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 retinol- and cellular retinoic acid-binding proteins, for the nuclear retinoid 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.

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-didehydretinol was oxidized to 3,4-didehydroretinol and 3,4-
didehydroretinoic acid according to the procedure of Mayer et al. (22).
Retinoids were dissolved in methanol/chloroform (3:1 vol/vol) at a concentration of 3 x 10^{-6} M with 10^{-5} M butylated hydroxytoluene (BHT) (Sigma Chemical Co.) 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 S2, Duca,
and SLA were established in our laboratory from the peripheral blood of healthy donors. The cell lines were grown in RPMI 1640 supplemented with 10% FCS, 1-glutamine (2 mM), and antibiotics. The cell lines were tested regularly for mycoplasma infections and were consistently negative. The human teratocarcinoma cell line N-tera 2, the human breast carcinoma cell line BT-20, the human Burkitt lymphoma cell line Raji, and the human hepatoma 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 Blazer et al. (2). Cells taken from their exponential growth phase were washed twice and seeded for or without retinoids at graded cell concentrations in serum

Serum-containing Medium

The following oligonucleotide primers were used: as upstream primer for the human retinoic acid receptor (RAR) alpha (15) RA 1: 5'-GCT TGG CTG AGC AGA AG-3'; for hRAR beta (4) RA05: 5'-GGA GAA AGC TCT CAA AGC ATG C-3' and for the RAR gamma (19) RA 5: 5'-AAG GAG ATG GCC TCT CTG TC-3'. The oligonucleotide primer RA2: 5'-CCC ACT TAC AAG CTC 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 a, B, and g, respectively. Oligonucleotide primer pairs RBPI (upstream) 5'-CTC TGG GAC GTG GTA CGC CA-3' and RBBP2 (downstream) 5'-TCC TGG AGA AAG GAG GCT AGC AGC-3' were used for the detection of retinol-binding protein (RBP) (9). CRBPI (upstream) 5'-GAG AGA AGA TGG TGG TC-3' and CRBBP2 (downstream): 5'-CTT GGA TAC TCT GTT CCA GA-3' were used for the detection of cellular retinol-binding protein (CRBP I) (8). These pairs form an amplified 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 RNR a (21) RXRII (upstream): 5'-TCA ATG GCC TCC TCA AAG TC-3' and RXR2 (downstream): 5'-ATG ACG GCC CAC TCT TG-3' form an amplified fragment of 255 bp (nucleotide position 413-668). For cellular retinoid acid-binding protein (CRABP) (35) a pair of oligonucleotide
times primers corresponding to the mouse sequence was used: CRABPI (upstream) 5'-TGG GAG AAT GAG ACA GAG-3' and CRABBP2 (downstream) 5'-ACC ACG TCA TCG GCG CAA A-3'; resulting in an amplified product of 124 bp (nucleotide position 69-193).

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 mg/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 using a FAC200 flow cytometer (Ortho Diagnosti-
cs, Westwood, MA) as described (18, 19). The red (600 to 640 nm) and green (515 to 575 nm) luminescence emissions from each cell were opti-
cally separated, measured by separate photomultipliers, and the data col-
lected and stored in a Compaq Deskpro 386 computer. The number of cells in G1, S, and G2+M cell cycle compartments were calculated using inter-
active computer programs.

Polymerase Chain Reaction (PCR) Amplification

RNA was extracted in a single-step procedure by using RNazol (Cinna/Biotex, Friendswood, TX). In a 20-pl 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, 20 pmol oligo dT (15), 25 U RNSic inhibitor (Boehringer Mannheim, Indianapolis, IN) and 200 U of MMLV reverse transcriptase (Bethesda Research Laboratories, Gaithers-
burg, 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 mM 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 cy-
cle conditions: Denaturation for 1 min at 94°C, annealing 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

FC5 is used in most cell culture systems at a concentration

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%.

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%.

of 5–10%. 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 eight-fold 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 G2/M phases, suggesting that there was no preferential cell death (loss of cytoplasm) in any specific phase of the cell.

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 48 h of culture. (C) Cells with $10^{-6}$ M retinol. (D) Cells without retinol.
S/2 Cells Transcribe mRNAs 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. 4 d) or RXR-α (Fig. 4 g). S/2 cells expressed mRNA for cellular retinol-binding protein I (CRBP) (Fig. 4 a) and cellular retinoic acid–binding protein I (CRABP) (Fig. 4 b), but not for serum retinol-binding protein (RBP) (Fig. 4 c). There was also message for the nuclear DNA binding proteins, RAR-α (Fig. 4d), RAR-γ (Fig. 4 f), and RXR-α (Fig. 4 g), but no transcript was detectable for RAR-β in S/2 cells (Fig. 4 e). Dr. W. Blaner confirmed by RNase protection assays that S/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 S/2 cells. If 10^{-6} M retinol was given to 200,000 S/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^{-7} to 2 \times 10^{-6} 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, 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. 6 A) were tested for their growth-sustaining activity on S/2 cells in comparison with the natural retinoids, all-trans retinol and all-trans retinoic acid (Fig. 6 B). None of the derivatives was able to replace retinol in the dose range tested.

Figure 3. Effect of retinol deprivation on RNA and DNA content. 5/2 cells were washed and seeded at a density of 50,000/ml in HB 101 (A) without, and (B) with 10^{-6} M retinol. After 48 h the cells were stained with acridine orange and 5,000 cells/sample were analyzed by flow cytometer. Scattergrams represent distribution of cells with respect to their DNA and RNA content. 2n corresponds to diploid, 4n to tetraploid DNA content. The boxed dots with very low RNA content correspond to nuclei.
between 10^{-9} and 10^{-6} M. Ro 40-6055 and Ro 13-7410 produced an increase in thymidine uptake at a concentration of 1-3 \times 10^{-7} M compared to the negative control, but this effect was 8 to 20 times lower than the optimal response to retinol. None of the derivatives at a concentration of 3 \times 10^{-7} M was able to reverse the decline in the thymidine uptake on days 2 and 3 of culture (Fig. 6 D) due to cell death, as described above. When the synthetic retinoids (at 3 \times 10^{-7} M concentration) were mixed with 10^{-6} M retinol, the growth stimulatory capacity of the latter was not inhibited (Fig. 6 D).

**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 1-3 days compared with 1 day 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 standards for 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 retinylpalmitate, respectively. The cells contained two additional major groups of more hydrophilic retinyl esters. The cells contained two additional major groups of more hydrophilic retinoid 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 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 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.

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%.

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, 17, 30). 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, 17, 30).
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, retinyl-oleate; 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 lyses, leaving intact nuclei behind. Thus apoptosis is precluded, and we have confirmed that the characteristc "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 retinyl esters 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^-4 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^-4 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
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 TTPNB, AM80, and Etretine, 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 (13, 17, 30). In fact, in some systems retinol appears to be completely inactive. Retinoic acid analogues, especially TTPNB, are more potent inducers of differentiation than retinoic acid itself (1, 13, 18, 31, 34). TTPNB induces transcriptional activity of RAR but not of RXR (21). In the context of our investigation on growth promotion by retinoids, it is noteworthy that retinoic acid and its synthetic analogues are inactive despite the presence of the corresponding cytoplasmic and nuclear receptors thought to mediate retinoic acid effects. Retinoic acid does not impede the action of retinol, as mixtures of the two exhibit the same dose-dependent growth stimulation as retinol alone.

Retinoic acid, but not retinol, has been shown in hamster tissues and a number of cell lines to induce its own catabolism (25, 27). It has been argued that 10T1/2 cells fail to undergo transformation in response to retinoic acid because it is more rapidly cleared and degraded than retinol (27). It has been further proposed that retinol would constitute the reservoir from which a constant supply of short-lived retinoic acid would be produced. Rapid turnover would explain why so little retinoic acid can be detected. We have tested this hypothesis in 5/2 and Raji cells and find that it does not apply. On the contrary, in these cell lines retinol, with a t1/2 of 1 d, is metabolized faster than retinoic acid (t1/2, 3 d). Thus, instability cannot be the explanation for the lack of growth stimulation by retinoic acid.

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^{-5} M, we were unable to detect retinoic acid or didehydroretinoic acid. Instead, we found relatively large amounts of retinylesters and two 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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