# 1α,25-Dihydroxyvitamin $D_3$ Induces Maturation of the Human Monocyte Cell Line U937, and, in Association with a Factor from Human T Lymphocytes, Augments Production of the Monokine, Mononuclear Cell Factor

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**Abstract.** The monocyte factor, interleukin 1, or other factors homologous with interleukin 1, modulates functions of a variety of cells, including T and B lymphocytes, synovial cells, and chondrocytes. We have reported that a human monocyte cell line, U937, produces interleukin 1 when incubated with a soluble factor from lectin-stimulated T lymphocytes. We have also shown that U937 cells have a specific cytosolic receptor for 1α,25-dihydroxyvitamin $D_3$ (1α,25[OH]$_2$$D_3$). We now report that 1α,25(OH)$_2$$D_3$ ($10^{-11}$–$10^{-10}$ M) induces maturational changes in the U937 cells similar to those produced by conditioned medium from lectin-stimulated T lymphocytes (increase in Fc receptors and OKM1 binding and decrease in proliferation), but does not induce monokine production as measured by mononuclear cell factor activity. 1α,25(OH)$_2$$D_3$ is 200–300-fold more effective than 25-hydroxyvitamin $D_3$, which is consistent with the known biological potency of these vitamin $D_3$ metabolites. 1α,25(OH)$_2$$D_3$ and the lymphokine together markedly augment maturational effects and, in addition, augment monokine production. The specificity of the interaction is further demonstrated by the lack of augmentation of monokine production with 1β,25-dihydroxyvitamin $D_3$ in the presence of lymphokine. These interactions of a classical hormone and the hormonelike product(s) of the immune system with U937 cells serve as a model for human monocyte/macrophage differentiation and suggest a role for these interactions in some aspects of inflammation.

## Introduction

The effects of the hormone, 1α25-dihydroxyvitamin $D_3$ (1α,25[OH]$_2$$D_3$)[1] are primarily on cells in intestine, bone, and kidney (1–3). Specific cytosolic receptors for 1α,25(OH)$_2$$D_3$ are found in these cells as well as cells from several other normal (3, 4) and neoplastic (5, 6) tissues. The significance of 1α,25(OH)$_2$$D_3$ receptors in tissues other than intestine, bone, or kidney, however, has not been established. Recently, several investigators reported effects of 1α,25(OH)$_2$$D_3$ on cellular replication and differentiation. 1α,25(OH)$_2$$D_3$ receptors are present in human melanoma cell lines and the addition of hormone to the culture medium caused a dose-dependent decrease in their rate of growth (7). Abe et al. (8) demonstrated maturation of the murine M-1 cell line towards mature macrophages, while Miyaura et al. (9) induced the human myeloid cell line HL-60 to differentiate into granulocytes with 1α,25(OH)$_2$$D_3$. 1α,25(OH)$_2$$D_3$ either stimulated or inhibited proliferation of ROS 17/2.8 rat osteoblastic osteosarcoma cells depending upon

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1. *Abbreviations used in this paper:* 25(OH)$D_3$, 25-hydroxyvitamin $D_3$; 1α,25(OH)$_2$$D_3$, 1α,25-dihydroxyvitamin $D_3$; 1β,25(OH)$_2$$D_3$, 1β,25-dihydroxyvitamin $D_3$; $PGE_2$, prostaglandin $E_2$.

the state of maturation of the cells (10). A concentration-dependent biphasic effect of $1\alpha,25(OH)_2D_3$ on cell growth was demonstrated to a greater extent when the ROS 17/2.8 cells were cultured in serum-free medium (11). That these in vitro observations may have biological significance in vivo was suggested by three recent communications. The first, reported by Bar-Shavit et al. (12) noted that macrophages from vitamin $D_3$-deficient mice functioned abnormally and that the defect was corrected by $1\alpha,25(OH)_2D_3$ repletion in vitro and in vivo. The second dealt with another mouse model in which vitamin D-deficient animals were thyroparathyroidectomized and given exogenous $1,25(OH)_2D_3$, which increased osteoclast activity and numbers in the absence of parathyroid hormone (13). The third extended the observations on the in vitro differentiation of M1 cells by demonstrating prolonged survival of $1\alpha,25(OH)_2D_3$-treated mice inoculated with M1 cells (14).

We have studied monocyte products and their role in the tissue destruction accompanying inflammatory processes and have recently utilized a human monocyte cell line, U937, originally described by Sundström and Nilsson (15). The cells possess monocyte characteristics such as Fc receptors, nonspecific esterase staining, phagocytosis of latex beads, and secretion of lysozyme (16). Upon exposure to crude lymphokine the cells exhibit increased Fc receptors and increased antibody-dependent cellular cytotoxicity (17–19). They develop receptors for the chemotactic tripeptide *N*-formyl-methionyl-leucyl-phenylalanine, in association with directional migration (20), as well as an enhanced ability to kill intracellular pathogens (21). We have demonstrated that a soluble factor of $M_r$ ~45–60,000 from lectin-stimulated cloned human T lymphocytes when incubated with U937 cells induces production of mononuclear cell factor (22) and lymphocyte-activating factor activities (23), (Amento, E. P., J. T. Kurnick, and S. M. Krane. Manuscript submitted for publication) which are ascribed to interleukin 1. We have also observed that the U937 cells possess specific high affinity cytosolic receptors for $1\alpha,25(OH)_2D_3$ (24, 25). We therefore asked whether $1\alpha,25(OH)_2D_3$ would have specific effects on the differentiation and function of U937 cells either alone or in conjunction with the lymphokine produced by T lymphocytes.

We now report that $1\alpha,25(OH)_2D_3$ causes changes in the shape, adherence, Fc receptor number, and surface antigen expression of U937 cells consistent with a maturational event. These changes are not accompanied by monokine secretion. The addition of $1\alpha,25(OH)_2D_3$ to lymphokine-stimulated U937 cells, however, augments monokine production.

## Methods

*Cell culture.* U937 cells (kindly provided by Dr. R. Synderman, Duke University Medical Center) were grown in stationary suspension culture in Iscove's modified Dulbecco's medium (Gibco Laboratories, Grand Island, NY), which contained 10% fetal calf serum (Bioproducts, Inc., Warrenton, OR), 50 μM 2-mercaptoethanol, 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco Laboratories) (complete medium) at 37°C in a humidified atmosphere of 5% $CO_2$ and 95% air.

T lymphocytes and their lectin-stimulated soluble factor were obtained as previously described (22). Briefly, normal human peripheral blood lymphocytes were exposed to tetanus toxoid (1 floculation unit/ml) (State of Massachusetts), and proliferating cells separated on Percoll-density gradients (26) and maintained in culture with interleukin 2 (T cell growth factor)-containing medium. Periodic reexposure to tetanus toxoid and antigen-presenting cells was necessary to maintain interleukin 2 responsiveness and to assure expansion of the antigen-specific T lymphocyte population (27). Flow cytometer analysis (Ortho Spectrum III, Ortho Diagnostic Systems, Inc., Westwood, MA) using fluoresceinated monoclonal anti-T lymphocyte subset reagents showed that all of the cells expressed the T3, T4, and T11a surface antigens and were negative for OKT8 binding (kindly provided by Ortho Pharmaceutical, Raritan, NJ).

T lymphocytes (all OKT4 positive) were washed free of their growth-supporting medium and resuspended at a density of $5 \times 10^5$ cells/ml in Iscove's modified Dulbecco's medium containing 50 μM 2-mercaptoethanol and penicillin/streptomycin with or without 10% fetal calf serum, with or without 1 μg/ml of phytohemagglutinin (Leucoagglutinin, Pharmacia, Uppsala, Sweden) for 24 h.

*Vitamin D metabolites.* The $1\alpha,25(OH)_2D_3$ and 25-hydroxyvitamin $D_3$ (25[OH]$D_3$) used in these studies were gifts from Dr. Milan Uskoković (Roche Laboratories, Nutley, NJ) and Dr. John Babcock (Upjohn Co., Kalamazoo, MI), respectively. $1\beta,25(OH)_2D_3$ was synthesized as described (28). All compounds were reconstituted in ethanol and stored in concentrated solutions at −20°C. The vitamin D metabolites were freshly diluted in the appropriate medium before each experiment. The alcohol concentration in the test conditions never exceeded 0.1% and appropriate controls were performed to exclude an effect of the ethanol on target cells.

*U937 cell stimulation.* U937 cells in log phase growth were incubated at $1\text{–}5 \times 10^5$ cells/ml with the conditioned medium from phytohemagglutinin-stimulated T lymphocytes, vitamin $D_3$ metabolites, or conditioned medium plus vitamin $D_3$ metabolites. Incubations were performed in Iscove's modified Dulbecco's medium in the presence or absence of 10% fetal calf serum or in a defined medium that contained bovine serum albumin (6 mg/ml) (Pentex, fraction V, Miles Laboratories, Inc., Research Products Div., Ekhart, IN) and transferrin (5 μg/ml) (Sigma Chemical Co., St. Louis, MO) (defined medium) in either 24-well or 96-well trays (Costar, Data Packaging, Cambridge, MA) or 100-mm plates (Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, CA).

Cell viability was assessed by trypan blue exclusion. U937 cell proliferation under experimental conditions was determined by cell counts (Coulter counter, Coulter Electronics, Inc., Hialeah, FL) and by [$^3$H]thymidine incorporation. Cell numbers and [$^3$H]thymidine incorporation were assayed initially in parallel to validate the thymidine incorporation data. After appropriate culture periods 1 μCi/well of [$^3$H]thymidine (67 Ci/mmol, New England Nuclear, Boston, MA) was added to U937 cells for 4 h and the cells were collected with an automatic cell harvester on glass filter strips (MASH-11, Flow Laboratories, Inc., McLean, VA), dried, and counted in Omnifluor (New England Nuclear) using a Packard Tricarb scintillation counter (Packard Instrument Co., Inc., Downers Grove, IL).

*Analysis of cell surface changes.* Unstimulated and stimulated U937 cells were assessed for surface antigens by first preincubating washed cells with heat-aggregated and monomeric human IgG (1:1 ratio, 25 μg/ml in phosphate-buffered saline) for 45 min to block nonspecific binding of the monoclonal antibodies to Fc receptors as we have described (29). Cells were washed twice between all steps. Approximately $1 \times 10^6$ cells

were incubated with each monoclonal antibody for 30 min and then stained with fluorescein-conjugated goat anti-mouse IgG (heavy and light chain specific) (1:40, Tago, Inc., Burlingame, CA). Fc receptor binding was determined in a similar fashion with fluoresceinated goat anti-human IgG (1:40, Tago, Inc.). All incubations were performed at 4°C. The cells were fixed in 1% paraformaldehyde before analysis with an automated laser-activated flow cytometer (Ortho Spectrum III). The data are presented as linear fluorescence histograms with fluorescence intensity indicated along the abscissa and number of cells on the ordinate.

*Mononuclear cell factor assays.* Cell-free supernates of stimulated U937 cells were either used immediately or frozen at −20°C until assayed. Mononuclear cell factor assays were performed as previously described (22). Briefly, synovial tissue from patients with rheumatoid arthritis was obtained during joint surgery. After enzymatic dispersion of the synovial lining cells, the adherent cell population was prepared and maintained in culture as described (30). Before bioassay, adherent synovial cells were removed with trypsin (trypsin-EDTA, Gibco Laboratories) and placed in 24-well trays at $5 \times 10^4$ cells/well. Supernatant medium from stimulated U937 cells and control samples was then diluted 1:5 in Dulbecco's modified Eagle's medium (Gibco Laboratories), which contained 10% fetal calf serum, penicillin/streptomycin and incubated with the adherent synovial cells for 3 d. Prostaglandin $E_2$ ($PGE_2$) production from the synovial cells was determined by radioimmunoassay (31) (the antibody to $PGE_2$ was generously provided by Dr. L. Levine, Brandeis University).

## Results

*1α,25(OH)$_2$D$_3$ effects upon U937 cell proliferation.* U937 cells proliferate in complete medium with a doubling time of ~27 h. At an initial culture density of $\sim 1 \times 10^5$ cells/ml, maximum cell density of $1.25 \times 10^6$/ml was attained within 6 d. In suspension culture the cells had a pleomorphic appearance with numerous projections. When maximum cell density was reached and exhaustion of nutrient occurred, the cells rounded up and remained viable for 1–2 d before undergoing rapid cell death. The addition of 1α,25(OH)$_2$D$_3$ to U937 cells in complete medium caused a dose-dependent decrease in cell proliferation. In the presence of 10% fetal calf serum, inhibition of cell proliferation was apparent at $10^{-8}$ M 1α,25(OH)$_2$D$_3$ ($1.17 \times 10^5$ cells/ml vs. U937 cells alone at $2.4 \times 10^5$ cells/ml after 48 h of incubation). In addition, the physical appearance of the cells was altered. They adhered to, and spread on, the plastic culture dishes. The changes were similar to those seen when U937 cells were incubated with conditioned medium from lectin-stimulated T lymphocytes. The changes were subtle at $10^{-9}$ M, but readily apparent at 1 and $2 \times 10^{-8}$ M (Fig. 1). In contrast, 25(OH)D$_3$, at $10^{-8}$ and $10^{-7}$ M, had no discernable effect on U937 cells in the presence of 10% fetal calf serum. Replicate cultures had excellent viability as assessed by trypan blue exclusion when cells were incubated with 1 and $2 \times 10^{-8}$ M 1α,25(OH)$_2$D$_3$. In fact, viability was consistently greater in the presence of 1α,25(OH)$_2$D$_3$. Viability was not enhanced when the cells were incubated with 25(OH)D$_3$ at $10^{-8}$ or $10^{-7}$ M.

*Synergism between 1α,25(OH)$_2$D$_3$ and lymphokine.* Since the effects upon U937 cell proliferation caused by 1α,25(OH)$_2$D$_3$ were similar to those we had previously demonstrated with T lymphocyte-conditioned medium, we asked whether the incubation of 1α,25(OH)$_2$D$_3$ and lymphokine would enhance the changes. When U937 cells grown in complete medium were incubated with an amount of T lymphocyte-conditioned medium that did not produce a significant decrease in cell proliferation (as measured by [$^3$H]thymidine incorporation by U937 cells), the addition of 1α,25(OH)$_2$D$_3$ to the culture caused a dose-dependent decrease in proliferation, which was synergistic with the effect of the lymphocyte medium (Table I). When [$^3$H]thymidine incorporation by U937 cells was similar to control cells in the presence of the lymphocyte-conditioned medium and 73% of control cells when incubated with $10^{-8}$ M 1α,25(OH)$_2$D$_3$, the combination of both conditioned medium and hormone decreased thymidine incorporation by U937 cells to 52% of control values.

The addition of conditioned medium plus a 1α,25(OH)$_2$D$_3$ to U937 cells increased the number of adherent cells without a discernible difference in morphology (Fig. 1). Although lymphocyte-conditioned medium or 1α,25(OH)$_2$D$_3$ alone caused the cells to adhere, this effect was not pronounced at the concentrations chosen. However, the combination of lymphocyte-conditioned medium and 1α,25(OH)$_2$D$_3$ produced a marked increase in adherence, which required scraping to transfer cells.

*1α,25(OH)$_2$D$_3$ effects in defined medium.* In our studies described thus far, the effects of 1α,25(OH)$_2$D$_3$ were examined in the presence of 10% fetal calf serum. The presence of serum with its vitamin D$_3$-binding protein reduces the effective concentration of 1α,25(OH)$_2$D$_3$ in the culture medium. To observe the influence of the hormone in the absence of vitamin D$_3$-binding protein, bovine serum albumin (6 mg/ml) and transferrin (5 μg/ml) were substituted for serum in order to increase the free 1α,25(OH)$_2$D$_3$ available to the cell.

Altered cell shape, increased adherence, and decreased cell proliferation were observed with $10^{-11}$ M 1α,25(OH)$_2$D$_3$ and for the first time with 25(OH)D$_3$ at $10^{-9}$ M. The effect of vitamin D$_3$ metabolites on cell proliferation was demonstrable in serum-free medium at a lower concentration than in medium that contained 10% fetal calf serum. This effect of the hormone was further enhanced in the presence of albumin and transferrin (Fig. 2). Daily additions of hormone for 3 d rather than a single addition at the initiation of culture decreased [$^3$H]thymidine incorporation in proportion to the increased concentration. A comparison of the concentrations required to produce a 50% inhibition of [$^3$H]thymidine incorporation by U937 cells showed that 1α,25(OH)$_2$D$_3$ was 200–300-fold more effective than 25(OH)D$_3$, consistent with the known biological potency of these vitamin D$_3$ metabolites. The concentration of 1α,25(OH)$_2$D$_3$ and 25(OH)D$_3$, which caused a 50% inhibition of [$^3$H]thymidine incorporation by U937 cells in the presence of albumin and transferrin, was $2 \times 10^{-11}$ and $5 \times 10^{-9}$ M, respectively. A decrease in the magnitude of the response was observed when cells were maintained in serum-free medium. 50% inhibition of [$^3$H]thymidine incorporation by U937 cells occurred with 1α,25(OH)$_2$D$_3$ at $3 \times 10^{-10}$ M and with 25(OH)D$_3$

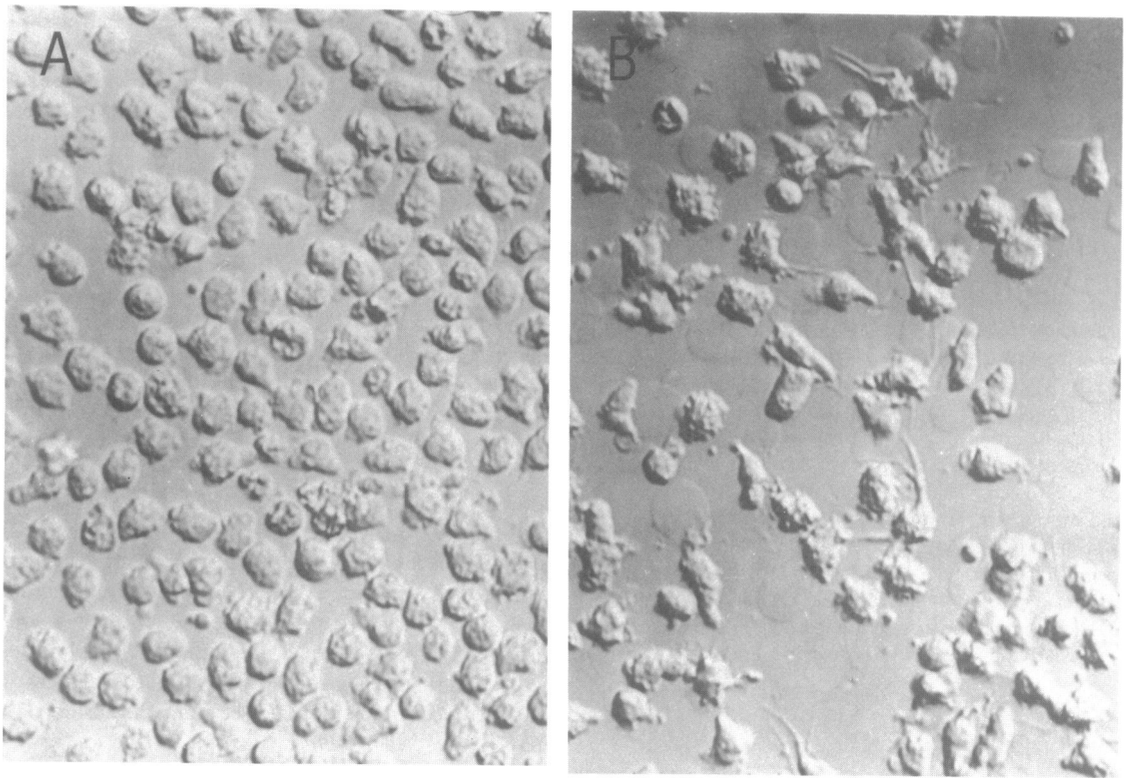


*Figure 1.* Photomicrographs of U937 cells in the absence and presence of $1\alpha,25(OH)_2D_3$. U937 cells at $5 \times 10^5$/ml were incubated for 72 h alone (*A*) or with $1\alpha,25(OH)_2D_3$ (*B*), $10^{-8}$ M in Iscove's modified Dulbecco's medium with 10% fetal calf serum. U937 cells incubated with conditioned medium from lectin-stimulated T lymphocytes or lymphokine plus $1\alpha,25(OH)_2D_3$ were morphologically indistinguishable from those incubated with hormone alone (although the conditions could be readily distinguished by the greater number of adherent cells seen with the combination of lymphokine and hormone). Photographs were obtained using wet preparations fixed in 1% paraformaldehyde, examined with a Zeiss IM 35 inverted-stage microscope equipped with Nomarski optics (× 387).

at $7 \times 10^{-8}$ M. Decreased proliferation due to a lack of nutrients could presumably account for the lesser effect.

*Effects of $1\alpha,25(OH)_2D_3$ on cell maturation as evaluated by the detection of monocyte-related surface antigens and Fc receptors.* We previously observed that the cell membrane changes that occurred when U937 cells were incubated with medium from lectin-stimulated T lymphocytes included, among others, increased numbers of Fc receptors and increased binding of monoclonal antibodies that defined monocyte-related surface antigens (29). A similar change was found when U937 cells were incubated with $1\alpha,25(OH)_2D_3$ in the presence or absence of fetal calf serum (Fig. 3). The presence of Fc receptors was evaluated by determining the binding of aggregated and monomeric human or mouse IgG to U937 cells with the flow cytometer. Preincubation of the cells with aggregated mouse IgG completely blocked binding of human IgG. In the same manner, nonspecific binding of mouse monoclonal antibodies to human cell surface antigens was blocked by preincubating the U937 cells with human IgG. The incubation of $1\alpha,25(OH)_2D_3$ ($10^{-8}$ M) with U937 cells caused an increase in Fc receptors as well as OKM1 binding. The addition of the hormone to U937 cells cultured with a low stimulatory concentration of lymphocyte-conditioned medium caused a marked increase in Fc receptors and OKM1 binding (Fig. 3, *A–D*).

The OKM1 antibody defines a cell surface antigen found on monocytes, granulocytes, and natural killer cells (32). We previously showed that the OKM1 antibody defines a monocyte-related antigen on U937 cells (29). To further clarify the antigen expression on the cell membrane of U937 cells incubated with $1\alpha,25(OH)_2D_3$, we utilized the monoclonal antibody designated Leu M-3 (Becton, Dickinson & Co., Oxnard, CA). Leu M-3 binds to 80–90% of peripheral blood monocytes and fluid macrophages. In contrast to OKM1 it exhibits minimal binding to granulocytes and does not bind to null cells (33). Leu M-3 did not bind to U937 cells before stimulation (Fig. 4 *A*); however, when U937 cells were incubated with $1\alpha,25(OH)_2D_3$ ($10^{-8}$ M) a shift in fluorescence intensity was detected as demonstrated by an increase in median fluorescence from 10 to 17, signifying the expression of a new surface antigen (Fig. 4 *B*). The addition of hormone to U937 cells cultured together with a substimulatory concentration of lymphocyte-conditioned medium, caused a marked increase in Leu M-3 binding (Fig. 4 *D*). These data

*Table I. Effect of 1α,25(OH)$_2$D$_3$ on [$^3$H]Thymidine Incorporation by U937 Cells*

| Addition to U937 cells | [$^3$H]Thymidine |
|---|---|
| | *cpm* × 10$^{-3}$* |
| None | 240±5.8 |
| 1α,25(OH)$_2$D$_3$ | |
| $10^{-10}$ M | 227±9.4 |
| $10^{-9}$ M | 214±8.8‡ |
| $5 \times 10^{-9}$ M | 176±3.4§ |
| $10^{-8}$ M | 172±1.8§ |
| T lymphocyte-conditioned medium | 232±4.4 |
| T lymphocyte-conditioned medium plus 1α,25(OH)$_2$D$_3$ | |
| $10^{-10}$M | 222±13.4 |
| $10^{-10}$ M | 198±5.3 |
| $5 \times 10^{-9}$ M | 153±8.9‖ |
| $10^{-8}$ M | 126±3.2¶ |

U937 cells at $1 \times 10^5$/ml were incubated at 200 μl/well in 96 plates for 3 d with 1α,25(OH)$_2$D$_3$ ($10^{-10}$–$10^{-8}$ M), or 10% (vol/vol) of cell-free medium conditioned by phytohemagglutinin-stimulated T lymphocytes, or a combination of 1α,25(OH)$_2$D$_3$ and lymphokine in Iscove's modified Dulbecco's medium that contained 10% fetal calf serum. [$^3$H]Thymidine incorporation was assayed on the third day with the addition of 1 μCi of [$^3$H]thymidine/well for 4 h.

* Values shown are means±SD for triplicate samples. Statistical significance of a difference from U937 cells alone is indicated as (‡) $P < 0.02$, and (§) $P < 0.001$; and for a difference from the same concentration of 1α,25(OH)$_2$D$_3$ in the absence of T lymphocytes-conditioned medium as (‖) $P < 0.02$, and (¶) $P < 0.001$. The results are representative of three experiments.

indicate a synergistic effect of lymphokine and 1α,25(OH)$_2$D$_3$ on the expression of two monocyte surface antigens defined by the monoclonal antibodies OKM1 and Leu M-3.

*Effects of 1α,25(OH)$_2$D$_3$ and lymphokine on mononuclear cell factor production by U937 cells.* Medium from U937 cells incubated with 1α,25(OH)$_2$D$_3$ in the presence of either 10% fetal calf serum, conditioned medium from phytohemagglutinin-stimulated T lymphocytes, or a combination of conditioned medium and hormone was then assayed for mononuclear cell factor activity (Table II). Although 1α,25(OH)$_2$D$_3$ caused changes in U937 cells similar to those observed with T lymphocyte-conditioned medium, no mononuclear cell factor activity was demonstrable when U937 cells were incubated with 1α,25(OH)$_2$D$_3$ alone. The addition of 1α,25(OH)$_2$D$_3$ together with conditioned medium from lectin-stimulated T lymphocytes, however, augmented monokine production by the U937 cells. 25(OH)D$_3$ at $10^{-7}$ M, in the presence of 10% fetal calf serum, did not potentiate monokine production by U937 cells stimulated with T lymphocyte-conditioned medium. In a parallel experiment, after 8 d of incubation neither 25(OH)D$_3$ ($10^{-7}$ M) nor 1β,25(OH)$_2$D$_3$ ($10^{-8}$ M) augmented mononuclear cell factor production by U937 cells exposed to T lymphocyte-conditioned medium (Table III).

## Discussion

We have demonstrated that 1α,25(OH)$_2$D$_3$ causes concentration-dependent alterations in U937 cells consistent with monocyte maturation. In addition to changes in cell shape and adherence properties, the hormone causes decreases in proliferation, increases in Fc receptors, and in the expression of monocyte-related surface antigens. These changes are similar to those in our previous observations using conditioned medium from lectin-stimulated cloned T lymphocytes (both OKT4 and OKT8 positive), 100% OKT4 positive noncloned T lymphocytes, and peripheral blood T lymphocytes (22, 29). Monokine production as assayed by stimulation of $PGE_2$ production from adherent rheumatoid synovial cells, however, is not induced by 1α,25(OH)$_2$D$_3$ alone, but the presence of 1α,25(OH)$_2$D$_3$ in combination with conditioned medium from lectin-stimulated T lymphocytes augments mononuclear cell factor production. The effects of 1α,25(OH)$_2$D$_3$ on the U937 cell are probably mediated through a specific cytosolic receptor, since 1β,25(OH)$_2$D$_3$ was not capable of altering U937 cells nor augmenting mononuclear cell factor production.

The inability of $10^{-7}$ M 25(OH)D$_3$ to affect U937 cells in 10% fetal calf serum may be due to several factors. The vitamin D-binding protein in serum binds 25(OH)D$_3$ to a greater degree than 1α,25(OH)$_2$D$_3$ (34), thereby lowering the concentration of free hormone available for entry into the cell. In addition,

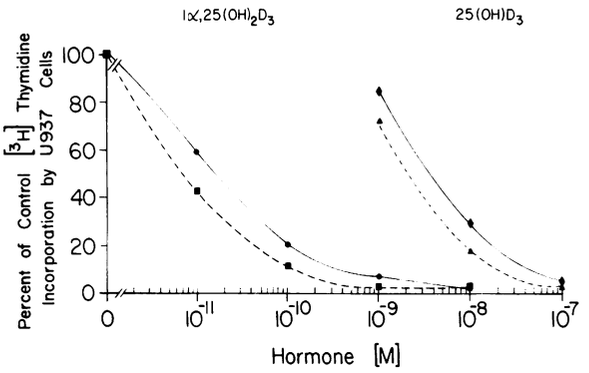


*Figure 2.* Effects of 1α,25(OH)$_2$D$_3$ and 25(OH)D$_3$ on [$^3$H]thymidine incorporation by U937 cells in defined medium. U937 cells at $2 \times 10^5$/ml were incubated for 3 d (200 μl/well) in 96-well plates in Iscove's modified Dulbecco's medium containing bovine serum albumin (6 mg/ml) and transferrin (5 μg/ml) with 1α,25(OH)$_2$D$_3$ ($10^{-11}$–$10^{-9}$ M) (●, ■) or 25(OH)D$_3$ ($10^{-9}$–$10^{-7}$ M) (♦, ▲) added once at the initiation of culture (——) or in daily additions (– – –). [$^3$H]Thymidine incorporation was assayed on the third day by the addition of 1 μCi of [$^3$H]thymidine/well for 4 h. Values shown are means for triplicate samples. The results are representative of three experiments.

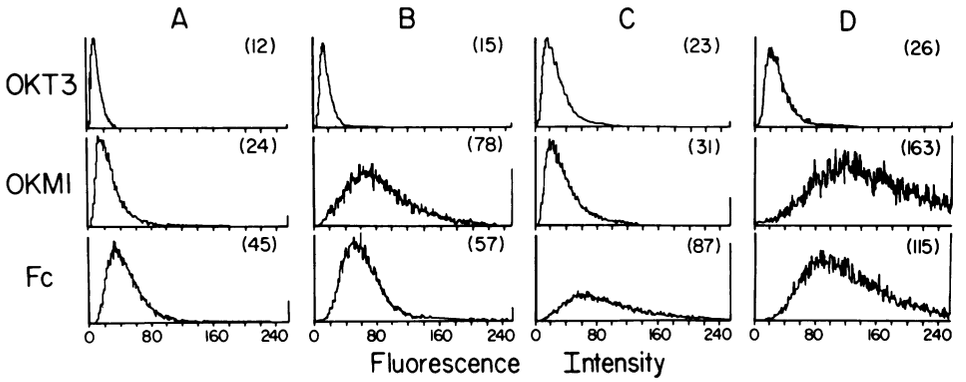


*Figure 3.* Modulation of surface antigens and Fc receptors on U937 cells. U937 cells at 2–4 × $10^5$/ml were incubated in Iscove's modified Dulbecco's medium, 10% fetal calf serum for 48 h alone (*A*), with $1\alpha,25(OH)_2D_3$ ($10^{-8}$ M) (*B*) or conditioned medium from phytohemagglutinin-stimulated T lymphocytes (7.5% vol/vol) (*C*) or lymphokine and $1\alpha,25(OH)_2D_3$ (*D*). OKT3, OKM1, and Fc binding were determined with the flow cytometer and the results expressed as histograms of fluorescence intensity on the horizontal axis and cell number on the vertical axis. Binding of OKT3 served as negative control. Binding of the monoclonal antibodies to Fc receptors was blocked by preincubating the U937 cells with monomeric and aggregated human IgG before measurements of binding of the mouse monoclonal antibodies to specific surface antigens. The median fluorescent channel for each condition is indicated in parentheses. Cell changes could be seen at 24 h, although to a lesser extent than at 48 h. The median fluorescent channel for cells at 24 h of incubation in conditions corresponding to those in the figure are: OKT3, *A* (10), *B* (13), *C* (17), and *D* (21); OKM1 *A* (37), *B* (44), *C* (30), and *D* (53); and Fc binding *A* (37), *B* (40), *C* (82), and *D* (88). The results at 48 h of incubation are representative of six separate experiments.

$1\alpha,25(OH)_2D_3$ has a greater affinity for its cytosolic receptor than $25(OH)D_3$.

To more closely approximate physiologic conditions, experiments were performed with a defined medium of albumin and transferrin. We reasoned that cells outside of the vascular compartment were not in serum and therefore the concentration of free $1\alpha,25(OH)_2D_3$ was presumably greater in the extravascular space. In tissue culture, at any total concentration of $1\alpha,25(OH)_2D_3$ the concentration of free $1\alpha,25(OH)_2D_3$ would be greater in the system utilizing albumin and transferrin rather than 10% fetal calf serum. Indeed, in the absence of serum, changes in cell morphology and decreases in cell proliferation are seen with $1\alpha,25(OH)_2D_3$ at $10^{-11}$–$10^{-10}$ M. Measurements of cell proliferation in serum-free medium or in a defined medium containing albumin and transferrin demonstrate a 200–300-fold greater effectiveness of $1\alpha,25(OH)_2D_3$ vs. $25(OH)D_3$ in stimulating maturation of U937 cells as assessed by decreases in cell division. These changes occur at concentrations of the active hormone that are within the physiological range ($10^{-10}$–$10^{-11}$ M). The difference in efficacy between the two active vitamin $D_3$ metabolites is consistent with their biological potency.

Mononuclear cell factor shares structural and functional homologies with lymphocyte-activating factor (or interleukin 1). Purified murine lymphocyte-activating factor has mononuclear cell factor activity, and conversely mononuclear cell factor from human peripheral blood monocytes is active in lymphocyte-activating factor assays (35). Subsequent work confirms these observations and demonstrates other activities shared by these substances (36, 37). We have previously shown that lymphocyte-activating factor activity is not detected in crude medium from T lymphocyte-stimulated U937 cells, although mononuclear cell factor activity is induced. However, both lym-

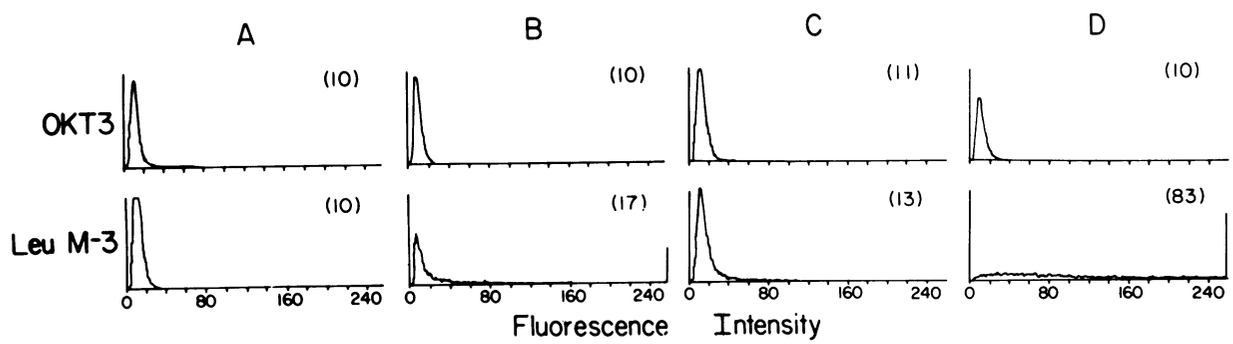


*Figure 4.* Modulation of monocyte surface antigens on U937 cells. U937 cells at 2–4 × $10^5$/ml were incubated in medium containing 10% fetal calf serum for 48 h alone (*A*), with $1\alpha,25(OH)_2D_3$ ($10^{-8}$ M) (*B*), conditioned medium from phytohemagglutinin-stimulated T lymphocytes (7.5% vol/vol) (*C*), or lymphokine and $1\alpha,25(OH)_2D_3$ (*D*). OKT3 and Leu M-3 binding were determined and Fc receptors blocked as described in Fig. 3. The median fluorescent channel for each condition is indicated in parentheses. The results are representative of two separate experiments.

*Table II. Effects of $1\alpha,25(OH)_2D_3$ and Conditioned Medium from Phytohemagglutinin-stimulated T Lymphocytes on Mononuclear Cell Factor Production by U937 Cells*

| Addition to U937 cells | Adherent synovial cells $PGE_2$* |
|---|---|
| | *ng/ml* |
| None | <2.2 |
| $1\alpha,25(OH)_2D_3$ | <2.4 |
| T lymphocyte-conditioned medium | 17.5±7.2 |
| $1\alpha,25(OH)_2D_3$ plus T lymphocyte-conditioned medium | 50.1±8.8 |
| $25(OH)D_3$ | 2.3±0.7 |
| $25(OH)D_3$ plus T lymphocyte-conditioned medium | 13.7±1.3 |

Synovial cells in their second passage were plated at $5 \times 10^4$ cells/well 4 d before bioassay. They were incubated for 3 d with medium from $1 \times 10^5$ U937 cells alone, or U937 cells cultured with $1\alpha,25(OH)_2D_3$ ($10^{-8}$ M) or $25(OH)D_3$ ($10^{-7}$ M), or medium from phytohemagglutinin-stimulated T lymphocytes (10% vol/vol) (all OKT4 positive, noncloned) or a combination of T lymphocyte-conditioned medium and vitamin $D_3$ metabolite as indicated for 3 d in 1 ml of medium. Samples were then diluted 1:5 in Dulbecco's modified Eagle's medium containing 10% fetal calf serum before being added to synovial cells in a final volume of 0.4 ml/well. Basal $PGE_2$ levels were <2.2 ng/ml in unstimulated synovial cells. $PGE_2$ production by synovial cells incubated with phytohemagglutinin alone, conditioned medium from T lymphocytes not stimulated by lectin, or the combination of both was not different from basal levels. The addition of $1\alpha,25(OH)_2D_3$ or $25(OH)D_3$ to cultures of U937 cells containing conditioned medium from nonstimulated T lymphocytes and their subsequent addition to synovial cells did not alter $PGE_2$ production by the synovial cells.

* Values shown are means±SD for triplicate samples. The results are representative of three experiments.

phocyte-activating factor and mononuclear cell factor activities are coeluted after chromatographic removal of an inhibitor of lymphocyte proliferation present in the crude medium (23) (Amento, E. P., J. T. Kurnick, and S. M. Krane. Manuscript submitted for publication).

One possible explanation for our findings that $1\alpha,25(OH)_2D_3$ augments mononuclear cell factor (interleukin 1) production by lymphokine-stimulated U937 cells is the increased cell viability achieved when $1\alpha,25(OH)_2D_3$ is added to the T lymphocyte-conditioned medium. In preliminary experiments we have maintained the adherent subpopulation of differentiated and nonreplicating U937 cells for 21 d after incubation for the first 8 d with T lymphocyte-conditioned medium plus $1\alpha,25(OH)_2D_3$. Mononuclear cell factor activity was present in medium from the 21-d-old cultures (last medium change on day 12), although none was detected in parallel cultures initially incubated with conditioned medium from lectin-stimulated T lymphocytes in the absence of $1\alpha,25(OH)_2D_3$. Whether this protective effect of the vitamin D metabolite is due to stabilization of membrane components, decrease in toxic by-products of U937 cell activation, or interference with the action of a hypothetical toxic T lymphocyte product, remains to be ascertained.

The observations that certain features of monocyte maturation can take place in the absence of induction of monokine production provide an opportunity to explore the nature of the signals involved in these two dissociable events. The T lymphocyte-conditioned medium may contain two distinct activities—one that promotes maturation, the second that stimulates production of monokines. Meltzer (38) has reported that lymphokine-rich supernatant fluids contain both priming and trigger activities when assayed for tumor cytotoxicity on mouse peritoneal macrophages. Studies involving a variety of inducing agents have been performed utilizing the human promyelocytic leukemia cells, HL-60 (39). For example, exposure of the cells to dibutryl cyclic AMP causes plasma membrane differentiation without morphological maturation (40). Recently Olsson et al. (41) have reported that both HL-60 and U937 cells can be primed with retinoic acid to respond to previously substimulatory concentrations of a T lymphocyte-derived differentiation factor or agents that increase cellular cyclic AMP content. It cannot be stated whether or not the effects of $1\alpha,25(OH)_2D_3$ and retinoic acid are mediated through a similar mechanism.

The physiological relevance of the inhibition of cellular replication in U937 cells associated with alterations of surface antigens consistent with cellular differentiation has not yet been

*Table III. Effects of $1\alpha,25(OH)_2D_3$ and Conditioned Medium from Phytohemagglutinin-stimulated T Lymphocytes on Mononuclear Cell Factor Production by U937 Cells*

| Addition to U937 cells | Aherent synovial cells $PGE_2$* |
|---|---|
| | *ng/ml* |
| None | <2.2 |
| $1\alpha,25(OH)_2D_3$ | <2.2 |
| $1\beta,25(OH)_2D_3$ | <2.2 |
| $25(OH)D_3$ | <2.2 |
| T lymphocyte-conditioned medium | 3.3±0.4 |
| $1\alpha,25(OH)_2D_3$ plus T lymphocyte-conditioned medium | 76.1±23.3 |
| $1\beta,25(OH)_2D_3$ plus T lymphocyte-conditioned medium | 3.9±1.4 |
| $25(OH)D_3$ plus T lymphocyte-conditioned medium | 8.5±6.4 |

Synovial cells in their second and third passage (equal numbers) were plated at $5 \times 10^4$ cell/well 3 d before bioassay. They were incubated for 3 d with medium from $1 \times 10^5$ U937 cells alone, or U937 cells cultured with $1\alpha,25(OH)_2D_3$ ($10^{-8}$ M), $1\beta,25(OH)_2D_3$ ($10^{-8}$ M), or $25(OH)D_3$ ($10^{-7}$ M), or medium from phytohemagglutinin-stimulated T lymphocytes (10% vol/vol) (all OKT4 positive, noncloned), or a combination of lymphokine and vitamin $D_3$ metabolite as indicated for 8 d in 1 ml of medium. Samples and controls were assayed as indicated in Table II.

* Values shown are means±SD for triplicate samples. The results are representative of two experiments.

determined. However, these changes can be documented in serum-free medium as well as in defined medium at physiologically relevant concentrations of $1\alpha,25(OH)_2D_3$. In view of our previous findings of a specific cytosolic receptor for $1\alpha,25(OH)_2D_3$ in U937 cells (24, 25), as well as in peripheral blood monocytes and activated, but not resting T lymphocytes (42), and the work of others (7–14), it is likely that this hormone has a role beyond that currently recognized in intestine, bone, and kidney. This possibility is further supported by our observations that interleukin 1 production by lymphokine-stimulated U937 cells is augmented in the presence of $1\alpha,25(OH)_2D_3$. Thus, interactions between classical hormones and some of the hormonelike products of cells of the immune system may play a role not only in monocyte differentiation but in aspects of inflammation in general.

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