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Differentiation Induction of Human Leukemia Cells (HL60) by a Combination of 1,25-Dihydroxyvitamin D_3 and Retinoic Acid (*All Trans* or 9-*Cis*)

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$1,25(OH)_2D_3$ and two stereoisomers of retinoic acid, *all trans* and 9-*cis* retinoic acid, are regulators of cell proliferation and differentiation. The aim of this study was to evaluate the effects of a combination of $1,25(OH)_2D_3$ and retinoic acid (*all trans* or 9-*cis*) on proliferation and cell differentiation of the human promyelocytic leukemia cell line HL60, and to test the reversibility of the induced differentiation. Cell proliferation was inhibited as expected by $1,25(OH)_2D_3$ and *all trans* retinoic acid alone (IC_{50} of cell survival was 4×10^{-7} M, 9×10^{-6} M and 9×10^{-7} M for $1,25(OH)_2D_3$, *all trans* and 9-*cis* retinoic acid, respectively). Combination of $1,25(OH)_2D_3$ and either form of retinoic acid resulted in a partially additive decrease in cell proliferation. $1,25(OH)_2D_3$ induced a monocytic differentiation (100% CD14+ cells with 10^{-7} M $1,25(OH)_2D_3$), while retinoic acid led to a predominantly granulocytic differentiation (36 and 42% CD67+ cells with 10^{-6} M *all trans* and 9-*cis* retinoic acid, respectively). Additive effects on differentiation were observed upon combination of subtherapeutical doses of the drugs, achieving a mainly monocytic population, demonstrating the dominant role of $1,25(OH)_2D_3$ in determining the direction of differentiation. The effects on proliferation and differentiation of the solitary drugs were reversible, while the proliferation arrest and differentiation induced by the combination persisted and even progressed after withdrawal of the drugs. We conclude that $1,25(OH)_2D_3$ and retinoic acid (*all trans* or 9-*cis*) exert additive effects on inhibition of proliferation and induction of cell differentiation of HL60 cells, leading to a persistent differentiation, even after drug withdrawal.

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INTRODUCTION

The classical role of the steroid hormone $1,25(OH)_2D_3$ on calcium homeostasis has long been recognized. This hormone acts through an intracellular receptor leading to gene-regulating functions mediating various biologic responses. The receptor has been cloned and found to belong to the steroid/thyroid hormone superfamily [1]. The $1,25(OH)_2D_3$ receptors are not only present in classical target tissues, regulating calcium homeostasis but are also found in a wide variety of "non-classical" target tissues, including normal or malignant keratinocytes, breast cancer or prostatic cells and cells belonging to the immune system. For this reason the influence of $1,25(OH)_2D_3$ on cell growth has been examined. A vast amount of literature exists on the *in vitro* effects of $1,25(OH)_2D_3$ on the proliferation and induction of differentiation of benign and malignant cells [2, 3].

The active form of vitamin D, $1,25(OH)_2D_3$, is an important inhibitor of cell proliferation and stimulator of cell differentiation, when added to the human promyelocytic leukemia cell line HL60 [4-6]. Similar effects are observed with other substances, such as phorbol myristate acetate (PMA), dimethyl sulfoxide (DMSO), *all trans* retinoic acid and, as recently demonstrated, also 9-*cis* retinoic acid [7-20]. Several of these agents are already used *in vivo* [21-33]. $1,25(OH)_2D_3$ and retinoic acid are both hormones that

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achieve their effects through interactions with their respective receptors, members of the same superfamily of nuclear steroid hormone receptors [34–37]. Recently, interactions between the vitamin D receptor (VDR) and one of the retinoid receptors, retinoic X receptor (RXR), have been described [38–39]. These interactions, namely the formation of heterodimers between activated VDRs and RXRs, are probably important steps in the activation of intracellular pathways by $1,25(OH)_2D_3$. The presence of the natural ligand for RXR, 9-*cis* retinoic acid, appears not to be absolutely necessary to allow the heterodimer formation, but seems to be a facilitating factor [38]. A first aim of the present study was therefore to evaluate the effects of combining 9-*cis* retinoic acid or *all trans* retinoic acid with $1,25(OH)_2D_3$ on cell proliferation and differentiation of HL60 cells.

A second point of interest was the direction of differentiation induced by the combination of $1,25(OH)_2D_3$ and retinoic acid (*all trans* or 9-*cis*). Previous reports demonstrated that $1,25(OH)_2D_3$ induced monocytic, while retinoic acid mainly induced granulocytic differentiation [4–7, 9]. The direction induced by a combination of $1,25(OH)_2D_3$ and retinoic acid will be a reflection of the priority of the intracellular action pathways.

Finally, the last aim was to study the reversibility of the effects induced by $1,25(OH)_2D_3$ and retinoic acid alone or in combination. Due to incomplete differentiation induced by each drug alone, a return to leukemic cells can be observed *in vitro* and *in vivo*, necessitating continuous application of the drugs to maintain their effect [9, 40]. Achieving terminal and irreversible differentiation and growth arrest of the leukemia cells by a harmless, short term treatment is therefore a major goal in designing research and future treatment strategies [41–57]. In the present study, we present evidence for the achievement of prolonged differentiation and growth arrest induced by a combination of $1,25(OH)_2D_3$ and retinoic acid (*all trans* or 9-*cis*).

MATERIALS AND METHODS

Cell line and culture conditions

The human promyelocytic leukemia cell line, HL60, was obtained from the American Type Culture Collection (ATCC, Rockville, MD). Cells were seeded at 1×10^5/ml in 80 cm^2 Falcon (Becton Dickinson, Erembodegem, Belgium) tissue chambers in RPMI-1640 medium supplemented with 20% heat-inactivated fetal calf serum (Gibco, Paisley, Scotland) and Gentamycin 50 μg/ml (Gibco, Paisley, Scotland). Final volume was 25 ml.

$1,25(OH)_2D_3$ and 9-*cis* retinoic acid were a gift from M. Uskokovic (Hoffman-La Roche, Nutley, NJ) and *all trans* retinoic acid was purchased from Sigma (St Louis, MO). These drugs were added to the cell culture in ethanol (final concentration below 0.2%) simultaneously at the start of the experiments. Control cultures treated with vehicle alone showed no evidence of cell differentiation (data not shown). Cells were incubated for 4 or 6 days for the reversibility tests in a humidified atmosphere of 5% CO_2 in air at 37°C. After 4 or 6 days, the Falcon flasks were gently shaken to loosen adherent cells. Cells were washed twice in RPMI, consequently counted and assayed as described below. In the reversibility experiments, cells were then seeded at 2×10^6/ml and incubated in the above described medium and conditions in the absence of the test-substances for another 48 or 96 h.

Cell proliferation assays

After 4 days of incubation and for the reversibility assays after 6 days of incubation or 48 and 96 h after withdrawal of the test drugs, the culture flasks were gently shaken to loosen adherent cells and cells were counted. Trypan blue exclusion was used to distinguish living cells. Cell proliferation was assessed by measuring methyl[^{3}H]thymidine incorporation. Cells were seeded at 1×10^6/ml in the medium described above in a 96-well microtiter plate in a final volume of 200 μl per well. Triplicate cultures were performed. 1 μCi methyl[^{3}H]thymidine (ICN, Asse, Belgium) was added per well and cells were harvested after 7 h incubation at 37°C, 5% CO_2, on filter paper (Whatman 934AH, Maidstone, U.K.) by a semiautomatic cell harvesting system and their radioactivity was counted in a liquid scintillation counter (Coulter electronics Ltd, Dunstable Beds, England).

NBT reduction assay

Superoxide production was assayed by NBT-reducing activity. HL60 cells at 1×10^6 cells/ml were mixed with an equal volume of freshly prepared solution of phorbol 12-myristate 13-acetate (200 ng/ml) and NBT (4-nitroblue tetrazolium) (2 mg/ml) and incubated for 30 min at 37°C. The percentage of cells containing black formazan deposits was determined using a hemacytometer.

Flow cytometry

The presence of cell surface markers was evaluated by a broad panel of monoclonal antibodies: CD14, HLADR (Becton Dickinson, Erembodegem, Belgium), CD36, CD67, CD71 (Serotec, Oxford, England) and CD24 (Janssen Pharmaceuticals, Geel, Belgium). CD14 and HLADR were studied as markers for monocytic differentiation. CD14 seems to be one of the most monocyte specific antigens and is mainly expressed on mature monocytes and macrophages. It seems to play a role in T cell activation as an adhesion molecule. Another important molecule for T cell activation by monocytes and macrophages, that is upregulated when differentiation towards monocytes occurs, is the class II HLADR molecule, playing a crucial role in Ag presentation. As specific granulocyte surface markers, we used

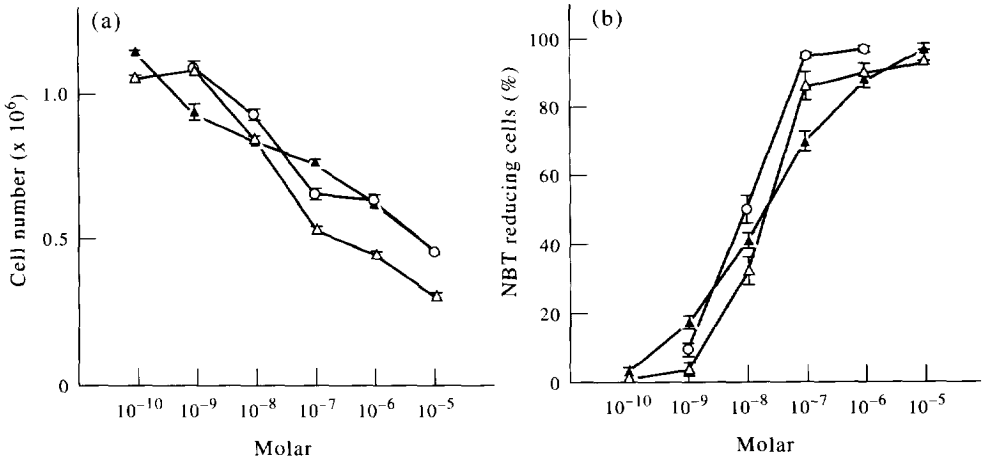

Fig. 1. Dose-dependent inhibition of proliferation and stimulation of differentiation of HL60 cells by $1,25(OH)_2D_3$ (○), *all trans* (△) and 9-*cis* retinoic acid (▲). HL60 cells (1×10^5/ml) were incubated for 4 days with increasing concentrations of the test drugs. Cells were counted and NBT reducing capacity was assayed. All experiments were performed at least 3 times and mean values ± SD are shown.

CD24, present on B cells and granulocytes, and CD67, a maturation (and activation) marker for granulocytes.

After 4 or 6 days of incubation with the differentiating agents and 48 and 96 h after withdrawal), cells were washed twice with ice cold PBS and incubated for 20 min at 4°C with the monoclonal antibodies. Consequently cells were washed twice with ice cold PBS and fixed with 1% paraformaldehyde. Cells were analysed on a Facsstar Plus flowcytometer (Becton Dickinson, Erembodegem, Belgium).

Morphology

Cytospins were made, using a cytocentrifuge and slides were stained with May-Grünwald-Giemsa. Slides were scored by 2 independent investigators by light microscopy.

RESULTS

Effect of $1,25(OH)_2D_3$, all trans and 9-cis retinoic acid on cell growth

A first parameter of cell growth was the number of living cells (recognized by trypan blue exclusion) remaining in culture. These data [Fig. 1(a)] clearly demonstrate the existence of a dose-dependent inhibition of cell survival after incubation with $1,25(OH)_2D_3$, *all trans* and 9-*cis* retinoic acid alone (IC_{50} was 4×10^{-7} M, 9×10^{-6} M and 9×10^{-7} M for $1,25(OH)_2D_3$, *all trans* and 9-*cis* retinoic acid, respectively). An additive effect by combining $1,25(OH)_2D_3$ and one of both forms of retinoic acid was observed [Fig. 2(a)]. These data were completely paralleled by the data of thymidine incorporation (data not shown), illustrating inhibition of active cell proliferation. Both forms of retinoic acid were slightly more active than

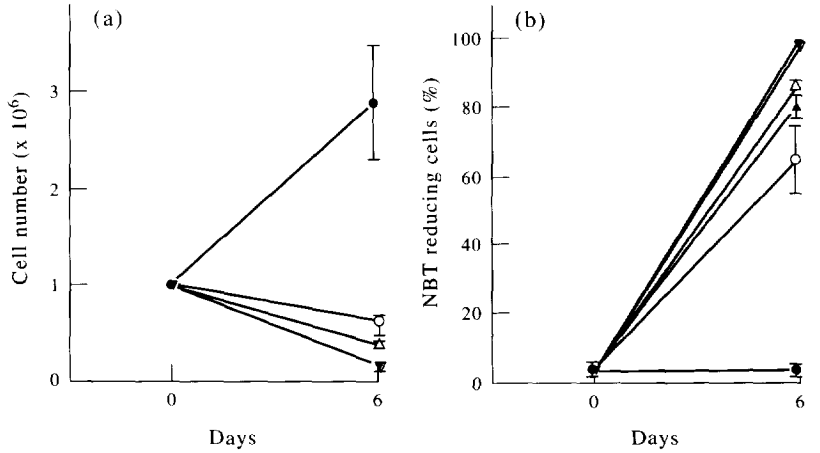

Fig. 2. Partial additive effect of $1,25(OH)_2D_3$ and retinoic acid (*all trans* or 9-*cis*) on cell proliferation and differentiation of HL60 cells. HL60 cells (1×10^5/ml) were incubated for 6 days with $1,25(OH)_2D_3$ (10^{-8} M) or retinoic acid (10^{-7} M) (*all trans* or 9-*cis*) alone, or with a combination of both drugs. Again cell count, thymidine incorporation and NBT reduction was performed. Experiments were performed in 3-fold and mean values ± SD are shown. ●, control; ○, $1,25(OH)_2D_3$; ▲, 9-*cis*; △, *all trans*; ▽, $1,25(OH)_2D_3$ and 9-*cis*; and ▼, $1,25(OH)_2D_3$ and *all trans* retinoic acid.

$1,25(OH)_2D_3$ in inhibiting cell proliferation and clear additive effects could be seen upon combination of either form of retinoic acid with $1,25(OH)_2D_3$. Combination of both retinoic acid isomers alone and of all 3 drugs together had no supplementary additive effect (data not shown). True effects on cell proliferation had to be distinguished from direct toxic effects.

To assess reversibility of the effects on cell proliferation, HL60 cells were incubated for 6 days with $1,25(OH)_2D_3$ and/or retinoic acid and cell proliferation was evaluated at 0, 48 and 96 h after withdrawal of the drugs. Cell count as well as thymidine incorporation [Fig. 3(a and b)] clearly demonstrated reversibility of the growth arrest induced by $1,25(OH)_2D_3$ or retinoic acid alone (increase of cell proliferation in 96 h after withdrawal of $1,25(OH)_2D_3$ is 2.5-fold and of *all trans* and 9-*cis* retinoic acid was 3.1- and 4-fold, respectively), while a full, irreversible growth arrest and even decrease in cell number was achieved by combining $1,25(OH)_2D_3$ and retinoic acid (*all trans* or 9-*cis*) (a reduction in cell number of 50 or 30% was observed with the combination of $1,25(OH)_2D_3$ and *all trans* or 9-*cis* retinoic acid, respectively).

Effects of $1,25(OH)_2D_3$, all trans and 9-cis retinoic acid on cell differentiation

Expression of cell surface markers. A broad panel of cell surface markers were used to determine the nature and extent of differentiation induced by the different substances.

As Fig. 4 demonstrates, $1,25(OH)_2D_3$ induced a dose dependent monocytic differentiation (CD14 expression in 100% of cells incubated with 10^{-7} M $1,25(OH)_2D_3$), whereas retinoic acid mainly induced granulocytic features, also with, however, some monocytic characteristics (14/16% CD14 and 36/42% CD67 positive cells induced by 10^{-6} M *all trans*/9-*cis* retinoic acid, respectively). The direction of differentiation induced by the combination $1,25(OH)_2D_3$ and retinoic acid (*all trans* or 9-*cis*) was clearly dominated by $1,25(OH)_2D_3$, since CD14 was present on almost 100% of cells, but here again granulocytic characteristics were present, especially in the combination of $1,25(OH)_2D_3$ and 9-*cis* retinoic acid (Table 1).

Reversibility of the induced differentiation was tested, by analyzing HL60 cells, preincubated for 6 days with $1,25(OH)_2D_3$ and/or RA, 0, 48 and 96 h after withdrawal of the drugs or their combination. Differentiation induced by $1,25(OH)_2D_3$ and retinoic acid alone proved to be reversible, especially the monocytic differentiation by $1,25(OH)_2D_3$ alone (Fig. 5). The presence of CD14, the main monocytic marker, decreased in $1,25(OH)_2D_3$ incubated cells (10^{-8} M) from 91% (0 h) to 23% (96 h after withdrawal) paralleled by the disappearance of HLADR (9% at 0 h to 2% at 96 h after withdrawal). The granulocyte markers on the retinoic acid treated cells also decreased, but to a lesser extent (13/31% CD67 positive cells and 8/28% CD24 positive cells at 0 h for *all trans* and 9-*cis* retinoic acid, respectively, was reduced to 10/18% CD67 positive and

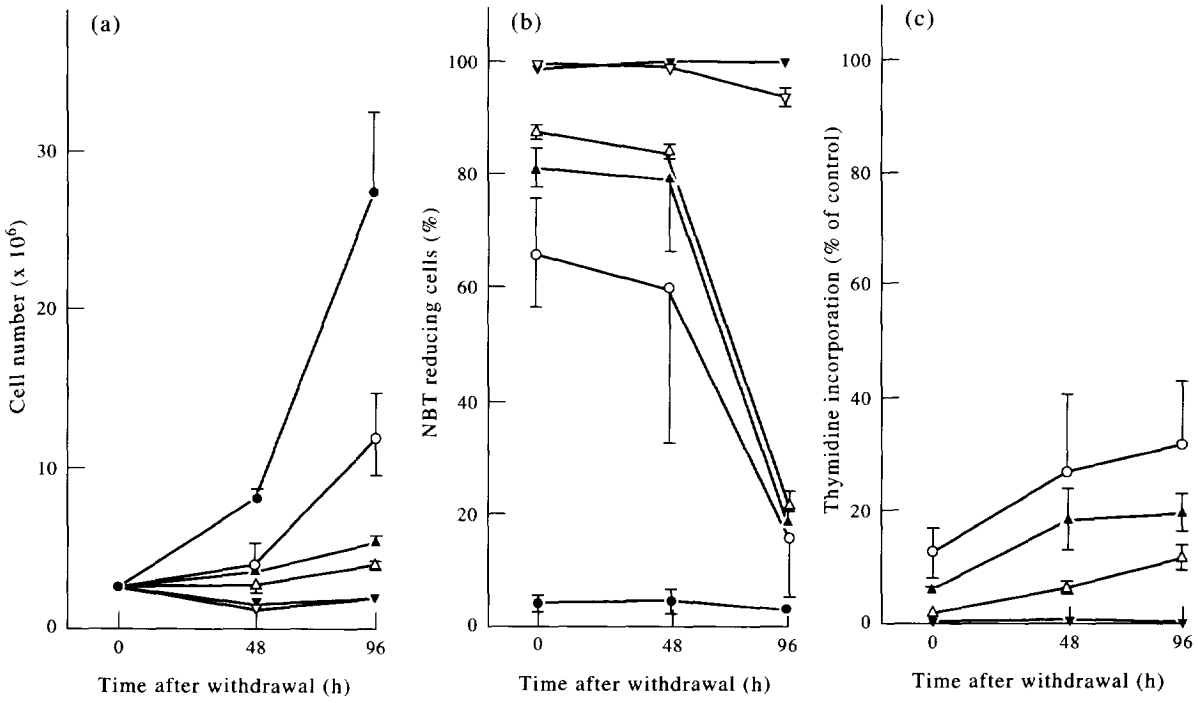

Fig. 3. Reversibility of proliferation arrest and differentiation induced by $1,25(OH)_2D_3$ or retinoic acid or a combination of both drugs. Cell count, thymidine incorporation and NBT reducing capacity are shown. ●, control; ○, $1,25(OH)_2D_3$; ▲, 9-*cis*; △, *all trans*; ▽, $1,25(OH)_2D_3$ and 9-*cis*; and ▼, $1,25(OH)_2D_3$ and *all trans* retinoic acid. Combinations of $1,25(OH)_2D_3$ and retinoic acid (*all trans* or 9-*cis*) induce irreversible growth arrest and end stage differentiation.

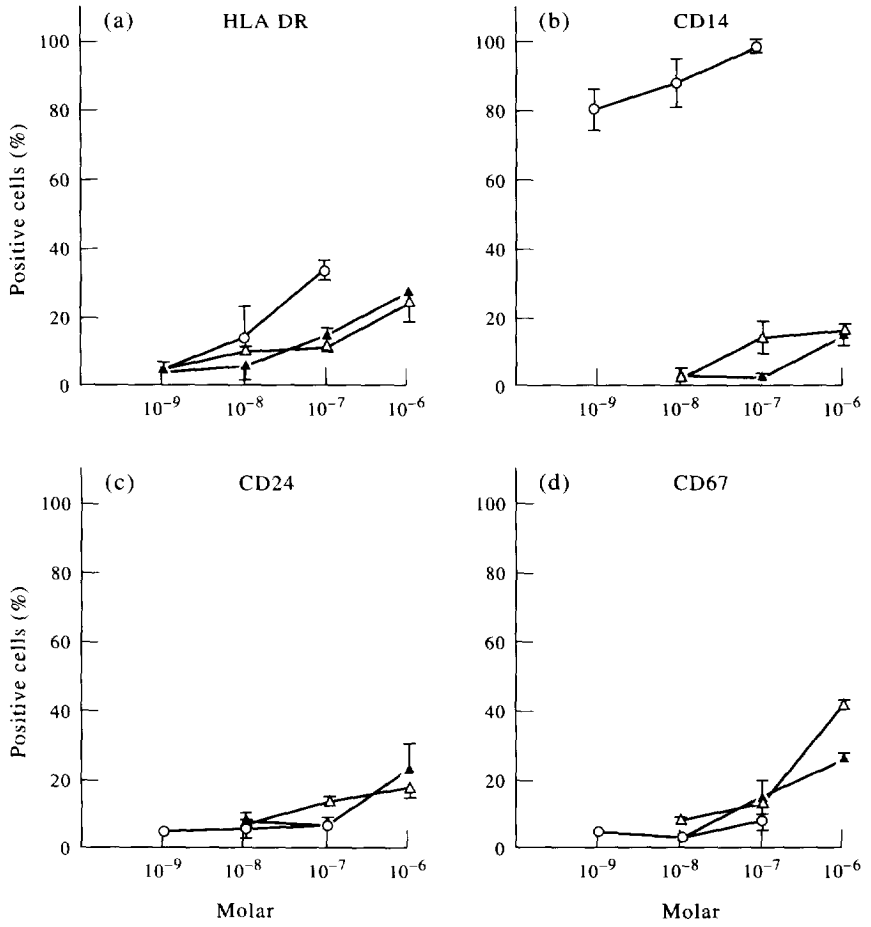

Fig. 4. Cell surface markers induced by $1,25(OH)_2D_3$ or retinoic acid (*all trans* or 9-*cis*). HL60 cells (1×10^5/ml) were incubated for 4 days with $1,25(OH)_2D_3$ (○), *all trans* (△) and 9-*cis* retinoic acid (▲) in increasing concentrations. The number of differentiated cells increased with increasing concentrations of either drug. $1,25(OH)_2D_3$ induced preferentially monocytic markers, while retinoic acid induced granulocytic or mixed features.

3/15% CD24 positive cells at 96 h after withdrawal of the drugs). A remarkable and consistent finding was that after withdrawal of retinoic acid (*all trans* as well as 9-*cis*), the weak CD14 expression that was already present after 6 days incubation, increased to a considerable level (6/26% CD14 positive cells at the moment of withdrawal of *all trans*/9-*cis* retinoic acid, respectively, increased to 19/35% positive cells 96 h after withdrawal).

The combination of $1,25(OH)_2D_3$ and retinoic acid induced an irreversible and even progressing differentiation. However, after drug withdrawal, a change in surface markers could be observed, with a switch from a mainly monocytic (100/92% CD14 positive cells and 21/64% CD67 positive cells at withdrawal of *all trans*/9-*cis* retinoic acid, respectively) to a cell population expressing mixed cell surface markers (CD67 presence increased to 80/76% of *all trans*/9-*cis*

Table 1. Cell surface marker analysis of HL60 cells

	Control	$1,25(OH)_2D_3$ (10^{-8} M)	*all trans* RA (10^{-7} M)	9-*cis* RA (10^{-7} M)	$1,25(OH)_2D_3$ + *all trans* RA	$1,25(OH)_2D_3$ + 9-*cis* RA
CD14	3 ± 1	91 ± 8	6 ± 3	26 ± 3	100 ± 1	92 ± 1
HLADR	3 ± 1	9 ± 5	7 ± 3	17 ± 2	18 ± 6	40 ± 2
CD24	5 ± 1	8 ± 2	8 ± 1	28 ± 8	28 ± 9	57 ± 8
CD67	4 ± 1	8 ± 3	13 ± 3	31 ± 4	21 ± 2	64 ± 3

HL60 cells (1×10^5/ml) were incubated for 6 days with $1,25(OH)_2D_3$ (10^{-8} M), *all trans* (10^{-7} M), 9-*cis* retinoic acid (10^{-7} M) and a combination of these drugs. After 6 days cells were washed and incubated with a panel of monoclonal antibodies. Analysis was performed on a Facstar Plus. $1,25(OH)_2D_3$ induced monocytic differentiation, both isomers of retinoic acid induced a mainly granulocytic differentiation (with also some monocytic characteristics) and the combination of $1,25(OH)_2D_3$ and the retinoic acids induced a mainly monocytic differentiation, and also, especially with 9-*cis* retinoic acid, important granulocytic characteristics. Mean values ± SD are shown.

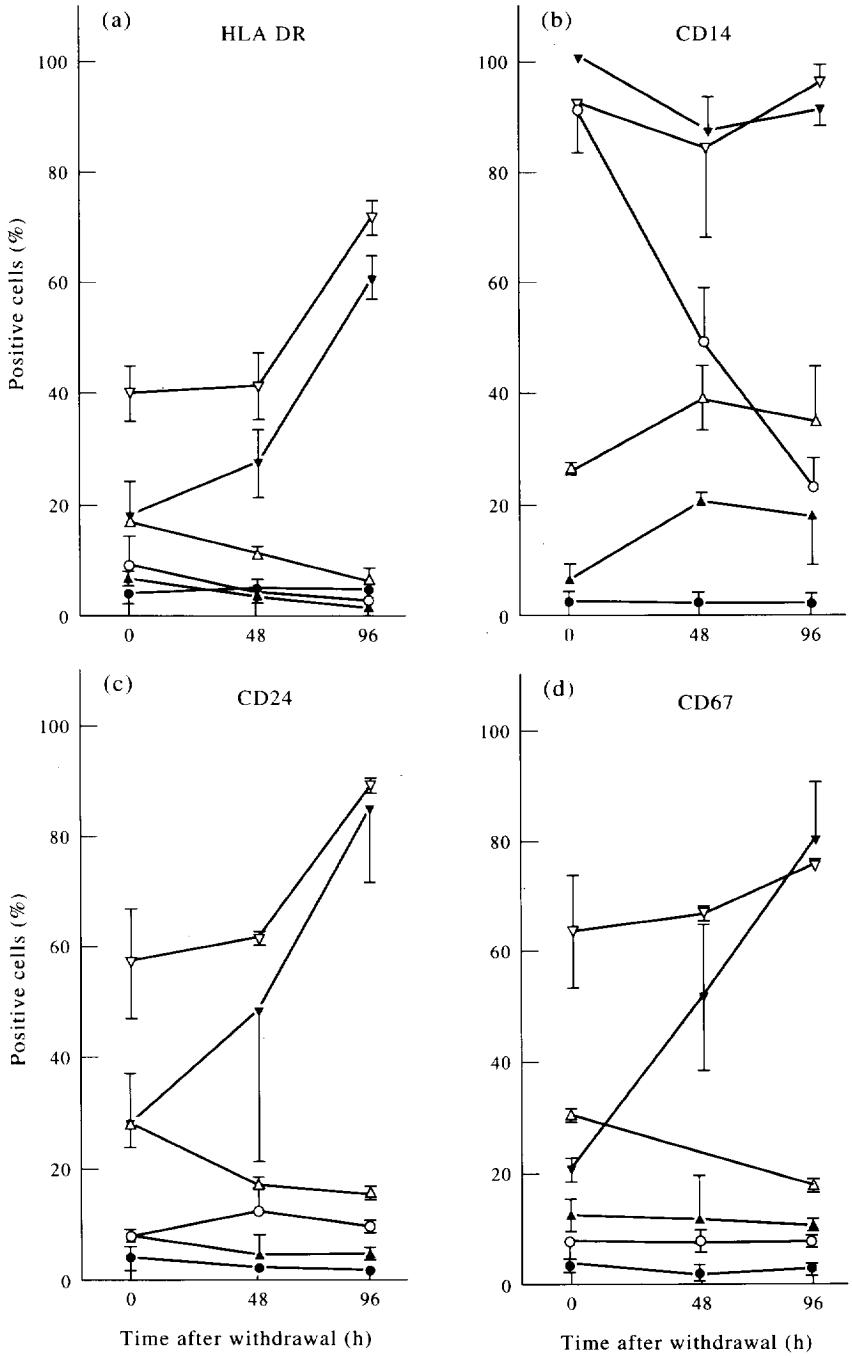

Fig. 5. Reversibility of differentiation induced by $1,25(OH)_2D_3$ or retinoic acid (*all trans* or 9-*cis*). Cells were analyzed with a spectrum of monoclonal antibodies after 6 days of incubation with either drug, alone or with a combination of drugs. Analyses were also performed 48 and 96 h after withdrawal of the original drugs. ●, control; ○, $1,25(OH)_2D_3$; ▲, 9-*cis*; △, *all trans*; ▽, $1,25(OH)_2D_3$ and 9-*cis*; and ▼, $1,25(OH)_2D_3$ and *all trans* retinoic acid. Cell surface markers induced by $1,25(OH)_2D_3$ alone, disappeared quickly after withdrawal of the drug. Also the granulocytic markers induced by retinoic acid diminished after withdrawal, with, however, an increase in CD14. After incubation with the combination of $1,25(OH)_2D_3$ and retinoic acid (*all trans* or 9-*cis*), cell surface markers remained present and even increased after withdrawal of the drugs, suggesting irreversible and even progressing differentiation.

retinoic acid incubated cells 96 h after withdrawal of the drugs).

Functional differentiation of HL60 cells. The functional capacity of the HL60 cells was tested via the NBT reduction test. This test is rather aspecific, since granulocytes as well as monocytes will stain positive. Figure 1(b) demonstrates the dose dependent induction of functional differentiation by $1,25(OH)_2D_3$ and retinoic acid (*all trans* or 9-*cis* retinoic acid) and Fig. 2(b) also shows additive effects here between $1,25(OH)_2D_3$ and retinoic acid. NBT reducing activity was increased from 65, 81 and 88% with $1,25(OH)_2D_3$,

all trans and 9-*cis* retinoic acid alone, respectively, to 100% or upon combination of $1,25(OH)_2D_3$ with *all trans* retinoic acid or 9-*cis* retinoic acid. In Fig. 3(c) the persistence of this functional maturation induced by a combination of $1,25(OH)_2D_3$ and retinoic acid is demonstrated. While the NBT reducing capacity of the drugs alone was lost (from 65 to 4% for $1,25(OH)_2D_3$ and 81/88% to 19/21% for *all trans*/9-*cis* retinoic acid, respectively), almost all cells incubated with the combination maintained a full functional capacity.

Morphology

Cytospin samples, stained with May-Grünwald Giemsa confirmed the direction and extent of differentiation of the HL60 cells induced by the different substances and their combinations (Fig. 6). The HL60 cells differentiate towards monocytes with $1,25(OH)_2D_3$ and granulocytes with retinoic acid. Combinations of $1,25(OH)_2D_3$ and retinoic acid induce mainly monocytic characteristics. After withdrawal of the drugs, $1,25(OH)_2D_3$ or retinoic acid treated cells regain the leukemic phenotype and cell divisions appear more frequently. Cells in the combined treatment group remain well differentiated, with mixed phenotypes appearing, and apoptotic cells can be seen.

DISCUSSION

Promyelocytic leukemias are characterized by a differentiation arrest at an immature, still proliferating cell stage [9, 11, 27, 29]. Treatment perspectives therefore not only include (new) cytostatic drugs but more interestingly differentiation-inducing agents [4–20]. Clinical results with these differentiating drugs are encouraging [21–33], but their application remains limited mainly through their toxicity, especially after long term treatment [9]. This long term treatment is necessary to maintain cell differentiation and to prevent the return to the original malignant state. Combination of several of the differentiating agents could increase their efficacy in inducing terminal, irreversible differentiation and growth arrest of the leukemic cells and allow for dose reduction, thereby decreasing their side effects.

In the present study, we investigated the effects of combining $1,25(OH)_2D_3$ and retinoic acid (*all trans* or 9-*cis*) on the human promyelocytic leukemia cell line, HL60.

$1,25(OH)_2D_3$, the active form of vitamin D, and retinoic acid both achieve their cellular effects through interaction with their proper receptors that are members of the steroid/thyroid hormone superfamily [34–37]. This family not only includes the vitamin D receptor (VDR) and retinoic acid receptor (RAR), the receptors for $1,25(OH)_2D_3$ and *all trans* retinoic acid, respectively, but also the retinoic X receptor (RXR) (the true receptor for 9-*cis* retinoic acid), the thyroid receptor and other steroid hormone receptors. A major part of the cellular effects of $1,25(OH)_2D_3$ is mediated through interactions between the activated VDR and the RXR [38, 39]. $1,25(OH)_2D_3$ can indeed work along two distinct pathways, the first being through the formation of homodimers between 2 activated VDRs, that will then bind to DNA and initiate a cascade of cellular events. Recently, however, a second and more generally used pathway has been described, whereby the activated VDR forms a heterodimer with an RXR and then interacts with vitamin D responsive elements in the DNA, leading to gene transcription (e.g. osteopontin). The presence of the natural RXR ligand, 9-*cis* retinoic acid, is not absolutely necessary to allow heterodimer formation, but seems to be a permissive factor [58–60]. The main point of interest of our study was therefore to examine whether the presence of this ligand would modify the intracellular action of $1,25(OH)_2D_3$.

We found indeed an additive effect between $1,25(OH)_2D_3$ and 9-*cis* retinoic acid on proliferation and differentiation of HL60 cells, but also *all trans* retinoic acid potentiated the actions of $1,25(OH)_2D_3$. These experiments do not allow us to draw any conclusions on the exact mechanisms of interaction between $1,25(OH)_2D_3$ and both forms of retinoic acid. Indeed, the observed potentialization of the $1,25(OH)_2D_3$ effect by retinoic acid could just be the result of the independent action of both substances through their own receptor, but the stimulation of heterodimer formation (VDR–RXR) by the presence of the natural ligand for RXR, 9-*cis* retinoic acid, could be an important additional factor. This is suggested mainly by the fact that addition of 9-*cis* retinoic acid potentiates the $1,25(OH)_2D_3$-effect more efficiently than *all trans* retinoic acid. *All trans* retinoic acid can namely also interact with the RXR, but only after isomerization into 9-*cis* retinoic acid. Additional experiments are needed to elucidate the importance of those intracellular interactions.

Previous reports on combinations of *all trans* retinoic acid and $1,25(OH)_2D_3$ in leukemia are conflicting, with some reports demonstrating either synergism [61] or no additive effect, depending on the cell line used [62–64] while others even showed clear antagonism [64]. The additive effect of $1,25(OH)_2D_3$ and retinoic acid on differentiation observed in our experiment contradicts the results of Öberg *et al.* [64]. In these experiments, preincubation of HL60 cells with *all trans* retinoic acid antagonized the differentiating action of $1,25(OH)_2D_3$. This antagonism could be explained, as the authors suggest, by the downregulation of VDR by *all trans* retinoic acid. In our experiments, however, both drugs were added simultaneously, thus allowing for direct interactions. The importance of simultaneous addition to observe additive and even synergistic effects between

retinoic acid and $1,25(OH)_2D_3$ was also described by others [62].

Confirming previous findings [4–7], the direction of the induced differentiation was mainly monocytic for $1,25(OH)_2D_3$. The differentiation induced by the retinoic acids was mainly granulocytic, but, especially when using higher doses of 9-*cis* retinoic acid, monocytic characteristics also arose. The induction of mixed monocytic/granulocytic features on HL60 cells by retinoic acid has already been observed previously, since it has been demonstrated that retinoic acid could induce the c-fms (monocyte colony-stimulating factor receptor) gene in HL60 cells [65]. After withdrawal of retinoic acid from the culture supernatant, this monocytic differentiation direction became even more apparent. Upon combination of $1,25(OH)_2D_3$ and retinoic acid (*all trans* or 9-*cis*), the main direction of differentiation was clearly monocytic. Others have also demonstrated this predominance of $1,25(OH)_2D_3$ over retinoic acid in determining the direction of differentiation [62, 63]. This monocytic differentiation may be a gain in clinical applications of retinoic acid in the treatment of promyelocytic leukemias, since the most severe complication of treatment with retinoic acid is the "retinoic acid syndrome", characterized by a pulmonary leukostasis, coinciding with a rapid increase in the number of mature granulocytes [66, 67].

The most important advantage of the combined use of $1,25(OH)_2D_3$ and retinoic acid is that the achieved differentiation stage is persistent, with even progression of differentiation to more mature stages and cell death, after removal of the drugs from the culture

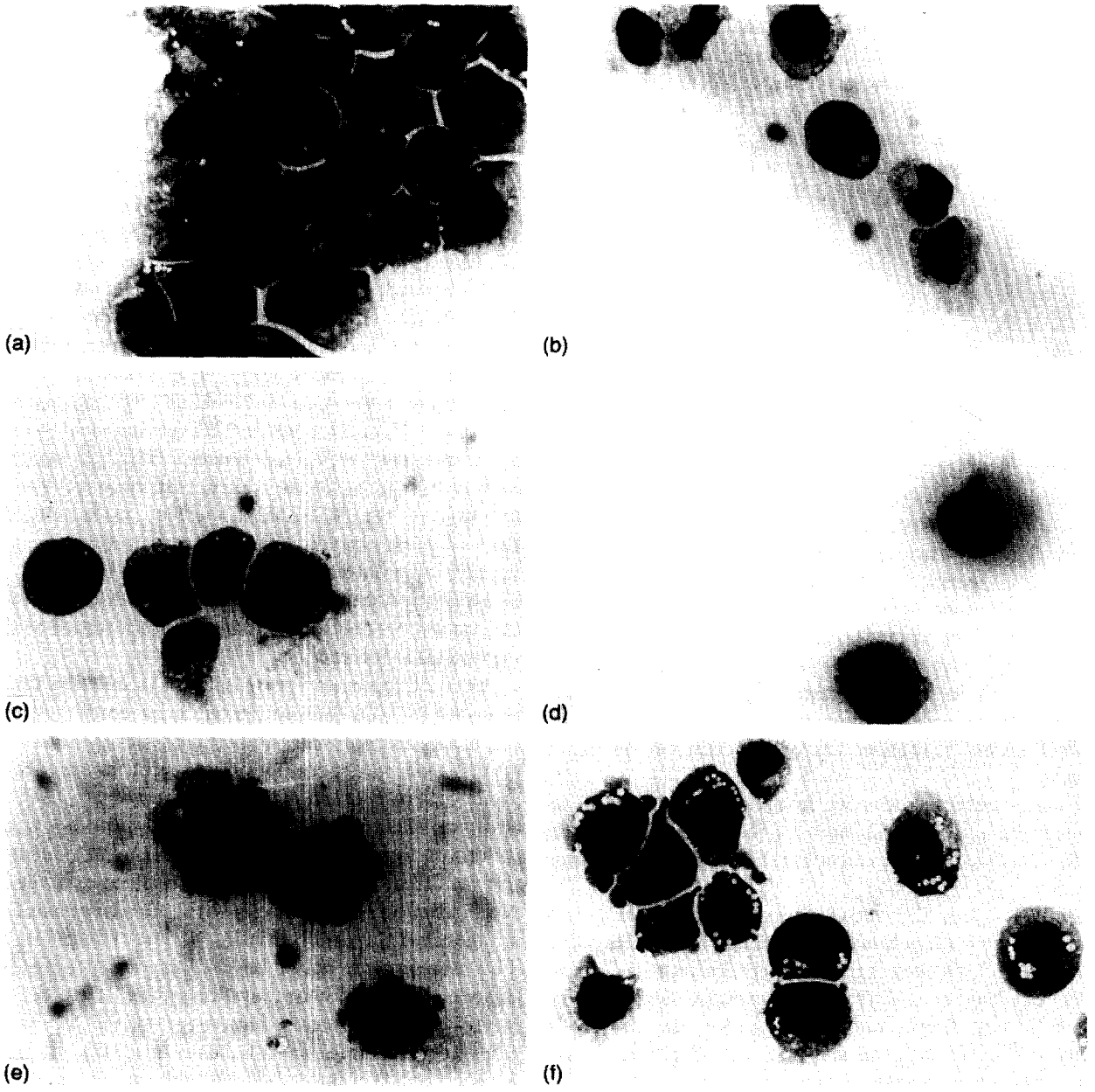

Fig. 6(a–f)—*legend opposite*

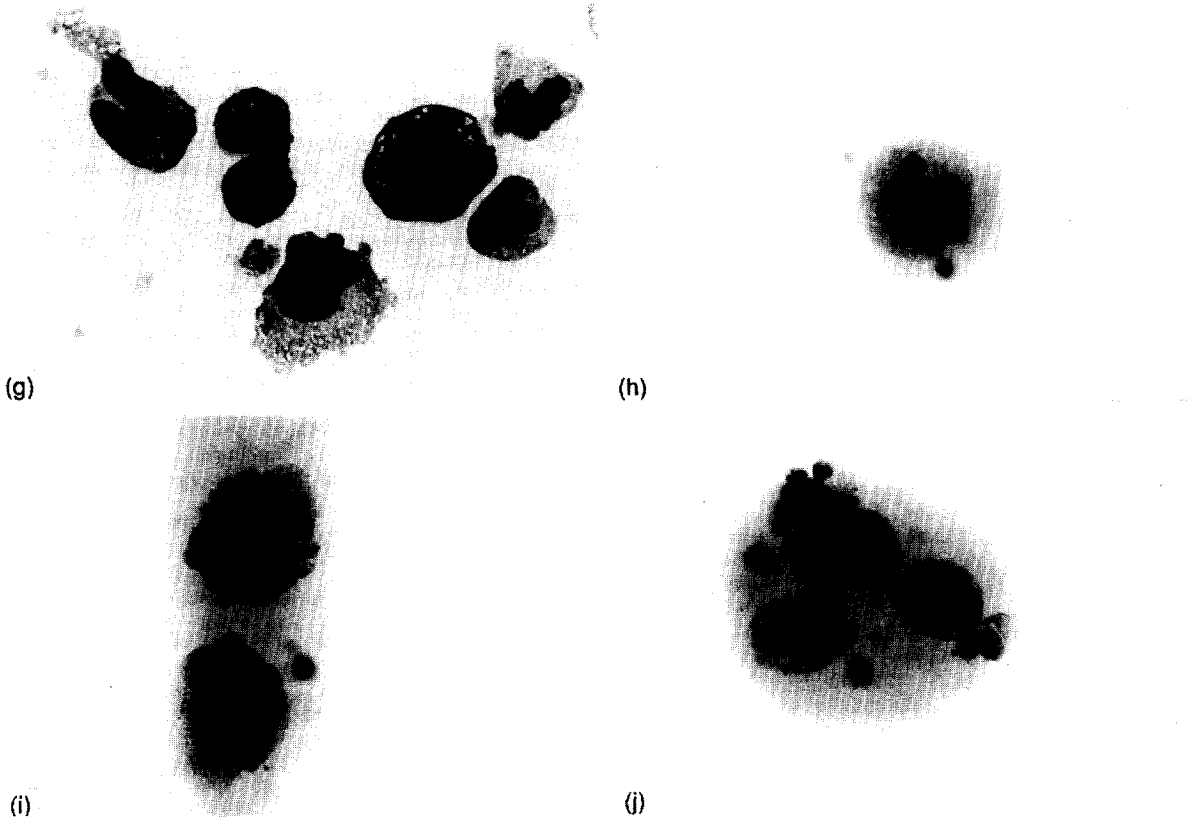

Fig. 6. Morphology of HL60 cells incubated with $1,25(OH)_2D_3$ (10^{-8} M) or retinoic acid (*all trans* or 9-*cis*, 10^{-7} M) or a combination of both drugs. Cells were stained with May-Grünewald-Giemsa (×1250) after 6 days incubation and 48 and 96 h after withdrawal of drugs. The original leukemic cells (a) differentiate towards monocytes with $1,25(OH)_2D_3$ (b) and granulocytes with retinoic acid (c). Combinations of $1,25(OH)_2D_3$ and retinoic acid induce mainly monocytic characteristics (d and e). After withdrawal of the drugs, $1,25(OH)_2D_3$ (f) or retinoic acid (g) treated cells regain the leukemic phenotype and cell divisions appear more frequently. Cells in the combined treatment group remain well differentiated, with mixed phenotypes appearing (h and i), and apoptotic cells (j) can be seen.

medium. This progression of differentiation, even days after withdrawal of the initial drugs, can probably be explained by the achievement of a certain differentiation stage, where cytokine production arises enabling further differentiation [68]. Irreversible differentiation of leukemic cells by a safe and short term treatment is the final goal in the treatment of leukemic patients. Up to now, this final differentiation could only be maintained through long term and repetitive administration of drugs (e.g. retinoic acid) [9]. Upon withdrawal, frequent recurrences are noted, indicating that leukemic cells still persisted in the bone marrow. Also *in vitro* it has been proved previously that the effects of differentiating agents such as $1,25(OH)_2D_3$ and retinoic acid are reversible, partly because of true reversion of more differentiated cells to less differentiated cells and partly due to overgrowth by remaining undifferentiated leukemic cells [9]. A combination of differentiating agents, however, may provide a solution.

In conclusion, these data demonstrate that a combination of $1,25(OH)_2D_3$ and retinoic acid (*all trans* or 9-*cis*) leads to an additive inhibition of proliferation of HL60 cells and more importantly, induction of persistent differentiation towards monocyte like cells. After removal of the hormones, cell growth does not recur and differentiation progresses even further. Clinical application in myeloid leukemias could be considered, especially by using new synthetic analogs of $1,25(OH)_2D_3$, with less calcemic effects and even more potent differentiating capacities [69–71].

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REFERENCES

1. Baker A. R., McDonnell D. P., Hughes M., Crisp T. M., Mangelsdorf D. J. and Haussler M. R.: Cloning and expression of full-length cDNA encoding human vitamin D receptor. *Proc. Natn. Acad. Sci. U.S.A.* 85 (1988) 3294–3298.
2. Walters M. R.: Newly identified actions of the vitamin D endocrine system. *Endocrine Rev.* 13 (1992) 719–764.
3. Bikle D. D. and Pillai S.: Vitamin D, calcium, and epidermal differentiation. *Endocrine Rev.* 14 (1993) 3–19.
4. McCarthy D. M., San Miguel J. F., Freake H. C., Greene P. M., Zola H., Catovsky D. and Goldman J. M.: 1,25-Dihydroxyvitamin D3 inhibits proliferation of human promyelocytic leukaemia

(HL60) cells and induces monocyte-macrophage differentiation in HL60 and normal human bone marrow cells. *Leuk. Res.* 7 (1983) 51–55.
5. Murao S., Gemmell M. A., Callaham M. F., Anderson N. L. and Huberman E.: The control of macrophage cell differentiation in human promyelocytic (HL60) leukemia cells by 1,25-dihydroxyvitamin D3. *Cancer Res.* **43** (1983) 4989–4996.
6. Mangelsdorf D. J., Koeffler H. P., Donaldson C. A., Pike J. W. and Haussler M. R.: 1,25-Dihydroxyvitamin D3-induced differentiation in a human promyelocytic leukemia cell line (HL60): receptor-mediated maturation to macrophage-like cells. *J. Cell. Biol.* **98** (1984) 391–398.
7. Breitman T. R., Selonick S. E. and Collins S. J.: Induction of differentiation of the human promyelocytic leukemia cell line (HL60) by retinoic acid. *Proc. Natn. Acad. Sci. U.S.A.* 77 (1980) 2936–2940.
8. Collins S. J., Robertson K. A. and Mueller L.: Retinoic acid-induced granulocytic differentiation of HL60 myeloid leukemia cells is mediated directly through the retinoic acid receptor (RAR-α). *Molec. Cell. Biol.* **10** (1990) 2154–2163.
9. Warrell R. P. Jr., de Thé H., Wang Z-Y. and Degos L.: Acute promyelocytic leukemia. *New Eng. J. Med.* **329** (1993) 177–189.
10. Rovera G., Santoli D. and Damsky C.: Human promyelocytic leukemia cells in culture differentiate into macrophage-like cells when treated with a phorbol diester. *Proc. Natn. Acad. Sci. U.S.A.* **76** (1979) 2779–2783.
11. Gale R. P. and Foon K. A.: Acute myeloid leukemia: recent advances in therapy. *Clin. Haemat.* **15** (1986) 781–810.
12. Matsuhima Y., Kawachi E., Tanaka H., Kagechika H., Hashimoto Y. and Shudo K.: Differentiation-inducing activity of retinoic acid isomers and their oxidized analogs on human promyelocytic leukemia HL60 cells. *Biochem. Biophys. Res. Commun.* **189** (1992) 1136–1142.
13. Joyce D. A. and Steer J. H.: Differentiation of the U-937 promonocytic cell line induced by phorbol myristate acetate of retinoic acid: effect of aurothiomalate. *Agents Actions* **37** (1992) 305–310.
14. Dulaney A. M. and Murgatroyd R. J.: Use of trans-retinoic acid in the treatment of acute promyelocytic leukemia. *Ann. Pharmacother.* 27 (1993) 211–214.
15. Sakashita A., Kizaki M., Pakkala S., Schiller G., Tsuruoka N., Tomosaki R., Cameron J. F., Dawson M. I. and Koeffler H. P.: *9-cis*-retinoic acid: effects on normal and leukemic hematopoiesis *in vitro*. *Blood* **81** (1993) 1009–1016.
16. Hofmann S. L.: Retinoids "differentiation agents" for cancer treatment and prevention. *Am. J. Med. Sci.* **304** (1992) 202–213.
17. Parkinson D. R., Smith M. A., Cheson B. D., Stevenson H. C. and Friedman M. A.: *Trans*-retinoic acid and related differentiation agents. *Semin. Oncol.* **19** (1992) 734–741.
18. Baez A. and Sepulveda J.: Myeloid differentiation of HL60 cells induced by anti-tumor drug 3-nitrobenzothiazolo[3,2-a]quinolinium. *Leuk. Res.* **16** (1992) 363–370.
19. Miller W. H. Jr., Jakubowski A. and Warrell R. P. Jr.: *9-cis* retinoic acid (RA), the RXR ligand, is an effective cytodifferentiating agent for acute promyelocytic leukemia (APL) cells. *Blood* **80** (1992) Suppl. 108a, abstract.
20. Joyce D. A. and Steer J. H.: Differentiation of the U-937 promonocytic cell line induced by phorbol myristate acetate or retinoic acid: effect of aurothiomalate. *Agents Actions* **37** (1992) 305–310.
21. Sun G. L., Huang Y. G., Chang X. F., Jiang G. S. and Zhang T.: Clinical study on all-*trans* retinoic acid in treatment of 544 cases of acute promyelocytic leukemia treated. *Clin. J. Hematol.* 13 (1992) 135–137.
22. Castaigne S., Chomienne C. and Daniel M. T.: All-*trans* retinoic acid as a differentiation therapy for acute promyelocytic leukemia. I. Clinical results. *Blood* **76** (1990) 1704–1709.
23. Warrell R. P. Jr., Frankel S. R. and Miller W. H. Jr.: Differentiation therapy of acute promyelocytic leukemia with tretinoin (all-*trans*-retinoic acid). *New Engl. J. Med.* **324** (1991) 1385–1393.
24. Chen Z. W., Xue Y. Q. and Zhang R. I.: A clinical and experimental study on all-*trans* retinoic acid-treated acute promyelocytic leukemia patients. *Blood* **78** (1991) 1413–1419.
25. Fearon E. R., Burke P. J., Schiffer C. A., Zehnbauer B. A. and Vogelstein B.: Differentiation of leukemia cells to polymorphonuclear leukocytes in patients with acute nonlymphocytic leukemia. *New Engl. J. Med.* **315** (1986) 15–24.
26. Daenen S., Vellanga E., van Dobbenburgh O. A. and Halie M. R.: Retinoic acid as antileukemic therapy in a patient with acute promyelocytic leukemia and Aspergillus pneumonia. *Blood* **67** (1986) 559–561.
27. Koeffler H. P.: Induction of differentiation of human acute myelogenous leukemia cells: therapeutic implications. *Blood* **62** (1983) 709–721.
28. Huang M. E., Ye Y. C. and Chen S. R.: Use of all-*trans* retinoic acid in the treatment of acute promyelocytic leukemia. *Blood* 72 (1988) 567–572.
29. Warrell R. P, Jr., de Thé H., Wang Z. Y. and Degos L.: Acute promyelocytic leukemia. *Medical Progress* **329** (1993) 177–190.
30. Hamidah H. N., Cheong S. K. and Ainoon O.: *In vivo* evidence of *all-trans* retinoic acid inducing maturation in acute promyelocytic leukaemia. *Acta Haematol.* **89** (1993) 50–51.
31. Chen Z. X., Tao R. T. and Xia X. M.: The present status in *all-trans* retinoic acid (ATRA) treatment for acute promyelocytic leukemia patients: further understanding and comprehensive strategy are required in the future. *Leuk. Lymphoma* 8 (1992) 247–252.
32. Goss G. D. and McBurney M. W.: Physiological and clinical aspects of vitamin A and its metabolites. *Crit. Rev. Clin. Lab. Sci.* **29** (1992) 185–215.
33. Degos L.: Approaches for an explanation of the differentiating effect of *all trans* retinoic acid in acute promyelocytic leukemia. *Leukemia* **6** (1992) 44–46.
34. Petkovich M., Brand N. J., Krust A. and Chambon P.: A human retinoic acid receptor which belongs to the family of nuclear receptors. *Nature* **330** (1987) 444–450.
35. Beato M.: Gene regulation by steroid hormones. *Cell* **56** (1989) 335–344.
36. Hashimoto Y. and Shudo K.: Retinoids and their nuclear receptors. *Cell Biol. Rev.* **25** (1991) 209–235.
37. Evans R. M.: The steroid and thyroid hormone receptor superfamily. *Science* **240** (1988) 889–895.
38. Carlberg C., Bendik I., Wyss A., Meier E., Sturzenbecker L. J., Grippo J. F. and Hunziker W.: Two nuclear signalling pathways for vitamin D. *Nature* **361** (1993) 657–660.
39. Green S.: Promiscuous liaisons. Nuclear hormone receptors. *Nature* **361** (1993) 590–591.
40. Bar-Shavit Z., Kahn A. J., Stone K. R., Trial J., Hilliard T., Reitsma P. H. and Teitelbaum S. L.: Reversibility of vitamin D-induced human leukemia cell-line maturation. *Endocrinology* **118** (1986) 679–686.
41. Sporn M. B. and Roberts A. B.: Interactions of retinoids and transforming growth factor-β in regulation of cell differentiation and proliferation. *Molec. Endocr.* 5 (1991) 3–7.
42. Kliewer S. A., Umesono K. K., Mangelsdorf D. J. and Evans R. M.: Retinoid X receptor interacts with nuclear receptors in retinoic acid, thyroid hormone and vitamin D_3 signalling. *Nature* 355 (1992) 446–449.
43. Huang M. E., Ye Y. C. and Chen S. R.: All-*trans* retinoic acid with or without low dose cytosine arabinoside in acute promyelocytic leukemia: report of 6 cases. *Chin. Med. J. (Engl.)* **100** (1987) 949–953.
44. Breitman T. R. and He R. Y.: Combinations of retinoic acid with either sodium butyrate, dimethyl sulfoxide, or hexamethylene bisacetamide synergistically induce differentiation of the human myeloid leukemia cell line HL60. *Cancer Res.* 50 (1990) 6268–6273.
45. Bollag W.: Retinoids and interferon: a new promising combination? *Br. J. Haematol.* **79** (1991) 87–91.
46. Hassan H. T. and Rees J.: Triple combination of retinoic acid plus actinomycin D plus dimethylformamide induces differentiation in human acute myeloid leukaemic blasts in primary culture. *Cancer Chemother. Pharmac.* **26** (1990) 26–30.
47. Tohda S., Minden M. D. and McCulloch E. A.: Interactions between retinoic acid and colony-stimulating factors affecting the blast cells of acute myeloblastic leukemia. *Leukemia* 5 (1991) 591–597.
48. Valtieri M., Boccoli G., Testa U., Barletta C. and Peschle C.: Two-step differentiation of AML-193 leukemic cell line: terminal maturation is induced by positive interaction of retinoic acid with granulocyte colony-stimulating factor (CSF) and vitamin D3 with monocyte CSF. *Blood* 77 (1991) 1804–1812.

49. Sakashita A., Nakamaki T., Tsuruoka N., Honma Y. and Hozumi M.: Granulocyte colony-stimulating factor, not granulocyte-macrophage colony-stimulating factor, co-operates with retinoic acid on the induction of functional *n*-formyl-methionyl-phenylalanine receptors in HL60 cells. *Leukemia* 5 (1991) 26–31.
50. Testa U., Masciulli R., Tritarelli E., Pustorino R., Mariani G., Martucci R., Barberi T., Camagna A., Valtieri M. and Peschle C.: Transforming growth factor-β potentiates vitamin D3-induced terminal monocytic differentiation of human leukemic cell lines. *J. Immunol.* 150 (1993) 2418–2430.
51. Zhang L., Nakaya K., Yoshida T. and Kuroiwa Y.: Induction by bufalin of differentiation of human leukemia cells HL60, U937 and ML1 toward macrophage/monocyte-like cells and its potent synergistic effect on the differentiation of human leukemia cells in combination with other inducers. *Cancer Res.* 52 (1992) 4634–4641.
52. Huang F., Zhao H. P., Gao X. Z., Dai M. M. and Fan L. L.: Recombinant human G-CSF and retinoic acid in synergistically inducing granulocyte differentiation of human promyelocytic leukemic cells. *Chin. Med. J. Engl.* 105 (1992) 707–712.
53. Morita K., Nishikawa M., Kobayashi K., Deguchi K., Ito M., Nakano T., Shima H., Nagao M., Kuno T. and Tanaka C.: Augmentation of retinoic acid-induced granulocytic differentiation in HL60 leukemia cells by serine/threonine protein phosphatase inhibitors. *FEBS Lett.* 314 (1992) 340–344.
54. Hassan H. T., Zyada L. E., Ragab M. H. and Rees J. K.: New synergistic combinations of differentiation inducing agents in the treatment of acute myeloid leukaemia. *Eur. J. Clin. Pharmac.* 41 (1991) 531–536.
55. Hassan H. T.: Recombinant human interleukin-6 enhances the antiproliferation and differentiation inducing effects of retinoic acid in HL60 human myeloid leukaemic cells. *Clin. Exp. Pharmac. Physiol.* 18 (1991) 847–851.
56. Takeuchi K., Ishihara H., Tsuneoka K. and Shikita M.: Barbiturates enhance retinoic acid or 1,25-dihydroxyvitamin D3-induced differentiation of leukemia HL60 cells. *Biochem. Biophys. Res. Commun.* 178 (1991) 263–268.
57. Santini V., Colombat P., Dewel R., van Gurp R., Touw I. and Lowenberg B.: Induction of granulocytic maturation in acute myeloid leukemia by G-CSF and retinoic acid. *Leuk. Res.* 15 (1991) 351–360.
58. Levin A. A., Struzenbecker L. J. and Kazmer S.: *9-cis* Retinoic acid stereoisomer binds and activates the nuclear receptor RCR. *Nature* 355 (1992) 359–361.
59. Heyman R. A., Mangelsdorf D. J. and Dyck J. A.: *9-cis* Retinoic acid is a high affinity ligand for the retinoid X receptor. *Cell* 68 (1992) 397–406.
60. Lehmann J. M., Long L. and Fanjul A.: Retinoids selective for retinoid X receptor response pathways. *Science* 258 (1992) 1944–1946.
61. Brown G., Bunce C. M., Rowlands D. C. and Williams G. R.: All-trans retinoic acid and 1,25-dihydroxyvitamin D3 co-operate to promote differentiation of the human promyeloid leukemia cell line HL60 to monocytes. *Leukemia* 8 (1994) 806–815.
62. Taimi M., Chateau M. T., Cabane S. and Marti J.: Synergistic effect of retinoic acid and 1,25-dihydroxyvitamin D3 on the differentiation of the human monocytic cell line U937. *Leuk. Res.* 15 (1991) 1145–1152.
63. Tanaka Y., Shima M., Yamaoka K., Okada S. and Seino Y.: Synergistic effect of 1,25-dihydroxyvitamin D3 and retinoic acid in inducing U937 cell differentiation. *J. Nutr. Sci. Vitaminol. Tokyo* 38 (1992) 415–426.
64. Öberg F., Botling J. and Nilsson K.: Functional antagonism between vitamin D3 and retinoic acid in the regulation of CD14 and CD23 expression during monocytic differentiation of U-937 cells. *J. Immunol.* 150 (1993) 3487–3495.
65. Hsu H. C., Yang K., Kharbanda S., Clinton S., Datta R. and Stone R. M.: *All trans* retinoic acid induces monocyte growth factor receptor (c-fms) gene expression in HL60 leukemia cells. *Leukemia* 7 (1991) 458–462.
66. Frankel S. R., Eardley A., Lauwers G., Weiss M. and Warrell R. P. Jr.: The "retinoic acid syndrome" in acute promyelocytic leukemia. *Ann. Intern. Med.* 117 (1992) 292–296.
67. Vosburgh E. Pulmonary leukostasis secondary to all-trans retinoic acid in the treatment of acute promyelocytic leukemia in first relapse. *Leukemia* 6 (1992) 608–610.
68. Taimi M., Defacque H., Commes T., Favero J., Caron E., Marti J. and Dornand J.: Effect of retinoic acid and vitamin D on the expression of interleukin-1β, tumour necrosis factor-α and interleukin-6 in the human monocytic cell line U937. *Immunology* 79 (1993) 229–235.
69. Rebel V. I., Ossenkoppele G. J., van de Loosdrecht A. A., Wijermans P. W., Beelen R. H. and Langenhuijsen M. M.: Monocytic differentiation induction of HL60 cells by MC 903, a novel vitamin D analogue. *Leuk. Res.* 16 (1992) 443–451.
70. Dusso A. S., Negrea L., Gunawardhana S., Lopez-Hilker S., Finch J., Mori T., Nishii Y., Slatopolsky E. and Brown A. J.: On the mechanisms for the selective action of vitamin D analogs. *Endocrinology* 128 (1991) 1687–1692.
71. Bouillon R., Allewaert K., Xiang D. Z., Tan B. K. and Van Baelen H.: Vitamin D analogs with low affinity for the vitamin D binding protein: enhanced *in vitro* and decreased *in vivo* activity. *J. Bone Min. Res.* 6 (1991) 1051–1057.