Retinoids Are Important Cofactors in T Cell Activation

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Summary

Murine thymic T cells depleted of antigen-presenting cells proliferate poorly in response to crosslinking anti-CD3 monoclonal antibodies or conconavalin A when cultured in conventional fetal calf serum-containing serum. However, in a serum-free medium formulated to contain, in addition to basic ingredients, insulin, transferrin, albumin, linoleic acid (ITLB), and retinol, proliferation is vigorous. The presence of retinol is critical, because when omitted, cells do not become activated. The subsets of T cells proliferating with the assistance of retinol cofactor are both $CD4^+$ and $CD8^+$ thymic T cells, and $CD4^+$ peripheral T cells. Mature $CD8^+$ T cells of lymph nodes can also be activated in ITLB medium plus retinol, provided that interleukin 2 (IL-2) is added. Retinol needs to be present at the time when T cell receptor triggering is initiated, suggesting that early activation events (G_0 to G_1 transition) are dependent on retinol. It is currently less clear whether or not subsequent events associated with G_1 to S phase transition also require the presence of retinol. 14-hydroxy-retroretinol (14HRR) is a metabolic product of retinol in lymphocytes, and this retinoid effectively supports T cell activation in conjunction with a mitogen in lieu of retinol. Thus, while retinol and its intracellular product, 14HRR, are unable to activate T cells on their own, they are important cofactors. The requirement for retinol in CD3-mediated T cell activation cannot be satisfied by retinoic acid or ILs-1, 2, 4, and 6, and tumor necrosis factor-α whereas interferon γ can substitute for retinol. Our experiments are compatible with the idea that retinol, in the course of cellular activation, is converted to 14HRR, which is needed as intracellular messenger. If substantiated by molecular studies now underway, our data should lead to the description of a new signal pathway distinct from the retinoic acid signal pathway observed in nonlymphoid cells, but perhaps functioning by a similar mechanism, i.e., ligand-assisted transcriptional regulation.

The activation of resting T cells is initiated by interaction of the TCR with antigen peptide bound to MHC on APCs. Pairs of ligand receptor structures on the interacting cells contribute secondary signals (for review see references 1 and 2). Although these molecular interactions have been described in considerable detail, the intracellular events ensuing are still poorly understood. However, the emerging overall picture presents multiple, interactive signal cascades that converge on the nucleus to effect transcriptional activation. As a general rule, these events are mediated by two different chemical classes of molecules, proteins and small lipophilic molecules, that shuttle to the nucleus to regulate transcription. For example, the protein products of the *rel* gene family (e.g., NF-kB) translocate upon activation from the cytoplasm to the nucleus and regulate transcription (3). Small lipophilic molecules including the steroids, vitamin D, thyroid hormone, and several forms of retinoic acids, bind to and activate their specific receptors belonging to the superfamily of steroid receptors for transcriptional activation (3–5).

To study the requirements of T cell activation, cellular immunologists customarily use culture media supplemented with FCS. Because FCS contains a number of growth factors and hormones, including steroids, vitamin D and retinoids, it is desirable to reduce this complexity. Several serum components appear to be indispensable while others may be inhibitory, as documented recently for platelet-derived growth factor (6). The essential ones include albumin, thought to play a role in the stabilization and transport of fatty acids and possibly other lipids, transferrin for regulation of iron metabolism, and insulin, ostensibly for control of carbohydrate metabolism (7, 8). Our laboratory has recently described retinol as a further serum constituent necessary for the growth of B lymphocytes (9). Both human and murine-activated B cells perish rapidly in culture when deprived of retinol, and this may be related to earlier findings that vitamin A-deficient

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mammals exhibit severe defects in lymphopoiesis and immune function (10–12). The essential role of retinol for the immune system has recently been highlighted in epidemiological studies where even mild vitamin A deficiency was associated with immune dysfunction (13).

We have hypothesized that retinol serves in lymphocytes as a precursor for one or more intracellular retinoid derivatives that might mediate the retinol effects, possibly through participation in signal transduction. The analogy supporting this hypothesis is retinoic acid. This molecule is derived from retinol, passes into the nucleus of target cells, and binds to specific retinoic acid receptors, leading to increased transcription of selected genes. Although this mechanism is well documented for a variety of tissues and cell types, it does not apply to B lymphocytes. B cells neither produce detectable levels of retinoic acid, nor respond to it (14). We therefore performed a biochemical analysis of the intracellular retinoids of B cells and found several known retinoids (e.g., retinol and retinyl esters) and at least two hitherto undescribed retinoids. Because one of these, 14-hydroxy-retro-retinol (14HRR),[1] was capable of supporting the proliferation of B cells in the absence of an external supply of retinol, we have speculated that this compound might serve as an intracellular mediator of retinol effects by a pathway distinct from that of retinoic acid (15). T lymphocytes also synthesize 14HRR (as indeed many other cell types studied by us), and we were therefore led to study whether T cell activation is critically dependent on 14HRR or its precursor molecule, retinol. The results reported here support this assumption.

Materials and Methods

Reagents and Culture Medium. The following antibodies were purified by protein A-Sepharose chromatography: Anti-CD3ε, clone 1452C11 (16), anti-I-A^d, clone MKD6 (17); anti-IE^d, clone 13/18 (19); anti-Lyt2.2, cone 19/178 (20); anti-L3T4, clone GK1.5 (21) used as ascites fluid; and anti-IL-4M, clone 11B11 (18). Fluorochrome-labeled antibodies were from commercial sources: anti-CD4 PE (Becton Dickinson & Co., San Jose, CA); anti-CD8 FITC (Boehringer Mannheim, Indianapolis, IN); and normal rat IgG FITC and normal rat IgG PE for controls (Southern Biotechnology, Birmingham, AL).

Retinoids. All-*trans* retinol, all-*trans* retinal, and all-*trans* retinoic acid were purchased from Sigma Chemical Co. (St. Louis, MO). The retinoids were dissolved at a concentration of 3×10^{-2} M in methanol or DMSO with 10^{-4} M butylated hydroxytoluene (Sigma Chemical Co.) added and stored in the dark at −20°C in a nitrogen atmosphere. Immediately before use, the stock solutions were diluted in serum-free medium. 14HRR was isolated from the pellets of HeLa cells fed with retinol by using a series of reversed phased HPLC columns as described (15). 14HRR was pure according to the retention time on an analytical C_{18} reversed phase column and the typieal UV absorption spectrum.

Interleukins and Growth Factors. Human rIL-1α was a gift from Hoffmann LaRoche Co. (Nutley, NJ); hrIL-2 and hrIL-6 were purchased from Boehringer Mannheim; murine rIL-4 was purchased from R&D Systems, Inc. (Minneapolis, MN); rTNF-α was donated by Genentech, Inc. (San Francisco, CA); mouse rIFN-γ was purchased from Genzyme Corp. (Cambridge, MA). Bovine insulin and human transferrin were purchased from Collaborative Research (Bedford, MA). Delipidated bovine albumin, all-*trans* retinol, linoleic acid, and Con A were bought from Sigma Chemical Co.

Mice. BALB/c mice of either sex were bred and housed in the Sloan-Kettering Laboratory Animal Facility. Our institution guarantees compliance with regulations promulgated by the Animal Welfare Act.

Preparation of Cells. Thymuses of 3–6-wk-old BALB/c mice were teased in serum-free RPMI medium supplemented with 1% BSA, 10^{-6} M linoleic acid, and antibiotics. Depletion of accessory cells was achieved by two cycles of complement-dependent lysis with a mixture of anti-IA^d (5 μg/ml) and anti-IE^d (1:500 diluted ascites fluid). Briefly, cells were incubated on ice for 30 min with Ia antibodies, spun down, resuspended in 1:40 diluted rabbit complement, and incubated at 37°C for 45 min. Incubations and two subsequent washes were carried out with RPMI medium containing 1% BSA. Cell viability was evaluated by trypan blue dye exclusion. To determine the extent of depletion of accessory cells, samples were stained with FITC-conjugated anti-Fc receptor antibody and analyzed by flow cytometry on a FACScan® instrument (Becton Dickinson & Co.). No FcR-bearing cells were detected by this procedure.

Mature T cells were obtained from pooled intestinal, axillary, inguinal, and submandibular lymph nodes of 4–10-week-old BALB/c mice. To fractionate cells into CD4 and CD8 subsets and at the same time remove APC, a combination of adherence and complement lysis was used as follows: The cell suspension in a 2 ml-vol. was applied to a nylon wool column (22) (0.6 g of washed nylon fibers in the barrel of a 10-ml syringe) and incubated at 37°C for 40 min. The nonadherent cells were recovered by washing with warm serum-free medium at a flow rate of 1 ml/min. The cells were then spun down and treated as described for thymocytes with two cycles of complement lysis, using either a mixture of MKD6, 13/18, and 19/178 (to obtain CD4-enriched T cells), or MKD6, 13/18, and GK1.5 (to obtain CD8-enriched T cells). The success of the enrichment procedures was monitored cytofluorimetrically using FITC-conjugated anti-CD8 and PE-conjugated anti-CD4. In either case, the T cell subsets were over 95% homogeneous. Analysis with FITC-conjugated anti-mouse IgG(k) revealed <2% contamination by B cells.

Proliferation Assays. Cells were cultured in serum-free medium, referred to as ITLB, containing RPMI 1640 supplemented with 8×10^{-7} M insulin (5 μg/ml), 7×10^{-8} M transferrin (5 μg/ml), 2×10^{-6} M linoleic acid, 2×10^{-6} M delipidated BSA (0.12 mg/ml), 2 mM L-glutamine, 1 mM sodium pyruvate, and antibiotics. They were seeded into 96-well flat-bottomed plates at varying cellular densities in a final volume of 100 μl. In control experiments, cells were cultured in medium containing 10% FCS or 3% human serum and 5×10^{-5} M 2-ME. T cells and thymocytes were activated with immobilized, purified mAb anti-CD3ε, or by the addition of Con A in the presence or absence of different concentrations of retinoids. Unless indicated otherwise, retinoids were replenished every 12 h to maintain a reasonably constant concentration of these labile compounds in culture. The optimal concentration for stimulation of T cells was determined for each batch of anti-CD3 mAb and Con A. The optimal range of Con A was particularly narrow in serum-free medium (0.5–0.2 μg/ml) and

[1] *Abbreviations used in this paper:* CRBP, cellular retinol-binding protein; 14HRR, 14-hydroxy-retro-retinol; RBP, retinol-binding protein; TTR, transthyretin.

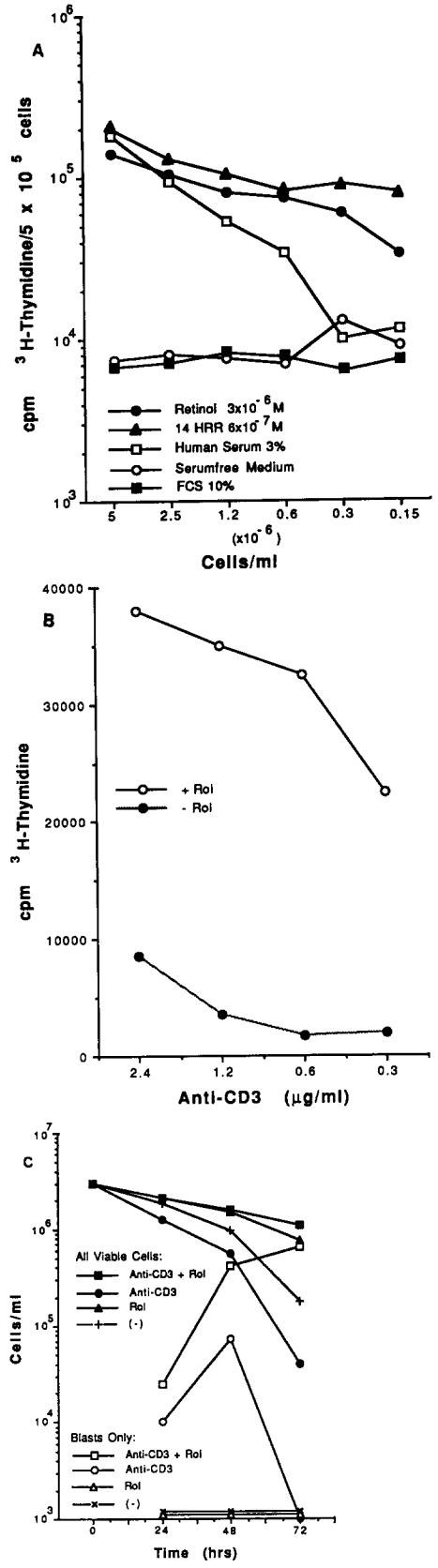

Figure 1. Retinoids are required cofactors for proliferation of anti-CD3ε activated thymocytes at low cellular density. (*A*) Purified BALB/c thymocytes were activated with immobilized anti-CD3ε antibody and cultivated for 4 d in ITLB medium with or without retinol (3×10^{-6} M), 14HRR (6×10^{-7} M) (fresh 14HRR was added every 12 h), human serum (3%) or FCS (10%) at the cellular densities shown. Proliferation was assayed by tritiated thymidine incorporation into cellular DNA. The SDs were <20%. (*B*) BALB/c thymocytes (10^6/ml) were added to microtiter plates coated with titrated amounts of anti-CD3ε antibody with 3×10^6 M retinol or without, as indicated. Proliferation was measured in hexaduplicate wells on day 3 by [³H]thymidine incorporation assay. (*C*) BALB/c thymocytes (3×10^6/ml) were activated with anti-CD3ε mAb as in *A*. The total number of viable cells was determined by counting trypan blue-excluding cells, and those of blast cells by counting viable large cells in six replicate wells. Because of the relatively low cell density required in the culture (see *A*), the numbers reported for blast cells are best estimates.

varied with the cell density used. To assess the costimulatory activities of retinoids with other lymphokines, various concentrations of IL-1, -2, -4, -6, IFN-γ and TNF-α were added to cultures. Cultures were carried out in duplicate. Plates were incubated at 37°C in a humidified 5% CO_2 atmosphere. Cellular proliferation was determined by [³H]thymidine uptake (0.5 µCi/well; New England Nuclear, Boston, MA) after the incubation times indicated for each experiment with a 4-h labeling pulse. Cells were harvested onto glass filters and [³H]TdR incorporation was determined by liquid scintillation counting. The data presented are the means of duplicate or six replicate cultures. The restriction to duplicate measurements was necessary to conserve scarce 14HRR. They were within 20% of each other. Each experiment was repeated at least twice.

Flow Cytometry. To determine the phenotype of blast cells generated in the thymocyte cultures stimulated with immobilized anti-CD3 antibody and retinoids, as well as the purity of CD4 and CD8 subsets isolated from pooled mouse lymph nodes, cells (10^5–10^6 cells/sample), stained with FITC-conjugated anti-CD4 antibody and PE-conjugated anti-CD8 antibody, were analyzed by two-color flow cytometry with a FACScan®(Becton Dickinson & Co.). Dead cells were eliminated by forward low-angle scatter. Isotype controls were included in all experiments. To determine the phenotypes of activated thymocytes, only blast cells were gated for collection and analysis.

Results

Stimulation of Thymocytes with Anti-CD3ε in Serum-free Medium is Dependent on the Presence of Retinoids. Thymocytes depleted of APC did not proliferate appreciably in response to crosslinking anti-CD3 mAb as the sole induction stimulus when cultured in FCS-containing medium (Fig. 1 *A*). They also failed to proliferate in serum-free medium ITLB in the absence of retinoids, unless very high doses of anti-CD3 antibody (in excess of 2 µg/ml) were employed (Fig. 1 *B*). However, in the presence of retinol at 3×10^{-6} M concentration or 14HRR at 6×10^{-7} M concentration, vigorous responses were elicited. These responses were positively correlated with cell density, but were independent over a wide dose range of the anti-CD3 concentration used to coat the plastic culture trays. Addition of 3% human serum also supported anti-CD3-initiated thymocyte proliferation, but these responses faded rapidly with decreased cell density. Growth curves of thymocyte cultures established by differential counts of cells in the presence of trypan blue showed a selective and

exponential proliferation of blast cells when stimulated with anti-CD3ε and retinol in combination. Anti-CD3ε alone produced only a modest and transient blast transformation whereas retinol on its own produced no discernible T cell activation (Fig. 1 *C*). When all viable cells were scored, it became evident that while the cultures as a whole declined, this decline was accelerated by anti-CD3ε stimulation (representing perhaps the well-known induction of apoptosis). It is surprising that retinol by itself appeared to maintain a higher state of viability than anti-CD3ε "only" stimulation, or omission of test reagents altogether. This rescue effect will have to be investigated in detail. The phenotypes of blast cells generated in the retinol- and 14HRR-supported cultures were determined by flow cytometric immunofluorescence as exclusively single-positive T cells, with the approximate 70% $CD4^+$ and 30% $CD8^+$ cells (data not shown).

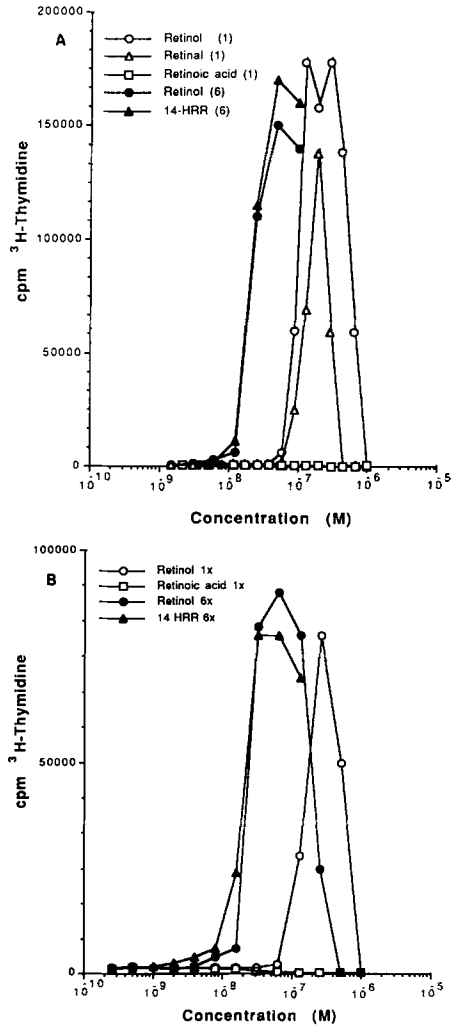

Figure 2. Dose responses elicited by different retinoids. Purified thymocytes (5×10^5 cells/well) were stimulated by TCR crosslinking with immobilized anti-CD3ε antibody (*A*) or 0.4 μg/ml Con A (*B*) in serum-free ITLB medium. The indicated amounts of retinoids were added either once at the beginning of the experiment (*1x*) or every 12 h thereafter (*6x*). DNA synthesis was measured after 72 h by tritiated thymidine uptake as described.

The proliferative responses of thymocytes were clearly dependent on the presence of all-*trans* retinol added at initiation of culture. Under these conditions, the optimal retinol concentration was between 3 and 1×10^{-6} M (Fig. 2 *A*). Because retinol decays in serum-free tissue culture medium with an estimated half-life of 24 h (14), we have replenished retinol twice daily and have found that with repeated feeding, five- to tenfold lower retinol concentrations were sufficient to sustain cell proliferation over the 3-d culture period (Fig. 1 *A*). We have described that retinol is metabolized by lymphocytes to 14HRR, and have hypothesized that this molecule serves as an intracellular mediator (15). To test this assumption in T cells, 14HRR was added instead of retinol and dose responses were recorded. A single addition of 14HRR given at the start was ineffective, probably because of the brief half-life of 14HRR of 4 h (data not shown). However, when provided at 12-h intervals, 14HRR was as potent as retinol in supporting T cell proliferation with a dose optimum of 5×10^{-7} M (Fig. 2 *A*). Among other retinoids tested, 13-*cis*-retinol (data not shown) and all-*trans* retinal were equally effective as all-*trans* retinol. However, all-*trans* retinoic acid was completely inactive over a wide dose range tested irrespectively of how often the cultures were fed.

The growth kinetics of anti-CD3-activated thymocytes in serum-free medium revealed exponential growth over a 4-d period (Fig. 3) that was totally dependent on the presence of either retinol (3×10^{-6} M) or 14HRR (6×10^{-7} M). Human serum (3%) was also capable of supporting exponential growth, although in the experiment with 10^5 cells per well shown in Fig. 3 the proliferative indices were only half of those obtained with retinoids. Human serum contains retinol at 2×10^{-6} M. Attempts to remove retinol from serum by delipidation and subsequently replenish it were unsuccessful.

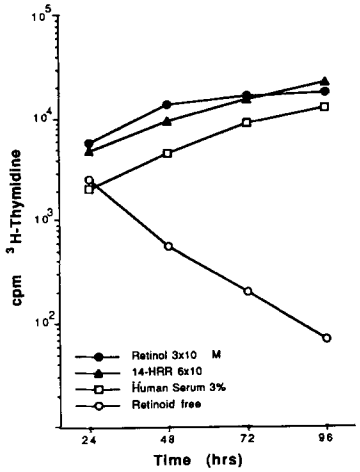

Figure 3. Growth curves of activated thymocytes with serum-containing or serum-free medium, and in the presence or absence of retinoids. Purified thymocytes (10^5 cells/well) were activated with anti-CD3 antibody in ITLB medium with or without retinol (3×10^{-6} M), 14HRR (6×10^{-7}, added every 12 h) or human serum (3%). DNA synthesis was measured daily by a 4-h pulse of tritiated thymidine. SDs were ≤12%.

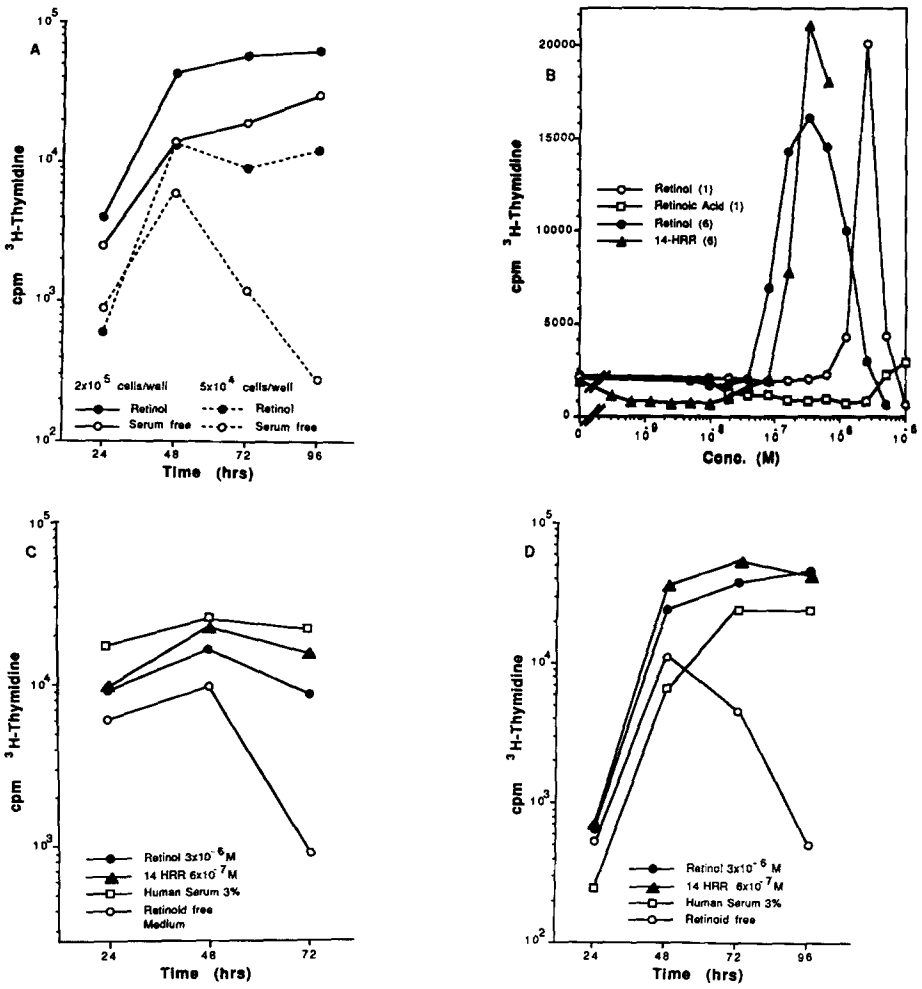

Figure 4. Retinoids are required cofactors for anti-CD3 activated peripheral T lymphocytes. Growth curves of lymph node T cells depleted of antigen-presenting cells (*A*) with and without 10^{-6} M retinol at 2×10^5 and 5×10^4 cells/well. (*B*) Dose–responses elicited by different retinoids in cultures of lymph node T cells depleted of antigen-presenting cells. Also shown are the growth curves of $CD4^+$ (*C*) and $CD8^+$ T lymphocytes (*D*) in the presence of either retinol, 14HRR, human serum, or in their absence. T lymphocytes, purified as described, 5×10^4/well, were activated with immobilized anti-CD3 antibody in ITLB medium. The indicated amounts of retinoids or human serum were added at initiation of cultures. Culture medium of $CD8^+$ T cells was supplemented with IL-2 (2 U/ml). 14HRR was added every 12 h. DNA synthesis was measured by a 4-h pulse with tritiated thymidine. SDs were ≤17%.

Table 1. *Growth-stimulating Effect of Retinol on Thymocytes in the Presence of Different Interleukins*

Stimulating agent	Dose	Growth-stimulating effect with or w/o retinol	
		No retinol	10^{-6} M retinol
	U/ml	*cpm*	
IL-1	10	959 ± 30	118,924 ± 3,051
IL-2	2	458 ± 156	77,343 ± 11,739
IL-4	5	991 ± 105	115,849 ± 20,630
IL-6	5	1,005 ± 815	130,590 ± 25,803
IFN-γ	40	81,876 ± 11,868	220,359 ± 27,726
TNF-α	12	464 ± 95	122,905 ± 11,577
IL-2 + IL-4	2/5	1,081 ± 103	103,025 ± 5,280
none	–	585 ± 207	79,247 ± 17,567

Proliferation of activated thymocytes in response to interleukins in presence and absence of retinol. Purified thymocytes (5×10^5 cells/well) were activated with immobilized anti-CD3 antibody in serum-free medium in the presence or absence of interleukins and retinol (3×10^{-6} M). Proliferation was assayed after 3 d by tritiated thymidine incorporation.

To test whether or not activation through the TCR is unique or whether other modes of stimulation lead to proliferation sustained by retinol, we have used Con A at the optimal concentration of 0.5 μg/ml in serum-free ITLB medium. Thymocyte proliferation was entirely retinol dependent, the cultures with 3×10^{-6} M retinol growing exponentially, and those without retinol perishing rapidly (Fig. 2 *B*).

Stimulation of Peripheral Lymphocyte Subsets Is Also Retinol Dependent. Because the phenotype analysis had implicated mature T cells among the thymocytes responsive to anti-CD3 activation in the presence of retinoid, we tested whether this finding also held true for peripheral T cells. Fig. 4 indicates that stimulation of lymph node T cells was dependent on the presence of retinol although at high cell density (2×10^6/ml) the dependence was less pronounced than at low density (5×10^5/ml or below) (Fig. 4 *A*). The dose–response curves for lymph node T cells were very similar to those for thymocytes (compare Figs. 2 and 4 *B*). Furthermore, 14HRR is effective over the same dose range as observed for thymocytes, whereas retinoic acid is nearly inert, except for a very modest stimulatory activity elicited at 10^{-5} M concentration.

Because a proportion of lymph node T cells proliferated upon activation by anti-CD3 independently of retinol, we tested whether these cells might belong to a particular subset. However, when $CD4^+$ and $CD8^+$ subsets were purified by negative immunoselection, and tested for proliferation in the presence or absence of retinol, they behaved no differently from unseparated cells, i.e., each subset responded to the activating signal (anti-CD3 for $CD4^+$ cells; anti-CD3 plus IL-2 for $CD8^+$ cells) only if retinol or 14HRR were present (Fig. 4, *C* and *D*).

Retinol Is Required at Onset of Culture. We have determined the kinetics of requirement of retinol by thymocytes and have found that the highest responses were elicited when retinol was supplied together with the activation signal. When delayed by 12 h, retinol still produced a growth supporting effect but this trailed behind by a margin of 4:1. A delay of 24 h caused complete failure of activation (Fig. 5).

Retinol Cannot Be Replaced by Interleukins. We have investigated whether lymphokines or cytokines known to impact on T cell activation can substitute for retinol or modulate its effect in anti–CD3-activated thymocytes. None of the four interleukins, IL-1, -2, -4, or -6, was capable of overcoming the requirement for retinol. However, among cytokines, interferon-γ can sustain thymocyte proliferation in the absence of retinol, whereas TNF-α does not. When testing for synergy between retinol and lymphokines, we found all except IL-2 to moderately enhance proliferation of thymocytes. Interferon-γ showed a clear additive effect with retinol (Table 1).

Discussion

Our results indicate that retinoids play a key role in the activation of murine T cells. Using minimal culture medium consisting of RPMI 1640 supplemented with insulin, transferrin, linoleic acid, delipidated serum albumin and all-*trans* retinol, activation of thymic and lymph node T cells is highly effective, whether these cells are stimulated by TCR cross-linking or unphysiologically by the lectin Con A. Retinol is not absolutely required because proliferation is initiated in its absence when the anti-CD3 density is very high, but even then proliferation reaches only one quarter the level of comparable cultures with retinol (Fig. 1 *B*). Thus it appears that a very strong TCR signal suffices to activate some T cells whereas retinol facilitates the activation of a much larger population. Retinol is needed at the initiation of culture and cannot be withheld for longer than a few hours without loss of proliferation capacity (Fig. 5). It is unclear from present experiments whether once activated, continued proliferation of normal T cells is also critically dependent on retinol in the culture medium. This issue is under investigation. 13-*cis*-retinol and retinal but not all-*trans* retinoic acid can substitute for retinol. A new retinoid, 14HRR recently discovered by us (15) was also capable of supporting activation and sustaining proliferation of T cells, provided that it was replenished twice daily to compensate for its rapid decay. Our experiments do not distinguish between defined stages in the activation process of resting T cells beyond a broad requirement during early and late events. The impact on early events is implied by the observation that a 12-h delay in retinol addition leads to stagnation, whereas the requirement for late events follows from the observation that a single addition of 14HRR does not enable sustained proliferation. Although retinol appears to be an important cofactor, its presence may not be absolutely required in situations where potent alternate second signals are given. For instance, interferon-γ proved quite

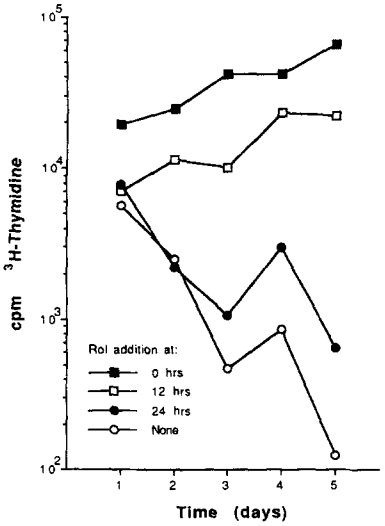

Figure 5. Retinol is required at the time of activation. Thymocytes (5×10^4/well) were activated by immobilized anti-CD3 mAb in medium ITLB. Retinol (2×10^{-6} M) was added at initiation of culture, or delayed by 12 and 24 h, respectively, or omitted altogether. [^{3}H]thymidine pulses of 4 h were used to measure DNA synthesis of hexaduplicate wells. (SDs were ≤20%).

efficient in anti–CD3-mediated activation in the absence of retinol (Table 1).

Retinol, an essential vitamin, circulates in blood as a stable complex with retinol-binding protein (RBP) and transthyretin (TTR) (23). Its concentration in plasma is closely regulated at 1–2 × 10^{-6} M, whereas intracellular concentrations vary between tissues and appear to depend on the extant concentration of cellular retinol-binding protein (CRBP) (24). Because the tissue distribution of CRBP is nearly universal (25), it is inferred that retinol is present ubiquitously as well (26). A general physiological purpose of retinol itself has not been discerned, but there is agreement that retinol is used as a metabolic precursor of other retinoids, including 11-*cis*-retinal functioning as the photoreceptor in vision (27), all-*trans* retinoic acid which has been implicated in differentiation (28) and morphogenesis (29), and 9-*cis* retinoic acid that has been found to activate the RXR receptor (30, 31).

Pursuing the hypothesis that retinol serves as a precursor of intracellular retinoid mediators, we have analyzed the metabolic products of retinol in B lymphocytes in previous studies (14, 15). B lymphocytes did not produce retinoic acids, but they synthesized a new class of retinoids, the retro-retinoids, hitherto seen in nature only in the form of anhydro-retinol (32). Retro-retinoids are characterized by a completely planar ring-to-tail configuration, rigidly enforced by the rearrangement of the carbon double bond system so as to fix the six-membered ring by a double bond to the polyene tail. 14-hydroxy-retro-retinol is the first naturally occurring retro-retinoid to be discovered in mammalian cells, and is 20–40 times more potent on a concentration basis in preventing necrotic cell death in B lymphocytes than its parent molecule, retinol.

Our results in this study show that retinol is an essential component in serum-free medium, without which T cells can be activated only superficially and T cell proliferation does not proceed. Serum is a customary supplement of culture media used for in vitro experimentation in cellular immunology, and the retinol herein might be part of the secret of why it is such an effective ingredient in growth medium. As implied by our results, however, components of serum other than retinol, albumin (as a transport protein of fatty acids), transferrin (to regulate iron metabolism), and insulin may not be needed for lymphocyte cultures. Indeed, the advantage of avoiding unknown influences by hormones and growth factors (notably PDGF) forms a compelling reason for experimentation in defined serum-free medium as discussed in detail by Daynes et al. (6).

In the serum-free medium composition used here, retinol is not protected by its physiological serum carrier proteins, RBP and TTR, is therefore labile, and decays with a half-life of <24 h in cell culture (14). The optimally effective dose of retinol is 2 × 10^{-6} M, when given once in a 3-d culture, or 2 × 10^{-7} M when provided at 12-h intervals. This dose range corresponds to the concentration of retinol in normal sera, i.e., 1–2 × 10^{-6} M.

The question of whether retinol mediates its effect on T cells through its metabolic product, 14HRR, cannot be answered definitely by the experiments shown in this study, but the arguments that follow support this mechanisms. First, 14HRR is capable of supporting T cell activation and proliferation in the absence of any extraneous source of retinol. Second, the dose–response curves for retinol and 14HRR in T cells are very similar, a finding of some concern, because a putative downstream mediator (i.e., 14HRR) might have been expected to be active at lower concentrations than its precursor. However, 14HRR is intrinsically a much more labile molecule than retinol. Previous analyses with B lymphocytes have demonstrated activity for 14HRR at 5 × 10^{-9} M concentration compared with 2 × 10^{-7} M for retinol, a 40-fold difference (15). Why T cells require higher concentrations of 14HRR is unclear. Third, 14HRR is a metabolic product of retinol on the basis of isotope-labeling experiments and because of the fact that 14HRR is an optically active compound, and therefore enzymatically derived (15). Fourth, 14HRR (in contrast to retinal) does not revert to retinol (our unpublished observations). Fifth, it is noteworthy that T lymphocytes neither respond to externally provided retinoic acid nor synthesize appreciable amounts of it (J. Buck and U. Hämmerling, unpublished results). Thus, 14HRR does not appear to be an intermediary compound in retinoic acid synthesis, an unlikely possibility on structural considerations, anyway.

Although these considerations leave unanswered the question whether 14HRR might be the intracellular mediator itself, they strongly suggest a regulatory retinol pathway distinct from that of retinoic acid observed in nonlymphoid cells. Retinoic acid has frequently been referred to as the active mediator of retinol effects, but our findings suggest alternative mediators and pathways. Having dismissed retinoic acid as an actual mediator in lymphocytes, it might be useful to recall the mechanism of retinoic acid action as a possible analogy by which 14HRR (or its active derivative) might function. Retinoic acid appears to be synthesized locally by unspecified regulatory cells and to pass into target cells where it is bound by a class of specific cytoplasmic retinoic acid binding proteins. The function of these cytoplasmic complexes is unknown. Retinoic acid then translocates to the nucleus and binds to one of three known specific nuclear receptor proteins, RAR-α, -β or -γ (33–36). Binding of the ligand, all-*trans* retinoic acid or 9-*cis* retinoic acid confers regulatory changes to the transcription of the respective gene(s) that bind RAR. The same principle governs gene activation by interaction of 9-*cis* retinoic acid with RXR (30, 31). Moreover, ligand-assisted transcriptional regulation is also the mechanism by which steroids function. Indeed, because retinoids and steroids are biochemically related as members of the same isoprenoid superfamily, it becomes increasingly clear that a large system of ligands has evolved from this chemical family to fulfill the demands of differential gene usage in complex organisms (37). The discovery of a parallel large family of genetically homologous and structurally related nuclear receptors emphasizes this point (38). By analogy, 14HRR might also be involved in ligand-assisted transcription. Whereas we have no direct evidence for this, in work to be

published elsewhere, we have observed that 14HRR facilitates the expression of immediate early genes in fibroblasts. If substantiated for T cells, this finding would handsomely explain the requirement for retinoids in T cell activation.

Throughout our work we have been concerned that our results might violate the precept of dual signaling in T cell activation (39, and for review see reference 40). According to this hypothesis, TCR occupancy must be accompanied by a second signal emanating under physiological conditions from an as yet undefined accessory cell/T cell interaction. The B7/CD28 pair of intereaction molecules might fulfill that function (41). Our findings, however, imply that the accessory signal, regardless of whether this is given by B7 or through another mechanism, is not obligatory because we obtained maximal activation of thymocytes and T cells in the absence of accessory cells by immobilized anti-CD3ϵ mAb or Con A alone. This finding runs counter to expectation, and must be ascribed to the unusual culture conditions employed by us, notably the addition of the retinol cofactor. In commonly used FCS-containing medium we find indeed no activation in the absence of APC, whether or not additional retinol is supplied. The dramatic difference in T cell stimulation in serum-free ITLB medium supplemented with retinol implies that FCS imparts inhibitory signals to T cells as suggested by Daynes et al. (6). On the other hand, we have not used protein-free conditions and cannot rule out the possibility that transferrin or insulin impart to T cells mitogenic signals that mimic a requisite secondary signal. The important question in our continuing investigation is now to determine how retinol and its metabolic product, 14HRR, are to be integrated into the intracellular signaling events alongside the other known biochemical consequences of TCR triggering: IP3 and DAG production.

We have attempted to determine whether retinol is required during the activation phase, G_0 to G_1, during the progression through S phase, or during both phases. Our results support the notion that retinol is needed for initial activation, because a delay in addition of retinol after the TCR signal was given caused a marked decrease in proliferation. The answer to the second question is less clear as retinol, once given to cells, cannot easily be removed by washing because of its lipid nature. However, a single dose of 14HRR given at initiation of culture, and decaying with a half-life of $\sim$4 h, was insufficient to drive T cell proliferation, speaking for a continuous requirement of retinol also for transition to S phase. Supporting this notion is also our published record concerning continuously growing lymphoid tissue culture lines that are dependent on retinol (9).

To complement the study of activation requirements in serum-free medium we have inquired into the role of exogenous lympho- and cytokines. The salient points of these experiments are that none of the interleukins tested (IL-1, -2, -4, and -6) nor TNF-α are substitutes for retinol. They are in agreement with the assumption that retinol needs to be physically present as a source for further metabolic modifications. However, this argument is partly negated by the observation that IFN-γ can circumvent the retinol requirement and initiate durable proliferation in the absence of retinol or 14HRR, whereas strong additive effects were seen when retinoids were present simultaneously with IFN-γ.

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