Differences in the Action and Metabolism between Retinol and Retinoic Acid in B Lymphocytes

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Abstract. We have previously reported on the dependency of activated B lymphocytes for retinol. Here we confirm and extend these findings that cells deprived of retinol perish in cell culture within days, displaying neither signs of apoptosis nor of cell cycle arrest. Cell death can be prevented by physiological concentrations of retinol and retinal, but not by retinoic acid or three synthetic retinoic acid analogues. To exclude the possibility that retinoic acid is so rapidly degraded as to escape detection, we have tested its stability in intra- and extracellular compartments. Contrary to expectation, we find that retinoic acid persists for longer ($t_{1/2}$ 3 d) in cultures than retinol ($t_{1/2}$ 1 d). Furthermore, despite the use of sensitive trace-labeling techniques, we cannot detect retinoic acid or 3,4-didehydroretinoic acid among retinol metabolites. However, retinol is converted into several new retinoids, one of which has the ability to sustain B cell growth in the absence of an external source of retinol, supporting the notion of a second retinol pathway. We have also determined which of the known retinoid-binding proteins are expressed in B lymphoblastoid cells. According to results obtained with polymerase chain reaction-assisted mRNA detection, they transcribe the genes for cellular retino- and cellular retinoic acid-binding proteins, for the nuclear retinoic acid receptors, RAR-alpha, -gamma, and RXR-alpha, but not RAR-beta. Our findings that B cells do not synthesize retinoic acid or respond to exogenous retinoic acid on the one hand, but on the other hand convert retinol to a novel bioactive form of retinol, suggest the existence of a second retinoid pathway, distinct from that of retinoic acids.

Abstract. We have previously reported on the dependency of activated B lymphocytes for retinol. Here we confirm and extend these findings that cells deprived of retinol perish in cell culture within days, displaying neither signs of apoptosis nor of cell cycle arrest. Cell death can be prevented by physiological concentrations of retinol and retinal, but not by retinoic acid or three synthetic retinoic acid analogues. To exclude the possibility that retinoic acid is so rapidly degraded as to escape detection, we have tested its stability in intra- and extracellular compartments. Contrary to expectation, we find that retinoic acid persists for longer ($t_{1/2}$ 3 d) in cultures than retinol ($t_{1/2}$ 1 d). Furthermore, despite the use of sensitive trace-labeling techniques, we cannot detect retinoic acid or 3,4-didehydroretinoic acid among retinol metabolites. However, retinol is converted into several new retinoids, one of which has the ability to sustain B cell growth in the absence of an external source of retinol, supporting the notion of a second retinol pathway. We have also determined which of the known retinoid-binding proteins are expressed in B lymphoblastoid cells. According to results obtained with polymerase chain reaction-assisted mRNA detection, they transcribe the genes for cellular retino- and cellular retinoic acid-binding proteins, for the nuclear retinoic acid receptors, RAR-alpha, -gamma, and RXR-alpha, but not RAR-beta. Our findings that B cells do not synthesize retinoic acid or respond to exogenous retinoic acid on the one hand, but on the other hand convert retinol to a novel bioactive form of retinol, suggest the existence of a second retinoid pathway, distinct from that of retinoic acids.

Although retinoic acid (32) and didehydroretinoic acid (33) have critical functions in developmental biology, the significance of ubiquitous presence of retinol in adult tissues is not yet understood. On the other hand, among retinoids that can enhance growth and induce differentiation, retinoic acids are qualitatively and quantitatively more potent than retinol or retinal (5, 13, 17, 30). While the discovery of retinoic acid receptors (4, 15, 19) as transcriptional regulators has provided a satisfactory explanation for the effectiveness of retinoic acids, the biological functions of retinol remain unclear.

We have previously analyzed the growth requirements of human lymphoblastoid cells in culture and have found that these cells are critically dependent for growth on a constant supply of retinol (6). In the absence of retinol, these cells perish within days, and the addition of other retinoids, e.g., retinoic acid, are unable to prevent cell death. These findings have prompted us to perform an analysis of retinoids in lymphocytes to investigate the hypothesis that retinoic acid is the intracellular mediator for the retinol activity. We have neither been able to identify intracellular retinoid acid among retinol metabolites, nor do we have evidence that an extracellular supply of retinoic acid can overcome the retinol block. However, we have identified two new retinoids in B lymphocytes. One of these is a substitute for retinol, and appears to be an intracellular mediator of the control of cell physiology by retinol.

Materials and Methods

Retinoids

Ro 10-1670 (Etretine), Ro 13-7410 (TTNPB), Ro 40-6085 (AM 580), and 3,4-didehydroretinol were generous gifts of Hoffmann-LaRoche, Inc. (Nut-
3.4-didehydroretinol was oxidized to 3.4-didehydroretinal and 3.4-didehydroretinoic acid according to the procedure of Mayer et al. (22). Retinoidesters were a gift of Dr. W. Blazer (Columbia University, New York). All other unlabeled retinoids used were purchased from Sigma Chemical Co. (St. Louis, MO). [3H]Retinol was purchased from American Chemical Co. (Arlington Heights, IL) and was >98% pure according to HPLC analysis. The retinoids were dissolved in methanol/chloroform (3:1 vol/vol) at a concentration of 3 x 10^{-5} M with 10^{-5} M butylated hydroxytoluene (BHT) (Sigma Chemical Co.) added and stored in the dark at -20°C in a nitrogen atmosphere. Immediately before bioassays, the stock solutions were diluted in serum-free medium.

**Cell Lines**

The human Epstein–Barr Virus (EBV)-transformed B-cell lines 5/2, Ducaf, and SLA were established in our laboratory from the peripheral blood of healthy donors. The cell lines were grown in RPMI 1640 supplemented with 8% FCS, L-glutamine (2 mM), and antibiotics. The cell lines were tested regularly for mycoplasma infections and were consistently negative. The human teratocarcinoma cell line N-teta-2, the human breast carcinoma line BT-20, the human Burkitt lymphoma cell line Raji, and the human hematopoietic line Hep G-2 were obtained from the tumor cell line depository of Sloan-Kettering Institute.

**Cell Proliferation Assay**

The assay system is a modification of the assay developed by Blazaret al. (2). Cells taken from their exponential growth phase were washed twice and seeded or without retinoids at graded cell concentrations in HB 101 medium (Hana Biologics, Berkeley, CA) or in FCS containing RPMI medium. Assays were done in 96-well microtiter plates in a final volume of 200 μl/well and in 25-mm tissue-culture flasks. The cells in the microtiter plates were cultured for 72 h and cell growth was determined by labeling for the last 16 h with 0.8 μCi of [3H]thymidine (6.7 Ci/mM sp act). Growth in the culture flasks was determined in three aliquots of 100 μl taken at 24-h intervals and cultured in the presence of 0.8 μCi of [3H]thymidine for an additional 6-h period. To determine the viable cell number nine aliquots per time point were differentially counted in a Neubauer chamber in the presence of trypan blue.

**Retinoid Metabolism**

Cells were washed and seeded at a density of 3 x 10^5 cells/ml in HB 101 medium. 10^{-6} M retinol or retinoic acid were added. In control experiments, retinoids were added to culture medium alone. After various incubation times, 1-ml aliquots were removed, cells and conditioned medium separated by centrifugation and immediately afterwards diluted according to the procedure of McLean et al. (23). In short, cell pellets suspended in PBS or equal volumes of medium (0.8 ml) were extracted with 320 μl of n-butanol/acetonitrile (1:1 vol/vol) containing 10^{-4} M BHT and 10 μg/ml retinyl acetate as internal standard. The organic component was separated by addition of 240 μl saturated K2HPO4 and centrifugation for 1 min in an Eppendorf centrifuge (Brinkmann Instruments, Westbury, NY). 150 μl of the organic phase was chromatographed by HPLC (600 E solvent delivery system and 991 photodiode array detector; Waters Associates, Milford, MA) using an analytical reversed-phase C18 HPLC column (Vydac; The Separation Group, Hesperia, CA). The eluate was acetonitrile/water (80:20) containing 0.01 M ammonium acetate (flow rate 1.5 ml/min). Concentrations of extracted retinol and retinoic acid were quantitated by their peak areas at 325 nm relative to the internal standard peak area and referenced to standard curves of authentic retinol and retinoic acid.

**Metabolites of Retinol**

Cells were washed and seeded at a density of 6 x 10^6 cells in 10 ml of HB 101 medium with 1 μCi/ml [3H]retinol added. After 24 h, the washed cell pellet was delipidated according to the described method of McLean et al. (23). Unlabeled reference retinoids (1 μM each) were added. The crude lipid extract was loaded on an analytical reversed-phase C18 column (Vydac) and eluted with a gradient of water/methanol/chloroform as shown in Fig. 8. The flow rate was 0.5 ml/min. The retention times of the reference retinoids were determined by their specific UV absorption patterns in the photodiode array detector. The radioactive elution pattern of [3H]retinol and its metabolites was determined on line with a Flo 140 Radio-Chromatography Detector (Radiomatic Instruments & Chemical Co., Tampa, FL) using 2.5 ml/min Flo-sciit V scintillation fluid (Radiomatic Instruments & Chemical Co.).

**RNA and DNA Staining**

To analyze cell progression through the cell cycle, cells were stained with acridine orange (Polysciences, Inc., Warrington, PA) (9, 10). In brief, 0.4 ml of acid detergent (0.1% Triton X-100; 0.08 N HCl; 0.15 M NaCl) was added to 0.2 ml of the cell suspension. 30 s later, 1.2 ml of acridine orange staining solution (60 μg/ml acridine orange, 10^{-7} M EDTA, 0.15 M NaCl, 0.1 M citrate-phosphate buffer at pH 6) was added to each sample. Cells were measured immediately after staining with a FAC200 flow cytometer (Ortho Diagnostics, Westwood, MA) as described (18, 19). The red (600 to 640 nm) and green (515 to 575 nm) luminescence emissions from each cell were optically separated, measured by separate photomultipliers, and the data collected and stored in a Compaq Deskpro 386 computer. The number of cells in G_{1}, S, and G_{2} M cell cycle compartments were calculated using interactive computer programs.

**Polymerase Chain Reaction (PCR) Amplification**

The following oligonucleotide primers were used: as upstream primer for the human retinoic acid receptor (H-RAR) alpha (15) RA 1: 5'-GCT TGG TGG GCC AGA AG-3'; for H-RAR beta (4) RA 5S: 5'-GCA GAA AGC TCT CAA AGC ATG C-3' and for the RAR gamma (19) RA 5': 5'-AGG GATG AGC GCC TCT CGT TC-3'. The oligonucleotide primer RA 2A: 5'-CCC ACT TAA GAC TTC TG-3' was used as downstream primer for all three RARs, resulting in amplified products of 192 bp (nucleotide position 365-557), 347 bp (nucleotide 408-755), and 295 bp (nucleotide 580-875) for RAR α, β, and γ, respectively. Oligonucleotide primer pairs RBP1 (upstream) 5'-CTC TGG GAC GTG GTA CGC CA-3' and RBP2 (downstream) 5'-TTC TGG AAA AGA GAG GCT ACG-3' were used for the detection of retinol-binding protein (RBP) (9). CRBP1 (upstream) 5'-GCT AGT GGA AGA TGG TGT TC-3' and CRBP2 (downstream): 5'-CTT GAA TAC TTC CTT GCA CA-3' were used for the detection of cellular retinol-binding protein (CRBP) (10). These primer pairs amplified a product of 236 bp (nucleotide 436-673) for RBP and 376 bp (nucleotide 145-521) for CRBP, respectively. The oligonucleotide primers for the newly described nuclear retinoid receptor RXR α (21) RXR 5' (upstream): 5'-CTA ATG AGG TGCC TCC TAA GC-3' and RXR 3' (downstream): 5'-ATG AGG GCC ACC TTC TG-3' form an amplified fragment of 255 bp (nucleotide position 413-668). For cellular retinoid–binding protein (CRBP) (35) a pair of oligonucleotide primers corresponding to the mouse sequence was used: CRABPI (upstream) 5'-TGT GAG AAT GAG AAG ACC ATG AT-3' and CRABP2 (downstream): 5'-ACC AGC TCA TCG GCG CCA AA-3'; resulting in an amplified product of 124 bp (nucleotide position 69-193).

Total RNA was extracted in a single-step procedure by using RNAzol (Cinna/Biotex, Friendswood, TX). In a 20-μl reaction, 1 μg of total RNA was reverse transcribed in 50 ml Tris HCl (pH 8.3), 75 mM KCl, 3 mM MgCl2, 10 mM DTT, 0.5 mM of each dNTP, 80 pmol oligo dT (15), 25 U RNase inhibitor (Boehringer Mannheim, Indianapolis, IN) and 200 U of MMLV reverse transcriptase (Bethesda Research Laboratories, Gaithersburg, MD). After incubation at 37°C for 1-2 h, the mixture was heated for 5 min at 95°C and 3 μl of 10X PCR-buffer (100 ml Tris-HCl (pH 8.3), 500 mM KCl, 20 mM MgCl2, 0.01% gelatin), 1.25 U of Taq DNA polymerase (Perkin Elmer-Cetus Corp., Emeryville, CA) and 50 pmol of each primer was added and the volume adjusted to 50 μl. The reaction mixture was overlaid with 45 μl of mineral oil and then subjected to 35 cycles of DNA amplification in a Perkin-Elmer thermocycler, using the following cycle conditions: Denaturation for 1 min at 94°C, reannealing for 45 s at 55°C, extension for 1 min at 72°C. A final extension step for 7 min at 72°C was added. Amplified products were analyzed by electrophoresis on a 1.5% agarose gel and visualized by ethidium bromide.

**Results**

**Retinol Is a Stimulator of Cell Growth in Low Serum-containing Medium**

FCS is used in most cell culture systems at a concentration

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1. Abbreviations used in this paper: BHT, butylated hydroxytoluene; CRABP, cellular retinoic acid binding protein; CRBP, cellular retinol binding protein; RAR, retinoic acid receptor; RBP, retinol binding protein; RXR, retinoid X receptor.
Washed cells (2,500/well) were incubated with or without retinol in RPMI medium with the indicated amount of FCS. DNA synthesis was measured by [3H]thymidine as described. The measurements were done in triplicate. The SDs were <15%.

The growth rate of the 5/2 human lymphoblastoid cell line decreases rapidly at serum concentrations <10% (Fig. 1), as is the case with most cell lines. After 3 d of culture the 5/2 cells (25,000 cells/ml) in 1.25% FCS containing medium incorporated only one-twenty-fifth the [3H]thymidine of that incorporated in 10% FCS-containing medium. At the optimal concentration of 10⁻⁶ M, retinol did not change the thymidine uptake in 10% FCS but caused an eightfold increase in 1% FCS-containing medium. This growth-enhancing effect of retinol was not limited to serum-containing medium but was also seen with serum-free medium (HB 101) as described previously (6) (see also Fig. 5).

**Cell Death in Retinol-deficient Medium Is Cell Cycle Independent and Is Not Apoptotic**

The decrease in thymidine uptake in the retinol deficiency is due to cell death (6; Fig. 2, A and B). After 2 d of retinol deprivation, 30%, and after 3 d, 90% of the 5/2 cells were trypan blue positive. Cell death was not due to apoptosis (29, 36) since retinol-deficient 5/2 cells showed neither the degradation of DNA to oligonucleosomal bands nor the chromatin condensation characteristic of apoptosis (data not shown). Retinol-deficient cells showed a disintegrating cytoplasm with fused vacuoles when stained with Wright-Giemsa (Fig. 2, C and D). The nuclei, with their nucleoli, remained intact for prolonged periods of time. This observation was affirmed by measuring the RNA and DNA contents of 5/2 cells grown in retinol-deficient (Fig. 3 A) or retinol-containing medium (Fig. 3 B). 90% of cells from the retinol-deficient cultures are contained in the boxed area, indicating that they had lost most of their RNA due to disintegration of the cytoplasm. Comparing the DNA content of the nuclei with that of the normal cells showed similar distributions between G₁, S, and Gₙ/M phases, suggesting that there was no preferential cell death (loss of cytoplasm) in any specific phase of the cell cycle.

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**Figure 1.** Effect of retinol on 5/2 cells in FCS containing medium. Washed cells (2,500/well) were incubated with or without retinol in RPMI medium with the indicated amount of FCS. DNA synthesis was measured by [3H]thymidine as described. The measurements were done in triplicate. The SDs were <15%.

**Figure 2.** Retinol deprivation leads to cell death. 5/2 cells were washed once and seeded at a density of 300,000/ml in HB 101 medium with and without 10⁻⁶ M retinol. The (A) trypan blue-negative and (B) trypan blue-positive cell number of nine aliquots was determined every 24 h. Means ± SDs are shown. In a repeat experiment cells were stained with Wright-Giemsa stain after 40 h of culture. (C) Cells with 10⁻⁶ M retinol. (D) Cells without retinol.
cycle (calculation not shown). The Burkitt lymphoma line Raji and two other EBV-transformed lines tested, showed the same pattern of nonapoptotic, cell cycle-independent death in retinol deficient medium (data not shown).

5/2 Cells Transcribe RNAs Specific for Retinol and Retinoic Acid-binding Proteins

Requisite in retinoid physiology are the participation of extra- and intracellular binding proteins. As a prelude to the study of the pharmacological fate of retinol in B lymphoblastoid cells, we wished to take stock of the known cellular retinoid-binding proteins and therefore measured expression of the respective messages. Using specific primers, cDNA was amplified with the PCR and the expression of seven specific mRNAs determined (Fig. 4). Using human genomic DNA as template for DNA amplification, no primer pair leads to the same PCR product as the amplified cDNAs (data not shown). This indicates that introns are within all the primer pairs, as it is described for RBP, CRABP, and RAR (11, 12, 28). The cDNA detected by PCR reflects therefore exclusively mRNA and is not compromised by genomic DNA eventually present in the RNA preparation. In control PCR experiments, we chose one cell line known to be positive and another negative for each particular mRNA. We were not able to locate cell lines negative for RAR-α (Fig. 4d) or RXR-α (Fig. 4g). 5/2 cells expressed mRNA for cellular retinol-binding protein I (CRBP) (Fig. 4a) and cellular retinoic acid-binding protein I (CRABP) (Fig. 4b), but not for serum retinol-binding protein (RBP) (Fig. 4c). There was also message for the nuclear DNA binding proteins, RAR-α (Fig. 4d), RAR-γ (Fig. 4f), and RXR-α (Fig. 4g), but no transcript was detectable for RAR-β in 5/2 cells (Fig. 4e). Dr. W. Blaner confirmed by RNase protection assays that 5/2 cells are expressing mRNA for CRBP and CRABP and Dr. W. Miller (Memorial Sloan-Kettering Cancer Center) confirmed by Northern blotting expression of mRNA for RAR-alpha and RXR-alpha (personal communication).

Bioactivity of Natural and Synthetic Retinoids

Retinol is optimally bioactive at concentrations from $3 \times 10^{-6}$ to $5 \times 10^{-7}$ M in serum-free HB 101 medium (Fig. 5) as well as in low serum containing medium (data not shown). Retinol is toxic to lymphoblastoid cells in serum-free or low serum medium at concentrations $>5 \times 10^{-6}$ M. This toxicity can be prevented by increasing the amounts of serum (data not shown). Retinol is comparable with retinol in bioactivity at a concentration range of $1.5 \times 10^{-7}$ to $1.2 \times 10^{-6}$ M, but is already toxic at $2 \times 10^{-6}$ M (Fig. 5). The bioactivity of retinol is explicable by its fast conversion to retinol by 5/2 cells. If $10^{-6}$ M retinol was given to 200,000 5/2 cells in HB 101 medium, 85% of cellular and extracellular retinol was converted to retinol in 30 min, and after 120 min no retinol was detectable anymore (data not shown). Retinoic acid, supposedly the most potent natural retinoid, showed opposite effects at different concentrations (Fig. 5). At concentrations of $1 \times 10^{-6}$ to $2 \times 10^{-5}$ M, there is a suppression of thymidine uptake. Between $5$ and $8 \times 10^{-6}$ M, retinoic acid shows 10–70% of the retinol effect dependent on the bioassay, and at $10^{-5}$ M retinoic acid is toxic. Retinyl-palmitate does not show any bioactivity (data not shown). In most cell differentiation studies in vitro (1, 31, 34) and in vivo (13, 18), several synthetic retinoid analogs are up to 1000-fold more active than retinoic acid. Retinol is usually unable to induce differentiation. The three synthetic retinoid acid analogues, Ro 10-1670 (Etretine), Ro 13-7410 (TTNPB), and Ro 40-6085 (Am 580) (for chemical structure see Fig. 6A) were tested for their growth-sustaining activity on 5/2 cells in comparison with the natural retinoids, all-trans retinol and all-trans retinoic acid (Fig. 6B). None of the derivatives was able to replace retinol in the dose range tested...
Figure 4. Electrophoretic analysis of amplified cDNA for (a) cellular retinal-binding protein (CRBP), (b) cellular retinoic acid-binding protein (CRABP), (c) retinol-binding protein (RBP), (d) retinoic acid receptor alpha (hRAR), (e) beta (hRAR), (f) gamma (hRAR), and (g) RXR, the newly described nuclear retinoid receptor. 1 14g of total RNA extracted from (1) NTera-2 cells, (2) Hep G-2 cells, (3) BT20 cells, and (4) 5/2 cells was reverse transcribed and the cDNA amplified by using a specific oligonucleotide primer pair.

Figure 5. Dose-response curves of different natural retinoids. Washed 5/2 cells (5,000/well) were incubated for 72 h in HB 101 medium. The indicated amount of retinoids was added at the beginning of the experiment (Ix) or added additionally after 24 and 48 h. (3x). DNA synthesis was measured by [3H]thymidine as described. The measurements were done in triplicate. The SDs were <15%.

Retinol Is Used Up Faster in Conditioned Medium than Retinoic Acid

It has been reported that for some cells retinoic acid can rapidly induce its own metabolism (26, 27). Such rapid consumption could explain the lack of bioactivity of retinoic acid in lymphoblastoid cells. Therefore we tested the stability of retinoids in 5/2 cell and Raji cell (data not shown) cultures or cell-free “mock” cultures (Fig. 7). We found that in the presence of lymphoblastoid cells extracellular retinol concentrations decline more rapidly than those of retinoic acid. The effective half-life of retinoic acid was ~3 d as compared with 1 d for retinol. In the absence of cells, retinol and retinoic acid proved equally stable, showing only an insignificant difference in the rate of decline (15% and 30%, respectively, over a 3-d period).

Metabolism of Retinol (Fig. 8)

To test for metabolites of retinol within cells, lymphoblastoid cells were incubated overnight with tritium-labeled retinol. The cells were harvested and delipidated. The crude lipid mixture was separated on a reversed-phase column and the eluting retinol and its metabolites compared to standard concentrations of reference retinoids. The most abundant intracellular retinoid was all-trans retinol (45 min). The preceding shoulder at 44 min corresponds to 13-cis retinol. The metabolites eluting between 66 and 78 min correspond to retinylesters. The three major retinyl ester peaks coelute with the standards for retinyl linoleate, retinyl oleate, and retinyl palmitate, respectively. The cells contained two additional major groups of more hydrophilic retinol metabolites with peak elution times of 32-35 min and 36-39 min. Both peak regions contained more than one retinol metabolite judged by the shape of the radioactive peaks. None of the available retinol isomers or known metabolites, including 3,4-didehydroretinol and retinoic acid, elutes at these positions in our analytical system. The corresponding retinoids thus might comprise new forms of retinol metabolites. Peak 36-39 min has the same bioactivity on lymphoblastoid cells as retinol (Table I) if fed daily to the cells. We failed to observe 3,4-didehydroretinol, retinoic acid, and 3,4-didehydro-
Figure 6. Retinol but not synthetic retinoic acid analogs enable 5/2 cells to grow. (A) Chemical structure of retinoids used. (B) Dose–response curves measured day 3. (C) Effect of 3 × 10^{-7} M retinoids measured day 1, 2, and 3. (D) Combination of retinoic acid analogues (3 × 10^{-7} M) with (open bars) and without (filled bars) 10^{-6} M retinol measured day 3. In (B–D) 5/2 cells were washed twice and seeded at a concentration of 150,000 cells/ml in HB 101 medium. Triplicate samples of 100 μl of cell suspension were removed daily and pulsed for 6 h with [3H]thymidine. Means are shown. The SDs were never >20%.

Figure 7. Rate of loss of retinol and retinoic acid in cell-conditioned and unconditioned medium. 1 μM retinoid was added at zero time to HB 101 medium containing no cells or 400,000/ml 5/2 cells. At the shown time points aliquots of 1 ml were taken, medium or conditioned medium separated by centrifugation from its cells, delipidated, and analyzed on HPLC.

Discussion

In mammalian species the main forms of naturally occurring retinoids are retinol and its oxidation products, retinal and retinoic acid and their cis/trans isomers. Retinoids enhance growth and induce differentiation in many in vitro and in vivo systems (5, 13, 17, 30). In most instances, retinoic acids are qualitatively and quantitatively more potent than retinol or retinal. During studies of growth control of lymphocytes, we have shown previously (6) and confirm in the current report that the reverse order applies to growth control, with retinol and retinal more potent than retinoic acid, which is essentially unable to support B cell growth. Several closely related nuclear receptors have been described for retinoic acid, but none so far for retinol or retinal. The retinoic acid receptor RAR with its three different subtypes (α, β, and γ) (4, 15,
Figure 8. Retinol metabolites in 5/2 cells. 5/2 cells (6 x 10^6 cells in 10 ml HB 101 medium) were incubated with all-trans-[3H]retinol (10 μCi/ml). After 24 h, retinoids were extracted from the washed cell pellet and unlabeled marker retinoids were added. The crude mixture was loaded on an analytical reversed-phase C18 column. Retinoids were eluted with the shown gradient of water/methanol/chloroform. The flow rate was 0.5 ml/min. DPM were determined with an on-line scintillation counter. Reference retinoids were the all-trans forms of 0, 3,4-didehydroretinoic acid; 1, all-trans-retinoic acid; 2, 3,4-didehydroretinol; 3, retinol; 4, retinyl linoleate; 5, retinyleoleate; 6, retinyl palmitate.

19) as well as the newly described receptor RXR (21) are retinoic acid inducible transcription factors of the steroid/thyroid hormone receptor supergene family. Direct binding of retinoic acid was shown for RAR but not for RXR (21).

In this study, we used human lymphoblastoid cells as a prototype for activated human B cells, but many other cell types, including thymocytes and 3T3 cells show a similar dependency on retinol (unpublished observation). The principal finding is that at a dilute cell density and below a certain concentration of retinol these cells cease to grow and die. The reason why they die is unclear. Cytological inspection of retinol-deprived lymphoblastoid cells offers few clues, except that events seem to develop in the cytoplasm, which eventually lyse, leaving intact nuclei behind. Thus apoptosis is precluded, and we have confirmed that the characteristic "ladder" of oligonucleosomal bands is not seen in agarose gels (29, 36). Analysis of the total DNA content of nuclei by flow cytometry informs us that they derived from cells with a distribution from diploid to tetraploid, similar to that of intact cells of an exponentially growing culture. Hence, a blockage in any particular phase of the division cycle in retinol-deprived cells is unlikely to contribute to cell death. It is unclear why lymphoblastoid cells at high density (>10^5/ml) survive retinol starvation apparently intact. However, when such high-density, retinol-deprived cells are transferred to low density, retinol-free conditions, cell death is accelerated. It is conceivable that intracellular stores of vitamin A in the form of retinylesters are used up, rendering the cells even more sensitive to retinol deprivation.

Dose–response relationships in retinol stimulation show that conditions comparable to physiological ones are attained in cell culture. The normal concentrations of retinol in human and bovine plasma is 1.0–1.5 x 10^-6 M (16), and 10^-6–10^-7 M was the optimal range required by cells in serum-free medium. Most frequently used media formulations contain 10% FCS, corresponding to a retinol concentration of 10^-7 M, and interestingly 10^-7 M retinol is also a concentration sustaining growth in serum-low or serum-free medium, provided that it is replenished daily. The need for repeated feeding may be due to the different forms in which retinol is delivered to cells, i.e., as a stable complex with the serum protein RBP in FBS-containing serum versus a semi-stable complex with albumin in serum-free medium.

Our survey of proteins concerned with retinoid transport, storage, and function showed mRNAs of several of them expressed in 5/2 cells. The use of oligo dT to prime reverse transcription and the sizes of the PCR products observed indicate that only fully transcribed, polyadenylated and processed mRNA is amplified by the PCR. 5/2 cells express message for CRBP and CRABP. CRBP is thought to play a

Table I. Comparison of Growth-stimulating Effect between Retinol and the Retinol Derivative Peak 36–39 min

| Stimulating agent | Growth-stimulating effect (cpm) |
|------------------|---------------------------------|
| Plain medium     | 362 ± 193                       |
| Retinol (10^-6 M)| 38,645 ± 4,357                  |
| Peak 36–39 min   | 31,421 ± 3,713                   |

Washed 5/2 cells (5,000/well) were incubated for 72 h in serum-free HB101 medium. DNA synthesis was assessed by a 16-h [3H]thymidine uptake. The experiment was done in triplicate. The data correspond to mean ± SD. Peak 36–39 min was prepared according to the following procedure: 2,000 ml of 5/2 cells (600,000/ml) were grown with 10^-6 M retinol added to HB101 medium. After 16 h, retinoids were eluted from the washed cell pellet. The crude mixture was loaded onto a semipreparative reversed-phase C18 column. Retinoids were eluted with the gradient of water/methanol/chloroform shown in Fig. 8. The flow rate was 2 ml/min. The peak corresponding to 36–39 min was collected and used 1/100 in the illustrated experiment. The retinoids were reeled daily.
specific role in cellular uptake of retinol (7). The role of CRABP in lymphoblastoid or other cells is unclear. This protein has high affinity for retinoic acid and its synthetic analogues TTNPB, AM80, and Ettetine, and none for retinol (18). The presence of CRABP poses the question of its function in cells that have not been found to produce measurable levels of retinoic acid. In other systems retinoic acid has been found to originate from cells different from those which respond to it. The role of retinoic acid, providing directly or indirectly positional information to developing cells in the chick wing bud is one pertinent example (32). The proposed role of CRABP in this system is to modulate the morphogen gradient (20). Lymphoblastoid cells, however, are not known to depend on retinoic acid, yet they express specific binding protein. Similar puzzles concern the retinoic acid receptors RAR alpha and gamma, as well as RXR, whose mRNAs are expressed in 5/2 cells. In the absence of measurable amounts of retinoic acid, and assuming that retinoic acid is indeed the natural ligand for each, their functional potential in lymphoblastoid cells is unclear.

As mentioned before, in most biological test systems for retinoids employed to date, especially those measuring the differentiation potential, retinoic acid is superior in potency to retinol (5, 13, 17, 30). In fact, in some systems retinol appears to be completely inactive. Retinoic acid analogues, especially TTNPB, are more potent inducers of differentiation than retinoic acid itself (1, 13, 18, 31, 34). TTNPB induces morphological differentiation effects via one of the "orphan" receptors (14) of the steroid and thyroid hormone receptor superfamily. We also investigated whether lymphoblastoid cells are able to convert retinol to retinoic acid or didehydroretinoic acid, by using radioisotope-labeled all-trans-retinol. Within the limits of the sensitivity of our assay procedure, estimated to be able to detect levels as low as 10^{-4} M, we were unable to detect retinoic acid or didehydroretinoic acid. Instead, we found relatively large amounts of retinylesters and new retinoids at elution times 32-35 min and 36-39 min. Judged by spectral criteria and the isotope content, these two hitherto unidentified compounds are likely to be members of the family of retinoids. We speculate that one of them, eluting at 36-39 min, may constitute the intracellular mediator of retinol. We base this possibility on the observation that among the retinol metabolites detected in 5/2 cells, solely the 36-39-min peak contained the activity to sustain retinol-deprived 5/2 cells in culture. Experiments are in progress to assess whether this novel retinoid exerts its growth-controlling effects via one of the "orphan" receptors (14) of the steroid/retinoic acid receptor superfamily.

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