Anhydroretinol: A Naturally Occurring Inhibitor of Lymphocyte Physiology

By Jochen Buck,‡ Felix Grün, Fadila Derguini,§ Yq Chen,‡
Shoji Kimura, Noa Noy,* and Ulrich Hämmerling

From the Department of Immunology, Memorial Sloan-Kettering Cancer Center, New York
10021; the Departments of *Medicine and ‡Pharmacology, Cornell University Medical College,
New York 10021; and the §Department of Chemistry, Columbia University, New York 10027

Summary

Vitamin A (retinol) is an essential cofactor for growth of B lymphocytes in culture and for activation of T lymphocytes by antigen receptor–mediated signals. 14-hydroxy-4,14-retro-retinol (14-HRR) a metabolite of retinol, has been implicated as the intracellular mediator of this effect. Anhydroretinol (AR) is a retinol derivative with retro structure produced in activated human B lymphocytes and the insect cell lines SF 21 and Schneider S2. AR reversibly inhibits retinol- and 14-HRR-dependent effects and blocks B lymphocyte proliferation as well as activation of resting T lymphocytes. The intracellular signaling pathway blocked by AR in T cell activation is distinct from the calcineurin/interleukin 2 pathway inhibitable by cyclosporine A or FK-506.

In vertebrates, many different processes as diverse as growth, vision, and reproduction are vitamin A (retinol) dependent (1). Retinol is the source for a number of metabolites that are adapted to specific functions, such as 11-cis retinal in vision (2) or 9-cis and all-trans retinoic acid for differentiation of a number of cellular systems (3). The general mechanism of action of retinoids presumably is connected with their specific binding proteins. The retinoic acids serve as ligands for a group of nuclear receptor molecules termed RAR and RXR (4–6). These receptors represent transcription factors akin to the much larger family of steroid receptors (7). They have in common a modular structure that includes zinc finger- and ligand-binding domains. On the basis of genetic homologies, a number of "orphan" receptors have recently been identified, so-called because of similar modular design, but lack of knowledge of the respective ligands. Thus it is possible that a large family of lipophilic molecules evolved from primordial compounds in parallel to the superfamily of steroid/retinoic acid receptors.

Retinol can be regarded as a pro-hormone from which several effector molecules are derived. In whole animal experiments, retinol could not be replaced by retinoic acid to reverse all symptoms of vitamin A deficiency (8). For instance, neither the profound immune dysfunction nor male sterility resulting from vitamin A deprivation was ameliorated by retinoic acid repletion (9–11). Thus other metabolites of retinol, effective in the development of lymphoid cells and spermatocytes, have long been postulated. The first such effector molecule, 14-hydroxy-retro-retinol (14-HRR)[1] (12), was discovered among the endogenous retinoids of normal and transformed lymphocytes. It had the outstanding ability to substitute for retinol in its role as a required cofactor for activation and growth of lymphocytes in culture, and is presumed to be the active mediator of the retinol effect in this system (13). 14-HRR has been found in a variety of tissues and species extending as far back in evolution as fruit flies (our unpublished results). This remarkable conservation might point to a central physiological function. In fact, many transformed lymphoid cells deprived of 14-HRR after retinol withdrawal cease to proliferate and die (14, 15). However, at this time the mechanism of action of 14-HRR is unknown, although by analogy to the retinoic acids, the role as a ligand for a cytoplasmic or nuclear receptor is more than likely. This concept received strong support in this report with the discovery of a second endogenous retinoid of lymphocytes with retro structure, anhydroretinol (AR), which functions as a competitive inhibitor of 14-HRR and which might bind to the same receptor protein.

Materials and Methods

Cells and Cell Cultures. The lymphoblastoid cell line 5/2 was derived in our laboratory from a healthy volunteer by transformation with EBV. It was propagated in RPMI 1640 medium supplemented with 7% fetal bovine serum. Where indicated as serum-free culture, the medium contained RPMI 1640 supplemented with 8×10^{-7} M insulin, 7×10^{-8} M transferrin, 2×10^{-6} M linoleic acid, 2×10^{-6} M delipidated BSA, 2 mM L-glutamine, 1 mM sodium pyruvate, and antibiotics (referred to as ITBL medium). Invertebrate cell lines were obtained as follows: S2M3 from Dr. Robert DeLotto (Memorial Sloan-Kettering Cancer Center); and SF 21 and SF 9 from Dr. Philip W. Majerus (Washington University, St. Louis, MO). They were grown in Grace's insect medium/10% FCS at 26°C.

Retinoids. All-trans-retinol was purchased from Sigma Chemical Co. (St. Louis, MO). [^{3}H]retinol was purchased from Dupont/New England Nuclear (Boston, MA). AR was isolated from the cell line SF 21 as follows: 10 liters of cell suspension were incubated with 10^{-5} M retinol overnight at 26°C. The cell pellet was extracted by the method of McClean et al. (16). The extract was chromatographed on a preparative C$_{18}$ HPLC column using a water/methanol/chloroform gradient. The three cis-trans isomers of AR eluting at 92% methanol/8% water were pooled. A second run on an analytical C$_{18}$ column yielded pure AR. Its UV spectrum showed a characteristic vibronic fine structure with maxima at 351, 368, and 390 nm (17, 18). Mass spectroscopy and proton nuclear magnetic resonance confirmed the structure (Derguini, F., manuscript in preparation).

Synthetic AR produced by acid catalyzed dehydration of all-trans retinol (18) was used for bioassays. Synthetic AR was purified by the same HPLC chromatographic procedure described above. 14-hydroxy-retro-retinol was isolated from pellets of HeLa cells as described (12).

B Cell Proliferation and T Cell Activation Assays. These were carried out as described in detail by Garbe et al. (13).

Retinoid Binding Proteins. Human cellular retinol binding protein 1 (CRBP-1) and cellular retinoic acid binding protein 1 (CRABP-1) were gifts from Drs. W. S. Blaner (Columbia University) and J. Napoli (State University of New York, Buffalo, NY), respectively.

Results and Discussion

Among the metabolites of retinol extracted from B lymphoblastoid cells and chromatographically separated on an HPLC C$_{18}$ column with a water/methanol/chloroform gradient (Fig. 1 C), we noted the presence of a retinoid with relatively hydrophobic properties, eluting in the particular gradient system used at minute 29. Two minor companions at minutes 28 and 27 are likely cis/trans isomers of the same compound. The three peaks exhibited nearly identical UV absorption spectra with vibronic fine structure and maxima at 351, 368, and 390 nm indicating a retro-retinoid skeleton with six double bonds. A chance observation of overproduction of an identical retinoid in a subline of the Drosophila S2M3 cell line allowed us to isolate this retinoid in quantity and to determine its structure as AR on the basis of mass and nuclear magnetic resonance spectroscopy, as will be described elsewhere (Derguini, F., manuscript in preparation) (see structure 3 in Fig. 1). AR synthesis is dependent on an
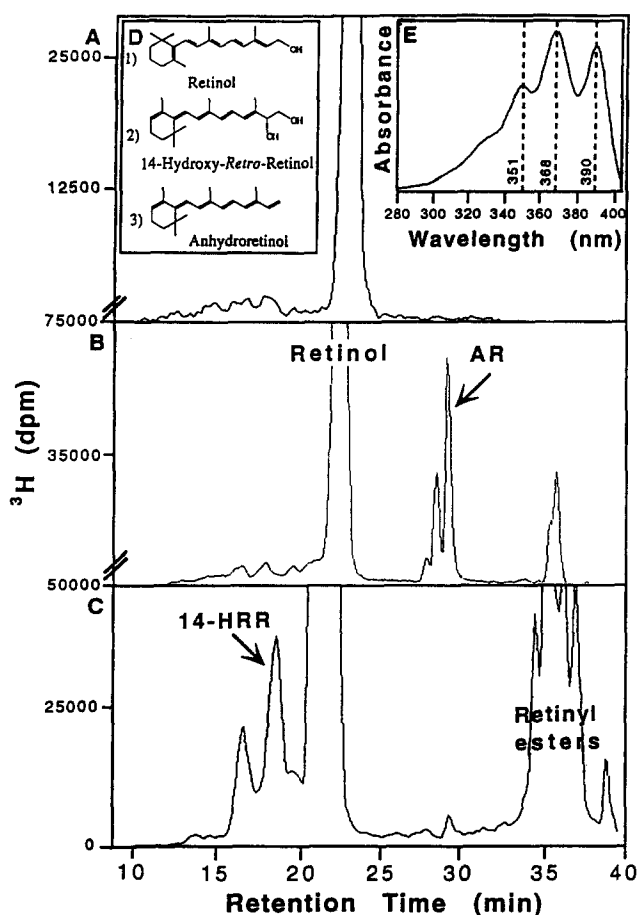

Figure 1. High pressure liquid chromatography of retinoids from insect SF 9 (*A*), SF 21 (*B*), and human lymphoblastoid 5/2 cells (*C*). Cells (10×10^6 cells in 10 ml of RPMI/7% FCS medium or Grace's insect medium/7% FCS) were incubated with 10 μCi [^{3}H]retinol; sp act, 49.3 Ci/mmol. After 4 h (*A* and *B*) or 16 h (*C*), retinoids were extracted from the washed cell pellet according to the procedure of McClean et al. (16). Retinoids were separated on an analytical C$_{18}$ reversed-phase column and detected with an on-line scintillation counter using the methodology described by Buck et al. (12). AR was the predominant metabolite in SF 21 (*B*) but was undetectable in SF 9 cells (*A*), a subclone of SF 21. Lymphoblastoid 5/2 also produced AR (*C*). Note that the small shifts in peaks are due to use of different C$_{18}$ columns, each one, however, calibrated by standards. (*D*) Structures of retinol, 14-hydroxy-retro-retinol, and anhydroretinol. The peak eluting at minute 29 is the all-trans AR, whereas the companion peaks at minutes 27 and 28 represent cis-isomers. All isomers yield spectra with characteristic vibronic fine structure similar to the spectrum of all-trans AR (*E*).

active metabolic state. It does not occur when cells are kept at +4°C, fixed with glutaraldehyde, or energetically blocked with sodium azide (data not shown). Moreover, the SF 21 cell line of *Spodoptera frugiperda* produces AR as the predominant retinol metabolite, whereas a subclone of this line, SF 9, does not produce detectable amounts of AR. Thus, AR does not appear to arise from spontaneous dehydration but is enzymatically derived. In the test tube, however, AR is easily synthesized from retinol by acid-catalyzed dehydration (18).

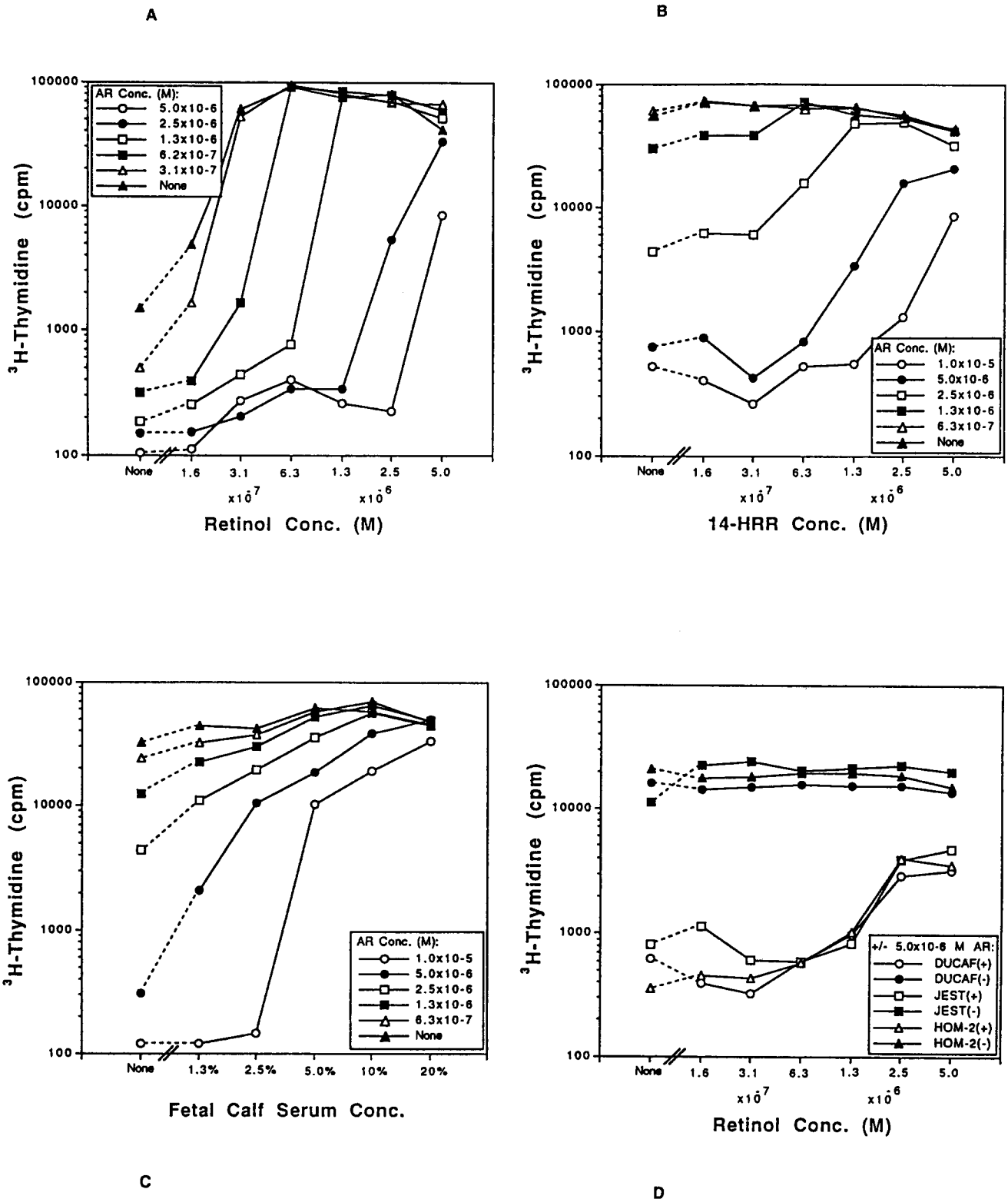AR was in fact the first retro-retinoid discovered in nature.

Figure 2. AR as reversible inhibitor of B lymphocyte proliferation. AR was produced from *all-trans* retinol by hydrochloric acid catalyzed dehydration as described (18), and the *all-trans* isomer was purified to homogeneity by reversed-phase high pressure liquid chromatography. Human B lymphoblastoid cells (e.g., cell line 5/2) were cultured in medium RPMI 1640 with 7% FCS. On the day of the assay, cells were washed and transferred to serum-free medium, RPMI 1640 supplemented with ITLB medium as described (13). Test reagents at indicated concentrations were added to triplicate cultures of 5×10^3 cells/well in 96-well plates (final volume of 200 μl/well) and cell proliferation tested 1 (*B–D*) or 3 d later (*A*) by [^{3}H]thymidine incorporation into DNA. The agonists used were *all-trans* retinol (*A*), 14-HRR (*B*), and FCS (*C*). Serum contains $1–2 \times 10^{-6}$ M *all-trans* retinol. (*D*) Three additional human lymphoblastoid cell lines, Hom 2, Jest, and Ducaf were tested under the conditions specified for *A*.
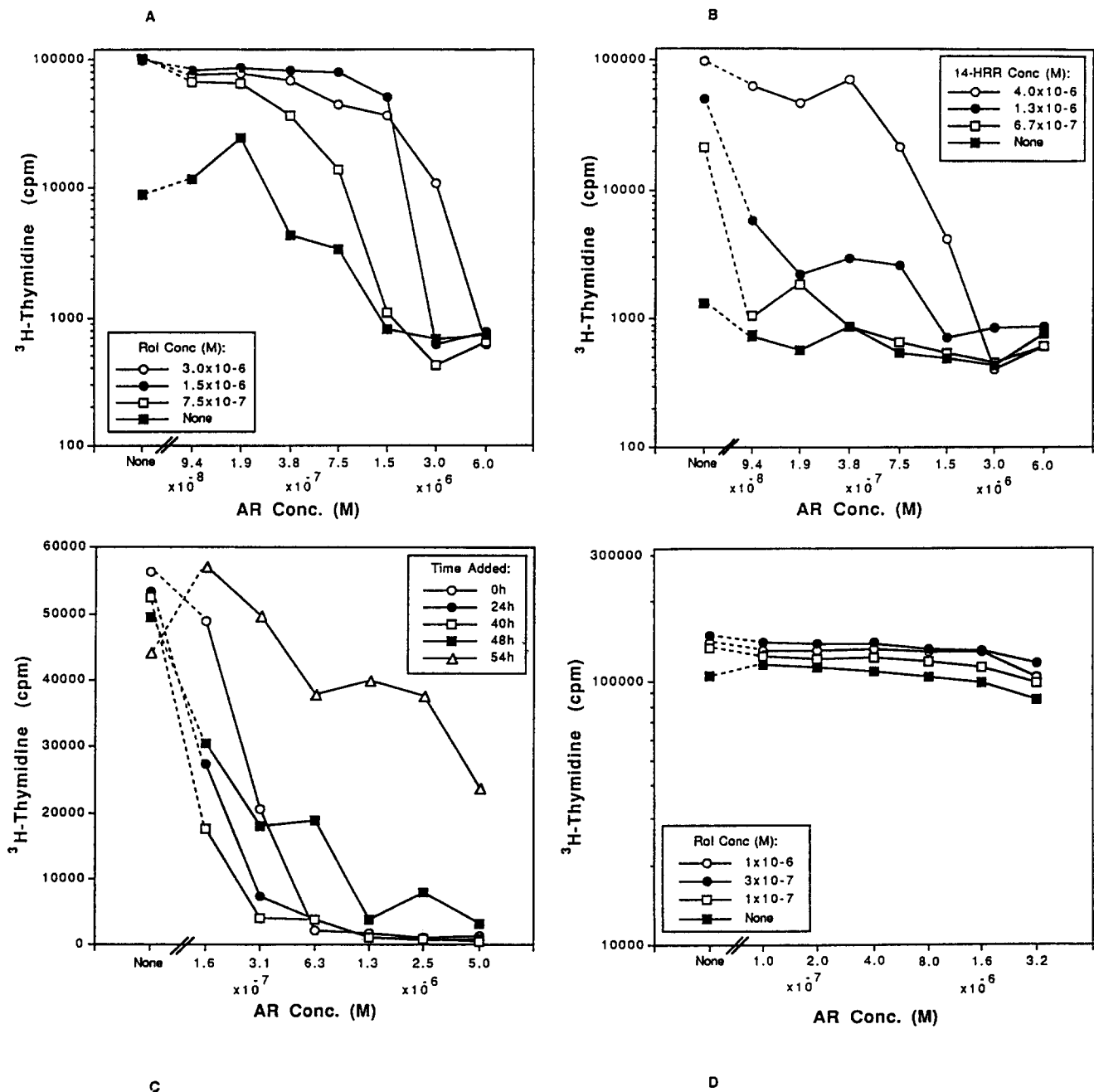
Figure 3. AR as antagonist of induction of thymocyte proliferation, but not of IL-2 production. Murine thymocytes (2 × 10⁵/well in triplicates) were cultured in ITLB medium in 96-well plates coated with 10 μg/ml anti-CD3ε mAb (24) as described (13). The retinoid agonists, retinol (*A*) or 14-HRR (*B*), were added along with AR at different doses in checkerboard fashion. Cell proliferation was tested on day 3 by [³H]thymidine incorporation into DNA. (*C*) The time of addition of AR at different concentrations was varied from 0 to 54 h after initiation of cultures with anti-CD3ε plus 2 × 10⁻⁶ M retinol. The results of cell proliferation assays performed at 72 h are shown. Culture supernatants of anti-CD3ε plus retinol/AR–stimulated thymocytes were tested after 18 h for their IL-2 content by the ability to stimulate the cell line CTLL (25). CTLL proliferation was measured by [³H]thymidine incorporation into DNA (*D*). SD of triplicate measurements were <10%. The CTLL cell line was tested in the presence of rIL-2 for responsiveness to retinol and AR over broad dose ranges and found independent of either retinoid (data not shown).

It was found in fish and whale liver oils in 1939 (19) and later in transformed mouse fibroblasts (20). Its biological function, however, remained obscure. Owing to the close structural similarity to 14-HRR, we tested the effect of AR on proliferation of lymphoblastoid B cells. AR did not support B cell proliferation on its own (Fig. 2 *A*). On the contrary, AR proved to behave as a competitive inhibitor, leading to cell death. The inhibitory effect was dose dependent, but de-

pended on the retinol status of the cultures and on the time of measurement. Inhibition was most pronounced, requiring a minimum dose of AR of 3×10^{-7} M and a culture time of 3 d to essentially kill all cells, when no other retinoid was added to the medium (Fig. 2 A), whereas 1 d after onset of culture complete inhibition required a 10-fold-higher dose of AR (Fig. 2 B). The inhibitory effect of AR was counteracted effectively by retinol (Fig. 2 A) or 14-HRR (Fig. 2 B), and in both cases, the relative concentrations of agonist (retinol or 14-HRR) and antagonist (AR) determined the outcome, i.e., proliferation versus cell death. The inhibition curves shown are typical for reversible inhibition of a pharmacological agent and imply competition for a common receptor molecule. AR was also capable of reversibly inhibiting lymphoblastoid B cells grown under more physiological conditions in serum-containing medium (Fig. 2 C). Several other randomly selected lymphoblastoid B cell lines were also found to be sensitive to inhibition with AR (Fig. 2 D). So far we have encountered no exception among many more lines tested, including freshly EBV-transformed B lymphocytes, indicating a general dependence among B cells on 14-HRR as growth-promoting agonist and corresponding susceptibility to AR as an antagonist.

Activation of resting thymocytes by TCR-generated signals in serum-free medium was previously shown to be dependent on retinoid cofactors, retinol, or 14-HRR (13). To test whether AR would interfere with this process as well, murine thymocytes were stimulated in ITLB medium with immobilized anti-CD3ε antibody and retinol or 14-HRR as cofactors, and various concentrations of AR. Inhibition of thymocyte activation was pronounced, depending on the relative concentrations of agonist (i.e., retinol or 14-HRR) and AR, as illustrated by the series of dose–response curves in Fig. 3, A and B. The interrelationship between agonist and antagonist was comparable to that observed in B lymphocytes. AR not only prevented the activation of thymocytes, but was also able to reduce the thymidine uptake of cycling thymocytes if given 2 d after activation (Fig. 3 C).

Cyclosporine A and FK 506 block IL-2 release and the expression of IL-2 receptor and prevent the activation of CD4⁺ T lymphocytes (21, 22). We knew from previous unpublished work that both IL-2 production and IL-2 receptor expression were unaffected by the presence or absence of retinol or 14-HRR. Therefore, as expected, AR did not interfere with IL-2 release, as shown in Fig. 3 D. This is an important control experiment to indicate that the AR effect on proliferation is not due to generalized toxicity.

A common feature in the physiology of retinoids is the use of intracellular and extracellular binding proteins, often specialized for particular retinoids, for protection against oxidative degradation and for transport. All three retinoids tested bind to RBP of plasma and to CRBP-1 (Table 1), but the affinities of retinol and AR are 10-fold higher than those of 14-HRR (23). Given these circumstances, competitive inhibition at the level of CRBP-I may explain the retinol-AR antagonism. For instance, if the CRBP-1/retinol complex were required for 14-HRR biosynthesis, its disruption by the AR antagonist might interfere with this step. However, we found

Table 1. *Dissociation Constants of Retinoids Bound to Extra- or Intracellular Binding Proteins*

Retinoid	Dissociation constant		
	RBP	CRBP-1	CRABP-1
		μM	
Retinol	0.15	0.013	No binding
14-HRR	0.12	0.21	No binding
AR	0.020	0.023	No binding

No binding, no significant binding could be detected.
Dissociation constants of retinoids to serum RBP, CRBP-1, and CRABP-1 were measured by fluorimetric titration. Apo-protein (1–3 μM) was titrated with the appropriate all-trans-retinoid dissolved in ethanol, and the titration was followed by measuring the increase in the fluorescence of the retinoids upon binding. Reaction mixtures included 150 mM NaCl, 0.1 mM DTT, and 25 mM Hepes, pH, 7.0 and 7.4, respectively. Retinoid fluorescence was measured at the excitation and emission maxima for the different retinoids: i.e., excitation, 330, 350, and 368 nm; emission, 480, 515, and 570 nm for retinol, 14-HRR, and AR, respectively. Data analyses were performed as described by Cogan et al. (26). Human RBP was purified from human serum by means of a transthyretin column (27).

no change in the metabolic rate of 14-HRR synthesis from ³H-labeled retinol in the presence of a 100-fold excess of AR (data not shown). Moreover, the promyelocytic cell line HL-60 fails to express CRBP-1 but is nonetheless susceptible to reversible growth inhibition by AR (Eppinger, T., J. Buck, and U. Hämmerling, unpublished observations). We conclude that CRBP-1 is not involved in the biological retinol-AR antagonism. Moreover, the facts that 14-HRR binds to CRBP-1 with appreciably lower affinity (Table 1) and that AR also reversibly inhibits cultures of lymphocytes when 14-HRR is provided as an agonist (Figs. 2 B and 3 B) imply antagonism at a point downstream of, or in addition to, CRBP-1. A separate 14-HRR/AR binding protein/receptor, as yet to be defined, is inferred.

The demonstration that AR acts as antagonist to retinol and, especially, to 14-HRR contributes to the understanding of the mechanism of action of these retinoids. First, the pharmacological reversibility is best explained by competition of two structurally closely related compounds for the same receptor. Second, 14-HRR and AR occur in mammalian and insect cells, thus suggesting conservation of a complex family of *retro*-retinoids over a long evolutionary period. Third, AR has been observed by us as a natural product of B lymphocytes and therefore the positive signaling action of 14-HRR and the negative one of AR may be meaningful for lymphocyte physiology. To our knowledge, 14-HRR and AR constitute the first naturally occurring agonist/antagonist pair of retinoids (or other small lipophilic messenger molecules) to be described. Fourth, the *retro*-retinoid pathway is essential for T cell activation, but appears to operate independently of the calcineurin/IL-2 pathway. AR might presage the development of new types of immunosuppressive and/or antiproliferative agents.

References

1. Sporn, M.B., A.B. Roberts, and D.S. Goodman, editors. 1984. The Retinoids. Volumes 1 and 2. Academic Press Inc., Orlando, FL.
2. Wald, G. 1968. Molecular basis of visual excitation. *Science (Wash. DC).* 162:230.
3. Roberts, A.B., and M.B. Sporn. 1984. Cellular Biology and biochemistry of the retinoids. *In* The Retinoids. M.B. Sporn, A.B. Roberts, and D.S. Goodman editors. Vol. 2. Academic Press, Orlando. 209–286.
4. Giguere, V., E.S. Ong, P. Segui, and R.M. Evans. 1987. Identification of a receptor for the morphogen retinoic acid. *Nature (Lond.).* 330:624.
5. Petkovich, M., N.J. Brand, A. Krust, and P. Chambon. 1987. A human retinoid receptor which belongs to the family of nuclear receptors. *Nature (Lond.).* 330:444.
6. Mangelsdorf, D.J., E.S. Ong, J.A. Dyck, and R.M. Evans. 1990. Nuclear receptor that identifies a novel retinoic acid response pathway. *Nature (Lond.).* 345:224.
7. Evans, R.M. 1988. The steroid and thyroid hormone receptor superfamily. *Science (Wash. DC).* 240:889.
8. Wolbach, S.B., and P.R. Howe. 1925. Tissue changes following deprivation of fat-soluble A vitamin. *J. Exp. Med.* 42:753.
9. David, C.Y., and J.L. Sell. Effect of retinol and retinoic acid nutrition on the immune system of chicks. *J. Nutr.* 113:1914.
10. Bieri, J.G., E.G. McDaniel, and W.E. Rogers. 1969. Survival of germfree rats without vitamin A. *Science (Wash. DC).* 163:574.
11. Howell, J.M., J.N. Thompson, and G.A.J. Pitt. 1963. Histology of the lesions produced in the reproductive tract of animals fed a diet deficient in vitamin A alcohol but containing vitamin A acid. *J. Reprod. Fertil.* 5:159.
12. Buck, J., F. Derguini, E. Levi, K. Nakanishi, and U. Hammerling. 1991. Intracellular signaling by 14-hydroxy-4,14-*retro*-retinol. *Science (Wash. DC).* 254:1654.
13. Garbe, A., J. Buck, and U. Hammerling. 1992. Retinoids are important cofactors in T cell activation. *J. Exp. Med.* 176:109.
14. Buck, J., G. Ritter, L. Dannecker, V. Katta, B. Chait, and U. Hammerling. 1990. Retinol is essential for the growth of activated human B cells. *J. Exp. Med.* 171:1613.
15. Buck, J., A. Myc, A. Garbe, and G. Cathomas. 1991. Differences in the action and metabolism between retinol and retinoic acid in B lymphocytes. *J. Cell Biol.* 115:851.
16. McClean, S.W., M.E. Rudel, E.G. Gross, J.J. DiGiovani, and G.L. Pede. 1982. Liquid-chromatographic assay for retinol (vitamin A) and retinol analogs in the therapeutic trials. *Clin. Chem.* 28:693.
17. Shantz, E.M., J.D. Cawley, and N.D. Embree. 1943. Anhydro ("cyclized") vitamin A. *J. Am. Chem. Soc.* 65:901.
18. Edisburry, J.R., A.M. Gillam, I.M. Heilbron, and R.A. Morton. 1932. Absorption spectra of substances derived from vitamin A. *Biochem. J.* 26:1164.
19. Embree, N.D. 1939. The occurrence of cyclized vitamin A in fish liver oils. *J. Biol. Chem.* 128:187.
20. Bhat, P.V., L.M. DeLuca, S. Adamo, I. Akalovsky, C.S. Silverman-Jones, and G.L. Peck. 1979. Retinoid metabolism in spontaneously transformed mouse fibroblasts (Balb/c 3T12-3 cells): enzymatic conversion of retinol to anhydroretinol. *J. Lipid Res.* 20:357.
21. Handschumacher, R.E., M.W. Harding, J. Rice, R.J. Drugge, and D.W. Speicher. 1984. Cyclophilin: a specific cytosolic binding protein for cyclosporine A. *Science (Wash. DC).* 226:544.
22. Sawada, S., G. Suzuki, Y. Kawase, and F. Takaku. 1987. Novel immunosuppressive agent, FK 506 in vitro effects on the cloned T cell activation. *J. Immunol.* 139:1797.
23. Ong, D.E., and F. Chytil. 1980. Purification of cellular retinol- and retinoic acid-binding proteins from rat tissue. *Methods Enzymol.* 67:288.
24. Leo, O., M. Foo, D.H. Sachs, L.E. Samelson, and J.A. Bluestone. 1987. Identification of a monoclonal antibody specific for a murine T3 polypeptide. *Proc. Natl. Acad. Sci. USA.* 84:1374.
25. Gillis, S., and K.A. Smith. 1977. Long term culture of tumour-specific cytotoxic T cells. *Nature (Lond.).* 268:154.
26. Cogan, U., M. Kopelman, S. Mokady, and M. Shinitzky. 1976. Binding affinities of retinol and related compounds to retinol binding protein. *Eur. J. Biochem.* 65:71.
27. Fex, G., C.-B. Laurell, and E. Thulin. 1977. Purification of prealbumin from human and canine serum using a two-step affinity chromatographic procedure. *Eur. J. Biochem.* 75:181.