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Activation of Retinoid X Receptors Induces Apoptosis in HL-60 Cell Lines

LÁSZLÓ NAGY,[1,2] VILMOS A. THOMÁZY,[1,3] GREGORY L. SHIPLEY,[1] LÁSZLÓ FÉSÜS,[2] WILLIAM LAMPH,[4] RICHARD A. HEYMAN,[4] ROSHANTHA A. S. CHANDRARATNA,[5] AND PETER J. A. DAVIES[1]*

Department of Pharmacology, Medical School, University of Texas—Houston, Houston, Texas[1]; Departments of Biochemistry[2] and Pathology,[3] University Medical School, H-4012 Debrecen, Hungary; and Department of Cell Biology, Ligand Pharmaceuticals, San Diego,[4] and Retinoid Research, Departments of Chemistry and Biology, Allergan Incorporated, Irvine,[5] California

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Retinoids induce myeloblastic leukemia (HL-60) cells to differentiate into granulocytes, which subsequently die by apoptosis. Retinoid action is mediated through at least two classes of nuclear receptors: retinoic acid receptors, which bind both all-*trans* retinoic acid and 9-*cis* retinoic acid, and retinoid X receptors, which bind only 9-*cis* retinoic acid. Using receptor-selective synthetic retinoids and HL-60 cell sublines with different retinoid responsiveness, we have investigated the contribution that each class of receptors makes to the processes of cellular differentiation and death. Our results demonstrate that ligand activation of retinoic acid receptors is sufficient to induce differentiation, whereas ligand activation of retinoid X receptors is essential for the induction of apoptosis in HL-60 cell lines.

Apoptosis, the regulated or programmed death of cells, is an important physiological process controlling the growth of both normal and neoplastic tissues (6). Although much interest has recently been directed to the role of apoptosis in both physiological and pathological processes, very little is known of the molecular mechanisms that underlie its expression. To address some of these issues, we have investigated the induction of differentiation and cell death in human myeloid leukemia (HL-60) cells (13).

Normal hemopoietic maturation involves the progressive differentiation of precursor cells into terminally differentiated forms (i.e., neutrophils, monocytes, and macrophages). These mature forms subsequently undergo spontaneous apoptosis, a process that maintains homeostasis in the face of a continually proliferating pool of stem cells (24, 51). These events appear to be recapitulated in certain leukemic cell lines. HL-60 cells are a line of leukemic myeloid precursor cells that can be induced to undergo differentiation in response to a variety of biological and chemical agents (12). HL-60 cells not only differentiate but also are capable of undergoing apoptosis in response to the appropriate stimuli (37). We have investigated the ability of one class of biological response modifiers, retinoids, to induce both the differentiation and death of HL-60 cells.

Retinoids were chosen for these studies because there is ample evidence in the literature that retinoic acid induces both the differentiation and the death of HL-60 cells (11, 37). Thus, in HL-60 cells, it is possible to study the relationship between the induction of a differentiated phenotype and the expression of the apoptotic program in response to a single class of pharmacologic agents. Interpretation of the effects of retinoic acid on cellular function is complicated by the fact that there are at least two classes of retinoid receptors that may be activated by retinoic acid (35). One class, the RARs, are directly activated by ATRA (22), whereas the second class of retinoid receptors, the RXRs (34), are activated by an ATRA isomer, 9-*cis* RA (26, 33). The regulatory profile of these compounds is complex, because 9-*cis* RA not only activates RXRs but also binds to and activates RARs (25). RARs and RXRs coexist in most cells (35), including HL-60 cells (20, 23), and thus the effects of retinoic acid on cellular differentiation and death may reflect activation of either RARs or RXRs or both.

To dissect the molecular pathways involved in the effect of retinoic acid on HL-60 cells, we have taken advantage of the recent development of receptor-selective synthetic retinoic acid analogs (5, 7, 10, 18, 23, 27, 32) that preferentially activate either RARs or RXRs. Using these selective retinoids and HL-60 cell sublines that differ in their pattern of retinoid responsiveness, we have delineated the role of the different classes of retinoid receptors in the induction of differentiation and death in HL-60 cells. Our results demonstrate that ligand activation of endogenous RARs is sufficient to induce differentiation of the HL-60 cells but that induction of apoptosis requires ligand activation of endogenous RXRs.

MATERIALS AND METHODS

Abbreviations. AcNPV, *Autographa californica* nuclear polyhedrosis virus; AGN191701, (*E*)-5-[2-(5,6,7,8-tetrahydro-3,5,5,8,8-pentamethylnaphthalen-2-yl) propen-1-yl]-3-thiophenecarboxylic acid; ATRA, all-*trans* retinoic acid; Am80, 4-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenylcarbamoyl)benzoic acid; cAMP, cyclic AMP; CHAPS, 3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate; DMSO, dimethyl sulfoxide; IgG, immunoglobulin G; NBT, nitro blue tetrazolium; 9-*cis* RA, 9-*cis* retinoic acid; PBS, phosphate-buffered saline; RAR, retinoic acid receptor; RXR, retinoid X receptor; RARE, retinoic acid receptor response element; RXRE, retinoid X receptor response element; SD, standard deviation; SEM, standard error of the mean; TPA, tetradecanoyl phorbol acetate; TRE, thyroid hormone response element; TTNPB, (*E*)-4-[2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1-propenyl]benzoic acid; 3-met TTNPB, (*E*)-4-[2-(5,6,7,8-tetrahydro-3,5,5,8,8-pentamethyl-2-naphthalenyl)-1-propenyl]benzoic acid.

Cell culture and evaluation of cellular morphology. The parental clone of HL-60 cells (clone CCL 240) was obtained from the American Type Culture Collection, Rockville, Md. A clone of HL-60 cells resistant to the differentiating effects of ATRA, the HL-60 R cell line, was a kind gift of Steven Collins, Fred Hutchinson Cancer Center, Seattle, Wash. Both HL-60 CCL 240 and HL-60 R cells were cultured in suspension in RPMI 1640 medium (Gibco BRL Life Technologies, Inc., Gaithersburg, Md.) supplemented with 10% fetal bovine serum (Irvine Scientific Inc., Santa Ana, Calif.). A second clone of HL-60 cells

* Corresponding author. Mailing address: Department of Pharmacology, University of Texas—Houston, P.O. Box 20708, Houston, TX 77225. Phone: (713) 792-5904. Fax: (713) 792-5911. Electronic mail address: pdavies@farmr1.med.uth.tmc.edu.

resistant to the differentiating activity of retinoic acid, HL-60 CDM-1 cells, a kind gift of Diane Lucas (Walter Reed Army Medical Center, Washington, D.C.), was cultured in suspension in RPMI 1640 medium supplemented with insulin (5 μg/ml), transferrin (5 μg/ml), and sodium selenite (2 nM). Retinoids or an equivalent concentration of ethanol (<0.1, vol%) was added to cells in RPMI 1640 supplemented with insulin, transferrin, and sodium selenite at 2×10^5 cells per ml in 3 ml of medium in six-well tissue culture plates (Corning Glass Works, Corning, N.Y.). Standard cell culture conditions were used. After the indicated times, 0.05 to 0.1 ml of cells was taken to make cytospin preparations, which were fixed in 100% methanol and stained with Diff-Quick stain (Dade Diagnostics, Inc., Aguada, P.R.) according to the manufacturer's protocol. Cells were scored as differentiated if they showed features of metamyelocytes or more differentiated forms and as apoptotic if there was evidence of nuclear pycnosis and fragmentation, cytoplasmic condensation, and basophilia (see Fig. 2, side panels). Differential counts (at least 200 cells per sample) were performed on the cytospin preparations.

Retinoids. ATRA, TTNPB, 3-met TTNPB, Am80 (28), 9-*cis* RA, and AGN191701 were obtained from Allergan Inc., Irvine, Calif., and Ligand Pharmaceuticals, San Diego, Calif. Their ability to activate RARs and RXRs was assayed by a transactivation assay (10, 22, 34) and a ligand-binding assay (2, 25).

NBT reduction assay. HL-60 cells grown in RPMI 1640 medium for 72 h were counted, and 2×10^5 cells were aliquoted into 0.2 ml of RPMI 1640 medium with 0.8 ml of 0.125% NBT and 20 μl of TPA (10^{-5} g/liter). The cells were incubated at 37°C for 30 min, centrifuged at $1,100 \times g$ for 7 min at room temperature, and resuspended in 200 μl of PBS. Cytospins from aliquots of the samples were stained with Safranin-O (one part 2.5% Safranin-O in ethanol to 4 parts H_2O) for 5 min. The percentage of NBT-positive cells in each preparation was determined.

DNA fragmentation assay. HL-60 cells (2×10^5/ml in T-25 flasks) were incubated with retinoids as indicated and collected by centrifugation at $220 \times g$ for 10 min at 25°C. Cell pellets were lysed by the addition of 200 μl of 20 mM Tris-HCl (pH 7.4)–0.4 mM EDTA–0.4% Triton X-100, transferred to a microcentrifuge tube, and centrifuged at $15,000 \times g$ for 5 min at 4°C. Supernatants were collected and adjusted to 0.5 M NaCl (final concentration), and 250 μl of isopropanol was added. After overnight incubation at −20°C, samples were centrifuged at $15,000 \times g$ for 15 min, and the pellets were washed once with 70% ethanol and centrifuged again. Pellets were resuspended in a buffer containing 10 mM Tris-HCl (pH 7.5) and 1 mM EDTA. After addition of 6× DNA loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol, 40 vol% sucrose in water), samples were separated in a 1.5% agarose gel containing 0.5 μg of ethidium bromide per ml and visualized under UV light.

RNase protection assay. Total RNA was prepared by a one-step method (Tri-Reagent; Molecular Research Center, Inc., Cincinnati, Ohio) according to the manufacturer's instruction. Ten micrograms of total RNA was used per probe in the assay. [^{32}P]UTP-labeled receptor subtype-specific antisense riboprobes of human RARα, RARβ, RARγ, RXRα, RXRβ, and γ-actin were generated by inserting 100- to 150-bp-long fragments of their respective cDNAs into a pGEM4Z vector. The assays were performed as described before (26). Autoradiograms were scanned and digitalized, and the relative intensity of the protected bands was determined by using the PDQUEST Quantity One Scanning and Analysis Program (PDI, Inc., Huntington Station, N.Y.) on a Sun workstation. The intensities were normalized for the number of radioactively labeled uridine residues in each probe and for the intensity of the bands of an internal γ-actin control.

Transactivation assay. The biological properties of the retinoids were characterized in a cotransfection assay by examining their ability to interact with retinoid receptors and modulate gene expression. The receptor expression vectors pRS-hRARα, pRS-hRARβ, pRS-hRARγ, pRS-hRXRα, pRS-hRXRβ and pRS-hRXRγ used in the cotransfection assay have been described previously (10, 22, 34). A basal reporter plasmid, pMTV-LUC, containing two copies of the TRE-palindromic response element, pMTV-TREp-LUC (50), was used in all transfections for RARα, RARβ, and RARγ. The reporter plasmid pTK-CRB PII-LUC, containing the RXRE from CRBPII (36), was used for the RXRα, RXRβ, and RXRγ transfections.

CV-1 cell transfections were performed as described before (34, 36) and modified for automation in 96-well plates (8). Briefly, for CV-1 cells, the plasmids were transiently transfected by the calcium phosphate method with 10 ng of a receptor expression plasmid vector, 50 ng of the reporter luciferase plasmid, 50 ng of pRS-β-gal (β-galactosidase) as an internal control, and 90 ng of carrier plasmid. Cells were transfected for 6 h and then washed to remove the precipitate. The cells were incubated for 36 h with and without the test retinoid. All of the transfections were performed on a Beckman Biomek Automated Workstation, and cell extracts were then prepared as described by Berger et al. (8) and assayed for luciferase and β-galactosidase activities. All determinations were performed in triplicate in at least two independent experiments and were normalized for transfection efficiency by using β-galactosidase as the internal control.

Ligand-binding assay. For binding studies, retinoid receptors and a baculovirus expression system were used (2, 25). The methods for growth, purification, and assays of recombinant viruses followed the protocol outlined by Summers and Smith (49). The recombinant plasmids were cotransfected into Sf21 cells with wild-type AcNPV DNA, and the recombinant viruses were plaque purified. For the control extracts, wild-type AcNPV-infected cells were used. The baculovirus-infected cells were disrupted by Dounce homogenization (Kontes Co., Vineland, N.J.) in 10 nM Tris (pH 7.6)–5 mM dithiothreitol–2 mM EDTA–0.5% CHAPS–1 mM phenylmethylsulfonyl fluoride. The KCl concentration was adjusted to 0.4 M after cell lysis. The cell lysates were centrifuged for 1 h at 4°C at $100,000 \times g$, and the supernatant was recovered as a high-salt whole-cell extract. For the saturation binding analysis, cell extracts (50 μg of protein) were incubated at 0°C for 2 h with ^{3}H-labeled 9-*cis* RA (10) in the presence and absence of a 200-fold excess of unlabeled ligand. Specific ligand binding to the receptor was determined by a hydroxyapatite binding assay according to the protocol of Wecksler and Norman (51).

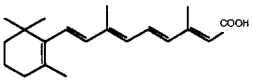
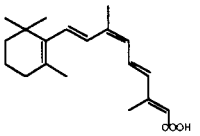
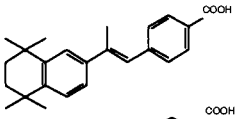
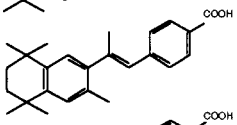
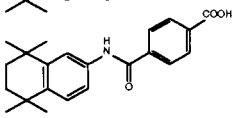
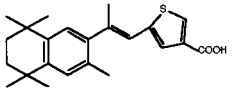
Western blot (immunoblot) analysis. Expression of BCL-2 protein was determined with the use of a monoclonal antibody, DAKO-bcl-2 124 (DAKO Corporation, Carpinteria, Calif.). Cell lysates were prepared as follows. Cells were collected by centrifugation at $500 \times g$ for 10 min. Pellets were resuspended in PBS and sonicated on ice. Protein concentration was determined by using the Bio-Rad protein assay (Bio-Rad Chemical Division, Richmond, Calif.). Cell lysates (50 μg of protein per lane) were size fractionated in sodium dodecyl sulfate–10% polyacrylamide gels prior to transfer to Immobilon-P Membranes (Millipore, Bedford, Mass.) by standard protocols. The membranes were blocked overnight with 5% dry cow's milk in PBS at 4°C. The monoclonal anti-BCL-2 antibody was used at a 1:1,000 dilution (1 μg/ml) in 1% dry milk in PBS, followed by a secondary antiserum, rabbit anti-mouse IgG (Pel-Freez Biologicals, Rogers, Ariz.) (10 μg/ml) diluted in 1% dry milk in PBS, and finally ^{125}I-protein A (0.99 mCi/ml; ICN Radiochemicals, Irvine, Calif.) at a concentration of 0.1 μCi/ml in 1% dry milk in PBS. The membranes were dried and exposed to film.

RESULTS

Receptor-selective retinoids. There are two major classes of retinoid receptors, RARs and RXRs (35). Each class of receptors includes at least three discrete receptor genes (RARα, RARβ, and RARγ or RXRα, RXRβ, and RXRγ) encoding distinct receptor proteins (35). The interaction of retinoids with these receptors can be assessed either directly, by measuring their ability to bind to the receptors (competitive ligand-binding assay), or indirectly, by measuring the ability of the retinoids to stimulate the transcriptional activity of the receptor following their transfection with a retinoid-responsive reporter gene into a suitable tissue culture cell (transactivation assay). Tables 1 and 2 compare the binding and transactivation activities of a series of a naturally occurring and synthetic retinoids that differ in their ability to interact with the different classes of retinoid receptors. The binding data represent the concentration of ligand necessary to reduce by 50% (50% inhibitory concentration [IC_{50}]) the binding of ^{3}H-9-*cis* RA to the retinoid receptors present in nuclear extracts of Sf-1 insect cells that had been transfected with baculovirus expression vectors containing one of the six retinoid receptors (see Materials and Methods). The transactivation activity is expressed as the concentration of the retinoid required to stimulate 50% of the maximal response (EC_{50}) in CV-1 cells transfected with a receptor expression vector and either an RAR-responsive reporter construct (pMTV-TREpal-Luc) or an RXR-responsive reporter construct (pTK-CRBPII-LUC) under the conditions described in detail in Materials and Methods.

9-*cis* RA is a prototypic receptor panagonist. It binds with high affinity to both RARs and RXRs and can stimulate the transcriptional activity of both classes of receptors. The effects of ATRA are more complicated. It can bind to all three RARs with an affinity comparable to that of 9-*cis* RA and can activate RAR-driven expression of the TRE-palindromic response element reporter construct. ATRA does not bind to RXRs, but it does show RXR-stimulating activity in in vivo transactivation assays (34). This activity is likely due to its conversion to 9-*cis* RA under in vivo culture conditions (25). TTNPB, on the other hand, is an RAR-specific retinoid. TTNPB binds with high affinity to all three classes of RARs and is a potent inducer of their transactivation activity. It neither binds to nor transactivates RXR receptors. Am80 is also an RAR-specific retinoid; it binds to and transactivates all three classes of RARs, show-

TABLE 1. Structures and transactivation properties of natural and synthetic retinoids[a]

Retinoid	Structure	Transactivation EC_{50} (nM)					
		RARα	RARβ	RARγ	RXRα	RXRβ	RXRγ
All-*trans* retinoic acid		350±31	80±9	10±2	900±70	1400±130	1100±85
9-*cis* retinoic acid		191±20	50±17	45±5	100±25	200±30	140±13
TTNPB		30±6	3±2	2±1	>5000	>5000	>5000
3-*methyl* TTNPB		340±30	230±28	180±15	1200±120	1175±150	1500±111
Am80		45±28	235±60	591±6	>5000	>5000	>5000
AGN191701		>5000	1990	979	215±23	180±41	105±54

[a] EC_{50} values were determined in cotransfection experiments as described in the text. Values are means for at least six determinations ± standard error. Values for ATRA, 9-*cis* RA, TTNPB, and 3-met TTNPB are from Boehm et al. (10).

ing a higher affinity for and a greater activity at RARα than either RARβ or RARγ. Like TTNPB, it neither binds to nor transactivates the RXRs. 3-met TTNPB is a retinoid receptor panagonist. It binds less well than TTNPB to RARs and shows proportionately less activity as a transactivator of RAR-dependent transcriptional activity. Unlike TTNPB, 3-met TTNPB also binds to the RXR receptors. It binds well to RXRα and RXRγ but poorly to RXRβ. 3-met TTNPB will also stimulate RXR-dependent activation of TK-CRBPII-Luc, although it is 10-fold less active than 9-*cis* RA in this assay.

AGN191701 is an RXR-selective retinoid. It binds to RXR receptors and will transactivate them. Although its affinity for these receptors in the binding assay is at least 10-fold lower than that of 9-*cis* RA, the two compounds are equivalent in their ability to transactivate cells transfected with RXRs and an RXRE reporter construct. AGN191701 does not bind to any of the RARs. It shows no transactivation activity in RARα-transfected cells and only a low level of transactivation activity in cells transfected with either RARβ or RARγ. This residual activity probably reflects ligand-induced activation of the RXR moiety of the RAR-RXR heterodimers present in RAR-transfected CV-1 cells.

Retinoid receptor profile of HL-60 cell lines. In the studies reported here, we evaluated the effects of receptor-selective retinoids on the induction of differentiation and death in several lines of human myeloid leukemia (HL-60) cells. To provide a baseline for the interpretation of these studies, we measured the abundance of transcripts for RARs α, β, and γ and

TABLE 2. Competition for ^{3}H-9-*cis* RA by RARs and RXRs[a]

Retinoid	Mean IC_{50} (nM) ± SEM					
	RARα	RARβ	RARγ	RXRα	RXRβ	RXRγ
ATRA	15 ± 2	13 ± 3	18 ± 1.8	>1,000	>1,000	>1,000
9-*cis* RA	7 ± 1.7	7 ± 1.3	17 ± 1.1	32 ± 3.5	12 ± 3	4 ± 2
TTNPB	36 ± 5.1	5 ± 2.3	26 ± 4.3	>1,000	>1,000	>1,000
3-met TTNPB	638 ± 75	1,169 ± 274	645 ± 120	32 ± 8	>1,000	100 ± 23
Am80	124	2,181	7,462	>10,000	>10,000	>10,000
AGN191701	>10,000	>10,000	>10,000	308	387	301

[a] Values for ATRA, 9-*cis* RA, TTNPB, and 3-met TTNPB are as reported by Boehm et al. (10). RARs and RXRs were prepared from Sf21 extracts. The values shown are the means for at least three determinations ± SEM.

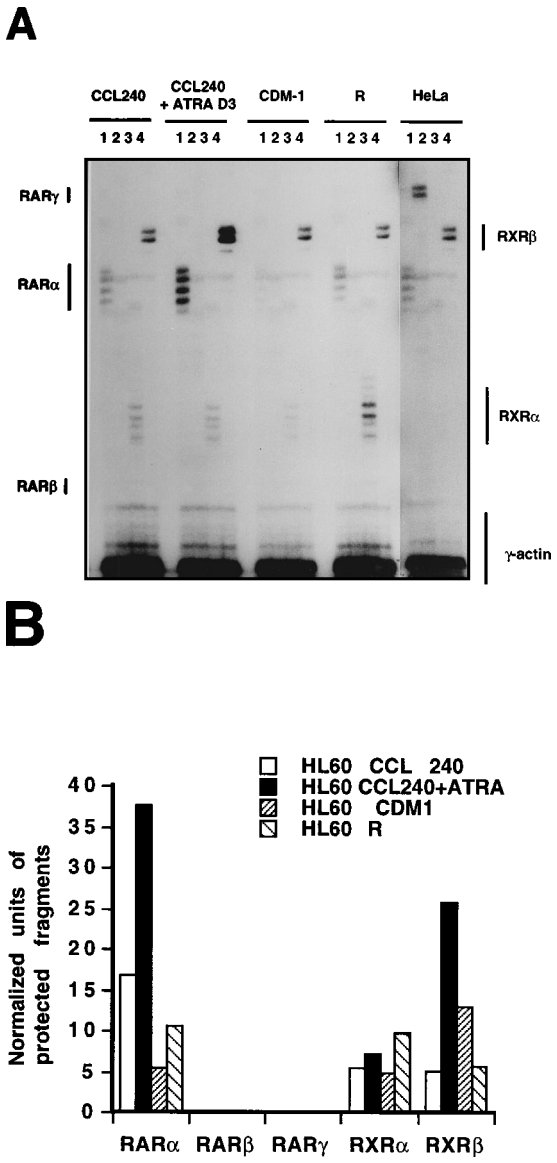


FIG. 1. Retinoid receptor profile of HL-60 cell lines. (A) Autoradiogram of an RNase protection assay performed on total RNA isolated from HL-60 CCL 240 cells (control and ATRA treated for 3 days), HL-60 CDM-1 and HL-60 R cells, and HeLa cells (used as a positive control for RAR and RXR expression). RNA from each cell line was hybridized to radiolabeled probes for human RARα (lane 1), human RARβ and RARγ (lane 2), human RXRα (lane 3), and human RXRβ (lane 4). A radiolabeled probe for human γ-actin was included in each assay. The mobility of specific protected fragments of each of the receptor transcripts is indicated by the solid bars on the right and left margins. (B) Relative abundance (in arbitrary units) of transcripts for each of the five retinoid receptors assayed in HL-60 CCL 240, CDM-1, and R cells and ATRA-treated HL-60 CCL 240 cells. Autoradiograms of fragments protected from RNase digestion were scanned, and the total protected radioactivity (in arbitrary units) was calculated. Values were corrected for the abundance of radiolabeled uridine residues in the probe oligonucleotides (based on the sequence) and were then normalized for recovery based on the intensity of the γ-actin band in each lane.

RXRs α and β in these cell lines by an RNase protection assay (RXRγ was not measured). Figure 1A shows the pattern of protected fragments for each of the radiolabeled receptor probes (the protected bands are identified by solid bars along the margins) following incubation with total RNA from either the parental HL-60 (CCL 240) cells or the same cells following treatment with ATRA for 3 days, two retinoic acid-resistant clones (HL-60 CDM-1 and HL-60R), and HeLa cells (used as positive control for RAR expression). Multiple protected bands differing by a single nucleotide are generated for each receptor probe as a result of partial degradation of the ends of the protected fragments ("breathing" of hybrids). A human γ-actin probe was used as an internal standard to normalize for transcript recoveries (see Materials and Methods).

Figure 1B summarizes the levels of transcripts for the five receptors assayed in the three HL-60 cell lines. HL-60 CCL 240 cells contained RARα, RXRα, and RXRβ. The level of RARβ and RARγ messages was undetectable. Three days of ATRA treatment resulted in a substantial change in the receptor profile of the parental (HL-60 CCL 240) cell line. The amount of RARα mRNA doubled, that of RXRβ mRNA increased fivefold, and that of RXRα mRNA remained unchanged. Messages for RARβ and RARγ remained undetectable. Comparison of receptor transcripts in HL-60 CCL 240, HL-60 CDM-1, and HL-60 R cells demonstrated that all three contained RARα transcripts and none contained RARβ or RARγ transcripts. The level of RARα transcripts in the HL-60 CDM-1 cells was lower than in either the HL-60 CCL 240 or HL-60 R cells. The lines contained RXRα and RXRβ. The levels of RXRα were similar in all three cell lines, whereas RXRβ transcript levels were higher in HL-60 CDM-1 cells than in either the CCL 240 or HL-60 R cell lines.

Effects of receptor-selective retinoids on cellular proliferation, differentiation, and death. The next step in our studies was to evaluate the effects of receptor-selective retinoids on the growth, differentiation, and apoptotic death of the "parental" HL-60 cell line CCL 240.

(i) Untreated cells. Control (solvent-treated) HL-60 CCL 240 cells, plated at 2×10^5/ml, grew exponentially, with a doubling time of ~24 h (Fig. 2A). Exponentially growing cultures had frequent mitotic figures and morphological features characteristic of a blastic leukemic cell line (Fig. 3A). There was little evidence of spontaneous differentiation, as measured by either a low abundance of morphologically differentiated cells (<5%, Fig. 2B) or a low frequency of cells competent to reduce the NBT dye (NBT^+ cells, Fig. 4A). There was also little evidence of spontaneous apoptosis in these exponentially growing cultures. Morphologic counts of apoptotic cells indicated that the basal apoptotic index was <5% (Fig. 2C and 3A). Apoptosis in myeloid leukemia cells is associated with fragmentation of DNA into nucleosomal multimers because of activation of a specific endonuclease (DNA laddering). There was no evidence of laddering in preparations of DNA from exponentially growing CCL 240 cells (Fig. 4B, lane 1).

(ii) ATRA-treated cells. ATRA is well known to induce growth arrest, differentiation, and apoptosis in HL-60 cells (11, 12, 14, 37). To provide a baseline for our studies on the activity of receptor-selective retinoids, we characterized the activity of ATRA in our assays of HL-60 functions. ATRA induced a complete inhibition of proliferative activity in CCL 240 cells within 2 days of its addition to the culture (Fig. 2A). Although at day 2 there was minimal evidence for morphologic differentiation, by day 4 numerous differentiated cells (detected by the appearance of cells with bands and segmented nuclei, decreased cellular volume, and a decrease in the nuclear/cytoplasmic volume ratio) were observed (Fig. 2B, right panel, and 3B). ATRA was a potent inducer of CCL 240 differentiation, with an EC_{50} of 5 nM (Fig. 3B). Morphologic differentiation was paralleled by an equivalent increase in the number of NBT^+ cells (Fig. 4A).

ATRA-induced differentiation of CCL 240 cells is associated with the appearance of apoptotic cells. Apoptotic HL-60 cells can be recognized by the appearance of nuclear condensation

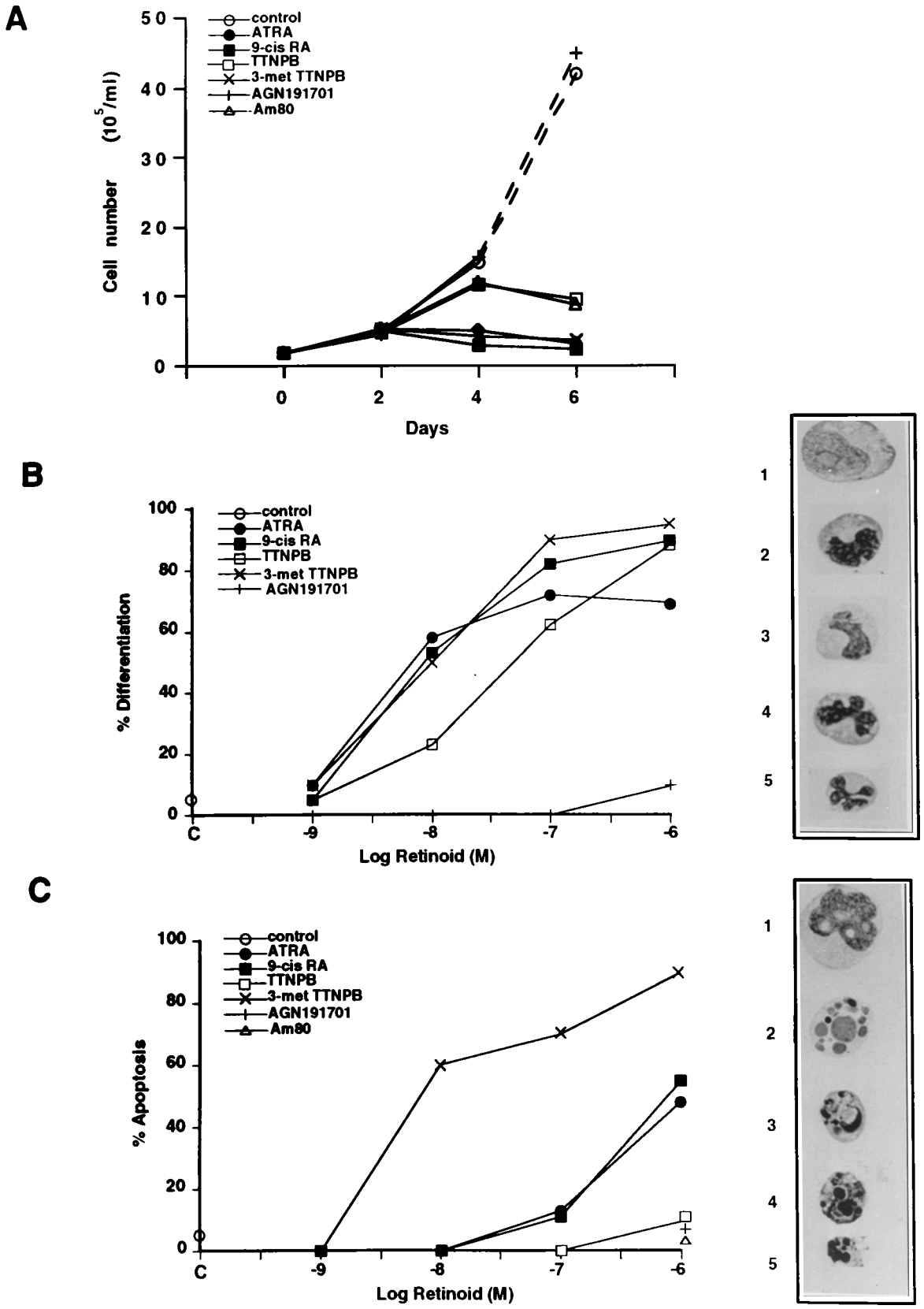


FIG. 2. Effect of receptor-selective retinoids on cellular proliferation, differentiation, and death. (A) Growth curves for HL-60 CCL 240 cells treated with the indicated retinoids at 1 μM. Retinoids were added at day 0, and the cell number was determined on days 2, 4, and 6. Cultures treated with solvent (control) and AGN191701 were diluted at day 4 to avoid overgrowth, and cell numbers were corrected for dilution (dashed line). The values shown are the means for three separate experiments; the SEM was ±15%. (B) Frequency of differentiated cells (expressed as a percentage of the total cell number) following treatment of HL-60 CCL 240

and fragmentation, increased basophilia of the cytoplasm, and a marked decrease in cellular volume (side panel to Fig. 2C). After 4 days of culture in ATRA, most cells are differentiated, and a few apoptotic cells can be detected (Fig. 3B). After 6 days, most of the differentiated cells have become clearly apoptotic (Fig. 3C). The apoptotic index with 1 μM ATRA is approximately 45%. It is notable that the EC_{50} for this effect (250 nM) is significantly higher than the EC_{50} for the induction of differentiation (5 nM).

(iii) 9-*cis* RA-treated cells. 9-*cis* RA is a retinoid receptor panagonist. Figure 2A demonstrates that 9-*cis* RA, like ATRA, produced complete growth inhibition within a few days of its addition to CCL 240 cells. This antiproliferative effect was paralleled by extensive differentiation (Fig. 2B). While 9-*cis* RA showed a potency comparable to that of ATRA as an inducer of differentiation, the actual degree of differentiation achieved in the culture was somewhat greater than that achieved with ATRA. Similarly, quantitative assays of NBT^+ cells showed that 9-*cis* RA induced almost complete differentiation of HL-60 cells (Fig. 4A). 9-*cis* RA was also comparable to ATRA in its ability to induce apoptosis (Fig. 2C). The EC_{50}s for the two compounds were very similar in these assays.

(iv) 3-met TTNPB-treated cells. Interpretation of the biological activity of ATRA and 9-*cis* RA is complicated by the fact that these compounds can be interconverted by enzymatic or non-enzymatic isomerization (9). 3-met TTNPB is a stable, aromatic retinoid that shares with 9-*cis* RA the ability to transactivate both RARs and RXRs (Table 1). We therefore investigated the effect of this synthetic retinoid panagonist on HL-60 cell differentiation and death.

Like 9-*cis* RA, 3-met TTNPB induces complete inhibition of proliferation within 48 h of its addition to HL-60 CCL 240 cells (Fig. 2A), and like 9-*cis* RA, it also induces complete differentiation (Fig. 2B). A striking feature of 3-met TTNPB action is that it is also a very potent inducer of apoptosis. Figure 2C demonstrates that 3-met TTNPB is significantly more active than 9-*cis* RA in inducing apoptosis: cultures treated with 10 or 100 nM 3-met TTNPB show extensive accumulation of apoptotic cells (Fig. 2C and 3E). The morphological evidence of retinoid-induced apoptosis is supported by the extensive laddering of DNA detected in 3-met TTNPB-treated cultures (Fig. 4, lane 3).

(v) TTNPB-treated cells. TTNPB is an aromatic retinoid that differs from 3-met TTNPB only in the lack of a methyl substituent at the 3 position of the tetramethyltetralin ring (Table 1). The absence of this methyl group, however, results in a marked increase in the potency of this retinoid as a transactivator of RARs and a reciprocal loss in RXR transactivation activity, qualifying TTNPB as an RAR-specific retinoid (34). Comparison of the activity of TTNPB and 3-met TTNPB therefore provides a useful index for assessing the relative roles of RAR and RXR activation in retinoid action. We therefore evaluated the ability of TTNPB to induce the differentiation and death of HL-60 cells.

Although TTNPB can inhibit the proliferation of HL-60 cells, the kinetics of this response is significantly different from the antiproliferative effects of ATRA, 9-*cis* RA, or 3-met TTNPB (Fig. 2A). In contrast to 3-met TTNPB, which induces growth arrest within 48 h, TTNPB requires at least 96 h to inhibit cellular proliferation. After 4 days of treatment with TTNPB, cultures show a high percentage of fully differentiated cells (Fig. 2B and 3D). This is reflected in the accumulation of NBT^+ cells (Fig. 4A). It is notable that the degree of differentiation attained in the TTNPB-treated cultures is somewhat lower than that achieved in cultures treated with retinoid panagonists such as 9-*cis* RA and 3-met TTNPB.

What is striking about the TTNPB-treated cultures is that although the cells differentiate, there is no evidence of apoptosis. Simple inspection of the TTNPB-differentiated cells (assessed 6 days after addition of retinoid to the cells [Fig. 2C and 3D]) indicates many "band" and segmented nuclear forms, indicative of differentiation, but no apoptotic cells. This is in striking contrast to parallel cultures exposed to equivalent or even lower doses of ATRA or 3-met TTNPB (Fig. 2C and 3C and E). The absence of apoptosis in the TTNPB-treated cultures was confirmed by analysis of cellular DNA for evidence of endonuclease activation (Fig. 4B, lane 2). There is no fragmentation of DNA in the TTNPB-treated cultures, further demonstrating that this retinoid induces differentiation but no apoptosis.

(vi) Am80-treated cells. The ability of TTNPB to induce differentiation but not death of HL-60 cells is not unique to this compound but is shared by other RAR-specific retinoids. Am80 is an aromatic retinoid with an internal amide (Table 1). Am80 is equivalent to TTNPB in its ability to bind to and to transactivate RARα, although it is significantly less active at RARβ and RARγ (Table 1). Like TTNPB, Am80 has no RXR-transactivating activity (Table 1). Am80 showed the same antiproliferative activity as TTNPB (Fig. 2A) and was equivalently active in inducing HL-60 cell differentiation (data not shown). As with TTNPB, there was no induction of apoptosis in Am80-treated cultures (Fig. 2C).

(vii) AGN191701-treated cells. AGN191701 is an aromatic retinoid analog of 3-met TTNPB in which the benzoic acid group is replaced with a 3-carboxy thiophene ring (Table 1). This substitution results in a retinoid that binds to and can transactivate RXRα, RXRβ, and RXRγ. This retinoid does not bind to RARs, although it has the ability to cause a modest increase in transactivation assays of RARβ and RARγ (probably via indirect activation of RAR-RXR heterodimers). AGN191701 neither binds to nor transactivates RARα, so in cells such as HL-60 cells, which express only RARα and RXRs. AGN191701 will function as an RXR-specific retinoid. We assayed the effect of AGN191701 on HL-60 cell function to determine to what extent the activity of retinoid receptor panagonists such as 3-met TTNPB could be replicated by an RXR-specific retinoid.

The results obtained when AGN191701 was used alone were strikingly simple. The compound had no antiproliferative activity; the cells grew exponentially for 6 days in the presence of 1 μM AGN191701 (Fig. 2A). There was no evidence of morphological differentiation (Fig. 2B) and no increase in the abundance of NBT^+ cells (Fig. 4A). Most importantly, there was no indication that in the CCL 240 cell line, AGN191701

cells with different concentrations of the indicated retinoids for 4 days. The side panel shows the typical morphology of cells undergoing granulocytic differentiation (1, myeloblast; 2, metamyelocyte; 3, band; 4 and 5, segmented polymorphonuclear cells). We scored cells as differentiated if they had reached or passed beyond the metamyelocyte stage (stage 2). The values shown reflect the means for three separate experiments; the SEM was ±15%. (C) Frequency of apoptotic cells (expressed as a percentage of the total cell number) following treatment of HL-60 CCL 240 cells for 6 days with different concentrations of the indicated retinoids. Differential cell counts were performed on stained cytospin preparations at day 6. The side panel shows the typical morphology of HL-60 cells undergoing apoptosis (1, differentiated cell; 2, nuclear fragmentation; 3 and 4, nuclear and cytoplasmic fragmentation and condensation; 5, apoptotic remnant). Cells showing fragmentation and condensation were scored as apoptotic. The values shown reflect the means for three separate experiments; the SEM was ±15%.

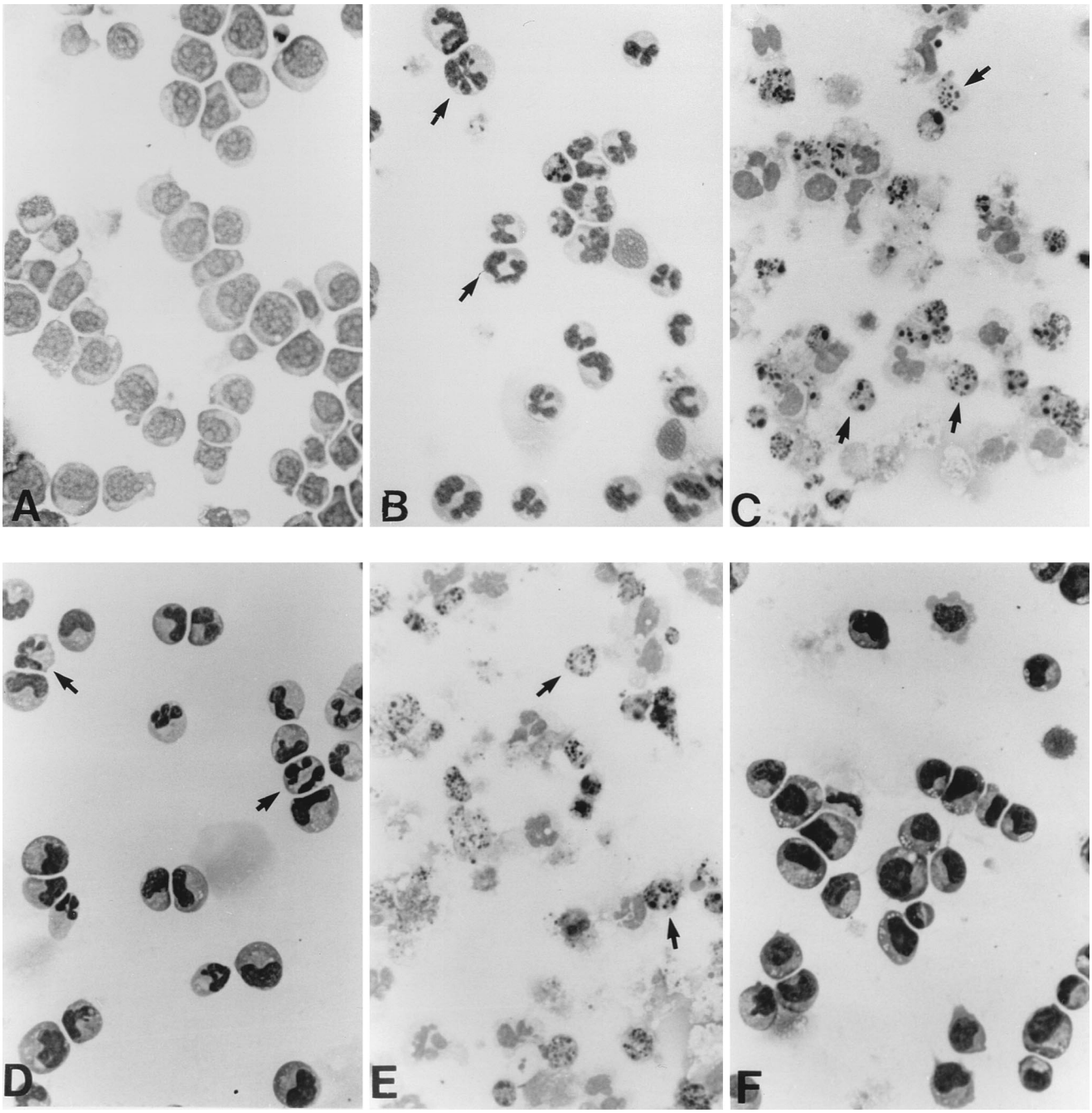


FIG. 3. Effect of retinoids on the morphology of HL-60 CCL 240 cells. (A) Control cells. (B) Cells following treatment with ATRA (1 μM) for 4 days; arrows identify typical differentiated cells with segmented nuclei. (C) Cells treated with ATRA (1 μM) for 6 days; arrows show typical apoptotic cells with fragmented nuclei. (D) Cells treated with TTNPB (1 μM) for 6 days; arrows show typical differentiated cells with segmented nuclei. (E) Cells treated with 3-met TTNPB (100 nM) for 6 days; arrows show typical apoptotic cells with fragmented nuclei. (F) Cells treated with AGN191701 (1 μM) for 6 days. Magnification, ×465.

could induce any apoptosis. The apoptotic index in the AGN191701-treated cultures was no different from that in controls (Fig. 2C), and there was no evidence of DNA fragmentation (data not shown). Thus, an RXR-specific retinoid with activity at an RXR comparable to that of 3-met TTNPB was not able to induce cellular differentiation or apoptotic cell death.

Differentiation and the induction of apoptosis. The preceding studies suggested that retinoids with RAR agonist activity could induce the differentiation of HL-60 cells but only compounds with retinoid receptor panagonist activity (compounds that activate both RARs and RXRs) could induce apoptosis. Furthermore, simple activation of endogenous RXRs by an RXR-specific retinoid such as AGN191701 was insufficient to induce apoptosis in HL-60 CCL 240 cells. This suggested that apoptosis is contingent on preceding differentiation. To test this possibility, we compared the ability of the RXR-specific retinoid AGN191701 to induce apoptosis in undifferentiated

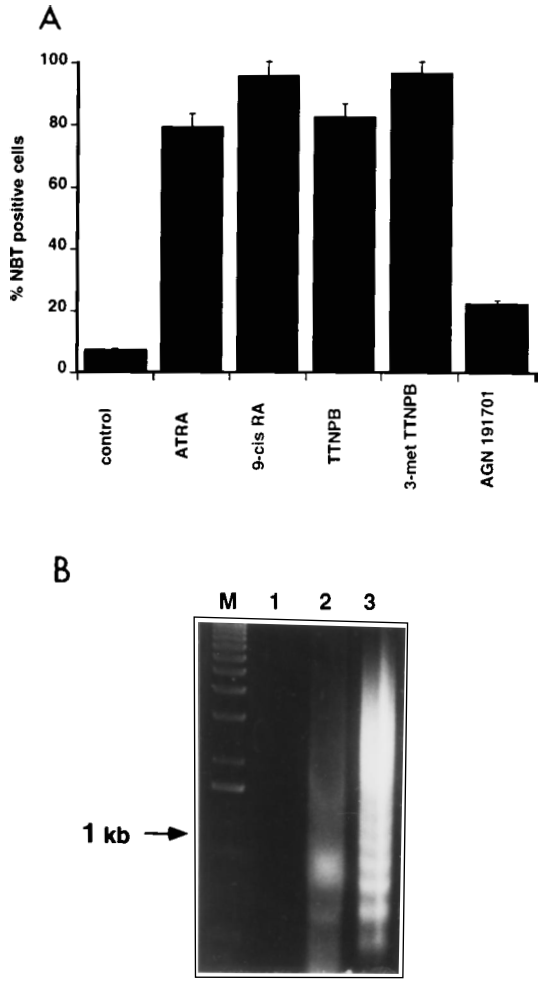


FIG. 4. Retinoid effects on the expression of markers of differentiation and apoptosis. (A) Frequency of differentiated cells (NBT^+ cells) in HL-60 CCL 240 cells treated with retinoid (1 μM) for 3 days. The values shown are the mean values for three experiments ± SD. (B) DNA fragmentation in retinoid-treated CCL 240 cells. DNA isolated from the nuclei of HL-60 CCL 240 cells treated for 6 days with solvent control (lane 1), 1 μM TTNPB (lane 2), or 1 μM 3-met TTNPB (lane 3) was fractionated on an agarose gel and stained with ethidium bromide. Lane M, molecular size markers.

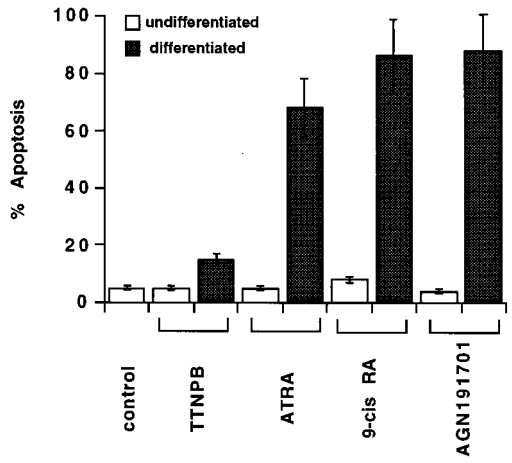


FIG. 5. Effect of retinoids on the induction of apoptosis in differentiated HL-60 cells. Undifferentiated (open bars) or differentiated (solid bars) HL-60 CCL 240 cells (induced by 3 days of treatment with 1 μM TTNPB) were treated with the indicated retinoids for 3 days, and the frequency of apoptosis was determined as described in the legend to Fig. 2. The mean values for three experiments and the SD are shown.

and differentiated HL-60 cells. Differentiation was induced by pretreating HL-60 CCL 240 cells for 3 days with TTNPB. The culture was then split into two parts; cells were either continued for an additional 3 days in the presence of TTNPB alone or exposed to either ATRA, 9-*cis* RA, or AGN191701. The results of this experiment (Fig. 5A) showed that although AGN191701 could not induce apoptosis in undifferentiated cells, it was very effective in inducing the apoptotic phenotype in differentiated cells. Treatment of undifferentiated cells with TTNPB, ATRA, 9-*cis* RA, or AGN191701 resulted in minimal apoptosis (<10%) (Fig. 2C). Treatment of differentiated cells with TTNPB produced only a slight increase in the apoptotic index (15%), but treatment of the same cells with AGN191701 resulted in extensive apoptosis (apoptotic index, 88%). AGN191701 was as effective as 9-*cis* RA or ATRA in inducing the death of differentiated HL-60 cells.

To determine the specificity of retinoid-induced differentiation as a prerequisite for apoptosis, we tested the ability of two other agents that induce HL-60 cell differentiation to sensitize them to RXR-induced apoptosis. Logarithmically growing cultures of HL-60 CCL 240 cells were pretreated for 72 h with either DMSO (1.25%) or TPA (10^{-6} M). Cells were then cultured for an additional 72 h with either an RXR-specific retinoid (AGN191701), RAR-specific retinoids (TTNPB and Am80), or retinoid receptor panagonists (9-*cis* RA and ATRA). There was no evidence of increased apoptosis in either the DMSO- or TPA-treated cultures when exposed to either the solvent or the various classes of retinoids. In all instances, the apoptosis index was less than 10%.

An independent way of evaluating the relationship between retinoid-induced differentiation and cell death in HL-60 cells is to compare the ability of receptor-selective retinoids to induce apoptosis in differentiation-resistant sublines of the parental HL-60 cell line. The HL-60 CDM-1 cell line is a differentiation-resistant line in which retinoic acid is capable of activating gene expression through RXRs, as measured by the induction of transglutaminase activity (7), although it does not induce morphologic differentiation. The HL-60 R cell line is a second differentiation-resistant cell line in which both retinoid-dependent gene expression and retinoid-induced differentiation are suppressed. Table 3 summarizes the effect of receptor-selective retinoids on the induction of apoptosis (measured by apoptotic index) in these cell lines. In these experiments, cells were exposed to retinoids in the presence of dibutyryl cAMP, conditions that have been shown previously to maximize the induction of retinoid-dependent gene expression (17, 30). In the

TABLE 3. Apoptosis in differentiation-resistant HL-60 cell lines[a]

Treatment	% Apoptosis		
	CCL 240	CDM-1	HL-60 R
None (control)	<5	<5	<5
TTNPB	<5	8	<5
9-*cis* RA	<5	92	<5
3-met TTNPB	<5	86	<5
AGN191701	<5	85	<5

[a] Cells were treated with 1 mM dibutyryl cAMP and retinoids (1 μM) or solvent (ethanol) as indicated, for 48 h. Cytospin preparations were made, and differential counts were performed as described in the text. The values shown are the means for at least three determinations; the SEM was <15%.

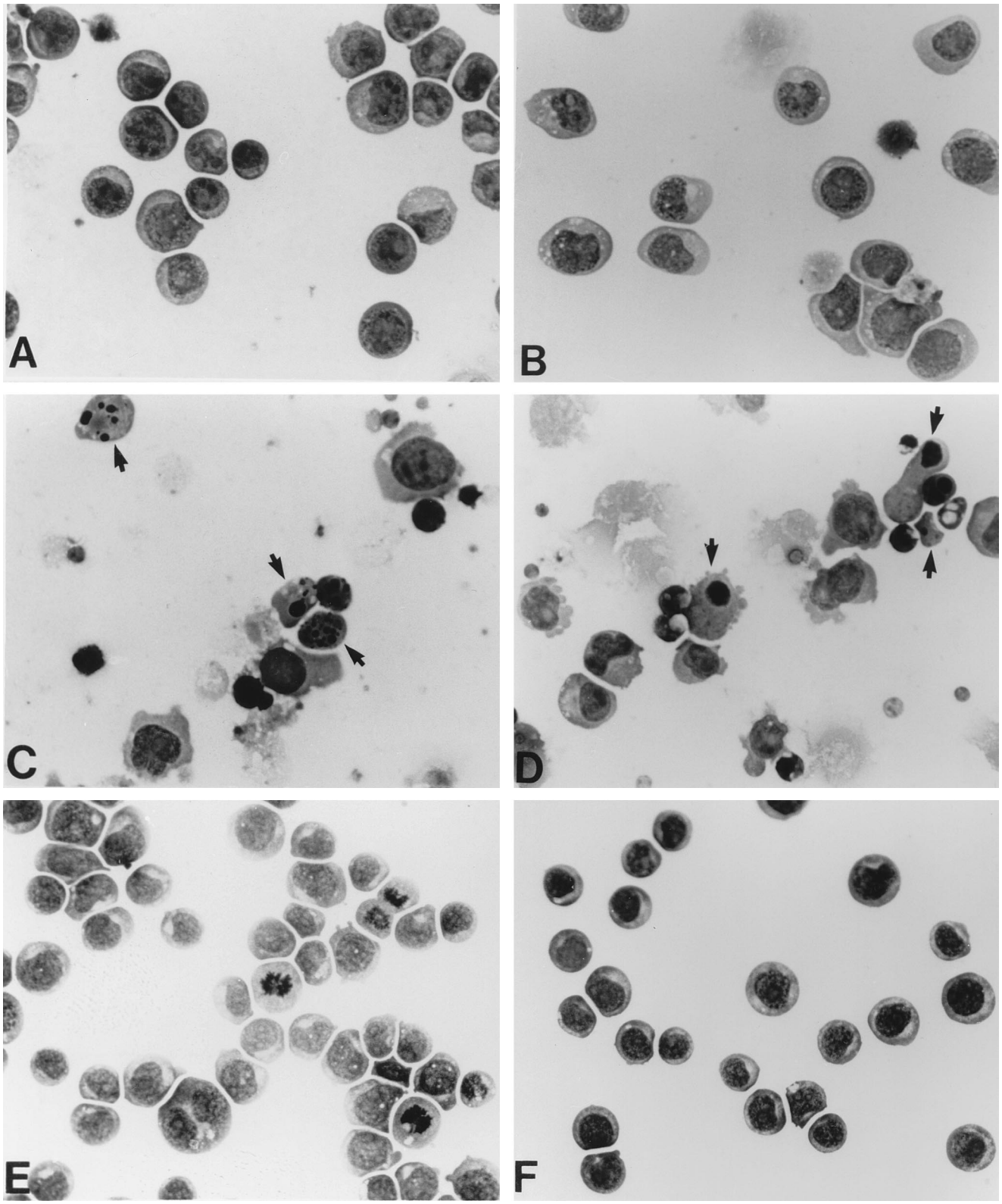


FIG. 6. Effect of retinoids on the morphology of HL-60 CDM-1 and HL-60 R cells. (A) Untreated CDM-1 cells. (B) CDM-1 cells treated with 1 μM ATRA (day 6). (C and D) CDM-1 cells treated with 1 μM AGN191701 for 3 days; arrows show typical apoptotic cells with pycnotic or fragmented nuclei. (E) Untreated HL-60 R cells. (F) HL-60 R cells treated with 1 μM AGN191701 for 3 days. All cultures were treated with 1 mM dibutyryl cAMP. Magnification, ×500.

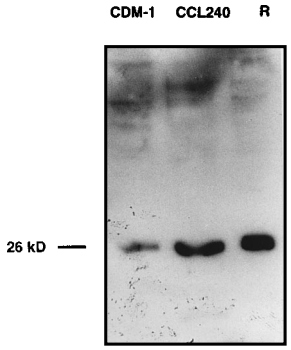


FIG. 7. BCL-2 protein levels in HL-60 cell lines. Cells were treated and processed as described in Materials and Methods. A Western blot of the BCL-2 levels in untreated HL-60 cell lines CDM-1, CCL 240, and R is shown.

HL-60 CDM-1 cell line, the retinoid panagonists 9-*cis* RA and 3-met TTNPB and the RXR agonist AGN191701 induced extensive apoptosis (apoptotic indices, >75%). The RAR-specific retinoid TTNPB did not induce apoptosis. Inspection of these cultures indicated that apoptosis developed in these cells without antecedent differentiation (Fig. 6). 3-met TTNPB- and AGN191701-treated cultures showed frequent apoptotic cells, with hyperchromic, condensed nuclei, extensive cytoplasmic blebbing, and cellular fragmentation characteristics of myeloid cell apoptosis. Few differentiated forms were detected, even in cells in the early stages of apoptosis.

Treatment of the parental CCL 240 and HL-60 R cell lines with retinoids produced a different result. Exposure of these cells to the receptor-selective retinoids for 2 days in the presence or absence of dibutyryl cAMP resulted in no apoptosis (Table 3). Apoptosis could not be induced in the parental cell line by RXR agonists unless it reached a differentiated state induced by RAR agonists (Fig. 5). The HL-60 R cell line appears to be resistant to both the differentiation-inducing and apoptosis-inducing activity of retinoid receptor panagonists as well as RAR-specific and RXR-specific retinoids.

BCL-2 protein expression in HL-60 cell lines. BCL-2 is a membrane-associated protein thought to be involved in suppressing the expression of apoptosis in both normal and transformed cells. Differences in the susceptibility of the different HL-60 cell lines to retinoid-induced apoptosis prompted us to compare the levels of BCL-2 protein, measured by Western immunoblot, in the CCL 240, CDM-1 and HL-60 R cell lines (Fig. 7). The level of BCL-2 in the CDM-1 cell line (Fig. 7, lane CDM-1) is much lower (8 to 10%) than the level in either the CCL 240 or HL-60 R cells.

DISCUSSION

Apoptosis appears to be a physiological component of normal myeloid cell maturation (24, 53). A number of lines of experimental evidence suggest that there is extensive apoptosis during the maturation of erythroid and myeloid precursor cells (53), so that many cells are lost prior to the migration of mature cells from the marrow into the circulation. Cytokines such as erythropoietin, granulocyte-macrophage colony-stimulating factor, and interleukin-3 increase the number of mature cells entering the circulation by their ability to suppress intramedullary apoptosis (29, 46, 53). Once they are in the circulation, the lifetime of mature neutrophils is limited by their propensity to undergo spontaneous apoptosis (24, 44, 45). Little is known of the physiological signals that induce the expression of the apoptotic program during normal myelopoiesis.

Susceptibility to apoptosis is not limited to normal myeloid maturation but is also a feature of the maturation of leukemic cell lines (31, 37). Lanotte et al. (31) have shown that IPC-81 myeloid leukemia cells undergo apoptosis in response to agents that elevate intracellular cAMP levels. U937 cells, a human myeloid leukemia cell line, undergo apoptosis in response to interleukin-6 (2). HL-60 cells are another model system of myeloid apoptosis since these cells can undergo apoptosis in response to physiological or pharmacological stimuli (16, 37, 38). Of particular relevance to our studies is the report by Martin et al. (37) that ATRA is a potent inducer of apoptosis in HL-60 cells. Retinoids have been linked to the induction of apoptosis in several in vivo and in vitro models of cell death (3, 4, 17, 21), but very little is known of the molecular mechanisms involved in the retinoid-mediated induction of this process. We have used the HL-60 cell line as a model system to investigate the role of retinoid receptors in the induction of apoptosis in myeloid leukemia cells.

HL-60 cells contain both RARs and RXRs (20, 23). There is extensive evidence in the literature to suggest that ligand activation of RARs in HL-60 cells can induce cellular differentiation (14, 15, 43). A number of retinoic acid analogs that activate RARs have been reported to induce HL-60 differentiation (23, 28, 47), and an RARα receptor antagonist can block the process (5). Furthermore, mutations in RARα that block endogenous RAR function render cells resistant to RA-induced differentiation (42). HL-60 cells contain not only RARα but also significant levels of RXRα and RXRβ. Much less is known of the role of these RXRs in the induction of HL-60 cell differentiation. Our finding that AGN191701, an RXR-specific retinoid, does not induce HL-60 differentiation suggests that ligand activation of RXRs is not sufficient to induce the differentiation of these cells. The RXR receptor itself, however, may be an integral component of the differentiation pathway, since Robertson et al. (43) found that in retinoic acid-resistant cells, susceptibility to retinoic acid-induced differentiation could be restored by transfection of an RXRα expression vector.

Several years ago, Strickland et al. (48) noted that 3-met TTNPB, a retinoid receptor panagonist, was more potent and more effective than TTNPB, an RAR-specific retinoid, in inducing HL-60 differentiation. In the present studies, we have found that retinoid receptor panagonists were more effective than RAR-specific compounds in inducing differentiation of the cells. These studies suggest that the mediator of differentiation-inducing activity of retinoic acid in HL-60 cells is an RAR-RXR heterodimer (RARα complexed with either RXRα or RXRβ) and that ligand activation of the RAR moiety of this complex is sufficient to induce differentiation. Ligand activation of the RXR moiety is not sufficient to induce differentiation, although it may potentiate the differentiation-inducing activity of RAR-binding ligands.

Although ligand activation of the RXR (25, 33, 54) in HL-60 cells may not play an important role in the induction of cellular differentiation, it appears to play a very important role in the induction of apoptosis. A key finding in our studies has been the observation that while RAR-specific compounds can trigger cellular differentiation, they are unable to induce apoptosis. Apoptosis was observed only in cultures treated with agents capable of activating both RARs and RXRs (retinoid receptor panagonists). In these cultures, the appearance of differentiation was followed by the apoptosis of many of the differentiated cells. Among the receptor panagonists used in this study, 3-met TTNPB was the most active. The marked activity of this retinoid may be due to its ability to bind RXRα with high affinity or may reflect other, unrecognized properties. 9-*cis* RA

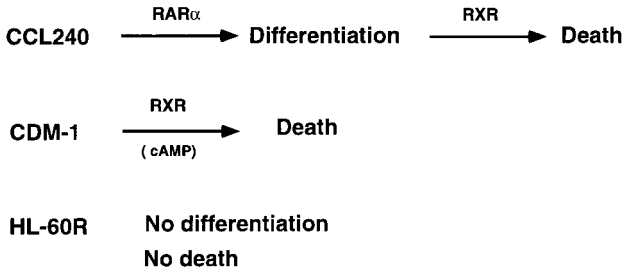


FIG. 8. Model of the relationship between retinoid-induced differentiation and apoptosis in HL-60 cell lines. The HL-60 CCL 240 cell line undergoes apoptosis in response to RXR agonists once it has been differentiated by RAR agonists. The HL-60 CDM-1 cell line undergoes apoptosis in response to RXR ligands. The HL-60 R cells, which are resistant to retinoids, do not undergo apoptosis.

and ATRA showed similar abilities to induce apoptosis. 9-*cis* RA binds to both RARs and RXRs with high affinity. ATRA binds to RARs but does not bind to RXRs (Table 2). Numerous studies have shown, however, that in in vivo studies, ATRA can activate RXRs (25, 33, 34). This activity is thought to be due to isomerization to 9-*cis* RA under the conditions of tissue culture (25), although alternative molecular explanations cannot be ruled out. In the HL-60 CCL 240 cell line, the RXR-specific retinoid AGN191701 was not capable of inducing the apoptosis of undifferentiated cells but could induce cell death once the cells had undergone RAR-induced differentiation. In the HL-60 CDM-1 cell line, AGN191701 induced extensive apoptosis without antecedent differentiation, although in these cells, the retinoid was most effective when combined with agents that increased intracellular cAMP levels. This effect on the RXR ligand appears to be specific, since it is not observed in the HL-60 R cell line, in which retinoid receptor function has been abrogated by a *trans*-dominant negative regulatory receptor mutation (42). If one assumes that the receptor species mediating the effects of the panagonist and RXR-specific retinoids on apoptosis is also an RAR-RXR heterodimer, then it is likely that ligand activation of the RXR moiety of this receptor alters the transcription of genes critical to the induction of apoptosis.

Several changes are detectable during retinoid-induced differentiation of HL-60 cells that may render them susceptible to RXR-induced apoptosis. The levels of BCL-2 in HL-60 cells decrease markedly during differentiation (19), and the levels of RXRβ transcripts increase. Expression of BCL-2 in HL-60 cells has been shown to suppress apoptosis (40, 41), so a reduction in the level of this protein may be necessary for the apoptotic response. The sensitivity of the CDM-1 cell line, which has very low basal rates of BCL-2 expression, to RXR-induced apoptosis is compatible with this conclusion. RAR-selective retinoids can suppress BCL-2 expression, whereas RXR-selective retinoids do not (39); however, it appears that simple suppression of BCL-2 expression is not sufficient to account for the whole process, since neither DMSO nor TPA, agents which decrease HL-60 BCL-2 expression (19), can render cells sensitive to RXR-induced apoptosis. It remains to be determined precisely how retinoid-induced differentiation is linked to the expression of apoptosis in these human myeloid leukemia cells.

The observation that ligand activation of retinoid receptor heterodimers may occur via activation of either the RAR or the RXR moiety may explain the progressive appearance of first morphologic differentiation and then apoptosis in myeloid cells undergoing terminal differentiation (Fig. 8). Early in the process, retinoic acid may primarily activate the RAR component of retinoid receptor heterodimers, activating the expression of genes linked to cellular differentiation (Fig. 8). As differentiation proceeds, however, progressive accumulation of retinoic acid metabolites that activate the RXR component of the heterodimer (i.e., 9-*cis* RA) and accumulation of the RXR receptors themselves could lead to activation of genes linked to apoptosis. The net effect would be a progressive increase in the frequency with which differentiating cells enter the apoptotic program. It is possible that differential activation of the RAR and RXR components of retinoid receptor heterodimers may represent a general mechanism of retinoid-dependent gene switching in retinoid-responsive cells and tissues.

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REFERENCES

1. **Afford, C. S., J. Pongracz, R. A. Stockley, J. Crocker, and D. Burnett.** 1992. The induction by human interleukin-6 of apoptosis in the promonocytic cell line U937 and human neutrophils. J. Biol. Chem. **267:**21612–21616.
2. **Allegretto, E. A., M. R. McClurg, S. B. Lazarchik, D. L. Clemm, S. A. Kerner, M. G. Elgort, M. F. Boehm, S. K. White, J. W. Pike, and R. A. Heyman.** 1993. Transactivation properties of retinoic acid and retinoid X receptors in mammalian cells and yeast: correlation with hormone binding and effects of metabolism. J. Biol. Chem. **268:**26625–26633.
3. **Alles, A. J., and K. K. Sulik.** 1990. Retinoic acid-induced spina bifida: evidence for a pathogenic mechanism. Development **108:**73–81.
4. **Alles, A. J., and K. K. Sulik.** 1989. Retinoic-acid induced limb-reduction defects: perturbation of zones of programmed cell death as a pathogenetic mechanism. Teratology **40:**163–171.
5. **Apfel, C., F. Bauer, M. Crettaz, L. Forni, M. Kamber, F. Kaufman, P. LeMotte, W. Pirson, and M. Klaus.** 1992. A retinoic acid receptor α antagonist selectively counteracts retinoic acid effects. Proc. Natl. Acad. Sci. USA **89:**7129–7133.
6. **Arends, M. J., and A. H. Wyllie.** 1991. Apoptosis: mechanisms and role in pathology. Int. Rev. Exp. Pathol. **32:**223–254.
7. **Beard, R. L., D. W. Gil, D. K. Marler, E. Henry, D. F. Colon, S. J. Gillett, P. J. A. Davies, and R. A. S. Chandraratna.** 1994. Structural basis for the differential RXR & RAR activity of stilbene retinoid analogs. Bioorg. Med. Chem. Lett. **4:**1447–1452.
8. **Berger, T. S., Z. Parandoosh, B. W. Perry, and R. B. Stein.** 1992. Interaction of glucocorticoid analogues with the human glucocorticoid receptor. J. Steroid Biochem. Mol. Biol. **41:**733–738.
9. **Blaner, W. S., and J. A. Olson.** 1994. Retinol and retinoic acid metabolism, p. 229–256. *In* M. B. Sporn, A. B. Roberts, and D. S. Goodman (ed.), The retinoids. Raven Press, New York.
10. **Boehm, M. F., M. McClurg, C. Pathirana, D. Mangelsdorf, S. K. White, J. Hebert, D. Winn, M. Goldman, and R. A. Heyman.** 1994. Synthesis of a high specific activity [^{3}H]-9-cis retinoic acid and its application for identifying retinoids with unusual binding properties. J. Med. Chem. **37:**408–414.
11. **Breitman, T. R., S. E. Selonic, and S. J. Collins.** 1980. Induction of differentiation of the human promyelocytic leukemia cell line (HL-60) by retinoic acid. Proc. Natl. Acad. Sci. USA **77:**2936–2940.
12. **Collins, S. J.** 1987. The HL-60 promyelocytic leukemia cell line: proliferation, differentiation, and cellular oncogene expression. Blood **70:**1233–1244.
13. **Collins, S. J., R. C. Gallo, and R. E. Gallagher.** 1977. Continuous growth and differentiation of human myeloid leukaemia cells in suspension culture. Nature (London) **270:**347–349.
14. **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. Mol. Cell. Biol. **10:**2154–2163.
15. **Collins, S. J., F. W. Ruscetti, R. E. Gallagher, and R. C. Gallo.** 1978. Terminal differentiation of human promyelocytic leukemia cells induced by dimethyl sulfoxide and other polar compounds. Proc. Natl. Acad. Sci. USA **75:**2458–2462.
16. **Cotter, T. G., S. V. Lennon, J. M. Glynn, and D. R. Green.** 1992. Microfilament-disrupting agents prevent the formation of apoptotic bodies in tumor cells undergoing apoptosis. Cancer Res. **52:**997–1005.
17. **Davies, P. J. A., J. P. Stein, E. A. Chiocca, J. P. Basilion, V. Gentile, V. Thomazy, and L. Fesus.** 1992. Retinoid-regulated expression of transglutaminases: links to the biochemistry of programmed cell death, p. 249–263. *In* G. Morriss-Kay (ed.), Retinoids in normal development and teratogenesis. Oxford University Press, Oxford.

18. **Delescluse, C., M. T. Cavey, B. Martini, B. A. Bernard, U. Reichert, J. Maignan, M. Darmon, and B. Shroot.** 1991. Selective high affinity retinoic acid receptor α or β-γ ligands. Mol. Pharmacol. **40:**556–562.
19. **Delia, D., A. Aiello, D. Soligno, E. Fontanella, C. Melani, M. A. Pierotti, and G. D. Porta.** 1992. bcl-2 proto-oncogene expression in normal and neoplastic human myeloid cells. Blood **79:**1291–1298.
20. **de The, H., A. Marchio, P. Tiollais, and A. Dejean.** 1989. Differential expression and ligand regulation of the retinoic acid receptor α and β genes. EMBO J. **8:**429–433.
21. **Fesus, L., P. J. A. Davies, and M. Piacentini.** 1991. Apoptosis: molecular mechanisms in programmed cell death. Eur. J. Cell Biol. **56:**170–177.
22. **Giguere, V., E. S. Ong, P. Seul, and R. M. Evans.** 1987. Identification of a receptor for the morphogene retinoic acid. Nature (London) **330:**624–629.
23. **Hashimoto, Y., H. Kagechika, and K. Shudo.** 1990. Expression of retinoic acid receptor genes and ligand-binding selectivity of retinoic acid receptors (RAR's). Biochem. Biophys. Res. Commun. **166:**1300–1307.
24. **Haslett, C.** 1992. Resolution of acute inflammation and the role of apoptosis in the tissue fate of granulocytes. Clin. Sci. **83:**639–648.
25. **Heyman, R. A., D. J. Mangelsdorf, 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 **68:**397–406.
26. **Janssen, K.** 1994. Ribonuclease protection assay, p. 4.7.1–4.7.2. *In* F. M. Ausubel et al. (ed.), Current protocols in molecular biology. John Wiley & Sons, New York.
27. **Jong, L., J. M. Lehmann, P. D. Hobbs, E. Harlev, J. C. Huffman, M. Pfahl, and M. I. Dawson.** 1993. Conformational effects on retinoid receptor selectivity: effects of 9-double geometry on retinoid X receptor activity. J. Med. Chem. **36:**2605–2613.
28. **Kagechika, H., T. Himi, K. Namikawa, E. Kawachi, Y. Hashimoto, and K. Shudo.** 1989. Retinobenzoic acids. 3. Structure-activity relationship of retinoidal azobenzene-4-carboxylic acids and stilbene-4-carboxylic acids. J. Med. Chem. **32:**1098–1108.
29. **Koury, M. J., and M. C. Bondurant.** 1990. Erythropoietin retards DNA breakdown and prevents programmed cell death in erythroid progenitor cells. Science **248:**378–381.
30. **Kruyt, F. A. E., G. Folkers, C. E. Brink, and P. T. Saag.** 1992. A cyclic AMP response element is involved in retinoic acid-dependent RARβ2 promoter activation. Nucleic Acids Res. **20:**6393–6399.
31. **Lanotte, M., J. B. Riviere, S. Hermouet, G. Houge, O. K. Vintermyr, B. T. Gjertsen, and S. O. Doskeland.** 1991. Programmed cell death (apoptosis) is induced rapidly and with positive cooperativity by activation of cyclic adenosine monophosphate-kinase I in a myeloid leukemia cell line. J. Cell. Physiol. **146:**73–80.
32. **Lehmann, J. M., L. Jong, A. Fanjul, J. F. Cameron, X. P. Lu, P. Haefner, M. I. Dawson, and M. Pfahl.** 1992. Retinoids selective for retinoid X receptor response pathways. Science **258:**1944–1946.
33. **Levin, A. A., L. J. Sturzenbecker, S. Kazmer, T. Bosakowski, C. Huselton, G. Allenby, J. Speck, C. Kratzeisen, M. Rosenberger, A. Lovey, and J. F. Grippo.** 1992. 9-Cis retinoic acid stereoisomer binds and activates the nuclear receptor RXRα. Nature (London) **355:**359–361.
34. **Mangelsdorf, D. J., E. S. Ong, J. A. Dyck, and R. M. Evans.** 1990. A nuclear receptor that identifies a novel retinoic acid response pathway. Nature (London) **345:**224–229.
35. **Mangelsdorf, D. J., K. Umesono, and R. M. Evans.** 1994. The retinoid receptors, p. 319–350. *In* M. B. Sporn, A. B. Roberts, and D. S. Goodman (ed.), The retinoids, 2nd ed. Raven Press, New York.
36. **Mangelsdorf, D. J., K. Umesono, S. A. Kliewer, U. Borgmeyer, E. S. Ong, and R. M. Evans.** 1991. A direct repeat in the cellular retinol-binding protein type II gene confers differential regulation by RXR and RAR. Cell **66:**555–561.
37. **Martin, S. J., J. G. Bradley, and T. G. Cotter.** 1990. HL-60 cells induced to differentiate towards neutrophils subsequently die via apoptosis. Clin. Exp. Immunol. **79:**448–453.
38. **Martin, S. J., and T. G. Cotter.** 1991. Disruption of microtubules induces an endogenous suicide pathway in human leukemia HL-60 cells. Cell Tissue Kinet. **23:**545–559.
39. **Nagy, L., V. A. Thomazy, R. A. Heyman, R. A. S. Chandraratna, and P. J. A. Davies.** Submitted for publication.
40. **Naumovski, L., and M. L. Cleary.** 1994. Bcl2 inhibits apoptosis associated with terminal differentiation of HL-60 myeloid leukemia cells. Blood **83:** 2261–2267.
41. **Park, J. R., K. Robertson, D. D. Hickstein, S. Tsai, D. M. Hockenbery, and S. J. Collins.** 1994. Dysregulated bcl-2 expression inhibits apoptosis but not differentiation of retinoic acid-induced HL-60 granulocytes. Blood **84:**440–445.
42. **Robertson, K. A., B. Emami, and S. J. Collins.** 1992. Retinoic acid-resistant HL-60R cells harbor a point mutation in the retinoic acid receptor ligand-binding domain that confers dominant negative activity. Blood **80:**1885–1889.
43. **Robertson, K. A., B. Emami, L. Mueller, and S. J. Collins.** 1992. Multiple members of the retinoic acid receptor family are capable of mediating the granulocytic differentiation of HL-60 cells. Mol. Cell. Biol. **12:**3743–3749.
44. **Savill, J., I. Dransfield, N. Hogg, and C. Haslett.** 1990. Vitronectin receptor-mediated phagocytosis of cells undergoing apoptosis. Nature (London) **343:** 170–173.
45. **Savill, J. S., A. H. Wyllie, J. E. Henson, M. J. Walport, P. M. Henson, and C. Haslett.** 1988. Macrophage phagocytosis of aging neutrophils in inflammation. J. Clin. Invest. **83:**856–875.
46. **Spivak, J. L., T. Pham, M. Isaacs, and W. D. Hankins.** 1991. Erythropoietin is both a mitogen and a survival factor. Blood **77:**1228–1233.
47. **Spruce, L. W., J. B. Gale, K. D. Berlin, A. K. Verma, T. R. Breitman, X. Ji, and D. Helm.** 1991. Novel heteroarotinoids: synthesis and biological activity. J. Med. Chem. **34:**430–439.
48. **Strickland, S., T. R. Bretman, F. Frickel, A. Nürrenbach, E. Hädicke, and M. B. Sporn.** 1983. Structure-activity relationship of a new series of retinoidal benzoic acid derivatives as measured by induction of differentiation of murine F9 teratocarcinoma cells and human HL-60 promyelocytic leukemia cells. Cancer Res. **43:**5268–5272.
49. **Summers, M. D., and G. E. Smith.** 1987. A manual of methods for baculovirus vectors and insect cell culture procedures. Tex. Agric. Exp. Stn. Bull. **155**.
50. **Umesono, K., V. Giguere, C. K. Glass, M. G. Rosenfeld, and R. M. Evans.** 1988. Retinoic acid and thyroid hormone induce gene expression through a common responsive element. Nature (London) **336:**262–265.
51. **Wecksler, W. R., and A. W. Norman.** 1979. An hydroxylapatite batch assay for the quantitation of 1α,25-dihydroxyvitamin D_3-receptor complexes. Anal. Biochem. **92:**314–323.
52. **Yamaguchi, Y., T. Suda, S. Ohta, K. Tominaga, Y. Miura, and T. Kasahara.** 1991. Analysis of the survival of mature eosinophils: interleukin-5 prevents apoptosis in mature human eosinophils. Blood **78:**2542–2547.
53. **Yu, H., B. Bauer, G. K. Lipke, R. L. Phillips, and G. V. Zant.** 1993. Apoptosis and hematopoiesis in murine fetal liver. Blood **81:**373–384.
54. **Zhang, X., J. Lehman, B. Hoffmann, M. I. Dawson, J. Cameron, G. Graupner, T. Hermann, P. Tran, and M. Pfahl.** 1992. Homodimer formation of retinoid X receptor induced by 9-cis retinoic acid. Nature (London) **358:**587–591.