of the murine myeloid leukemia M1 cell line to macrophages in culture (5). The pharmacologic utility of $1,25(OH)_2D_3$ in this latter process is demonstrated by its capacity to prolong the survival time of mice inoculated with M1 cells, in vivo (6).

The role of $1,25(OH)_2D_3$ as an agent responsible for mineral homeostasis has been extensively studied (7). Its mechanism of action within target cells is apparently steroid hormone–like, in that $1,25(OH)_2D_3$ first binds to a specific, high affinity cytosolic receptor protein which then translocates into the nucleus, binds to chromatin (8), and consequently initiates genomic expression of the necessary mineral-regulating proteins (9). In support of this theory are the findings that all known $1,25(OH)_2D_3$-responsive tissues such as the intestine (10), bone (11), and kidney (12), contain the cytosolic receptor. In addition, many other tissues and cultured cells have been shown to possess $1,25(OH)_2D_3$ receptors (13, 14), including some tumor cells (14–18).

In contrast to the current progress achieved in elucidating the role of $1,25(OH)_2D_3$ in mineral metabolism, its mechanism of action and biological significance in the differentiation of HL-60 cells are unknown. Preliminary evidence by Tanaka et al. (19) implicates the presence of cytosolic $1,25(OH)_2D_3$ receptors and suggests that differentiation may be occurring by a mechanism similar to that of $1,25(OH)_2D_3$'s classical steroid hormone action. However, Tanaka et al. (19) provided little direct physical and chemical identification of the $1,25(OH)_2D_3$ receptor protein in HL-60 cells. To further investigate $1,25(OH)_2D_3$-induced differentiation and the potential role of the receptor, we compared the effects of $1,25(OH)_2D_3$ on HL-60 and HL-60 Blast (a subclone of the HL-60 parent line incapable of differentiation), using a variety of biological and biochemical differentiation parameters. In accord with the preliminary data of Bar-Shavit et al. (20), but in contrast to the findings of Miyaura et al. (4), we show that $1,25(OH)_2D_3$ induces HL-60 (but not HL-60 Blast) to differentiate to monocytes and macrophages, and extensively describe this phenomenon. We also report the unqualified identification of the $1,25(OH)_2D_3$ receptor in HL60 cells and the existence of a positive correlation between $1,25(OH)_2D_3$-induced differentiation and the occurrence of occupied $1,25(OH)_2D_3$ receptors.

MATERIALS AND METHODS

Materials: 1α,25-Dihydroxy(23,24[n]-^{3}H)vitamin D_3 (120 Ci/mmol) was generated from 25-hydroxy(23,24[n]-^{3}H)vitamin D_3 (Amersham Corp., Arlington Heights, IL) as described (21). Nonradioactive $1,25(OH)_2D_3$ was a gift of Dr. Milan Uskokovic of Hoffmann-LaRoche (Nutley, NJ). DNA (Worthington Biochemical Corp., Freehold, NJ) and cellulose (Cellex N-1, Bio-Rad Laboratories, Richmond, CA) were coupled by a modification of the procedure of Alberts and Herrick (22). Formyl-L-methionyl-L-leucyl-L-phenylalanine, *N*-[phenylalanine-ring-2,6-^{3}H(N)]-([^{3}H]FMLP, 60 Ci/mmol) was secured from New England Nuclear (Boston, MA). Nonradioactive FMLP, *p*-chloromercuribenzene sulfonic acid (PCMBS), all-*trans*-retinoic acid (RA), and bovine serum albumin were purchased from Sigma Chemical Co. (St. Loius, MO). DMSO was purchased from Fischer Scientific Co. (Fairlawn, NJ) and ultrapure sucrose from Bethesda Research Laboratories (Rockville, MD). Whatman DE-81 filters are DEAE anion exchange filters obtained from Reeve Angel (Clifton, NJ). All other chemicals were of reagent grade or better.

Buffers: The following buffers were used: PBS (Dulbecco's phosphate-buffered saline), HPB (0.01 M Tris-HCl, pH 7.5, 2 mM EDTA, 0.5 mM EGTA, 0.15 mM spermine, 0.5 mM spermidine, 5 mM dithiothreitol, and KET (1 mM EDTA, 10 mM Tris-HCl, pH 7.4, and various concentrations of KCl; e.g. KET-0.1 = 0.1 M KCl in KET).

Cells: HL-60 (23), a continuous human myeloid cell line, and HL-60 Blast cells (24), a subclone of the HL-60 cell line, were generous gifts of Dr. R. Gallo of the National Cancer Institute, Bethesda, MD, and Dr. P. Majors, Harvard Medical School, Boston, MA, respectively.

Media and Culture Conditions: The cells were cultured in minimal essential medium–alpha supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. Media and sera were obtained from Grand Island Biological Co. (Grand Island, NY). Antibiotics were purchased from Eli Lilly Co. (Indianapolis, IN). Cells were grown at 37°C in a humidified 95% air–5% CO_2 atmosphere. Cells were subcultured twice weekly at a seeding density of 2×10^5 cells/ml for the HL-60 cell line and 1×10^5 cells/ml for the HL-60 Blast line. Tissue culture plastics were obtained from Costar, Data Packaging (Cambridge, MA).

Assessment of Cell Growth: To examine the effects of $1,25(OH)_2D_3$ on cell growth, 5 ml of cells at 10^5 cells/ml were seeded in 25-cm^2 flasks. Cells were seeded in the presence of various concentrations of $1,25(OH)_2D_3$ in culture medium supplemented with 10% fetal bovine serum. Control cultures contained the ethanol vehicle at 0.1% (vol/vol). After shaking to loosen any adherent cells, 100 μl of each cell culture were removed every 24 h for 4 d and cells were counted with a hemacytometer. Cell viability was determined via trypan blue exclusion.

[^{3}H]FMLP Binding: This assay was performed as described elsewhere (25), with modifications. Briefly, cells were seeded at 10^5 cells/ml in medium containing either varying concentrations of $1,25(OH)_2D_3$ or 10^{-6} M RA, a combination of both, 1% DMSO (vol/vol) as a positive control, or the ethanol vehicle alone. Cells were cultured for 6 d in the presence of the various differentiation-inducing compounds. On the sixth day, the cells were harvested by centrifugation and washed in PBS. 1×10^7 cells in 1 ml of PBS were mixed in microfuge tubes with [^{3}H]FMLP at a final concentration of 2.9×10^{-9} M. DMSO (0.1% final) or unlabeled FMLP (2.9×10^{-6} M final) was added to each tube. Cells were incubated with shaking every 5 min at 4°C for 1 h, then sedimented by centrifugation in an Eppendorf microfuge for 30 s. The supernatant was discarded and the pellets were washed once in PBS. The supernatant was discarded, and the pellet was dissolved in 1 ml of Ready-Solv HP/b (Beckman Instruments, Irvine, CA) and counted in a Beckman liquid scintillation counter. The difference in counts between samples with DMSO alone and those with excess unlabeled FMLP was defined as specific FMLP binding.

Cell Maturation: The HL-60 cells were cultured with or without $1,25(OH)_2D_3$ in medium (Flow Laboratories, Inc., Rockville, MD) containing 10% fetal calf serum, as described above. Cytocentrifuge preparations were made and the histochemical stains, Wright-Giemsa and nonspecific acid esterase, were used to help determine cell lineage (26, 27). Nonspecific acid esterase activity was determined by the ability of cells to hydrolyze α-naphthyl acetate and/or α-naphthyl butyrate.

Phagocytosis: The method developed for studying phagocytosis has been described in detail (28). Briefly, *Candida albicans* were opsonized in 20% human AB serum, and a 5:1 ratio of *Candida* to leukemic cells was incubated at 37°C for 30 min. After the incubation, the solution of the heat-killed opsonized *Candida* and leukemia cells was placed in trypan-eosin stain to confirm that the *Candida* had been phagocytized and were not merely adherent to the cell surface. Heat-killed *Candida* must be completely ingested to be protected from taking up the stain. The values obtained with this assay concurred with those using the Giemsa-staining technique (29).

NBT Reduction: The reduction of NBT was measured qualitatively. 1 ml of cells (2×10^6 cells/ml) was suspended in a medium containing 20% fetal calf serum, NBT (2%, dissolved in PBS), and 200 ng of freshly diluted 12-O-tetradecanoyl phorbol-13-acetate. The cell suspension was incubated at 37°C for 20 min, washed three times in cold PBS, cytocentrifuged, fixed in methanol, and stained 5 min with safranin (30, 31). The percentage of cells containing intracellular reduced blue-black formazan deposits was determined through light microscopy by differentially counting 200 cells.

Nuclear Extract Preparation: At least 300×10^6 cells were harvested in logarithmic growth, rinsed in fresh medium and resuspended in 10 ml of 1% fetal bovine serum in minimal essential medium–alpha. Cells were then labeled with 5 nM $1,25(OH)_2$[^{3}H]D_3 for 2 h at 37°C in a shaking water-bath. The following manipulations were done at 4°C. Cells were rinsed three times in ice-cold PBS with 1% bovine serum albumin, followed by centrifugation at 500 *g* for 5 min. The resulting pellet was resuspended in 5 ml of 0.01 M Tris-HCl, pH 7.5, 2 mM EDTA, 0.5 mM EGTA, 0.15 mM spermine, 0.5 mM spermidine, and 5 mM dithiothreitol and, after a 20-min incubation, the cells were lysed by Dounce homogenization. The lysate was centrifuged again for 15

min at 1,100 *g*, and the pellet was resuspended in 3 ml of KET-0.3 and centrifuged again for 5 min at 12,000 *g* after a 30-min incubation. This high salt extraction (0.3 M KCl) was used to recover the hormone-receptor complex from the nucleus (32). The supernatant from this extract was then used as described for DNA-cellulose chromatography or sedimentation analysis.

DNA-Cellulose Chromatography: DNA-cellulose chromatography was performed as outlined elsewhere (33). Briefly, 1 ml of the nuclear extract was diluted with 5 vol (vol/vol) of KET-0 to reduce ionic strength, and applied to a 10-ml DNA-cellulose column equilibrated with KET-0.05. After washing with KET-0.05, the labeled receptor complex was eluted with a 100-ml KET-0.05-0.5 linear gradient. All operations were performed at 4°C. 3-ml fractions were collected (flow rate = 1 ml/min), and a 0.5-ml aliquot of each fraction was counted in 5 ml of a liquifluor-based aqueous scintillation cocktail (33% efficiency) in a Beckman scintillation counter. Salt concentrations were determined with a conductivity meter (Radiometer, Copenhagen, Denmark).

Sedimentation Analysis: From the nuclear extract, 300-μl aliquots were incubated for 4 h at 4°C with one of the following: 8 μl of KET-0.3 plus 20 μl of fresh hybridoma culture medium; 8 μl of KET-0.3 plus 20 μl of SP2/0-4A5 clonal medium containing the anti-$1,25(OH)_2D_3$-receptor monoclonal antibody 4A5γ (34); or 100 μl of SP2/0-8D3 clonal medium containing the anti-$1,25(OH)_2D_3$-receptor monoclonal antibody 8D3μ (34). A 300-μl aliquot of nuclear extract was also incubated for 0.5 h at 4°C with 100 μl of 10 mM PCMBS in KET-0.3, pH 7.4. Each 400-μl incubate was layered onto a 4.6-ml gradient of 10–30% sucrose prepared in KET-0.3 with ultrapure sucrose using a Buchler gradient maker and Auto-Densi Flow apparatus. The gradients were then centrifuged at 2°C in an SW 50.1 rotor (Beckman Instruments, Inc.) at 265,000 *g* for 18 h. Four-drop fractions were collected from the top using a Gilson fractionator and counted in 5 ml of scintillation cocktail.

Saturation Analysis: At least 200 × 10^6 cells were harvested in logarithmic growth, washed two times with PBS, and resuspended in minimal essential medium-alpha containing 10% fetal bovine serum at 2 × 10^7 cells/ml. Aliquots of 0.5 ml (10^7 cells total) then were incubated for 4 h with $1,25(OH)_2[^3H]D_3$ at concentrations of 0.5 nM, 1.0 nM, 2.0 nM, 5.0 nM, and 10.0 nM ± 100-fold excess nonradioactive $1,25(OH)_2D_3$. All incubations were carried out in duplicate at 37°C in a shaking water-bath. All subsequent operations were performed at 4°C. At the end of the incubation, the cells were rinsed twice with ice-cold 1% bovine serum albumin in PBS and once with PBS alone, each followed by centrifugation for 5 min at 700 *g*. Each pellet was resuspended and lysed in 0.5 ml of 0.3 M KCl plus 5 mM DTT plus 0.5% Triton X-100 in 0.01 M Tris-HCl, pH 7.5, 2 mM EDTA, 0.5 mM EGTA, 0.15 mM spermine, 0.5 mM spermidine, and 5 mM dithiothreitol for 30 min with intermittent vortexing. The lysate was centrifuged for 10 min at 35,000 *g*, the supernatant was recovered, diluted in 6 vol (vol/vol) of 10 mM Tris-HCl in 1% Triton X-100, pH 7.4, and filtered through two 2.5-cm Whatman DE-81 filters via gravity (no applied suction) as originally described by Santi et al. (35). Each assay tube was rinsed twice more with 10 mM Tris in 1% Triton X-100, pH 7.4 to insure a quantitative yield. The filters were transferred to scintillation vials, dried in acetone, and counted for tritium in 5 ml of scintillation cocktail.

RESULTS

Growth Response Studies

HL-60 cells showed a time-dependent, biphasic dose response when grown with added $1,25(OH)_2D_3$ (Fig. 1*A*). Significant inhibition of growth could be seen as low as 10^{-8} M $1,25(OH)_2D_3$ and, within 4 d at 10^{-7} M, growth was slowed to <40% of control. However, a significant stimulation in growth above the control occurred as HL-60 cells were cultured with 10^{-9} M $1,25(OH)_2D_3$ (Fig. 1*A*). At concentrations of 10^{-10}–10^{-12} M $1,25(OH)_2D_3$ the growth of HL-60 cells remained unaffected (data not shown). To verify the validity of this biphasic response, we repeated the experiment twice, with the same results (data not shown). Contrary to HL-60, the HL-60 Blast cells showed no growth response to $1,25(OH)_2D_3$ at any concentration tested (Fig. 1*B*).

Functional and Morphological Studies

Table I represents the $1,25(OH)_2D_3$ dose responsiveness of HL-60 and HL-60 Blast cells to undergo various functional and morphological changes indicative of differentiating myeloid cells. Untreated HL-60 cells undergo little spontaneous differentiation (1–2%) as assayed by morphology, NBT reduction, phagocytic and nonspecific acid esterase activity. After 7 d of treatment with 10^{-10} M $1,25(OH)_2D_3$, significant differentiation occurred, as measured by the four test parameters. As the most sensitive assessment of differentiation, the NBT reduction assay demonstrated significant differentiation occurring at as low as 10^{-11} M $1,25(OH)_2D_3$. Phagocytosis exhibited the least response to $1,25(OH)_2D_3$ induction (Table I). Surprisingly, we also noticed that the HL-60 cells exposed to $1,25(OH)_2D_3$ became adherent to plastic, a characteristic of macrophages and not of granulocytes. Because this was in contrast to the $1,25(OH)_2D_3$-induced granulocytic differentiation reported by Miyaura et al. (4), we were prompted to investigate this phenomenon further. Of particular interest was that strong nonspecific acid esterase activity was induced in the HL-60 cells exposed to $1,25(OH)_2D_3$. Nearly all the HL-60 cells developed intense nonspecific acid esterase activity with exposure to 10^{-6} M $1,25(OH)_2D_3$. Nonspecific acid esterase activity is present in normal monocytes and macrophages, but not in neutrophils. Morphologically, the mature cells were ~13–15 μM in diameter; the nuclei were either large with an indented contour and delicate chromatin or reniform with a moderately dense chromatin. The cytoplasm was abundant, grayish in color, and contained few granules. Morphologically, the cells acquire characteristics of both monocytes and metamyelocytes. All mature-appearing cells were, however, nonspecific acid esterase–positive and none resembled mature granulocytes. The HL-60 cells began to show significant functional changes as early as 1 d after beginning treatment with $1,25(OH)_2D_3$ (data not shown). The percentage of differentiated cells increased in a dose-dependent manner, with concentrations as high as 10^{-6} M $1,25(OH)_2D_3$ causing >70% of the cells to demonstrate morphological and functional properties of differentiated cells. At this concentration, 60% of the cells became phagocytic. The HL-60 Blast cells were unresponsive at all $1,25(OH)_2D_3$ concentrations (Table I).

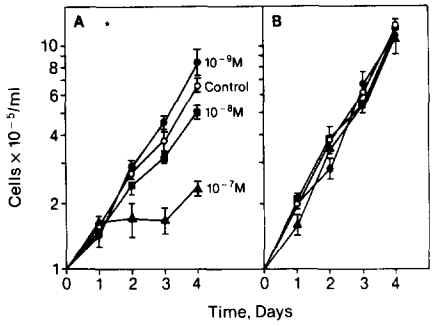

FIGURE 1 Dose-responsive growth of (*A*) HL-60 and (*B*) HL-60 Blast cells in $1,25(OH)_2D_3$. Cells were inoculated at 10^5 cells/ml in triplicate flasks on day 0 in the presence of various concentrations of $1,25(OH)_2D_3$. At the times indicated, each flask was sterilely sampled and counted as described in Materials and Methods. Cell viability was >99%. Control (0.1% ethanol), (○); 10^{-7} M $1,25(OH)_2D_3$, (▲); 10^{-8} M $1,25(OH)_2D_3$, (■); 10^{-9} M $1,25(OH)_2D_3$, (●). Each point represents the mean of triplicate counts (± standard deviation). In *A* all points for the day-3 and day-4 time points are significantly different from one another as statistically evaluated by the Student's *t*-test ($P < 0.05$). In addition, the difference between 10^{-7} M and 10^{-8} M $1,25(OH)_2D_3$-exposed HL-60 cells at the day-2 time point represents the same statistical significance ($P < 0.05$).

TABLE I

*Functional and Morphological Changes in HL-60 and HL-60 Blast Cells Induced by Various Concentrations of 1,25(OH)$_2$D$_3$**

Added concentration of 1,25(OH)$_2$D$_3$‡	Cell line	NBT reduction	Phagocytic cells	Morphology		
				Myeloblasts and promyeloblasts	Intermediate§ to mature	Nonspecific acid‖ esterase-positive
M		%	%	%	%	%
0	HL-60	2 ± 3	2 ± 2	99 ± 2	1 ± 2	2
	HL-60 Blast	0	1	100	0	0
10^{-11}	HL-60	10 ± 2	2 ± 3	95 ± 3	5 ± 4	3
	HL-60 Blast	0	0	100	0	0
10^{-10}	HL-60	18 ± 11	13 ± 7	82 ± 5	18 ± 7	10
	HL-60 Blast	0	0	100	0	0
10^{-9}	HL-60	37 ± 19	20 ± 7	66 ± 9	34 ± 6	25
	HL-60 Blast	0	0	100	0	0
10^{-8}	HL-60	64 ± 13	26 ± 4	45 ± 12	55 ± 9	54
	HL-60 Blast	0	0	100	0	0
10^{-7}	HL-60	82 ± 8	44 ± 9	32 ± 5	67 ± 6	82
	HL-60 Blast	3	5	96	4	0
10^{-6}	HL-60	86 ± 12	60 ± 3	27 ± 6	78 ± 14	98
	HL-60 Blast	1	0	100	0	0

* HL-60 and HL-60 Blast cells were cultured in the presence or absence of various concentrations of 1,25(OH)$_2$D$_3$ as detailed in Materials and Methods. After 7 d, cells were assessed for the various differentiation parameters. Cell viability was >99%. All data are expressed as the percentage of total cells assayed. At least 200 cells were assessed for each parameter. The HL-60 cell data represent the mean ± standard deviation of triplicate assays.

‡ Basal 1,25(OH)$_2$D$_3$ in 10% fetal bovine serum is 1.6×10^{-11} M.

§ Intermediate to mature cells include monocytes and macrophages.

‖ Represents the average of two experiments.

To better understand the 1,25(OH)$_2$D$_3$ induction process, we cultured the HL-60 cells with either 10^{-7} or 10^{-8} M 1,25(OH)$_2$D$_3$ for varying time periods, washed the cells, and assessed the ability of HL-60 cells to differentiate after a total of 7 d. Table II shows that morphological and functional changes begin to be minimally expressed after 7 d with a 1,25(OH)$_2$D$_3$ incubation as short as 18 h. The differentiation response becomes more pronounced the longer the hormone remains in the culture medium. These results suggest that the presence of 1,25(OH)$_2$D$_3$ is required and must be maintained over a long period of time relative to temporal events occurring at the molecular level.

[^{3}H]FMLP Binding Studies

To develop a dose-dependent biochemical marker of induced HL-60 differentiation, we used a modification of the [^{3}H]FMLP binding assay developed by Nelson et al. (36). Previous investigators have shown that development of chemotaxis coincides with the appearance of specific [^{3}H]FMLP binding in the DMSO-induced differentiated state of HL-60 cells (25). Fig. 2 shows the results of specific [^{3}H]FMLP binding to HL-60 cells after 6 d of treatment with various inducers of differentiation. Specific [^{3}H]FMLP binding in cells exposed to the various compounds was contrasted to specific [^{3}H]FMLP binding in unexposed control cells (see Fig. 2, inset). When treated with 1,25(OH)$_2$D$_3$, HL-60 cells responded in a dose-dependent manner. A maximal effect was seen at 10^{-7} M 1,25(OH)$_2$D$_3$ and binding decreased in a sigmoidal fashion (Fig. 3) until there was no detectable effect at 10^{-10} M. The effective dose at which 50% of specific [^{3}H]-FMLP binding can be induced (ED_{50}) was estimated to be 5.7 nM (Fig. 3). Strikingly, the ED_{50} for 1,25(OH)$_2$D$_3$-provoked NBT reduction (Table I) coincided with this value when plotted together with the FMLP binding data as shown in Fig. 3.

In comparison to 1,25(OH)$_2$D$_3$-induced cells, 1% DMSO-treated HL-60 cells produced a response similar to that with 10^{-9} M 1,25(OH)$_2$D$_3$ (Fig. 2). RA, a known inducer of HL-60 differentiation as measured by morphology and NBT reduction at micromolar amounts (37), did not induce [^{3}H]-FMLP binding at 10^{-6} M (Fig. 2). We also evaluated the ability of suboptimal doses of 1,25(OH)$_2$[^{3}H]D$_3$ and RA in concert to stimulate specific [^{3}H]FMLP binding in HL-60 cells. No synergistic response was detected at 10^{-10} M 1,25(OH)$_2$D$_3$ and 10^{-6} M RA together (Fig. 2). This evidence suggests that 1,25(OH)$_2$D$_3$-induced differentiation may be occurring through a mechanism separate from that of RA-induced differentiation. Unsurprisingly, the HL-60 Blast cells were unresponsive to induction of specific [^{3}H]FMLP binding when challenged with 10^{-8} M 1,25(OH)$_2$D$_3$ (Fig. 2).

Detection and Quantitation of 1,25(OH)$_2$D$_3$ Receptors

To approach the mechanism whereby 1,25(OH)$_2$D$_3$ induces differentiation in HL-60 cells, we first attempted to determine whether 1,25(OH)$_2$D$_3$ receptors were present in these cells. Aliquots of high salt nuclear extracts, prepared by first labeling intact HL-60 and HL-60 Blast cells with 1,25(OH)$_2$D$_3$ (see Materials and Methods), were analyzed by high salt sucrose density gradient centrifugation. As illustrated in Fig. 4*A*,

TABLE II

*Functional and Morphological Changes in HL-60 Cells Exposed to 1,25(OH)$_2$D$_3$ for Various Time Periods**

Length of 1,25(OH)$_2$D$_3$ exposure	Concentration of 1,25(OH)$_2$D$_3$	NBT reduction	Phagocytic cells	Myeloid cell type	
				Blast‡	Intermediate§ to mature
d	*M*	%	%	%	%
0		2	3	100	0
0.75	10^{-8}	7	4	92	8
	10^{-7}	16	11	88	12
1.0	10^{-8}	8	6	91	9
	10^{-7}	17	13	85	15
1.5	10^{-8}	10	8	92	8
	10^{-7}	22	16	81	19
2.0	10^{-8}	19	21	81	19
	10^{-7}	29	28	72	28
3.0	10^{-8}	38	21	58	42
	10^{-7}	52	42	49	51
4.0	10^{-8}	54	25	51	49
	10^{-7}	79	47	31	69

* HL-60 cells were cultured as detailed in Materials and Methods in the presence of 10^{-7} M or 10^{-8} M 1,25(OH)$_2$D$_3$ for various time periods. At the end of each time period, cells were washed free of 1,25(OH)$_2$D$_3$-supplemented medium, resuspended in fresh medium, and allowed to continue growing up to 7 d total. On d 7, cells were assessed for the various differentiation parameters. Cell viability was >99%. All data are expressed as the percentage of total cells assayed. At least 200 cells were assessed for each parameter.

‡ Blast cells include myeloblasts and promyeloblasts.

§ Intermediate to mature cells include monocytes and macrophages.

virtually all of the bound 1,25(OH)$_2$[^{3}H]D$_3$ migrates in a single 3.3S peak, characteristic of 1,25(OH)$_2$D$_3$ receptors (12, 14, 33). This peak is completely obliterated by the mercurial reagent, PCMBS (Fig. 4A), indicating dissociation of the 1,25(OH)$_2$D$_3$ hormone-receptor complex (38). Finally, to confirm the 3.3S macromolecule as the 1,25(OH)$_2$D$_3$ receptor, monoclonal antibodies to the 1,25(OH)$_2$D$_3$ receptor were incubated with the nuclear extract before sedimentation. As anticipated, the monoclonal antibody 4A5γ shifted the migration of the 3.3S macromolecule to the 7–8S position (Fig. 4*A*), a finding consistent with the sedimentation properties of the hormone-receptor-antibody complex (34). This observation represents unequivocal identification, in that the 4A5γ monoclonal antibody has been shown to interact with 1,25(OH)$_2$D$_3$ receptors from all sources tested, including human cells (34). In contrast, the 8D3μ monoclonal antibody is specific for chick intestinal receptor (34), and therefore has no effect on the sedimentation of this 3.3S macromolecule (Fig. 4*A*).

Fig. 4*B* shows a similar sedimentation pattern for the nuclear extract derived from HL-60 Blast. A PCMBS-dissociable 3.3S macromolecule that can be recognized by 4A5γ (but not 8D3μ) antibody demonstrates the presence of a small, yet detectable, amount of 1,25(OH)$_2$D$_3$ receptor in HL-60 Blast cells.

Using the 1,25(OH)$_2$D$_3$ receptor's inherent DNA-cellulose binding ability (12, 33), we chromatographically analyzed aliquots of the same nuclear extracts used in the sucrose density gradient centrifugation experiments. Fig. 5*A* illustrates the presence of an HL-60 1,25(OH)$_2$D$_3$-binding component that adsorbs to DNA under low ionic strength and elutes at a concentration of 0.23 M KCl. This elution pattern is, in part, characteristic of the 1,25(OH)$_2$D$_3$ receptor (33). A smaller receptor-like peak of weaker DNA binding elutes at 0.13 M KCl, and is at present not identified. Since there is no evidence on the sucrose gradients of a second peak of radioactivity, or of one that is unreactive to PCMBS or monoclonal antibody (Fig. 4*A*), this smaller peak may represent a proteolytic form of the receptor with altered DNA binding. Integrated analysis of these DNA-binding peaks yielded a 1,25(OH)$_2$D$_3$ receptor value of 0.28 pmol/10^8 cells.

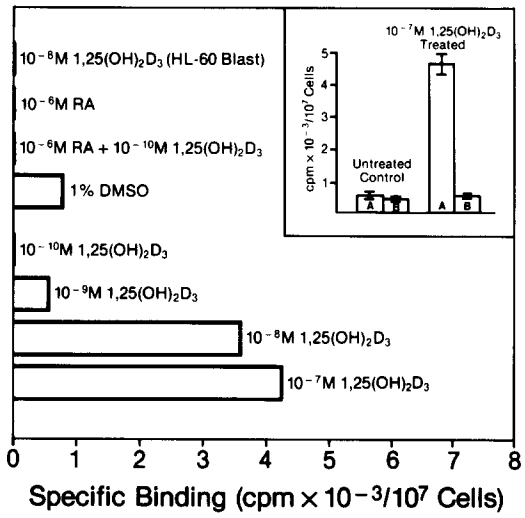

FIGURE 2 Specific [^{3}H]FMLP binding in HL-60 cells treated with various differentiation-inducing compounds. Each histogram represents the difference in [^{3}H]FMLP binding between treated and untreated control cells. The *inset* illustrates a typical experiment used to derive specific binding in HL-60 cells induced by 10^{-7} M 1,25(OH)$_2$D$_3$. Untreated (0.1% ethanol control) or treated cells were incubated with tritiated FMLP in the absence (*A*) or presence (*B*) of 1,000-fold excess radioinert FMLP. The data represent the mean ± standard deviation of triplicate assays. The difference between total (*A*) and nonspecific (*B*) [^{3}H]FMLP binding of the control cells was subtracted from the difference between total (*A*) and nonspecific (*B*) [^{3}H]FMLP binding of the treated cells and expressed in this Figure as induced specific [^{3}H]FMLP binding.

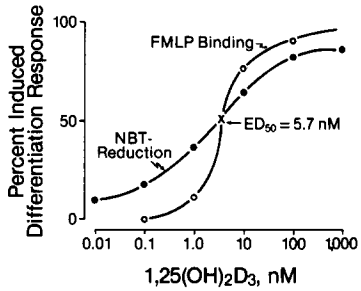

FIGURE 3 Dose response curves of 1,25(OH)$_2$D$_3$-induced differentiation events in HL-60 cells. The data are expressed as percent induced response at various concentrations of 1,25(OH)$_2$D$_3$ and derived from Table I (NBT reduction) and Fig. 2 ([^{3}H]FMLP binding). It is assumed that all cells have the potential to eventually differentiate and reduce NBT. Cells showing specific [^{3}H]FMLP binding (○); cells showing reduction of NBT (●); effective dose for a 50% induced differentiation response (×).

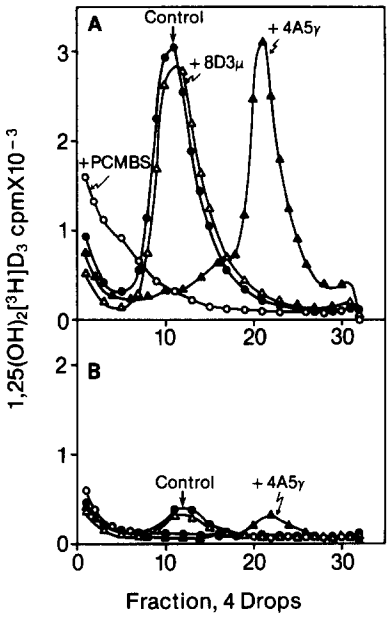

FIGURE 4 Sucrose density gradient analysis of $1,25(OH)_2[^3H]D_3$ binding in nuclear extracts of HL-60 (*A*) and HL-60 Blast (*B*) cells. 10–30% sucrose density gradient ultracentrifugation was performed as outlined in Materials and Methods. Previous to ultracentrifugation, samples were incubated in the presence (○) or absence (●) of PCMBS, the presence of 4A5γ monoclonal antibody (▲), or the presence of 8D3μ monoclonal antibody (△). Sedimentation markers were based on previously described analysis (34).

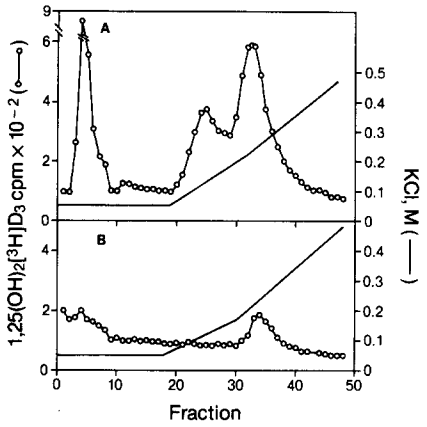

FIGURE 5 DNA-cellulose chromatography of $1,25(OH)_2[^3H]D_3$ binding in nuclear extracts of HL-60 (*A*) and HL-60 blast (*B*) cells. Chromatography was performed as described in Materials and Methods. After samples were applied to DNA-cellulose columns, fall-through radioactivity was monitored until a consistent baseline was established. DNA-binding components were then eluted with linear salt gradients and counted for radioactivity.

Fig. 5*B* shows that HL-60 Blast also contains a small, but measurable, amount of $1,25(OH)_2D_3$ binding component that elutes at 0.23 M KCl. This peak corresponds to 0.023 pmol of apparently functional $1,25(OH)_2D_3$ receptor/10^8 cells.

To provide insight into the role of the receptor in the mechanism of action of $1,25(OH)_2D_3$-induced differentiation, we sought to measure the specific uptake of $1,25(OH)_2[^3H]D_3$ into intact HL-60 cells under conditions that are normally used to grow these cells in culture. Cells were monitored for the specific uptake of various concentrations of $1,25(OH)_2[^3H]D_3$ in the presence of 10% fetal bovine serum in medium

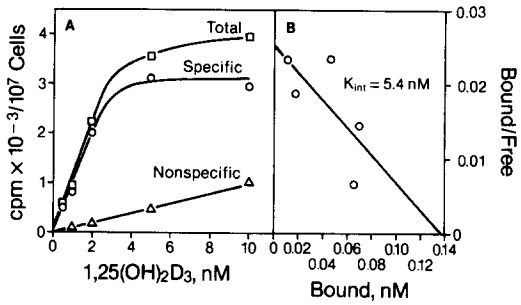

FIGURE 6 Determination of the equilibrium dissociation constant of $1,25(OH)_2[^3H]D_3$ internalization in intact HL-60 cells. Saturation analysis (*A*) was determined by incubating intact cells under normal growth conditions for 4 h with 10% serum along with various concentrations of tritiated $1,25(OH)_2D_3$ in the presence (△) or absence (□) of 100-fold excess nonradioactive $1,25(OH)_2D_3$. Specific binding (○) was transformed by Scatchard analysis and then the data line-fitted by linear regression. (*B*) to yield a K_{int} = 5.4 nM (abscissa intercept = 4,000 molecules/cell, $r = -0.71$).

and assayed on DEAE filters as described in Materials and Methods. Fig. 6*A* demonstrates that uptake by intact HL-60 cells is a specific and saturable process. Accordingly, Scatchard transformation of these data yields an equilibrium dissociation constant for $1,25(OH)_2D_3$ internalization (K_{int}) of 5.4 nM (Fig. 6*B*), and extrapolation to the abscissa predicts 4,000 $1,25(OH)_2D_3$ receptors molecules per HL-60 cell. The K_{int} (5.4 nM) is virtually identical to the ED_{50} (5.7 nM) for induction of specific [^{3}H]FMLP-binding and NBT reduction (Fig. 3).

DISCUSSION

Recently, Miyaura et al. and Abe et al. (4, 5) made the important observation that myeloid leukemia cells differentiate when challenged with the hormonal form of vitamin D_3, namely $1,25(OH)_2D_3$. This observation, coupled with the emergence of a wide variety of nontraditional target cells for $1,25(OH)_2D_3$ (14, 39), suggests that $1,25(OH)_2D_3$ may play a more fundamental role than previously appreciated. In the present communication, we report biological and biochemical characterization of the potent effect of $1,25(OH)_2D_3$ in causing the differentiation of human promyelocytic leukemia (HL-60) cells.

Physiologic levels of $1,25(OH)_2D_3$ in the culture medium of HL-60 cells induced the maturation of the cells as evidenced by their ability to reduce NBT (Table I, Fig. 3), by the appearance of receptor-like binding of the chemoattractant FMLP (Figs. 2 and 3), and by the inability to phagocytose *Candida* after 7 d exposure to $1,25(OH)_2D_3$ (Table I). In contrast to the data of others (4, 19, 40), we provide convincing evidence that HL-60 cells are differentiating toward monocytes and macrophages after exposure to $1,25(OH)_2D_3$. In a preliminary abstract, Bar-Shavit et al. (20) suggested that $1,25(OH)_2D_3$-induced HL-60 cells became adherent to plastic and that these adherent cells were capable of effective cell-bone attachment and degradation, all properties of osteoclast-related monocytes and macrophages. The role of $1,25(OH)_2D_3$ and the regulation of macrophage function is not without precedent. In a separate report Bar-Shavit et al. (41) have shown that the impaired phagocytic response of peritoneal macrophages from vitamin D_3-deficient mice can be corrected by $1,25(OH)_2D_3$. Interestingly, Miyaura et al. (4) observed that $1,25(OH)_2D_3$-exposed HL-60 cells become adherent to

plastic; however, they report all of these cells to be in various stages of granulocytic differentiation and none in the macrophage lineage. In addition to their becoming adherent to plastic, we show that the differentiated cells are strongly nonspecific acid esterase-positive (Table I). This enzyme is present in normal monocytes and macrophages, but not in granulocytes. Morphologically, most of the cells look like monocytes/macrophages and a lower percentage appear to have characteristics of metamyelocytes, but none of the cells resemble mature granulocytes. It is well established that HL-60 can be induced to macrophages after exposure to 10^{-7}–10^{-8} M 12-*O*-tetradecanoyl phorbol-13-acetate (42, 43). Assuming that normal myeloid cells can be stimulated to differentiate into monocytes/macrophages by $1,25(OH)_2D_3$, this sequence could comprise an important mechanism whereby $1,25(OH)_2D_3$ enhances bone resorption, in vivo. This would occur because monocytes can directly resorb bone mineral and because they are apparent precursors of bone-resorbing osteoclasts. HL-60 is likely a good model system to test this hypothesis.

The induction of membrane glycoproteins and receptors often comprises a key terminal step in cell differentiation. Recently, investigators showed that HL-60 cells increased the number of insulin receptors when induced to differentiate with $1,25(OH)_2D_3$, RA, DMSO, and 12-O-tetradecanoyl phorbol-13-acetate (40). A second membrane receptor, that for the *N*-formyl peptides, has been shown to be enhanced by DMSO treatment of HL-60 cells (25). The binding affinities for a series of *N*-formyl oligopeptides to receptors on the surface of human neutrophils correlates with their chemotactic potencies (44), indicating that there is a relationship between membrane receptors for the *N*-formyl peptides and the development of the chemotactic response. Although our data do not reflect conclusive changes in FMLP receptor number or binding affinities, the appearance of [^{3}H]FMLP binding can be used as a measure of the maturation of the chemotactic response in HL-60 cells exposed to $1,25(OH)_2D_3$. We have found that $1,25(OH)_2D_3$ is a more powerful inducer of [^{3}H]-FMLP binding than 1% DMSO in HL-60 cells (Fig. 3), intimating that $1,25(OH)_2D_3$ is extremely effective in creating motile behavior in myeloid leukemia cells. Surprisingly, RA, which like DMSO triggers granulocytic differentiation of HL-60 cells (37), did not increase [^{3}H]FMLP binding (Fig. 2). We have no explanation for this paradox, although the HL-60 cells we used do not display striking morphologic differentiation in the presence of RA and lack the cellular retinoid-binding protein (45).

At present a critical question regarding control of maturation of HL-60 cells by $1,25(OH)_2D_3$ is the molecular mechanism underlying this hormone-induced differentiation process. Tanaka et al. (19) have provided preliminary evidence that $1,25(OH)_2D_3$ functions in HL-60 cells via a steroid hormone-receptor mechanism by showing that the rank order of potency of various vitamin D_3 metabolites in inducing HL-60 differentiation corresponded with the relative ability of these metabolites to compete with $1,25(OH)_2D_3$ for binding to a putative cytosol receptor. In the present work, we unequivocally characterized $1,25(OH)_2D_3$ receptors in HL-60 cells after exposing intact cells to $1,25(OH)_2D_3$ in the presence of serum. Using this technique, we identified the $1,25(OH)_2D_3$ receptor by sucrose density gradients (Fig. 4), DNA cellulose chromatography (Fig. 5), selective chemical dissociation with PCMBS (Fig. 4), and specific immunochemical reactivity with monoclonal antibody (Fig. 4). The above intact cell labeling procedure also allows the determination of the true kinetics of $1,25(OH)_2D_3$ binding (i.e., K_{int}) that would be expected to occur in the bioresponse experiments. Thus, for the first time, binding and bioresponse data are comparable and the result is that the K_{int} of $1,25(OH)_2D_3$ in HL-60 at 4 h of incubation is coincident with the ED_{50} for the biological effects of the hormone on this cell at 6–7 d (Figs. 3 and 6). The fact that nuclear binding of receptor is a rapid event, whereas differentiation requires several days, may be reconciled by proposing that the $1,25(OH)_2D_3$ receptor must be maintained at some steady-state level within the nucleus over the long term. This then provides strong evidence for a correlation between events initiated by the $1,25(OH)_2D_3$ receptor and the cellular bioresponses to $1,25(OH)_2D_3$. As ancillary support to receptor mediation of these events we have found that the HL-60 subclone, HL-60 Blast, is not only insensitive to $1,25(OH)_2D_3$-induced differentiation (Figs. 1 and 2, Table I) but also has a much reduced $1,25(OH)_2D_3$ receptor copy number (8% of the parent line [Fig. 5]). It is therefore tempting to speculate that the differentiation process requires the presence of a critical concentration of $1,25(OH)_2D_3$ receptors. However, because HL-60 Blast cells have not responded to any tested inducer thus far, an equally plausible explanation is that these cells have simply lost their ability to differentiate.

In conclusion, HL-60 represents a cell line that possesses aggressive malignant proliferative potential in vivo but differentiates terminally when challenged with agents such as $1,25(OH)_2D_3$, in vitro. Possibly, the assays of the $1,25(OH)_2D_3$ receptor in peripheral leukocytes from patients with acute promyelocytic leukemia, or even preleukemic conditions, may be predictive of the ultimate response of the patient to adjunct therapy with exogenous $1,25(OH)_2D_3$. Promising results have already been obtained in the survival of mice inoculated with M-1 leukemic cells (6). Clearly, both an understanding of the mode of action of $1,25(OH)_2D_3$ in mediating the differentiation of hematopoietic cells and a test of its clinical effectiveness in leukemia are significant problems for the immediate future.

The authors greatly appreciate the expert technical assistance of Samuel L. Marion and Leslie Yelton.

This work was supported in part by National Institutes of Health grants AM-15781 and AM-30290 to M. R. Haussler, grant AM-32313 to J. W. Pike and funds from an Institutional Grant to the University of Arizona from the American Cancer Society. H. P. Koeffler is a Scholar of the Leukemia Society of America, Inc. and a member of the UCLA Jonsson Comprehensive Cancer Center and is supported by National Cancer Institute Grants CA 26038, CA 32737, and CA 33936.

Received for publication 4 April 1983, and in revised form 24 October 1983.

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