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Cell Biology

Differentiation of mouse myeloid leukemia cells induced by 1α,25-dihydroxyvitamin D_3

(vitamin D_3/1α-hydroxyvitamin D_3/macrophage/lysozyme activity/phagocytic activity)

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ABSTRACT **Mouse myeloid leukemia cells can be induced to differentiate into macrophages *in vitro* by 1α,25-dihydroxyvitamin D_3, the active form of vitamin D_3. The minimal concentration of 1α,25-dihydroxyvitamin D_3 to induce the cell differentiation was 0.12 nM. The degree of cell differentiation in various markers induced by 12 nM 1α,25-dihydroxyvitamin D_3 was nearly equivalent to that induced by 1 μM dexamethasone, the most potent known stimulator. Among several markers of the differentiation by 1α,25-dihydroxyvitamin D_3, phagocytic activity was induced within 24 hr, and this was followed by induction of lysozyme and locomotive activities. Similar changes were also induced by 0.01–1 μM 1α-hydroxyvitamin D_3. 25-Hydroxyvitamin D_3 and 24*R*,25-dihydroxyvitamin D_3 showed only weak inducing activity. These results suggest the possibility that, in addition to its well-known biological activities in enhancing intestinal calcium transport and bone mineral mobilization, 1α,25-dihydroxyvitamin D_3 is involved in the differentiation of bone marrow cells.**

The myeloid leukemia cell line (M1), originally established by Ichikawa (1) from an SL mouse with myeloid leukemia, is known to differentiate into mature macrophages and granulocytes *in vitro* when treated with conditioned media from various cell cultures (2), ascitic fluid of tumor-bearing animals (3), bacterial lipopolysaccharides (4), polyribonucleotides (5), or glucocorticoids (6, 7). Among various inducers, dexamethasone has been found to be the most potent stimulator (8). The differentiation can be detected by changes in cell morphology, adhesion of cells to the dish surface, increase in lysosomal enzyme activity, induction of phagocytic and locomotive activities, and the appearance of Fc and C3 receptors on the cell surface (2–8).

It is now well established that vitamin D_3 is metabolized first in the liver to 25-hydroxyvitamin D_3 [25(OH)D_3] and then in the kidney to 24*R*,25-dihydroxyvitamin D_3 [24*R*,25(OH)$_2D_3$] or 1α,25-dihydroxyvitamin D_3 [1α,25(OH)$_2D_3$] (9, 10). The latter metabolite is the active form of the vitamin in enhancing intestinal calcium transport (11) and bone mineral mobilization (12). 1α,25(OH)$_2D_3$, probably together with parathyroid hormone, causes multinuclear osteoclasts to appear in bone resorbing surfaces (13). As osteoclasts are thought to be derived from monocytes or macrophages (14, 15), we thought that M1 cells would be a good model to test whether vitamin D can induce this cell line to differentiate into macrophages.

This paper reports that 1α,25(OH)$_2D_3$ is much more potent than dexamethasone in inducing macrophages from M1 cells.

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MATERIALS AND METHODS

Cells and Cell Cultures. M1 cells, clone T22, were kindly donated by M. Hozumi (Saitama Cancer Center Research Institute, Saitama, Japan). They were cultured at 37°C under 5% CO_2/95% air in Eagle's minimal essential medium supplemented with twice the normal concentrations of amino acids and vitamins and 10% heat-inactivated calf serum (Chiba Serum Institute, Chiba, Japan). The cells were transferred every 2 to 3 days. Except for the study of cell fractionation, all cells (both adherent and nonadherent) were used for determining parameters of differentiation.

Hormone and Vitamin D Derivatives. Dexamethasone was purchased from Sigma and 25(OH)D_3 was from Philips–Duphar (Amsterdam). 1α,25(OH)$_2D_3$, 24*R*,25(OH)$_2D_3$, and 1α-hydroxyvitamin D_3 [1α(OH)D_3] were kindly donated by I. Matsunaga, Chugai Pharmaceutical, Tokyo.

Fractionation of the Cells by Discontinuous Density Gradient Centrifugation. For the study of cell fractionation, only adherent cells were used in the cultures treated with dexamethasone, 1α,25(OH)$_2D_3$, or 1α(OH)D_3 whereas all cells were used in the control culture. After the nonadherent cells and loosely adherent cells had been removed by gently rinsing three times with prewarmed culture medium, the cells attached to dishes were collected as adherent cells by pipetting with phosphate-buffered saline lacking Ca^{2+} and Mg^{2+} (P_i/NaCl) on ice. Cells were resuspended in P_i/NaCl and mixed with an equivalent volume of 2.43% Ficoll in Urografin. One milliliter of the mixture was layered on the top of Ficoll/Urografin density (*d*) gradients composed of 2.8 ml each of 2.43% (d = 1.054), 4% (d = 1.059), and 7% (d = 1.067) Ficoll/Urografin. The gradients were centrifuged at 1300 rpm for 15 min at 4°C and separated into four groups from the top of the gradient according to the method of Sakagami *et al.* (16). These were referred to as fractions I (d = 1.033–1.054), II (d = 1.054–1.059), III (d = 1.059–1.064), and IV (d = 1.064–1.067). Cells in each fraction were collected by recentrifugation and counted.

Determination of Lysozyme Activity. Lysozyme activity was determined by a modification of the method of Osserman and Lawlor (17) in which the lysoplate contained 1% Difco agar/0.5% heat-killed *Micrococcus lysodykticus* (Sigma)/50 mM

Abbreviations: 25(OH)D_3, 25-hydroxyvitamin D_3; 1α,25(OH)$_2D_3$, 1α,25-dihydroxyvitamin D_3; 1α(OH)D_3, 1α-hydroxyvitamin D_3; 24,25(OH)$_2D_3$, 24,25-dihydroxyvitamin D_3; P_i/NaCl, phosphate-buffered saline lacking Ca^{2+} and Mg^{2+}; *d*, density.

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NaCl/67 mM sodium phosphate, pH 5.9. After a 24-hr incubation at 30°C, the diameters of the zones cleared by lysozyme action were measured. One unit of activity was defined as the amount equivalent to 1 μg of egg white lysozyme (Seikagaku Kogyo, Tokyo) under the same conditions.

Measurement of Phagocytic Activity. Cells were collected by centrifugation and suspended in serum-free culture medium. The cells were then incubated for 4 hr with polystyrene latex particles (2 μl/ml; average diameter = 0.81 μm; Difco) at 37°C under 5% CO_2/95% air. After incubation, the cells were washed vigorously four times with P_i/NaCl, and the number of phagocytic cells among at least 200 viable cells was counted under a microscope.

Detection of Fc and C3 Receptors. The detection of Fc and C3 receptors was carried out as described by Lotem and Sachs (6). Sheep erythrocytes were washed three times with P_i/NaCl and suspended in the same buffer at 1×10^9/ml. Equal volumes of the sheep erythrocytes and rabbit antiserum diluted with P_i/NaCl were mixed and incubated at 37°C for 30 min. The antibody-coated erythrocytes obtained were washed three times with P_i/NaCl and suspended in serum-free culture medium at 1×10^9/ml. Equal volumes of antibody-coated erythrocytes and fresh mouse serum diluted with serum-free culture medium were mixed and incubated at 37°C for 30 min. The antibody and complement-coated erythrocytes were washed and resuspended as described for the antibody-coated erythrocytes. M1 cells (1×10^6) were mixed with 5×10^7 antibody- or antibody and complement-coated erythrocytes in a volume of 1 ml and centrifuged for 3 min at 500 × g. The sample was incubated without dispersing the pellet at 37°C for 30 min. The percentage of cells with a rosette in which at least four erythrocytes were bound was counted with a hemocytometer. At least 200 cells were counted.

Determination of Locomotive Activity. M1 cells (1×10^3) were inoculated in 3 ml of 0.33% soft agar on 4 ml of 0.5% hard agar base in a 6-cm glass dish. Two milliliters of culture medium containing dexamethasone or one of the various D_3 derivatives was poured onto 8-day-old colonies. The dispersed colonies were counted among at least 100 colonies for 9 days. The locomotive activity was expressed as the percentage of dispersed colonies in all colonies.

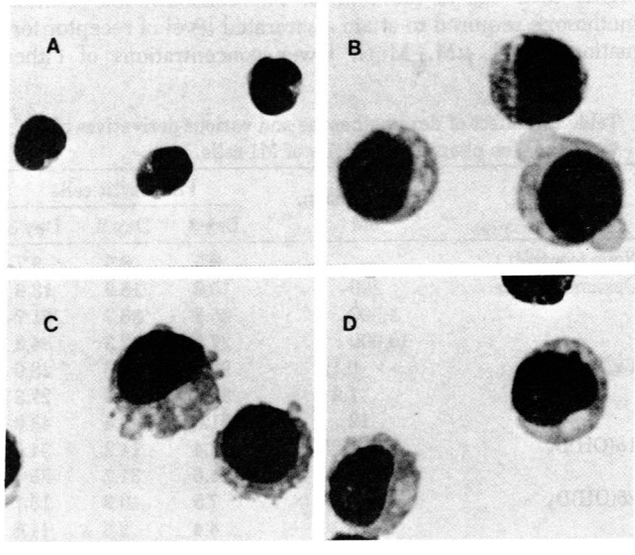

FIG. 1. Morphological changes in M1 cells treated with 1 μM dexamethasone (*B*), 12 nM 1α,25(OH)$_2$D$_3$ (*C*), or 1.25 μM 1α(OH)D$_3$ (*D*). (*A*) Control: cells incubated for 3 days with ethanol. (May–Grünwald–Giemsa; ×800.)

RESULTS

Effect of Vitamin D_3 Derivatives on Morphological Changes in M1 Cells. The morphological changes in M1 cells treated with dexamethasone or one of the vitamin D_3 derivatives were examined after staining with May–Grünwald–Giemsa. As shown in Fig. 1*A*, most of the untreated cells were myeloblastic with a large round nucleus and strongly basophilic cytoplasm. As with dexamethasone, treatment with 12 nM 1α,25(OH)$_2$D$_3$ or 1.25 μM 1α(OH)D$_3$ increased the number of cells in the intermediate stages between typical myeloblastic cells and mature macrophages and that of mature macrophage-like cells (Fig. 1 *B*, *C*, and *D*).

The differentiated cells were attached to the dish surfaces. Adherent cells in the hormone-treated cultures of M1 cells were collected and fractionated on Ficoll/Urografin density gradients. As Fig. 2 shows, most of the untreated cells were recovered in fraction III and the dexamethasone-treated cells were in fractions I and II. The 1α,25(OH)$_2$D$_3$ or 1α(OH)D$_3$-treated cells were also recovered in fractions I and II. Our previous investigations have shown that mature macrophage-like cells are present mainly in fraction I, intermediate cells are in fraction II, and myeloblastic cells are in fraction III (16).

Effect of Vitamin D_3 Derivatives on Cell Growth. The doubling time of M1 cells under the conditions described is 21 hr (16). Addition of dexamethasone or 1α-hydroxy derivatives of vitamin D_3 markedly inhibited cell growth in a time-dependent manner. The number of M1 cells decreased to 57% of the control culture when treated with 1 μM dexamethasone for 3 days

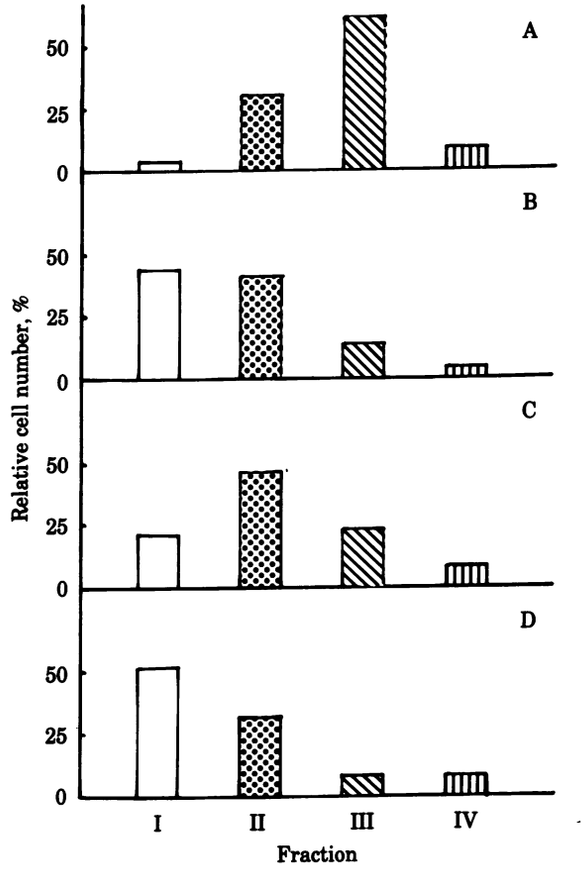

FIG. 2. Density-gradient distribution profiles of M1 cells treated with 1 μM dexamethasone (*B*), 12 nM 1α,25(OH)$_2$D$_3$ (*C*), or 1.25 μM 1α(OH)D$_3$ (*D*). (*A*) Control: cells incubated for 3 days with ethanol. Values are expressed as percent of cells in each fraction relative to all cells applied (*A*) or to all adherent cells applied (*B*, *C*, and *D*).

Table 1. Changes in total number of M1 cells and in number of adherent cells after treatment with dexamethasone or various derivatives of vitamin D_3

Addition	Concentration, nM	Total cells*	Adherent cells†
None (control)	—	100	5.2 ± 0.8
Dexamethasone	100	76 ± 2‡	25.0 ± 3.2‡
	1,000	57 ± 4‡	33.0 ± 5.4‡
	10,000	51 ± 6‡	31.1 ± 2.5‡
$1\alpha,25(OH)_2D_3$	0.12	103 ± 7	14.6 ± 4.0
	1.2	77 ± 2‡	17.9 ± 5.0
	12	57 ± 5‡	41.3 ± 8.0‡
$1\alpha(OH)D_3$	12.5	81 ± 8‡	19.1 ± 4.8
	125	71 ± 3‡	25.4 ± 3.4‡
	1,250	43 ± 6‡	26.5 ± 2.7‡
$25(OH)D_3$	62.5	114 ± 14	3.6 ± 0.8
	625	105 ± 1	14.8 ± 2.1‡
$24R,25(OH)_2D_3$	2.4	99 ± 13	6.5 ± 3.5
	24	94 ± 4	8.5 ± 2.8

Values are expressed as mean ± SEM of five experiments.
* Total cell numbers were counted 3 days after treatment with hormones; values are calculated as percentage of the control.
† Adherent cells are expressed as percent of total cells.
‡ Statistically significant ($P < 0.01$).

(Table 1). $1\alpha,25(OH)_2D_3$ and $1\alpha(OH)D_3$ also inhibited cell multiplication in a dose-dependent manner (Table 1). Both 1α-hydroxy derivatives of vitamin D_3 suppressed cell multiplication more specifically at concentrations lower than 1 μM. The minimal concentration of $1\alpha,25(OH)_2D_3$ to suppress cell growth was 1.2 nM. $1\alpha,25(OH)_2D_3$ was at least 100 times more potent on a molar basis than dexamethasone in suppressing cell growth.

In addition, $1\alpha,25(OH)_2D_3$ and $1\alpha(OH)D_3$, like dexamethasone, made M1 cells adhere to the dish surfaces. At concentrations as low as 0.1 nM, $1\alpha,25(OH)_2D_3$ was capable of increasing the percentage of adherent cells. The percentages of adherent cells in the cultures treated with 10 nM $1\alpha,25(OH)_2D_3$ or 1 μM $1\alpha(OH)D_3$ were similar to those treated with 1μM dexamethasone. Higher concentrations of $25(OH)D_3$ also slightly increased the number of adherent cells.

Effect of Vitamin D_3 Derivatives on Induction of Lysozyme Activity. The effects of various derivatives of vitamin D_3 on lysozyme activity, a typical biochemical marker of the differentiated macrophages, are shown in Figs. 3 and 4. Both dexamethasone and $1\alpha,25(OH)_2D_3$ markedly enhanced lysozyme activity but $1\alpha,25(OH)_2D_3$ was even more effective. The activity attained a maximum on the third day of incubation with 12 nM $1\alpha,25(OH)_2D_3$ at a level 3 times higher than that from treatment with 1 μM dexamethasone (Fig. 3). Concentrations of $1\alpha(OH)D_3$, $25(OH)D_3$, and $24R,25(OH)_2D_3$ higher than 1 μM also induced lysozyme activity but only slightly (Fig. 4).

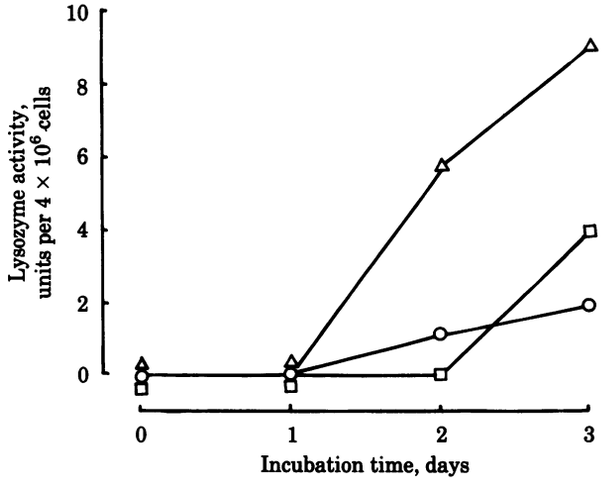

FIG. 3. Time course of lysozyme activity in M1 cells treated with 1 μM dexamethasone (○), 12 nM $1\alpha,25(OH)_2D_3$ (△), or 1.25 μM $1\alpha(OH)D_3$ (□). At indicated times, cells were harvested and their lysozyme activities were determined.

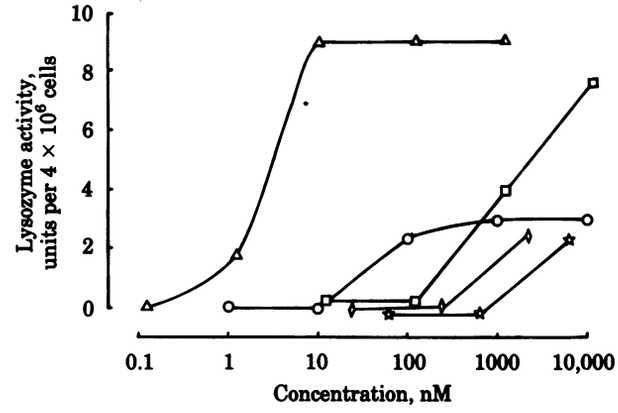

FIG. 4. Dose–response curves of lysozyme activity of M1 cells incubated for 3 days with various concentrations of dexamethasone (○), $1\alpha,25(OH)_2D_3$ (△), $1\alpha(OH)D_3$ (□), $25(OH)D_3$ (☆), or $24R,25(OH)_2D_3$ (◇).

Effect of Vitamin D_3 Derivatives on Induction of Phagocytic Activity. Treatment with 0.1 nM $1\alpha,25(OH)_2D_3$ significantly induced phagocytic activity as early as on the first day (Table 2). Further enhancement of phagocytic activity by $1\alpha,25(OH)_2D_3$, however, was not found during the remaining 2 days of cultivation. $1\alpha(OH)D_3$ (10 nM) was also capable of inducing phagocytic activity on the first day. The concentrations of 1α-hydroxy derivatives of vitamin D_3 that induced phagocytic activity appeared to be lower than those that enhanced lysozyme activity. Higher concentrations of $25(OH)D_3$ and $24R,25(OH)_2D_3$ affected the induction of phagocytic activity of M1 cells slightly.

Effect of Vitamin D_3 Derivatives on Formation of Fc and C3 Receptors. Table 3 shows that 1 μM dexamethasone increased the formation of Fc and C3 receptors to a level 3 times higher than that of the control. The concentration of dexamethasone required to attain a saturated level of receptor formation was 1 μM. Much lower concentrations of either

Table 2. Effect of dexamethasone and various derivatives of vitamin D_3 on phagocytic activity of M1 cells

Addition	Concentration, nM	Phagocytic cells Day 1	Day 2	Day 3
None (control)	—	0.5	8.9	9.7
Dexamethasone	100	15.6	18.9	18.6
	1,000	26.7	36.7	31.7
	10,000	27.1	37.3	34.3
$1\alpha,25(OH)_2D_3$	0.12	26.0	29.6	28.0
	1.2	35.0	44.4	27.3
	12	51.0	48.4	43.9
$1\alpha(OH)D_3$	1.25	1.4	14.2	34.0
	12.5	21.6	31.2	23.4
$25(OH)D_3$	62.5	7.5	10.9	13.7
	625	4.4	9.8	14.6
$24R,25(OH)_2D_3$	2.4	5.9	13.5	13.7
	24	14.0	14.4	17.6

Values are expressed as percent of total cells counted. These results were highly reproducible in independent sets of duplicate experiments.

Table 3. Effect of dexamethasone and various derivatives of vitamin D_3 on the appearance of Fc and C3 receptors

Addition	Concentration, nM	Fc receptor	C3 receptors
None (control)	—	6.9	7.6
Dexamethasone	100	7.8	15.6
	1,000	16.5	24.3
	10,000	15.8	23.8
$1\alpha,25(OH)_2D_3$	0.12	9.1	17.5
	1.2	11.1	12.7
	12	20.2	21.4
$1\alpha(OH)D_3$	1.25	8.7	10.6
	12.5	18.2	14.0
$25(OH)D_3$	62.5	3.6	4.8
	625	5.9	4.4
$24R,25(OH)_2D_3$	2.4	2.7	3.6
	24	2.4	7.7

M1 cells were incubated for 3 days with either vehicle (ethanol), dexamethasone, or various D_3 derivatives. Values are expressed as percent of total cells. These results were highly reproducible in independent sets of triplicate experiments.

$1\alpha(OH)D_3$ or $1\alpha,25(OH)_2D_3$ similarly induced the formation of receptors in a dose-dependent manner. $1\alpha,25(OH)_2D_3$ appeared to be 100-fold more potent than dexamethasone in inducing receptor formation. Higher concentrations of $25(OH)D_3$ and $24R,25(OH)_2D_3$ failed to induce receptor formation.

Effect of Vitamin D_3 Derivatives on Induction of Locomotive Activity. When 8-day-old colonies of M1 cells were treated with 1 μM dexamethasone for 3 days, ≈60% of the colonies became dispersed. Treatment with 12 nM $1\alpha,25(OH)_2D_3$ or 12.5 nM $1\alpha(OH)D_3$ also induced locomotive activity (Fig. 5). The percentages of colonies that achieved locomotive activity when treated with the 1α-hydroxy derivatives appeared to be less than those of colonies treated with dexamethasone. This conclusion was supported by the fact that, even at concentrations higher than 12 nM, $1\alpha(OH)D_3$ or 12.5 nM $1\alpha,25(OH)_2D_3$ failed to increase the percentages of dispersed colonies (data not shown). Maximal concentrations of dexamethasone, $1\alpha,25(OH)_2D_3$, and $1\alpha(OH)D_3$ made ≈95%, 60%, and 10% of all colonies locomotive, respectively. Within 24 hr after addition of 1 μM dexamethasone to day 5 cultures with colonies partially dispersed by 12 nM $1\alpha,25(OH)_2D_3$ or 12.5 nM $1\alpha(OH)D_3$, the percentages of dispersed colonies were markedly increased (Fig. 5).

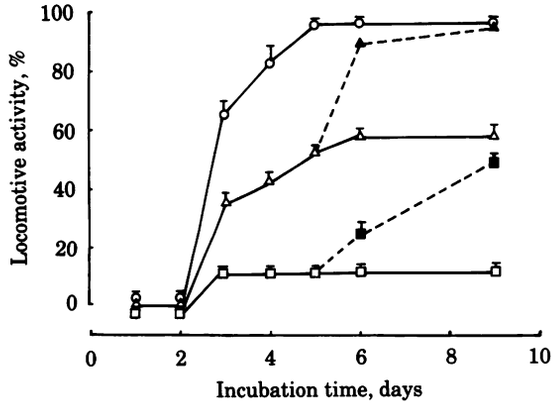

FIG. 5. Time course of change in locomotive activity induced by 1 μM dexamethasone (○), 12 nM $1\alpha,25(OH)_2D_3$ (△), or 12.5 nM $1\alpha(OH)D_3$ (□). In some experiments, 1 μM dexamethasone was added on the 5th day to colonies partially dispersed by $1\alpha,25(OH)_2D_3$ (▲) or $1\alpha(OH)D_3$ (■). Data are mean ± SEM of five experiments.

DISCUSSION

This paper reports the possibility that the active form of vitamin D_3 is involved in the differentiation of bone marrow cells. Like dexamethasone, $1\alpha,25(OH)_2D_3$ was capable of inducing differentiation of myeloid leukemia cells into macrophages. Parameters of differentiation such as morphological changes, cell adhesion on glass surfaces, induction of phagocytic activity, and the appearance of Fc and C3 receptors were induced by physiological plasma concentrations (0.1 nM) of $1\alpha,25(OH)_2D_3$, and lysozyme activity was induced by nanomolar concentrations of metabolite. The maximal effect, obtained with 12 nM $1\alpha,25(OH)_2D_3$, was nearly equivalent to that of 1 μM dexamethasone, the most potent known stimulator. Among various fat-soluble vitamins, only $1\alpha,25(OH)_2D_3$ induced all parameters of differentiation into macrophages. Retinoic acid (10 μM) has been reported to induce differentiation but only lysozyme activity (18). α-Tocopherol failed to induce differentiation of M1 cells (19).

Maximal concentrations of $1\alpha,25(OH)_2D_3$ and $1\alpha(OH)D_3$ made only 60% and 10% of all colonies locomotive, respectively, while dexamethasone caused almost all of the colonies to disperse. The addition of dexamethasone to the colonies partially dispersed by $1\alpha,25(OH)_2D_3$ made the residual undispersed colonies locomotive within 24 hr. These results suggest that the action of $1\alpha,25(OH)_2D_3$ in inducing locomotive activity of M1 cells is selective, while that of dexamethasone is not.

The mechanisms of induction of differentiation-associated properties in M1 cells by $1\alpha,25(OH)_2D_3$ are most interesting. We first examined the effect of Ca^{2+} in the culture medium. Not only dexamethasone but also $1\alpha,25(OH)_2D_3$ induced differentiation of M1 cells in culture media containing 0.7–5.0 mM Ca^{2+} (data not shown). It has been reported that dexamethasone first associates with a cytoplasmic protein receptor in M1 cells (20–22). The cytoplasmic receptor–dexamethasone complex then moves to the nucleus. RNA and protein syntheses are required for the induction of phagocytic activity in M1 cells by dexamethasone (23). Induction by dexamethasone of lysosomal enzyme activities in M1 cells has also been reported to be inhibited by treatment with puromycin (8). It is possible to consider similar mechanisms in the differentiation of M1 cells by $1\alpha,25(OH)_2D_3$.

It is interesting that 10 nM $1\alpha(OH)D_3$ is also effective in inducing differentiation in such parameters as inhibition of cell growth, stimulation of cell adhesion, induction of phagocytic activity, and receptor formation. It has been reported that $1\alpha(OH)D_3$ has to be converted to $1\alpha,25(OH)_2D_3$ in the liver before it can be effective in stimulating intestinal calcium transport and bone mineral mobilization (24, 25). Preliminary experiments, however, showed that $1\alpha(OH)[^3H]D_3$ was not converted to $1\alpha,25(OH)_2[^3H]D_3$ by M1 cells during 3 days of cultivation (data not shown), suggesting that $1\alpha(OH)D_3$ *per se* may be effective in inducing the differentiation of M1 cells. As 10 nM $25(OH)D_3$ and $24R,25(OH)_2D_3$ had only weak activity in inducing differentiation, the hydroxyl function at C-1 may be more important than that at C-25 in inducing differentiation. These results appear consistent with the experimental fact that the $1\alpha,25(OH)_2D_3$-specific cytosol receptor found in intestine can be competed for by a 100-fold excess of $1\alpha(OH)D_3$ or a 1000-fold excess of $25(OH)D_3$ (26, 27).

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