Growth Control or Terminal Differentiation: Endogenous Production and Differential Activities of Vitamin A Metabolites in HL-60 Cells

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Summary

Vitamin A (retinol) is a prohormone that exerts its pleiotropic biological effects after conversion into multiple metabolites. In this report we describe the identification of three endogenous, retinol-derived effector molecules, 14-hydroxy-*retro*-retinol (14-HRR), anhydroretinol (AR), and retinoic acid (RA) and a putative storage form of retinol, retinylesters (RE) in the human promyelocytic leukemia cell line HL-60. Exogenous application of the retinol metabolites in retinol-depleted serum-free cultures of HL-60 allowed the identification of unique cellular functions for each metabolite: 14-HRR is a growth factor for HL-60. AR is a functional antagonist of 14-HRR with growth-inhibiting activity, and RA is a potent inducer of granulocyte differentiation accompanied by growth arrest. Finally, intracellular RE serves as storage form allowing continuous production of 14-HRR when no external retinol is available.

Vitamin A (retinol) was discovered nearly a century ago on the basis of the severe physiological defects that result from dietary deprivation. Mild vitamin A deficiency affects night vision, whereas severe depletion causes progressively worsening syndromes of keratitis of the skin and the eyes, loss of reproductive capacity, dysfunction of the immune system and eventually, death (1). Oversupply of vitamin A is also deleterious to animals in prenatal life, causing severe deformations (2). Biochemical studies have uncovered several metabolites as the molecular mediators that explain some, but not all, of the pleiotropic effects of vitamin A and the symptoms of vitamin A deficiency. Retinaldehyde is the universal chromophore in vertebrate eyes (3). Two forms of retinoic acid (RA)[1], *all-trans*-RA and *9-cis*- RA, have been linked to a mechanism of gene activation termed ligand-assisted transcriptional regulation, which these retinoids share with steroid, thyroid, and other small lipophilic hormones (4–6). RAs function as ligands for two groups of nuclear RA receptors, referred to as RAR and RXR receptors (7–9). RA is involved in the development of the embryo and morphogenesis (10), which may account for the severe teratogenic effects of vitamin A. RA isomers also have proven effective as drugs in the treatment of certain forms of leukemias and dermatological disorders (11–13).

Despite these advances in the understanding of RA action, it would be too narrow a view to attribute all effector functions of vitamin A to RA, since several manifestations of the vitamin A deficiency syndrome, among these the immunodeficiency and the loss of spermatogenesis, are not reversed by repletion with RA (14). Two other vitamin A metabolites, 14-hydroxy-*retro*-retinol (14-HRR) and anhydroretinol (AR), have recently been identified as putative effector molecules (15, 16). They are both members of the *retro*retinoid family, referring to their specific configuration of doublebonds which gives these molecules a spatial appearance distinct from retinol and RA. The function of 14-HRR appears to lie in the support of proliferation of activated B lymphocytes (17) and functional activation of thymocytes (18), both activities which RA cannot mediate. AR exhibits inhibitory properties on lymphocytes that can be reversed by 14-HRR and retinol (16). All three retinoids, 14-HRR, AR, and RA, are found side by side in HL-60 cells, prompting us to study their unique actions in relationship to growth or differentiation of these cells. Our results underscore the view that at least two different independent effector pathways are regulated by three metabolites with specific functions that are derived from the common prohormone, retinol.

Materials and Methods

Cells and Culture Conditions. Cells were grown in RPMI 1640 medium containing 10% FCS (Hyclone Laboratories, Logan, UT), 2 mM glutamine plus penicillin and streptomycin (GIBCO BRL, Gaithersburg, MD). HL-60 cells were obtained from the American Type Culture Collection (Rockville, MD), and tested for myco-

[1] *Abbreviations used in this paper:* AR, anhydroretinol; BHT, butylated hydroxytoluene; FBS, fetal bovine serum; 14-HRR, 14-hydroxy-*retro*-retinol; ITLB, linoleic acid; NBT, nitrobluetetrazolium; RA, retinoic acid; RE, retinylester.

plasma twice a month. The defined serum-free medium consisted of RPMI 1640 with 0.12% BSA, 5 μg/ml insulin, and 5 μg/ml transferrin, 10^{-6} M linoleic acid (Sigma Chemical Co., St. Louis, MO), and 2 mM L-glutamine. 1,25-dihyroxy-vitamin D3 (gift of Hoffmann-LaRoche, Inc., Nutley, NJ) was dissolved in ethanol.

Differentiation and Proliferation of Cells. Nitrobluetetrazolium (NBT; Sigma Chemical Co.) is a yellow dye that when reduced by oxygen radicals turns black/blue. For detection of differentiation, HL-60 cells were tested for their ability to reduce NBT. During a 30-min stimulation with 10^{-7} M PMA (Sigma Chemical Co.), cells were provided with NBT, 0.61 mM, and analyzed for dark staining under the microscope (19). The percentage of differentiated cells was given in relation to the total number of viable trypan blue negative cells. CD11b and CD14 expression was detected by staining cells with FITC- and PEG-conjugated mAbs CD11b and CD14 (Amac, Inc., Westbrook, ME) according to the manufacturer's manual and analysis on a FACS® (Becton Dickinson & Co., Mountain View, CA). Morphological evaluation was done under the microscope with Wright-Giemsa–stained cytocentrifuge slides. Proliferation was assessed by [^{3}H]thymidine uptake. 30,000 cells/well were seeded in 96-well plates (Falcon, Becton Dickinson, Lincoln Park, NJ) and 0.8 μCi [^{3}H]thymidine (NEN, Du Pont, Wilmington, DE) were added to a final volume of 200 μl/well for the last 4 h before harvest onto glass filters. [^{3}H]thymidine uptake was determined by liquid scintillation counting. Assays were done in triplicates. Cell growth curves were obtained by taking aliquots from cultures in 12-well cell culture cluster dishes (Costar, Cambridge, MA) and counting in a Neubauer chamber. Cell numbers were kept between 5×10^5 and 2×10^6 cells/mm^3. Viability was assessed by trypan blue exclusion. Assays were done in duplicates.

Preparation of Retinoids. Retinol (Sigma Chemical Co.) was dissolved in ethanol. RA (Sigma Chemical Co.) was dissolved in DMSO (Fisher Scientific, Fairlawn, NJ). The final concentration of DMSO in the medium was always <0.0001% and had no effect on differentiation of the cells. The retinoids were purified by HPLC before use. Handling of retinoids was under amber light, and storage under argon in the dark at −70°C. Radioactive retinol and RA were purchased from NEN and purified by HPLC before use. 14-HRR was prepared from lipid extracts of HeLa cells incubated overnight with 10^{-5} M retinol and purified to homogeneity by a series of three different HPLC reversed phase columns with different gradients as described (15). AR was synthesized by acid catalyzed dehydration of *all-trans*-retinol (20). Synthetic AR was purified by HPLC chromatography. To all retinoids in solution 0.1 mM butylated hydroxytoluene BHT (Sigma Chemical Co.) was added as well as to control solvents used in cellular assays.

HPLC of Retinoids. Lipid extracts were prepared according to (21) by suspending cells in 8 parts PBS adding 3.2 parts of butanol/acetonitrile 1:1, vortexing for 60 s, then adding 2.4 parts of saturated K_2HPO_4 solution, briefly vortexing again, and then ultracentrifuging at 14,000 rpm for 4 min. Two parts were removed from the supernatant and injected in the HPLC (M6000 solvent delivery pump, photodiode array detector; Waters Associates, Milford, MA) for UV detection or measurement of eluting radioactivity with an on-line scintillation counter (Radiomatic, Tampa, FL). The gradients went from water, 20 mM Tris buffer, pH 7.4, or 20 mM glycine buffer, pH 4.5, to methanol to chloroform for preparative and analytical columns, and from water to acetronitrile for the semipreparative column in various time frames. The flow rates were 1.0 ml/min for the analytical C18 (4.6 × 250 mm internal diameter), 4 ml/min for the semipreparative C18 (10 × 250 mm internal diameter), and 8 ml/min for the preparative C18 (22 × 250 mm internal diameter) reversed phase columns (Vydac; Separations Group, Hesperia, CA). Fractions of HPLC eluates were collected into polypropylene tubes (Falcon, Becton Dickinson) containing the adequate amount of BHT under protection from light.

Results

HL-60 Leukemia Cells Are Dependent on Vitamin A (Retinol) for Growth under Serum-free Conditions. A variety of cell lines including human EBV-transformed B cell lines are dependent on retinol for growth (17). Retinoic acid is not able to replace the need for retinol (22). To test whether this was also the case with the human leukemia cell line HL-60, these cells were cultured under serum-free conditions in medium containing BSA, transferrin, insulin, and linoleic acid (ITLB). Proliferation of cells was determined by counting cells in a Neubauer chamber while assessing viability by trypan blue exclusion. Cells freshly seeded in ITLB continued to grow exponentially regardless of the amount of retinol added to the cultures, for about 1 wk. Thereafter, however, we noted a marked difference in cultures with or without retinol. Proliferation of cultures with a retinol concentration of $\leqslant 10^{-8}$ M, when given every 3 d, ceased around day 7 after transfer to ITLB. By day 9 or 10, viable cells were no longer detectable (Fig. 1 *a*, Fig. 2 *a*). In contrast, cells of cultures with a higher retinol concentration continued to thrive. If cells of retinol-depleted cultures were replenished with the vitamin on day 6 the cells resumed growth in a dose-dependent fashion (Fig. 1 *b*). The dose optimum for growth was at 10^{-7} M if fed every 3 d (Fig. 1 *a*) and 10^{-8} M if fed every day (Fig. 2 *A*). Doses exceeding this concentration did not stimulate further proliferation. On the contrary, in the range of 1 to 5×10^{-6} M, which is optimal for growth in other cell lines, proliferation was significantly slower than in cultures containing 10^{-7} M retinol (17, 18). This was not due to overt toxicity, because the cells remained intact and viability was consistently >95% by trypan blue exclusion. These findings demonstrate that retinol is an important cofactor for both sustenance and control of proliferation of serum-deprived HL-60 cells.

High Doses of Retinol Can Induce Granulocytic Differentiation of HL-60 Cells. A possible explanation for the induction of growth arrest by high dose retinol was the generation of a nondividing progeny of differentiated cells. We therefore tested whether retinol itself can induce differentiation in HL-60 cells, as has been suggested previously (23). HL-60 are promyelocytic cells that preserve the potential to differentiate towards a variety of cell types including granulocytes, monocytes, and osteoclasts (24, 25). After differentiation in the granulocytic pathway, they express the ability to reduce the yellow dye NBT upon stimulation with phorbol esters and also express lineage-specific antigens such as CR3b (CD11b). By contrast, the CD14 antigen is expressed when cells are driven towards a monocyte-like phenotype (26). To study the effects of retinol, HL-60 cells were seeded in serum-free ITLB medium with different amounts of HPLC-purified retinol. Cell number and viability were determined by counting and trypan blue exclusion. Differentiation was followed by NBT

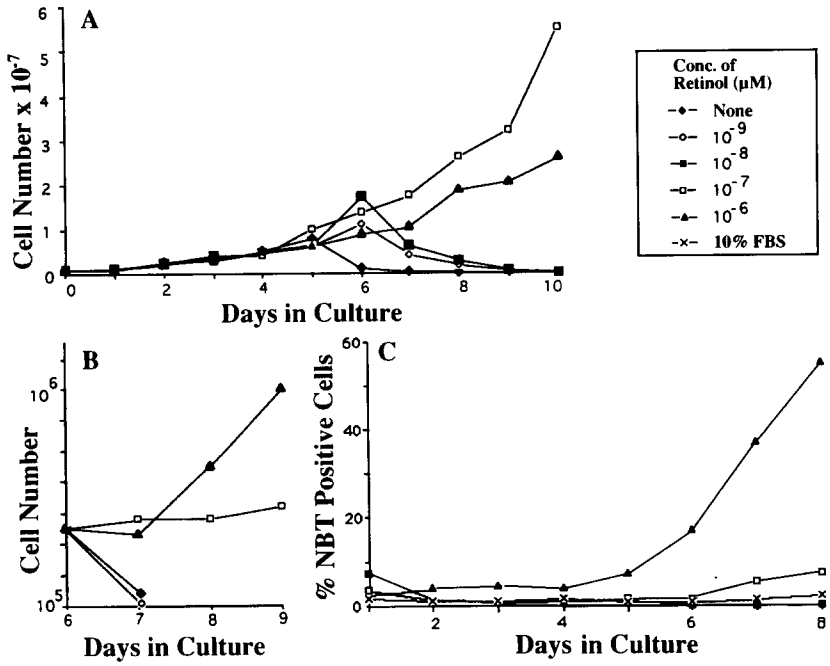

Figure 1. Growth and differentiation of HL60 promyelocytic leukemia cells under serum-free conditions dependent on the concentration of vitamin A. (*a*) HL 60 cells previously cultured in medium containing 10% FBS were reseeded in defined serum-free medium (ITLB) at day 0. HPLC-purified *all-trans*-retinol was added every 3 d to the cultures to yield final concentrations from 10^{-6} to 10^{-9} M in 10-fold dilution steps and solvent only was added to control cultures. Cell numbers and viability were determined each day by microscopic counting. Cell densities were kept between 5 × 10^5/ml and 2 × 10^6/ml by addition of medium accordingly. The total cell number of cultures is plotted over time in different retinol concentrations present. (*b*) Cells cultured in serum-free medium without retinol until day 6 were then replenished with the vitamin. Cell numbers were determined for the following 3 d in different retinol concentration provided to cultures. (*c*) The percentages of NBT positive, differentiated HL-60 cells in the cultures shown in *a* dependent on the retinol concentration provided in the medium and for comparison of cells cultured in 10% FBS.

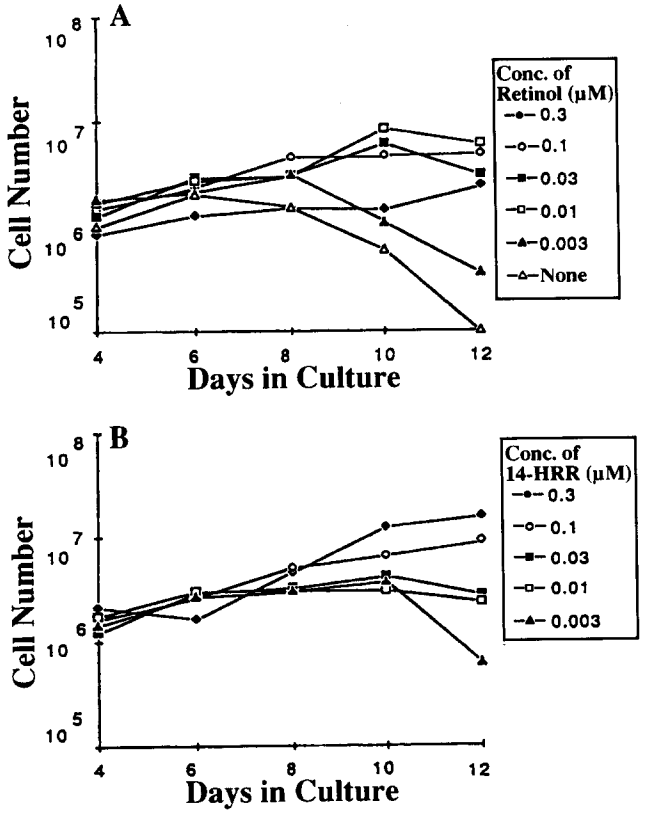

Figure 2. Growth of HL-60 under serum-free conditions with exogenously provided retinoids. HL-60 cells from stock cultures in 10% FBS were reseeded in serum-free ITLB medium and retinol (*A*), and 14-HRR (*B*) were added daily to the final concentrations shown in legend. Cell numbers and viability were determined by trypan blue exclusion and microscopic counting in cultures with different retinoid concentrations. The cell density was kept between 3 × 10^5 and 2 × 10^6 cells/ml by providing additional culture medium accordingly. Shown is the total cell number from days 4 through 12 of cultures dependent on the retinoid concentration.

reduction and by analyzing the proportions of cells expressing differentiation-specific antigens CD11b and CD14 by fluorescence flow cytometry. Below 10^{-7} M (if given every 3 d) or 3 × 10^{-8} M (if given daily), retinol did not induce more than background differentiation (i.e., <5% NBT positive cells, which occur spontaneously in culture with 10% fetal bovine serum [FBS]) in HL-60 cells. However, concentrations exceeding those optimal for growth induced differentiation. For instance, by day 8, 60% of the cells in 10^{-6} M retinol given every 3 d, or 3 × 10^{-7} M daily, were NBT positive (Fig. 1 *c*, Table 1). Thus the proportions of differentiated and undifferentiated cells on any given day was dependent on the concentration of retinol present in that culture. Cells induced to differentiate by high dose retinol were morphologically indistinguishable from those induced by RA, had acquired the ability to reduce NBT, and expressed CR3b but not CD14 (Table 1).

Retinol Derivatives Constitutively Produced in HL-60 Cells. To determine which metabolites of retinol were responsible for the cellular phenomena described above, we characterized the retinol metabolites produced in HL-60 cells. Initially, cells were trace-labeled with purified [^{3}H]retinol and lipid extracts from the cells analyzed by reversed phase HPLC for retinol metabolites. Several radioactive peaks were characterized by coelution with known standards on different HPLC gradients and by their behavior under changed separation conditions, such as neutral or acidic pH of the elution buffer. Larger numbers of cells were cultured and treated with cold

Table 1. *Phenotype of HL-60 Cells on Day 8 of Culture*

Concentration of inducer		Percent NBT positive	Percent CD11 positive	Percent CD14 positive
	μM			
Retinol	0.3	63	86	0.1
	0.1	22	51	0.2
	0.03	8.4	27	0.3
14-HRR	0.3	10	22	1.1
	0.1	9	23	1.0
	0.03	5	20	0.8
Control		9.8	*	*

HL 60 cells were cultured under serumfree conditions in the presence of different retinoids as described for Fig. 3. The percentages of differentiated cells on day 8 of the cultures as measured by the NBT assay and the proportion of cells expressing CR3b (CD11b) and CD14 are shown for different concentrations of retinol, and 14-HRR used.
* ND because of low numbers of viable cells in control cultures.

retinol under identical conditions as in the initial trace-labeling studies. UV-absorption characteristics of previously identified radioactive peaks were determined by photodiode array detection of HPLC eluates from lipid extracts of these cells and compared to the UV spectra of known standards. Using these techniques several distinct metabolites were identified: Retinol is metabolized by HL-60 cells to compounds both more lipophilic and hydrophilic than retinol itself (Fig. 3 *A*). A large proportion of retinol is converted into apolar substances that coelute with retinylesters (RE). Another group of metabolites eluting between retinol and RE on a reversed phase HPLC gradient corresponds to stereoisomers of compound 3 AR (16, 27). One of the more polar peaks was identified as 14-HRR (15) (Fig. 3, *A* and *C*, compound *2*). The structures are given in Fig. 6.

RA as a Candidate Mediator of Retinol-induced HL-60 Differentiation. To identify metabolites of retinol that mimic its differentiating effects, we exposed HL-60 cells to 6×10^{-6} M retinol for 2 d, a concentration that eventually induces granulocytic differentiation in the majority of cells, and chromatographed the lipid extract of these cells by HPLC as described in Materials and Methods on a preparative reversed phase C_{18} column with a linear water/methanol/chloroform gradient. 1-min fractions were collected for further testing. Dilutions of fractions 5–45 were added to HL-60 cells and the proportion of differentiated cells determined after 4 d, according to the criterion of NBT reduction. We found fractions 12–17 and fractions 27–29 to induce differentiation of HL-60 cells (Fig. 3 *B*). The latter fractions corresponded to authentic *all-trans*-retinol by retention time and UV absorption spectrum (data not shown). The second group conferring differentiating activity (fractions 12–17) comprised a minor peak in the UV absorption trace. To analyze these fractions in more detail we rechromatographed the pooled fractions on an analytical C_{18} reversed phase column. In fractions collected of this HPLC analysis, all differentiating activity was found within a fraction corresponding to a peak which displayed a UV absorption maximum at 340 nm identical to that of RA (inset, Fig. 3, *A* and *C*, compound *1*). This peak also coeluted with authentic RA and exhibited the same characteristic change in retention time as RA, i.e., from 16.5 to 14.5 min, when the pH of the elution buffer was changed from 7.5 to 4.5. In addition, with a gradient suitable for separation of RA isomers, the majority of bioactive material was identified as *all-trans* RA. In addition, we identified 13-*cis*- and *9-cis*-isomers of RA present in lower concentrations than the *all-trans* form. Some differentiating activity was also detected in the fraction corresponding to the *9-cis*-stereoisomer but not in the fraction containing *13-cis* RA (data not shown). Fractions 24 and 25 containing 14-HRR, and fractions 31–33 containing AR, did not induce differentiation.

Next we measured the intracellular concentration of RA in HL-60 cells as a function of the amount of retinol added to the cultures. RA levels in HL-60 cells grown in medium with 10% FCS alone, which contains $1–2 \times 10^{-7}$ M retinol, were below the level of detection in our assay ($<3 \times 10^{-9}$ M). Addition of 5×10^{-6} M exogenous retinol to the medium increased the intracellular level of RA in HL-60 cells to 1.5×10^{-7} M (Table 2) after 48 h.

14-HRR Mediates the Growth-promoting Activity of Retinol. We observed 14-HRR as a constitutively produced metabolite of retinol in HL-60 cells. To examine the effects of this compound on HL-60 cells, we evaluated the influences on cell proliferation and differentiation in HL-60 cell cultures. We compared serum-free cultures exposed to retinol, or 14-HRR, measuring growth by counting cells, viability by trypan blue exclusion, differentiation by NBT reduction, and expression of CD11b and CD14 by FACS® analysis. Retinoids were added daily since the half-life of 14-HRR in HL-60 cul-

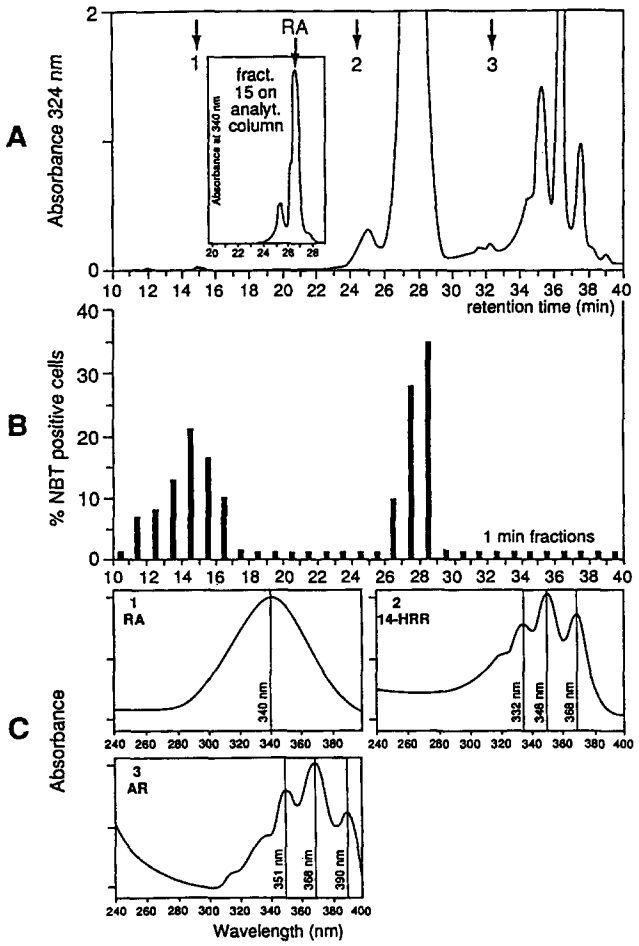

Figure 3. Retinol metabolism of HL 60 cells. (*A*) The lipid extract of 109 HL-60 cells grown for 2 d in medium containing 10% FBS and 5 μM retinol analyzed by reversed phase HPLC on a C_{18} preparative column with a linear gradient from water to methanol to chloroform. Shown is the UV absorbance at 324 nm dependent on the retention time. (*C*) (*Arrows 2* and *3*) Time points where UV-spectra of compounds 2 and 3 in 14-HRR and AR respectively, were obtained. (*Inset*) Reanalysis of fractions 12–17 (marked by *arrow 1* on preparative analysis) on an analytical C_{18} column using a different gradient by monitoring UV absorbance at 340 nm. Here RA could be identified according to retention time, shift under different pH, and UV absorption spectrum. (*B*) 1-min fractions of the eluate from the preparative HPLC analysis were collected and added in graded dilutions beginning with 1:20 to HL-60 cells in ITLB. Differentiation of cells in response to added fractions was determined by NBT reduction after 96 h. Shown is the percentage of NBT positive cells at a dilution of 1:100 for each fraction. (*C*) (2 and 3) UV absorption spectra obtained at time points of the HPLC analysis in Fig. 2 *A* marked correspondingly. (*1*) UV absorption at min 26.8 of the re-analysis of pooled fractions 12–17 on an analytical reversed phase HPLC-column. (1, 2, and 3) Correspond exactly to the published UV-absorption of RA, 14-HRR, and AR respectively.

tures was determined by use of radiolabeled [^{3}H]14-HRR as < 7 h. 14-HRR was able to sustain growth of HL-60 cells under these conditions with an efficiency equal to or exceeding that of retinol (Fig. 2). The growth suppression noted with high (but not toxic) doses of retinol (Fig. 1) was not found with the two polar retinol metabolites. Cell numbers at doses

Table 2. *All-trans-RA Levels in HL-60 Cells before and after 24 and 48 h of Incubation in 5 μM All-Trans-Retinol*

Treatment	Intracellular RA concentration
	μM
None	$\leqq 0.003$*
24 h	0.09
48 h	0.15

HL-60 were cultured in medium containing 10% FBS for 48 h in the presence of 5×10^{-6} M purified *all-trans*-retinol. Before incubation and after 24 and 48 h aliquots of cells were taken for HPLC analysis of cellular retinoids. 0.2 ml of cell pellet was extracted for 24 and 48-h values and 1 ml of cell pellet of untreated control cells. Retinoids were identified by correspondence of retention time with that of standard material on equilibrated HPLC column and characteristic UV absorption spectra as well as the pH-dependent characteristic shift of the retention time in the case of RA. The amount of RA present in the cells was determined by integration of the area of UV absorbance of the corresponding peak and multiplication with the known absorption constant. Indicated are the intracellular RA concentrations before and after 24 and 48 h of incubation in 5×10^{-6} M retinol.
* Limit of detection by method used.

optimal for growth were higher with 14-HRR than with retinol; thus 14-HRR was superior in its growth-promoting activities to that of their precursor, retinol. Conversely, with respect to cell differentiation, 14-HRR had much lower activity than retinol. 14-HRR produced the lowest proportion of differentiated cells only slightly above background as measured by NBT reduction and expression of differentiation-specific antigens, CD11b and CD14 (Table 1). These findings indicate a functional dichotomy of retinol metabolites, some serving as growth-promoting agents (14-HRR) and others inducing differentiation and growth arrest (RA) (see Fig. 6).

AR Is a Growth Inhibitor of HL-60. AR is the second member of the *retro*-retinoid family found in cells both of mammal and insect origin (16, 27). AR is present in HL-60 cells as well (see above and Fig. 3). To test the effects of exogenous AR on HL-60 cells, proliferation of cultures exposed to AR was determined. Thymidine uptake was measured after 20 h of exposure to AR and a pulse of [^{3}H]thymidine for the last 4 h. Cells freshly transferred to serum-free medium from a FBS-containing culture before measurement showed a 50% reduction of thymidine uptake by maximal doses of AR at 3×10^{-6} M (data not shown). When kept in ITLB for 3–4 d without retinol before exposure to AR, there was a marked growth suppression which was dose dependent and virtually complete at an AR concentration of 2.5×10^{-7} M (Fig. 4 *A*).

AR Inhibits Proliferation of HL-60 without Inducing Differentiation. Since both AR and RA caused a significant, if not complete, inhibition of cell growth (Fig. 4 *A*), it was of interest to ask whether this was due to the same mechanism and whether AR induces HL-60 differentiation. AR, retinol, and RA were added to cultures alone and in combination

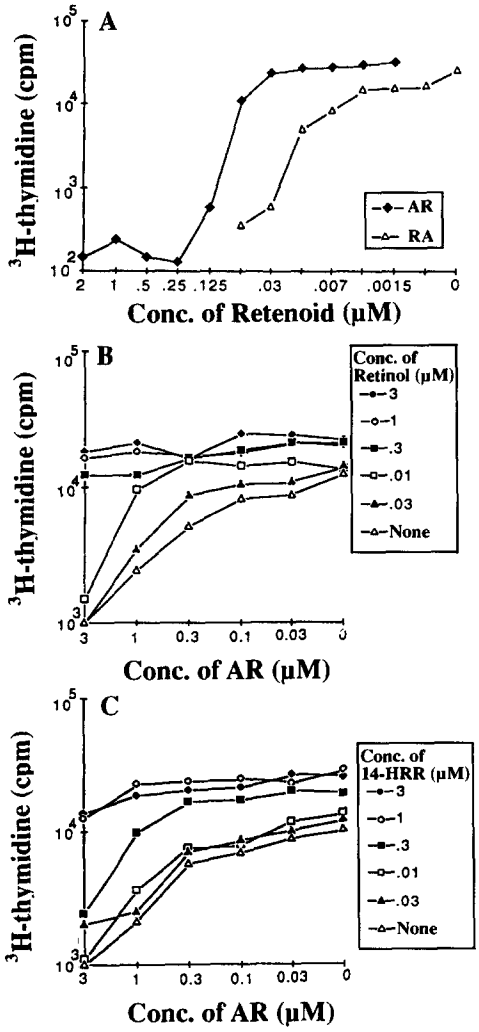

Figure 4. Growth suppression of HL-60 by AR and RA and antagonism of AR with retinol and 14-HRR. (*A*) HL-60 were cultured in serum-free ITLB medium without retinol for 4 d. AR and RA were added and proliferation was measured by [^{3}H]thymidine uptake during the last 4 h of a 24-h exposure dependent on RA and AR concentrations. (*B* and *C*) HL-60 cells were kept in serum-free ITLB without retinol for 4 d. Then they were reseeded on 96-well plates with 50,000 cells per well. Dilutions of AR and for each AR concentration titrations of retinol (*B*) and 14-HRR (*C*) were added. [^{3}H]thymidine uptake was measured during the last 4 h of the 20-h culture. Shown are cpm of [^{3}H]thymidine uptake at different retinol and 14-HRR concentrations over the AR concentration.

and differentiation was measured on day 4 dependent on the concentration of added retinoids. Again, NBT reduction after stimulation of differentiated cells with PMA as well as expression of CR3b (CD11b) were measured. RA led to differentiation of virtually all the cells at doses that caused significant growth inhibition. AR, in contrast, only led to 20% CD11b positive (Table 3) and 10% NBT-positive cells (Table 4) at concentrations that totally abolished growth. CD14, a monocyte-specific marker, was not found expressed after treatment with AR. Together, these data imply that growth inhibition of HL-60 by AR has to take place by means other than differentiation to granulocytic or monocytic progeny cells and is different from the mode by which RA inhibits growth of these cells.

AR Is a Reversible Inhibitor of Retinol- or 14-HRR–promoted Growth of HL-60 cells. We then tested whether AR would interfere with retinol and its cellular metabolites 14-HRR with respect to cell growth, thereby inhibiting proliferation of cells. Cells were kept in ITLB for 3 d and then treated with various concentrations of AR combined with retinol or 14-HRR. After 16 h of such exposure, proliferation of cells again was measured by [^{3}H]thymidine uptake. Retinol, as well as its metabolite 14-HRR (Fig. 4 *B*) was able to reverse the growth inhibition imposed by AR with similar relative efficiencies. The reversal was dose dependent and the ID_{50} of AR in this experiment rose from a concentration of 10^{-7} M in the absence of retinol to 3×10^{-6} M in the presence of 10^{-7} M retinol. 14-HRR acted similarly. These findings show that AR acts as a functional antagonist of 14-HRR and retinol with respect to cell proliferation and that its growth-suppressing activity is reversible.

AR Does Not Inhibit Differentiation of HL-60 Cells. Since retinol exerts two separate effects on HL-60 cells, growth and differentiation, and since we found AR to competitively inhibit retinol-mediated growth, we tested whether AR would also interfere with the differentiation-inducing activity of high dose retinol. Cells were therefore seeded in retinol, AR, or a combination of the two retinoids. When differentiation was measured by the ability of the cells to reduce NBT, retinol showed the expected dose-dependent activity (Table 4). When tested in combination, AR did not inhibit retinol-induced differentiation, unlike the retinol-mediated growth support, but rather seemed to act in an additive manner. The addition of 1.25×10^{-6} M AR to 2.5×10^{-6} M retinol increased the proportion of NBT-positive cells from 36 to 51%.

To look for effects of AR in combination with other differentiating agents, HL-60 cells were exposed to it together with RA or 1,25-dihydroxy-vitamin D_3, both known to induce different lineages of differentiation. In terms of induction of expression of CD11b, the minor activity observed with AR alone (Table 3) was additive to the activities of 1,25-dihydroxy-vitamin D_3 and RA. A more pronounced effect of AR was seen in combination with 1,25-dihydroxy-vitamin D_3 when induction of coexpression of CD14 with CD11b was measured, which rose from 34% double positive cells in 1,25-dihydroxy-vitamin D_3–treated cultures, to 62% in cultures which additionally were exposed to 10^{-6} M AR.

Intracellular RE Pools Are a Source for Retinol and Its Growth-promoting Metabolites, 14-HRR. Retinol is esterified efficiently with a variety of fatty acids to REs by HL-60 (see Fig. 3). In animals, REs are the storage form of retinol and are mainly found in the liver (14). To test whether the intracellular RE pool is used as a retinoid source when exogenous retinol is absent, we labeled intracellular retinoids by first starving HL-60 cells of retinol for 3 d and then exposing them overnight to a 5×10^{-6} M mixture of purified cold and [^{3}H]retinol. After such labeling, 55% of the cell-associated radioactivity eluted as REs on HPLC, 33% were present as retinol, and 3.5% as 14-HRR, respectively (Table 5). We followed the decay of retinol and REs over several days and found that

Table 3. *Influence of AR on RA or 2-OH Vitamin D_3–induced Differentiation Measured by Expression of Differentiation-specific Antigens CD11b and CD14*

	Percent CD11 positive				Percent CD14 positive			
AR	1.0	0.1	0.01	0	1	0.1	0.01	0
				μM				
2-OH-vitamin D_3								
0.01	99	91	86	83	62	42	38	34
0.001	91	70	66	96	17	13	13	7
RA 0.1	99	97	96	94	1.7	1.1	1.1	0.9
0.01	87	76	71	71	1.1	0.6	0.7	0.6
Control	23	10	7	7	1.2	0.7	0.8	0.7

HL-60 cells were coexposed to known differentiation inducing agents and AR in serum-free ITLB medium for 4 d. Expression of differentiation-specific antigens CD11b and CD14 was determined by FACS® analysis. Proportions of positive cells within a culture are given for either combinations of different concentrations of RA or 2-OH-vitamin D_3 with different amounts of AR, cultures treated with AR alone or untreated control cells. All CD14-expressing cells were coexpressing CD11b.

Table 4. *Differentiation of HL-60 by RA, AR, or Retinol as Measured by NBT Reduction Assay*

	Percent NBT positive							
Concentration of retinoid	5	2.5	1.25	0.6	0.3	0.15	0.08	0
μM								
retinol	100	36	22	11	7	7	1	3
AR	tx	10	8	2	1	4	2	3
RA						90	55	4
Combinations								
retinol + 1.25 μM AR	tx	51	15	10	8	10	8	8
AR + 2.5 μM retinol	tx	54	51	38	38	33	35	36

HL-60 cultures were exposed to retinol, RA or AR. Additionally, a fixed concentration of retinol was combined with graded amounts of AR and vice versa. The percent NBT positive cells was determined after 96 h of exposure and is given for the different inducer concentrations of retinol, AR, and RA alone or the retinol/AR combinations.
tx, toxic inducer concentrations.

without further exogenous supply, the half-lives were 7.7 h (retinol) and 48 h (REs). When external retinol was supplied, the decay was significantly slower (Table 5). In contrast, 14-HRR did not change its intracellular levels (Fig. 5). We observed in this experiment, that, whereas retinol and REs progressively disappeared from the cells, 14-HRR was maintained at relatively constant levels throughout a period of 6 d, which exceeded the half-life (< 7 h) by far. The cellular concentrations of 14-HRR only fell when retinol and REs had been exhausted, which in our experiments always coincided with the loss of proliferation of cell cultures. Thus, as long as retinol and REs are available, and 14-HRR is synthesized in proliferating cells and its cellular concentration appear to be tightly regulated.

Discussion

HL-60 cells have become a favored model system for the study of differentiation owing to the several developmental options these cells can execute. A variety of inducing agents can guide the cells selectively to different preordained endpoints. For instance, 1,25-dihydroxy-vitamin D_3 causes HL-

Table 5. *Cellular Retinoid Pools of HL-60 Cells*

	14-HRR	Retinol	RE	Percent total
		dpm		%
5 × 10^6 cells immediately after labeling	24,700	209,000	397,000	
Percent eluate	3.5	33.0	55.2	91.7*
5 × 10^6 cells grown in 10^{-7} M retinol for 48 h after labeling	31,300	147,000	246,000	
Percent eluate	5.2	24.6	38.1	67.9
5 × 10^6 cells grown without retinol for 48 h after labeling	34,000	28,000	109,000	
Percent eluate	12.8	9.5	39.0	61.3

Intracellular retinoid pool in cells cultured under serum-free conditions with or without vitamin A present in culture medium. HL-60 cells were grown in defined serum-free medium for 4 d and subsequently exposed to 5 μM cold retinol and additional 10 mCi/ml, 49.3 Ci/mmol retinol overnight for labeling of intracellular retinoids. Cells were then washed twice in medium alone and reseeded at 5 × 10^5 cells/ml in ITLB medium containing either 10^{-7} M retinol or no retinol. Lipid extracts of cells were analyzed by reversed phase HPLC on an analytical C_{18} column and radioactive peaks identified according to corresponding retention times of known standards in the equilibrated HPLC system as well as codetermination of the UV absorption spectra. Given are the integrated dpm of corresponding radioactive peaks for 14-HRR, retinol and RE as well as their proportion in percent total radioactive material recovered from cells of culture aliquots.
* Does not add to 100% because of unaccounted metabolites.

60 cells to differentiate to monocyte-like cells, whereas exposure to RA produces cells with a granulocytic phenotype.

We have investigated the HL-60 system with regard to endogenous retinoid production and the effects of these retinoids when applied exogenously. Previous experiments with retinoids have focused on RA, because of its marked and specific effect in driving the cells towards granulocyte differentiation. Other investigators have examined the effects of retinol and RE and have found both to induce granulocyte differentiation, albeit only when added in high concentrations (20, 28, 29). By comparison, RA is active at 10^{-8} M, a concentration agreeing with measured binding constants of the RA receptors. However, differentiation is not the only aspect of HL-60 cells governed by retinoids. We describe in this report that HL-60 cells are critically dependent on retinol for growth under serum-free conditions, a fact not previously appreciated, and that this effect is mediated by a distinct set of retinol metabolites.

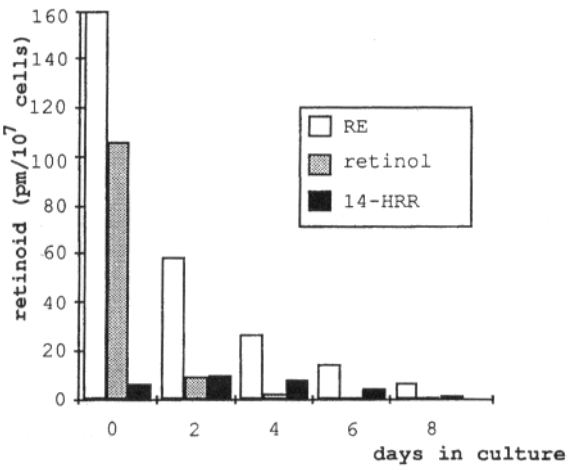

Figure 5. Cellular retinoids of HL-60 over the course of culture in serum-free ITLB. Intracellular retinoids of HL-60 cells were labeled by exposing cells to a mixture of cold and [^{3}H]retinol overnight. Cells were then washed and reseeded in ITLB medium. Lipid extracts of cells of culture-aliquots were HPLC analyzed immediately after reseeding and after 2, 4, 6, and 8 d of culture under retinol-free conditions. Shown are the amounts of labeled retinol derivatives recovered from cells of culture aliquots calculated from the specific activity of precursor [^{3}H]retinol, 14-HRR, retinol, and RE over time.

We identified four retinol metabolites in HL-60 cells, some of which are well-known and some discovered only very recently: 14-HRR, RA, AR, and a group of retinyl fatty acid esters (RE). Each of these has been identified as a metabolite of retinol on the basis of trace labeling with [^{3}H]precursor retinol. The molecular identities were defined by (*a*) position in a calibrated HPLC chromatogram and by coelution with authentic standards; and (*b*) by UV spectroscopic measurements, conforming in each case to the published absorption spectra. In the case of RA we also used the pH-dependent shift in retention time as an identifying characteristic. In a separate report, to be published elsewhere, we show that the production of all four retinoids depends on intact cells, or in the case of AR, can be inhibited by disruption of the electron transport with sodium azide, ruling out that the simple

dehydration reaction underlying conversion of retinol to AR could have occurred spontaneously.

We will now discuss the individual functions of each of the metabolites and the precursor retinol as well.

The first point is the role of vitamin A itself. This is normally provided to cultures as a constituent of the serum supplement, and in 10% serum, is present at a concentration of 1–2 × 10^{-7} M (30). It has been reported that HL-60 cells grow in serum-free, i.e., retinol-free, media (31, 19). Our findings are in agreement, but only insofar as short-term cultures are concerned. Extended culturing requires in our hands the addition of retinol in the 10^{-7} M range (Fig. 1). That HL-60 cells depend after all on an external source of vitamin A for growth may have escaped attention previously because the effects of vitamin A deprivation are not discernible immediately but gradually develop over a prolonged period, i.e., in some cases up to 10 d. This is plausibly explained by the high concentration of endogenous REs (Fig. 3). We have monitored these levels over time and find that the RE pool decays with a half life of 48 h under retinol-free culture conditions. Near total depletion of RE coincides with loss of proliferation and eventual cell death. Whereas the concentrations of retinol as well as REs declined rapidly in these cells, 14-HRR remained at constant steady-state level as long as the cells were proliferating. In contrast, exogenous 14-HRR decayed with a half-life of <7 h. Thus, the decline of RE is associated with continuous production of 14-HRR and the intracellular concentrations of which is maintained within close borders. The loss of proliferation is correlated with the exhaustion of 14-HRR, and this is likely to be a direct consequence of depletion of the endogenous RE and retinol pools, from which 14-HRR is synthesized. This leads to our second point.

The retinol metabolite, 14-HRR is sufficient to sustain proliferation in retinol-depleted HL-60 cultures. 14-HRR has not been found to be converted back into retinol (our unpublished results). 14-HRR can therefore be regarded as the effector molecule of the growth-promoting retinol activities. In support of this concept is the continuous proliferation of HL-60 cells in cultures supplemented with 14-HRR (Fig. 2) and our earlier published findings (18) with lymphocytes, that 14-HRR is both necessary and sufficient to support cell proliferation in the absence of exogenous retinol. The endogenous levels of 14-HRR are maintained in cells growing in retinol-free medium as long as the internal RE supply lasts and proliferation is sustained, as pointed out above.

The third point of discussion concerns the growth-inhibition mediated by retinol at high concentrations (Figs. 1 and 2). Confirming earlier reports on this phenomenon (23) we found that at high concentrations, typically >10^{-6} M, retinol induced the differentiation of HL-60 cells, and this appears to be directly related to the observed decline in proliferation. The phenotype of the differentiated cells identifies these as granulocytic cells, similar to those arising in response to RA. Since these cells are terminally differentiated cells with a limited potential for proliferation the decline of [^{3}H]thymidine incorporation as well as stagnating total cell numbers

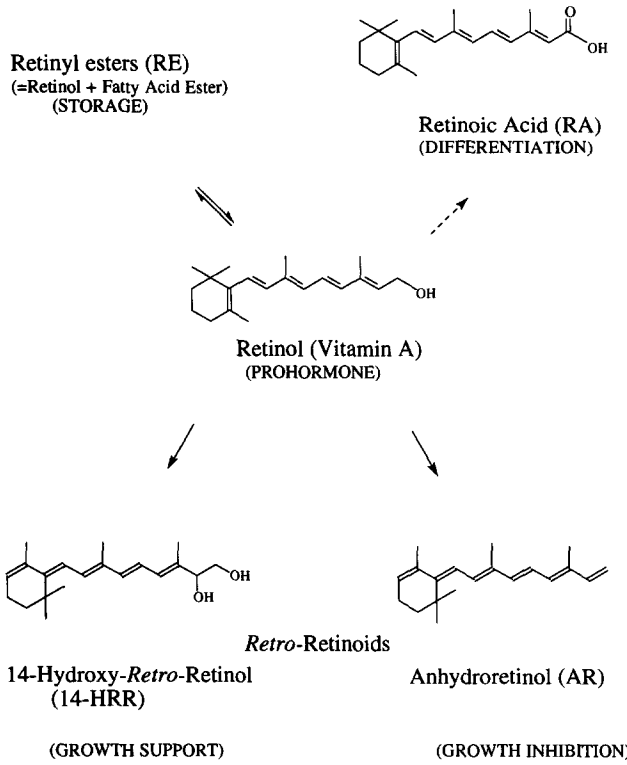

Figure 6. Retinoids in HL-60 cells. Intracellular pathways of retinol. Presented is our current model of cellular retinol metabolism leading to metabolites with specific activities for either growth support (*14-HRR*), negative growth regulation (*AR*), or differentiation (*RA*) via independent pathways.

in HL-60 cultures exposed to supranormal levels of retinol is satisfactorily explained by removal of a substantial portion of cells from the cell cycle.

We propose that the mechanism leading to differentiation involves the conversion of retinol to RA. When we assayed the cellular retinoids of HL-60 cells kept at 5 × 10^{-6} M retinol for 48 h for their inherent differentiation-inducing activities, we found that RA was present at a concentration of 1.5 × 10^{-7} M. In standard culture conditions (i.e., 10% FBS), the cellular RA level was below the limits of detection in our assay (3 × 10^{-9} M).

The molecular basis of RA action, in contrast to that of the proliferation-supportive effect of 14-HRR, is better understood. RA induces differentiation in HL-60 by means of binding to the nuclear receptor protein RAR-α (32, 33) which is thought to regulate the transcription of a set of target genes critical for differentiation (34, 35). Although our data do not establish a cause-effect relationship, the amount of RA generated intracellularly after exposure to elevated doses of retinol appears sufficient to saturate the RAR α receptor and initiate differentiation.

AR, as our fourth point, was described for the first time as a natural ingredient of fish oils in 1938 (36). No physiological role has been assigned to this substance. We have found AR in invertebrate and mammalian cells and tissues (16). HL-

60 cells synthesize AR constitutively, albeit at low concentration (Fig. 3). Although we have not been able to manipulate the level of endogenous AR and cannot comment on its physiological role, we have noted that exogenous AR exerts a striking inhibitory effect on the proliferation of HL-60 cells (Fig. 4 *A*). The result of this inhibition is cell death. It is similar to the antiproliferative effect of AR on lymphocytes (16). Pharmacologically, AR has all the hallmarks of a reversible inhibitor. Both retinol and 14-HRR compete with AR (Fig. 4, *B* and *C*). AR has the same basic *retro*retinoid structure as 14-HRR, inviting the speculation that competition for the same binding protein and displacement of positively acting 14-HRR by an inactive analog underlies the observed inhibition.

The effect of AR on the differentiation of HL-60 cells, and whether or not it may act in an additive manner with other inducers, is currently unresolved. We have recorded a small proportional increase of differentiated cells in AR-treated cultures, suggesting a weak differentiation-inducing capacity. However, another explanation may be simply a selective loss of the nondifferentiated HL-60 population that may be more sensitive to the cytostatic or even lethal effects of AR than the differentiated subpopulation within a culture treated with AR. The deleterious effects of AR may differ for cells at distinct stages of the cell cycle.

In summary, four endogenously produced retinoids of HL-60 cells have been linked to distinct physiologic tasks as illustrated in Fig. 6 and summarized in Table 5. REs serve as a storage form of retinol, to be used when external retinol is scarce. 14-HRR has been implicated in the control of proliferation as an essential cofactor, whereas AR appears to function as a competitive inhibitor of 14-HRR. The function of RA is control of differentiation towards granulocytes and this effect is largely independent of 14-HRR and AR.

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References

1. Wolbach, S.B., and P.R. Howe. 1925. Tissue changes following deprivation of fat-soluble A vitamin. *J. Exp. Med.* 42:753.
2. Kochhar, D.M. 1967. Teratogenic effect of retinoic acid. *Acta Path. Micro. Scand.* 70:398.
3. Wald, G. 1968. Molecular basis of visual excitation. *Science (Wash. DC).* 162:230.
4. De Luca, L. 1991. Retinoids and their receptors in differentiation, embryogenesis, and neoplasia. *FASEB (Fed. Am. Soc. Exp. Biol.) J.* 5:2924.
5. Evans, R.M. 1988. The steroid and thyroid hormone receptor superfamily. *Science (Wash. DC).* 240:889.
6. Green, S., and P. Chambon. 1986. A superfamily of potentially oncogenic hormone receptors [news]. *Nature (Lond.).* 324:615.
7. Mangelsdorf, D.J., E.S. Ong, J.A. Dyck, and R.M. Evans. 1990. Nuclear receptor that identifies a novel retinoic acid response pathway. *Nature (Lond.).* 345:224.
8. Levin, A.A., L.J. Sturzenbecker, S. Kazmer, T. Bosakowski, C. Huselton, G. Allerby, J. Speck, C. Kratzeisen, M. Rosenberger, A. Lovey, and J.F. Grippo. 1992. 9-Cis retinoic acid stereoisomer binds and activates the nuclear receptor RXR. *Nature (Lond.).* 355:359.
9. Heyman, R.A., D. Magelsdorf, J.A. Dyck, R.B. Stein, G. Eichele, R.M. Evans, and C. Thaller. 1992. 9-Cis retinoic acid is a high affinity ligand for the retinoid X receptor. *Cell.* 24:397.
10. Jessell, T.M., and D.A. Melton. 1991. Retinoids, homeoboxes, and growth factors: toward molecular models for limb development. *Cell.* 66:199.
11. Gollnick, H., R. Ehlert, G. Rinck, and C.E. Orfanos. 1990. Retinoids: an overview of pharmacokinetics and therapeutic value. *Methods Enzymol.* 190:291.
12. Huang, M., Y. Ye, and S. Chen. 1988. Use of all-trans retinoic acid in the treatment of acute promyelocytic leukemia. *Blood.* 72:567.
13. Warrell, R.J., S.R. Frankel, W.J. Miller, D.A. Scheinberg, L.M. Itri, W.N. Hittelman, R. Vyas, M. Andreeff, A. Tafuri, A. Jakubowski, et al. 1991. Differentiation therapy of acute promyelocytic leukemia with tretinoin (all-trans-retinoic acid). *N. Engl. J. Med.* 324:1385.
14. Roberts, A.B., and M.B. Sporn. 1984. Cellular Biology and biochemistry of the retinoids. *In* The Retinoids. M.B. Sporn, A.B. Roberts, and D.S. Goodman editors. Vol. 2. Academic Press, Orlando. 209–286.
15. Buck, J., F. Derguini, E. Levi, K. Nakanishi, and U. Hammerling. 1991. Intracellular signaling by 14-hydroxy-4,14-retroretinol. *Science (Wash. DC).* 254:1654.
16. Buck, J., F. Grün, S. Kimura, N. Noy, F. Derguini, and U. Hämmerling. 1993. Anhydroretinol: a naturally occurring inhibitor of lymphocyte physiology. *J. Exp. Med.* 178:675.
17. Buck, J., G. Ritter, L. Dannecker, V. Katta, B. Chait, and

U. Hammerling. 1990. Retinol is essential for the growth of activated human B cells. *J. Exp. Med.* 171:1613.
18. Garbe, A., J. Buck, and U. Hammerling. 1992. Retinoids are important cofactors in T cell activation. *J. Exp. Med.* 176:109.
19. Breitman, T.R., B.R. Keene, and H. Hemmi. Studies of growth and differentiation of human myelomonocytic leukemia cell lines in serum-free medium. *In* Methods for serum-free Culture of Neuronal and Lymphoid Cells. 1984. Alan R. Liss, Inc., New York. 215.
20. Edisbury, J.R., A.M. Gillam, I.M. Helibron, and R.A. Morton. 1932. Absorption spectra of substances derived from vitamin A. *Biochem. J.* 26:1164.
21. McClean, S.W., M.E. Rudel, E.G. Gross, J.J. DiGiovanni, and G.L. Pede. 1982. Liquid-chromatographic assay for retinol (vitamin A) and retinol analogs in therapeutic trials. *Clin. Chem.* 28:693.
22. Buck, J., A. Myc, A. Garbe, and G. Cathomas. 1991. Differences in the action and metabolism between retinol and retinoic acid in B lymphocytes. *J. Cell Biol.* 115:851.
23. Breitman, T.R., S.E. Selonick, and S.J. Collins. 1980. Induction of differentiation of the human promyelocytic cell line (HL-60) by retinoic acid. *Proc. Natl. Acad. Sci. USA.* 77:2936.
24. Bar-Shavit, Z., S.L. Teitelbaum, P. Reitsma, A. Horll, L.E. Pegg, J. Trial, and A.J. Kahn. 1983. Induction of monocyte differentiation and bone resorption by 1,25-dihydroxy-vitamin D3. *Proc. Natl. Acad. Sci. USA.* 80:5907.
25. McCarthy, D.M., J. SanMiguel, H.C. Freake, P.M. Green, H. Zola, D. Catovski, and J.M. Coldman. 1983. 1,25-dihydroxy-vitamin D3 inhibits proliferation of human promyelocytic leukemia (HL-60) cells and induces monocyte-macrophage differentiation in HL-60 and normal human bone marrow cells. *Leuk. Res.* 7:51.
26. Ferrero, D., S. Pessano, G.L. Pagliardi, and G. Rovera. 1983. Induction of differentiation of human myeloid leukemias: surface changes probed with monoclonal antibodies. *Blood.* 61:171.
27. Bhat, P.V., L.M. DeLuca, S. Adamo, I. Akalovsky, C.S. Silverman-Jones, and G.L. Peck. 1979. Retinoid metabolism in spontaneously transformed mouse fibroblasts (Balb/c 3T12-3 cells): enzymatic conversion of retinol to anhydroretinol. *J. Lipid Res.* 20:357.
28. Skrede, B., H.K. Blomhoff, E.B. Smeland, K.O. Wathne, K.R. Norum, and R. Blomhoff. 1991. Retinyl esters in chylomicron remnants inhibit growth of myeloid and lymphoid leukaemic cells. *Eur. J. Clin. Invest.* 21:574.
29. Wathne, K.O., K.R. Norum, E. Smeland, and R. Blomhoff. 1988. Retinol bound to physiological carrier molecules regulates growth and differentiation of myeloid leukemic cells. *J. Biol. Chem.* 263:8691.
30. Goodman, D.S. 1984. Plasma retinol-binding protein. *In* The Retinoids. Vol. 2. M.B. Sporn, A.B. Roberts, and D.S. Goodman, editors. Academic Press, Inc., New York. 41.
31. Breitman, T.R., S.J. Collins, and B.R. Keene. 1980. Replacement of serum by insulin and transferrin supports growth and differentiation of the human promyelocytic cell line, HL-60. *Exp. Cell Res.* 126:494.
32. Collins, S.J., K.A. Robertson, and L. Mueller. 1990. Retinoic acid-induced granulocytic differentiation of HL-60 myeloid leukemia cells is mediated directly through the retinoic acid receptor (RAR-alpha). *Mol. Cell. Biol.* 10:2154.
33. Nervi, C., J.F. Grippo, M.I. Sherman, M.D. George, and A.M. Jetten. 1989. Identification and characterization of nuclear retinoic acid-binding activity in human myeloblastic leukemia HL-60 cells. *Proc. Natl. Acad. Sci. USA.* 86:5854.
34. Hashimoto, Y., M. Petkovich, M.P. Gaub, H. Kagechika, K. Shudo, and P. Chambon. 1989. The retinoic acid receptors alpha and beta are expressed in the human promyelocytic leukemia cell line HL-60. *Mol. Endocrinol.* 3:1046.
35. Jetten, A.M., J.F. Grippo, and C. Nervi. 1990. Isolation and binding characteristics of nuclear retinoic acid receptors. *Methods Enzymol.* 189:248.
36. Embree, N.D. 1939. The occurrence of cyclized vitamin A in fish liver oils. *J. Biol. Chem.* 128:187.