

## RETINOL IS ESSENTIAL FOR GROWTH OF ACTIVATED HUMAN B CELLS

By JOCHEN BUCK, GERD RITTER, LUCIANA DANNECKER,  
VISWANATHAM KATTA,\* STEVEN L. COHEN,\* BRIAN T. CHAIT,\*  
AND ULRICH HÄMMERLING

*From the Department of Immunology, Memorial Sloan-Kettering Cancer Center; and the  
\*Mass Spectrometry Laboratory, The Rockefeller University, New York, New York 10021*

When Wolbach and Howe (1) studied vitamin A-deficient rats in the Twenties, they noted among the many histopathologic changes a marked atrophy of the thymus and loss of lymphoid cells in spleen and lymph nodes. Subsequent observations revealed that avitaminotic birds develop deficient bursas (2), and that avitaminotic rats reared conventionally, as opposed to under germ-free conditions, suffer from multiple, life-threatening infections (3). These findings may have signalled the possibility that lymphopoiesis is compromised in vitamin A deficiency, but a formal connection to the physiology of immune cells has not been established. Despite sporadic evidence in the literature (reviewed in references 4 and 5) that retinoids influence immune responses, to our knowledge systematic studies to uncover the mechanism underlying immune system enhancement have not been carried out.

In the course of studies of a growth factor for human, EBV-transformed B lymphocytes, we have isolated a lipid-like molecule by extraction of serum with organic solvents. We show in the present report that this low molecular weight factor consists of all-*trans* retinol. The finding that vitamin A supports the growth of cultured human B cells has not previously been reported. It may be related to growth stimulation of epithelial cell lines by retinoids although there are several reports to the contrary (for review, see reference 6), demonstrating growth inhibition by retinoids. We had previously isolated a protein on the basis of its ability to stimulate the proliferation of lymphoblastoid cells grown at low density (7). The bioactivity of this protein was markedly enhanced by a lipid cofactor. We now know that the protein factor represents the monomer subunit of prealbumin (PA)<sup>1</sup> (unpublished results), and that the lipid cofactor is retinol. PA (8) is one of two protein molecules of a complex endowed with the task of transporting retinol in the blood, the other being retinol-binding protein (RBP) (9). Retinol binds to RBP, conferring high binding affinity for PA

---

This work was supported by grants from the American Cancer Society (IM480) and the National Institutes of Health (CA-49933, CA-08748, and RR-00862). J. Buck was a Clinical Scholar in Biomedical Research supported by the Norman and Rosita Winston Foundation.

Address correspondence to Ulrich Hammerling, Department of Immunology, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York, NY 10021.

<sup>1</sup> *Abbreviations used in this paper:* BHT, butylated hydroxytoluene; CI, chemical ionization; EI, electron impact; m/z, mass to charge ratio; PA, prealbumin; RBP, retinol-binding protein; SAC, *Staphylococcus aureus* Cowan strain.

to the latter (10, 11). The trimolecular complex thus formed serves as the physiologically active form of vitamin A in blood plasma (9). We have searched for RBP in our B cell stimulatory factor and have confirmed that for full bioactivity RBP is indeed required. In the present report we give an account of our finding that retinol in conjunction with carrier proteins is essential for growth of activated B cells in culture.

### Materials and Methods

**Retinoids.** Retinoids were purchased from Sigma Chemical Co. (St. Louis, MO). The retinoids were dissolved at a concentration of  $3 \times 10^{-2}$  M in methanol/chloroform (3:1 vol/vol) with  $10^{-4}$  M butylated hydroxytoluene (BHT) (Sigma Chemical Co.) added and stored in the dark at  $-20^{\circ}\text{C}$  in a nitrogen atmosphere. Immediately before a bioassay, the stock solutions were diluted in serum-free medium (HB101; Hana Biologics, Berkeley, CA).

**Cells.** The human EBV-transformed lines 5/2, Ducaf, PLH, Pitout, Thomas, BH, and Mou were established in our laboratory from the peripheral blood of healthy donors. The cells were tested regularly for mycoplasma infections and were consistently negative. B cells were prepared from normal human spleens by Ficoll gradient centrifugation followed by OKT4 and OKT8 (Ortho Pharmaceutical, Raritan, NJ) antibody treatment plus rabbit complement and passage through a Sephadex G-10 column. Recovered cells contained  $>90\%$  sIg<sup>+</sup> cells.  $5 \times 10^5$  cells/ml were activated either with 0.03% of fixed *Staphylococcus aureus* Cowan strain (SAC) or 4  $\mu\text{g}/\text{ml}$  of F(ab)<sub>2</sub> fragments of rabbit anti-human Ig (Tago Inc., Burlingame, CA) in HB101 medium (Hana Biologics) 48 h before the beginning of the experiment.

**Standard B Cell Proliferation Assay.** The assay system is a modification of the assay developed by Blazar et al. (12). Lymphoblastoid cells grown for 18–24 h in serum-free HB101 medium at a cell density of  $4 \times 10^5$  cells/ml or B cells activated by SAC or anti- $\mu$  for 48 h were washed twice. The cells were then plated at graded cell concentrations in a final volume of 200  $\mu\text{l}$ /well of HB101 in 96-well microtiter plates and grown for 72 h. Cell growth was determined by labeling the cells for the last 16 h with 0.8  $\mu\text{Ci}/\text{well}$  of [<sup>3</sup>H]thymidine (sp act 6.7 Ci/mmol).

**Cell Growth Curves.** Lymphoblastoid 5/2 cells were washed once and seeded at a concentration of 300,000/ml with or without  $10^{-6}$  M retinol in HB101 medium in 25-cm<sup>2</sup> tissue culture flasks (Corning Glass Works, Corning, NY). Three aliquots of 100  $\mu\text{l}$  were removed at 24-h intervals, and thymidine uptake was determined by culturing in the presence of 0.8  $\mu\text{Ci}$  of [<sup>3</sup>H]thymidine for an additional 6-h period. To determine the viable cell number, nine aliquots per time point were removed and counted in a Neubauer chamber. Dead cells were determined by the uptake of trypan blue.

**Delipidation of Serum Protein.** LPS-free FCS was purchased from HyClone Laboratories (Logan, UT), and human plasma was produced from whole blood obtained from the New York Blood Center. The serum or plasma was stirred overnight at  $-20^{\circ}\text{C}$  in a 100-fold volume excess of diethylether/ethanol 1:3 (vol/vol). Precipitated proteins were washed twice in diethylether, dried, redissolved in PBS, and dialyzed extensively against PBS.

**Isolation of Serum Lipids.** 100 ml of human plasma or FCS was freeze dried. The dry powder was extracted twice with 40 ml of chloroform/methanol 2:1 (vol/vol) followed by two extractions with 40 ml of chloroform/methanol/water 10:10:1 (vol/vol). All solvents contained  $10^{-4}$  M BHT. The combined extracts were dried on a rotary evaporator. Lipids were dissolved in chloroform/methanol/water 10:10:1 (vol/vol) and stored at  $-80^{\circ}\text{C}$ . Immediately before a bioassay, aliquots of the lipid solution were evaporated to dryness and the residual lipids dispersed in HB101 medium by sonication.

**Lipid Separation on a Reversed-phase HPLC Column.** An HPLC system (M6000 solvent delivery pump, model 400 wavelength detector, data module, and data processor; Waters Associates, Milford, MA) was used. The crude lipid mixture was loaded on a semipreparative reversed-phase C<sub>18</sub> HPLC column (Vydac; The Separations Group, Hesperia, CA). Lipids were eluted with a linear gradient of water/methanol/chloroform. The gradient started with water/methanol 70:30 (vol/vol), went in 30 min to 100% methanol, and in another 30 min to methanol/chloroform 50:50 (vol/vol). The flow rate was 3 ml/min. The elution profile was monitored at wavelengths of 280 and 340 nm.

**Mass Spectrometry.** Bioactive lipid fractions were stored at  $-80^{\circ}\text{C}$  under nitrogen. Electron impact (EI) and either methane or isobutane chemical ionization (CI) mass spectra of the samples were obtained by loading the samples on a direct insertion probe of a VG 70-250 double-focusing mass spectrometer. The probe was slowly heated so that the antioxidant additive BHT volatilized first, followed by the lipid material.

**Isolation of Retinol-retinol Binding Protein Complex (13).** 40 mg of human prealbumin was covalently bound to 3 ml of CNBr-activated sepharose. 50 ml of human plasma was passed through the column. The column was washed with 30 ml of 0.04 M Tris HCl (pH 7.4)/0.5 M NaCl and eluted with 1-ml increments of distilled water. Purity was established by SDS-PAGE with silver stain using the PhastSystem by Pharmacia Fine Chemicals (Piscataway, NJ). The optical densities at 280 and 340 nm were determined.

## Results

**Serum as Growth Promoter of Activated Human B Cells.** Human B cells transformed by EBV grow in defined serum-free HB101 medium at high cell density ( $>100,000$  cells/ml). However, when removed from high cell density serum-free conditions after 1 d of culture, washed in serum-free medium, and reseeded in fresh HB101 medium at low density, the cells proliferate at a much lower rate and, if the average density falls below  $10^4$  cells per culture well ( $5 \times 10^4$  cells/ml), most of the cells die within 24 h. Suitable growth conditions are restored by the addition of human serum (Table I), FCS (data not shown), or our own conditioned medium of high density human lymphoblastoid cells grown in serum-free HB101 medium (12, 30, data not shown).

**Serum Lipids or Delipidated Serum Proteins Alone Possess Moderate Growth-stimulatory Capacity but Act in Synergy to Enhance B Cell Growth.** Serum lipids were isolated from human serum by solvent extraction with diethylether/ethanol or chloroform/methanol/water mixtures. The growth stimulatory effects of lipids and delipidated proteins (i.e., the residue after ether/ethanol extraction) for EBV-transformed cells were tested either alone or in combination. The results of Table I show that either component alone produces a definite, though modest increase in thymidine uptake. When the lipid and protein fractions were added together, a synergistic effect occurred that nearly matched the growth stimulation potential of whole serum.

**Characterization of the Growth-promoting Human Serum Lipid.** The bioactivity of lipids

TABLE I  
Comparison of Growth-stimulating Effect between Human Serum,  
Delipidated Serum Proteins, and Serum Lipids

Stimulating agent	Growth-stimulating effect <i>cpm</i>
Plain HB101	1,952 $\pm$ 138
Human serum (3%)	16,896 $\pm$ 232
Delipidated serum proteins	4,094 $\pm$ 772
Serum lipid	4,360 $\pm$ 266
Protein + lipid	13,211 $\pm$ 721

Washed 5/2 cells (1,500/well) were incubated for 72 h in serum-free HB101 medium. DNA synthesis was assessed by a 16-h [ $^3\text{H}$ ]thymidine uptake. The experiment was done in triplicate. Purified proteins and lipids were reconstituted to concentrations equivalent to those of 3% serum. The data correspond to the mean  $\pm$  SD.

decayed within hours at room temperature, but decay could be slowed by the addition of the antioxidant BHT and by storage of the sample in the dark at  $-80^{\circ}\text{C}$  in a nitrogen atmosphere. The lipid mixture was chromatographed on a reversed-phase  $\text{C}_{18}$  HPLC column (Fig. 1) with linear water/methanol/chloroform gradient. Analysis of the fractions in the bioassay showed that 80% of total activity was recovered in fraction 34. Corresponding to the main bioactivity peak was an optical absorption peak registering at the wavelengths of 280 and 340 nm. The material collected in this fraction fluoresced when exposed to UV light. The EI and CI mass spectra of the bioactive component (fraction 34 in Fig. 1) indicated the presence of a single compound with a molecular mass of 286 daltons. The EI spectrum, shown in Fig. 2 A, shows the molecular ion peak at mass to charge ratio ( $m/z$ ) 286, major fragment ion peaks at  $m/z$  268 and 255, and a series of low mass ions indicative of a highly unsaturated hydrocarbon. A computer search of the National Bureau of Standards library spectra showed a good fit with the spectrum of all-*trans* retinol, which is shown in Fig. 2 B. Accordingly, authentic all-*trans* retinol was applied to the bioassay, and a growth-enhancing effect was observed on activated human B cells similar in magnitude to the ones elicited by bioactive HPLC fraction 34 or the crude serum lipid (Table II, see below). When all-*trans* retinol was chromatographed on the HPLC  $\text{C}_{18}$  column, the bulk of the material was recovered at the same position (i.e., fraction 34) as the main bioactivity peak obtained from the lipid mixture. Fractions 30, 36, and 38–41 in Fig. 1 also contained bioactivity, but their biochemical nature has yet to be determined. A second series of experiments with FCS gave similar results, identifying all-*trans* retinol as the major bioactive component.

*Retinol-Retinol Binding Protein Complex Serves as Growth Factor.* In serum, the natural carrier of retinol is RBP complexed with PA (9). The trimolecular complex

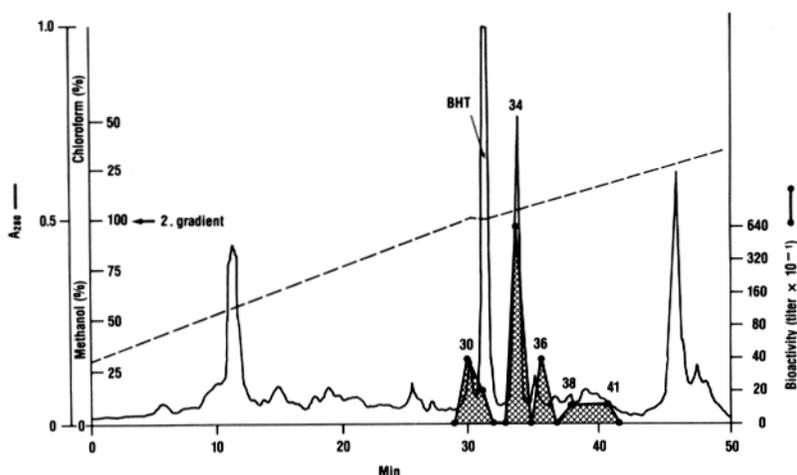


FIGURE 1. RP-HPLC. Crude lipids from 500 ml human serum were loaded on a semipreparative reversed-phase  $\text{C}_{18}$  HPLC column. Lipids were eluted with a linear gradient of water/methanol/chloroform. The flow rate was 3 ml/min. The elution was monitored by absorption at 280 nm (—), and each 1-min fraction was tested for bioactivity in the standard B cell growth assay as described in Materials and Methods.

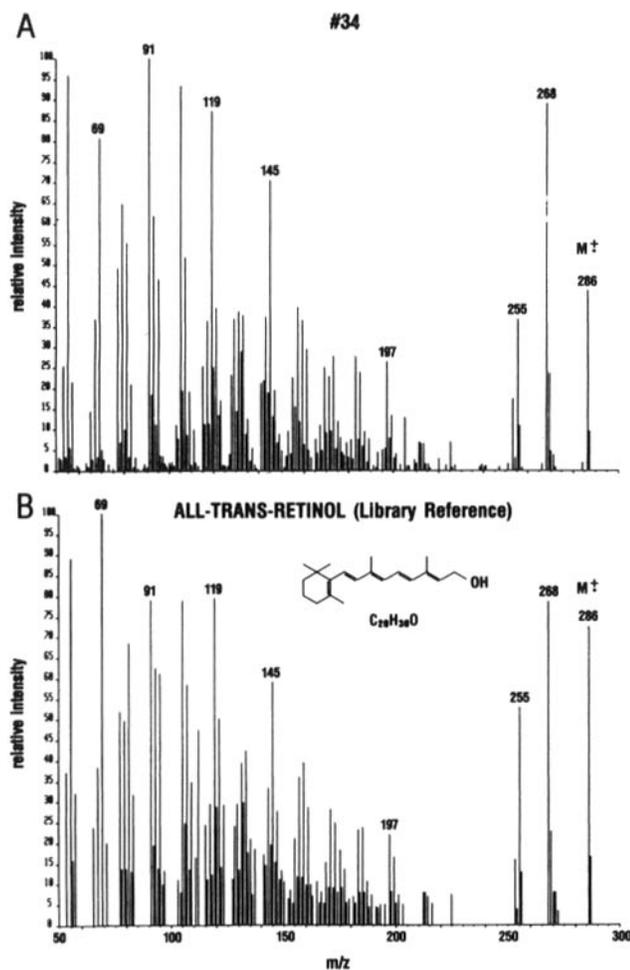


FIGURE 2. (A) EI mass spectrum of the bioactive fraction no. 34 (Fig. 1). (B) EI mass spectrum of all-*trans* retinol from the National Bureau of Standards library.

TABLE II  
 Comparison of Growth-stimulating Effect of Human Serum, Retinol,  
 and Purified Lipid on Activated Human B Cell Blasts

Stimulating agent	Growth-stimulating effect		
	SAC-blasts (2,000/well)	Anti- $\mu$ blasts (2,000/well)	EBV blasts (5/2 cell line) (1,500/well)
	<i>cpm</i>		
Plain medium	515 $\pm$ 53	383 $\pm$ 48	1,451 $\pm$ 366
3% human serum	1,780 $\pm$ 230	ND	10,446 $\pm$ 1,021
Fraction 34 (1/500)*	2,819 $\pm$ 337	4,783 $\pm$ 841	6,136 $\pm$ 213
Retinol ( $3 \times 10^{-7}$ M)	3,091 $\pm$ 421	4,375 $\pm$ 546	6,910 $\pm$ 1,015

Washed B cell blasts were incubated for 72 h in HB101 medium. DNA synthesis was assessed as described. The experiment was done in triplicate. The data correspond to mean  $\pm$  SD.

\* Final dilution of fraction 34.

is stable under isotonic conditions, but dissociation of the two proteins occurs in distilled water (13), retinol being retained by RBP. To test for the involvement of the natural carrier system, we have isolated RBP by use of a solid-phase PA column consisting of human PA attached to CNBr-activated Sepharose (14). Fresh human serum was passed through the affinity column, and bound proteins were eluted with water. The eluted proteins by SDS-PAGE proved to be 85% RBP, with some PA contamination (Fig. 3). The ratio of optical absorption at 280 nm to that at 340 nm (0.7) indicates that most RBP molecules were complexed to retinol (15). When assayed in cultures of lymphoblastoid cells (Table III), the RBP-retinol complex (holo-RBP) showed growth-enhancing activity at the physiological concentration of 40  $\mu\text{g}/\text{ml}$ , similar in magnitude to activities of crude serum lipids and of commercially available retinol. Delipidated RBP (apo-RBP) did not show any growth-enhancing activity. When the RBP-retinol complex was extracted with methanol/chloroform and the extract subjected to HPLC on a  $\text{C}_{18}$  column, a single peak was observed. This peak coeluted with fraction 34 (Fig. 1), had similar bioactivity, and showed the same molecular ion and fragmentation pattern as that of all-*trans* retinol (Fig. 2 B).

*Comparison of Retinol and Other Retinoids for Bioactivity.* Dose-response curves establish that all-*trans* retinol, tested in serum-free HB101 medium, is optimally active at a concentration of  $2 \times 10^{-6}$  to  $2.5 \times 10^{-7}$  M (Fig. 4), a range corresponding to the physiological concentration in serum ( $10^{-6}$  M, retinol) (16). At concentrations  $>4 \times 10^{-6}$  M, retinol is toxic to human B cells in serum-free HB101 (Fig. 4), as well as in 7% serum-supplemented medium (data not shown). All-*trans* retinal is comparable with retinol in bioactivity at a concentration range of  $1-5 \times 10^{-7}$  molar, but at  $2 \times 10^{-6}$  M, it displays toxicity. Retinoic acid, commonly assumed to be the most active retinol derivative in cell culture (6), has marginal bioactivity on B cells at  $10^{-5}$  M (15-25% growth-enhancing capacity as compared with retinol) and is toxic at higher concentrations. However, our results were not consistent, and in one-third of the experiments, retinoic acid did not show any growth-enhancing effect. At concentrations of  $4 \times 10^{-6}$  to  $6 \times 10^{-8}$  M, there is a noticeable suppression of thymidine incorporation. When retinoic acid was admixed to a stimulatory concentration of retinol, the growth stimulatory capacity of the latter was not inhibited (data not shown).

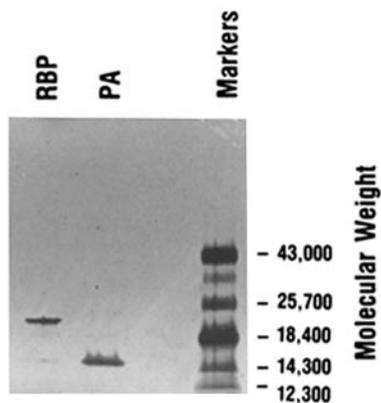


FIGURE 3. 10-15% gradient SDS-PAGE gel of RBP and PA. RBP was purified from human serum according to reference 13. Human PA and molecular weight markers are shown as reference. The protein bands are visualized by the silver staining technique.

TABLE III  
Comparison of Growth-stimulating Effect of Retinol, Holo-, and apo-RBP on Three Lymphoblastoid Cell lines

Stimulating agent	Growth-stimulating effect		
	5/2	Mou	BH
		<i>cpm</i>	
Plain medium	145 ± 4	837 ± 139	381 ± 100
Retinol	3,049 ± 356	1,360 ± 21	2,770 ± 105
holo-RBP	1,405 ± 86	2,263 ± 217	5,736 ± 253
apo-RBP	211 ± 31	801 ± 48	290 ± 37

Cells of EBV-transformed cell lines 5/2, Mou, and BH were washed and cultured at 2,500/well and incubated for 72 h in serum-free HB101 medium. DNA synthesis was assessed as described. The experiment was done in triplicate. The data correspond to mean ± SD. End concentration of retinol was  $10^{-6}$  M. 40 µg/ml of nondelipidated holo-RBP and delipidated apo-RBP were added. holo-RBP had an absorption ratio from OD<sub>340</sub>/OD<sub>280</sub> of 0.7.

**Retinol Prevents the Death of Lymphoblastoid Cells in Serum-free Medium.** The dependency on retinol was studied in detail with the human lymphoblastoid cell line 5/2. Retinol deprivation did not show an effect on the viable cell number (Fig. 5) or thymidine uptake (Fig. 6) during the first 24 h. However, on day 2, ~30%, and on day 3, ~90% of the lymphoblastoid cells in retinol-free medium were dead (trypan blue-positive cells). When 5/2 cells were harvested after 3 d of culture in retinol-containing medium and transferred to retinol-free medium, they showed once again a precipitous decline in viability and thus were still dependent on retinol (Fig. 6). The population crash began 1 d after the cells were put in retinol-free medium, and >90% of the cells were dead after 2 d. In contrast, cells seeded again in retinol-containing medium continued to grow exponentially.

**Retinol Enhances the Growth of Lymphoblastoid Cell Lines and of Activated Normal Human B Cell Blasts.** The growth of six other lymphoblastoid cell lines (cell lines Mou and

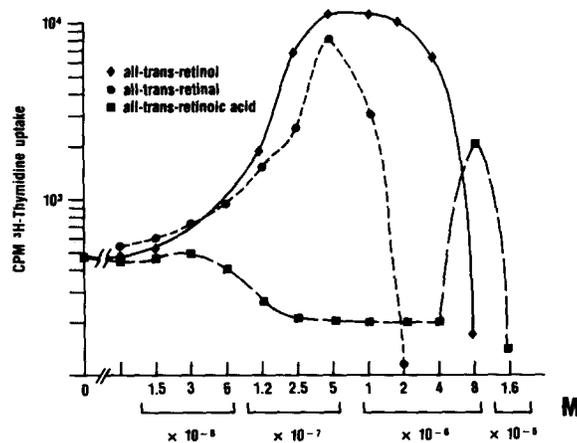


FIGURE 4. Dose-response curves of different retinoids. Washed 5/2 cells (5,000/well) were incubated for 72 h in HB101 medium with the indicated amounts of retinoids. DNA synthesis was measured by [<sup>3</sup>H]thymidine as described. The measurements were done in triplicate. The SDs were never >15%. Repeat experiments (10 for retinol and retinoic acid, five for retinal) yielded similar dose-response curves. One representative experiment is shown. Where retinol and retinal gave consistent results, retinoic acid did not, failing to show any stimulation in one-third of the experiments.

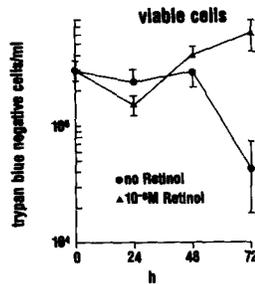


FIGURE 5. Growth curves of 5/2 cells in suspension culture with and without retinol. 5/2 cells were washed once and seeded at a density of 300,000/ml in HB101 with or without  $10^{-6}$  M retinol. The trypan blue-negative and trypan blue-positive (not shown) cell number of nine aliquots was determined every 24 h. Means  $\pm$  SDs are shown.

BH) (data shown in Table III); cell lines Ducaf, PLH, Thomas, and Pitout (data not shown), as well as the growth of two Burkitt lymphoma lines (data not shown) was enhanced 3-20-fold by the addition of  $10^{-6}$  M retinol to the serum-free HB 101 medium.

Normal human B cell blasts that were preactivated with anti- $\mu$  antibody or SAC required retinol as a costimulator for growth to the same extent as Epstein-Barr-transformed human B cells (Table II).

### Discussion

Our main finding is that for optimum growth in cell culture, human lymphoblastoid cells require retinol and its natural carrier proteins, notably retinol-binding protein. In reaching this conclusion, we have considered the following evidence. First, retinol occurs in fresh serum in a complex with RBP and PA (9). When retinol is removed with the serum lipids from the serum proteins by extraction with organic solvents, the growth-promoting properties of the supplemental serum are lost. But they are restored when authentic all-*trans* retinol is mixed back into the residual serum proteins. Thus, the effect is associated with retinol and not with an essential fatty acid. Second, the concentrations of retinol required for optimal growth are similar to those of fresh serum supplements. Third, all-*trans* retinol, isolated from human

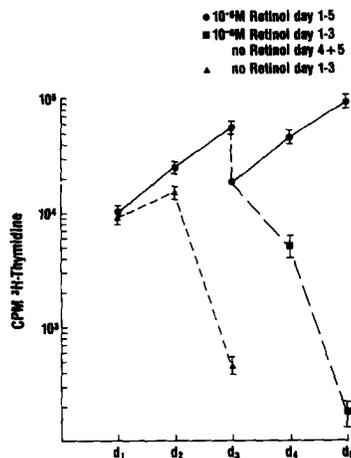


FIGURE 6. B lymphoblastoid cells show continued dependency on retinol. 5/2 cells were washed once and seeded at a concentration of 300,000/ml in HB101 medium with and without  $10^{-6}$  M retinol. The cells of retinol-containing medium were harvested on day 3, washed once, diluted 1:3 in HB101, and cultured for another 2 d with or without  $10^{-6}$  M retinol in HB101. Triplicate samples of 100  $\mu$ l of cell suspension were removed daily and pulsed for 6 h with [<sup>3</sup>H]thymidine. Means  $\pm$  SDs are shown.

serum, was positively identified by mass spectrometry and was as potent as all-*trans* retinol from commercial sources.

The dependency on retinol was demonstrated not only with the randomly picked lymphoblastoid cell line 5/2 but also with several other cell lines, including six other randomly selected lymphoblastoid and two Burkitt's cell lines. Culture of normal human B cells activated with either anti- $\mu$  antibody or heat-inactivated *S. aureus* bacteria were also found to be dependent on retinol in the medium. Thus, our finding may have general validity in B cell physiology.

Our analysis of a selected group of retinoids has revealed that all-*trans* retinol and all-*trans* retinal are able to support cell growth at concentrations as low as  $2 \times 10^{-7}$  M. The physiological concentration of retinol in human serum is  $\sim 10^{-6}$  M (9). Interestingly, all-*trans* retinoic acid proved to be the least active retinoid, showing only one-fifth of the activity of retinol at  $10^{-5}$  M, despite its reputation as a strong inducer of cell differentiation in cell culture. Perhaps growth promotion and induction of differentiation are distinct events in the life cycle of a cell, and at a given time point may even be mutually exclusive events. It is noteworthy that retinoic acid does not occur in serum at concentrations  $>10^{-8}$  M (16, 17), and thus, it is unlikely that the occasionally observed low stimulatory activity of retinoic acid at a concentration of  $10^{-5}$  M has any physiological meaning. On the other hand, we can only state that retinol and retinal represent the active forms of retinoids as extracellular mediators, but we do not know whether they are converted intracellularly to other derivatives or isomers, including retinoic acid. It is commonly assumed that in order to exert their effects, retinoids have to pass into the cells.

As already alluded to, retinol does not occur in free form in body fluids but is complexed with RBP, which in turn is bound to tetrameric PA, and it is therefore assumed that the bioactive form of retinol for extracellular actions is the trimeric complex. We have previously reported that a protein factor, provisionally named aBGF (7), and later shown by sequence identity to be the monomer of prealbumin, is capable of supporting B cell growth in suboptimal culture conditions, and we have suggestive evidence that RBP in some cultures increases the effectiveness of retinol (see Table III). These observations are consistent with the view that retinol requires carrier protein, but our experiments do not resolve this question since the synthetic tissue culture medium used (i.e., medium HB101), although free of serum, still contains serum proteins. In our experience, albumin preparations used as a supplement of HB101 are commonly contaminated with PA and RBP. Experiments to define the role of the physiological retinol carrier proteins require homogeneous materials and are currently in progress.

Our observation that retinol acts as growth promoter for human lymphoblastoid cells has not been reported previously. There is also a dearth of information on the *in vivo* effects of vitamin A on the immune response, although early observations in avitaminotic animals have suggested that vitamin A is required for normal lymphopoiesis. Reductions in the weights of thymus and lymph nodes in rats (1) as well as that of the bursa in birds (2) raised on a vitamin A-deficient diet are early manifestations of vitamin A deficiency disease, suggesting that lymphopoiesis is dependent on this vitamin. Frequent infections in such animals further suggest a malfunction of the immune system. Germ-free rats fed with a vitamin A-deficient diet have far fewer complications and attain a nearly normal life span, as compared with their

vitamin A-deficient littermates kept under conventional conditions, which do not survive the first 2 mo of life (3). Studies in chicks made vitamin A deficient by maintaining them from hatching on a diet of retinoic acid showed drastic reductions in the growth of the thymus and bursa (2). Thus, retinoic acid is inferior to retinol in maintenance of lymphoid tissue in chicks.

Systematic studies of the proliferative effect of vitamin A on cultured B lymphocytes are nonexistent. To place our findings in perspective with the known vitamin A physiology, one must therefore look farther afield. Best studied are epithelial cell cultures, since it has been known since the work of Wolbach and Howe (1) that vitamin A affects keratinocytes *in vivo*. Tissue explants and epithelial cell lines (6, 18) are regulated by retinoids. Besides cell growth, which requires retinoids, and delipidated serum as a medium supplement, the differentiation state of epithelial cells appears to be affected by vitamin A as well.

In other examples of cellular systems, where retinoids either enhance growth or induce differentiation, retinoic acid was found to be more potent than retinol or retinal (6). Cases in point are: (a) retinoic acid increases total DNA and RNA in newborn mouse epidermal cells in culture at a concentration of  $4 \times 10^{-10}$  M (18); (b) during embryogenesis, formation of a physiological retinoic acid gradient is decisive in determining the anterior/posterior orientation of the chicken wing (19, 20); (c) retinoic acid promotes differentiation in embryonal carcinoma cells (21), in HL-60 cells (22, 23), and numerous metaplasias in squamous epithelia (24); and (d) several closely related nuclear receptor molecules have been described for retinoic acid (25-27), and none so far for retinol or retinal.

One cellular system known to be controlled by retinol and where retinoic acid appears to play a subordinate role is spermatogenesis (28, 29). In this respect, our finding that B cell growth is dependent on vitamin A has its closest precedent in spermatogenesis. In both instances, retinoic acid is unable to substitute for the retinol or retinal forms of vitamin A.

### Summary

When EBV-transformed human B cells are removed from conventional cell cultures, washed, and seeded at a low cell density in serum-free medium, their growth potential is greatly diminished. Fresh serum restores the growth of low density B cell cultures. We have traced this restorative effect to an essential factor present in the lipid fraction of serum and have identified it as all-*trans* retinol. The identification is based on the close similarities of the factor isolated from serum with authentic all-*trans* retinol as revealed by mass spectrometry, HPLC chromatography, and the ability to stimulate the growth of lymphoblastoid cells in the bioassay. Retinol is active at concentrations equal to its concentration in serum. Retinol is also a requirement for growth in suspension cultures at cell densities of  $3 \times 10^5$ /ml. Cells removed at any time from such exponentially growing cultures and transferred to retinol-free medium cease to grow and consequently die, whereas in the continued presence of retinol, cell growth is unabated. All-*trans* retinal can substitute for retinol, but retinoic acid fails to stimulate the growth of lymphoblastoid cells at physiological concentrations. Normal human B lymphocytes also require retinol as a costimulator of proliferation after activation by anti- $\mu$  antibody or *Staphylococcus aureus* (Cowan strain) bacteria. In serum, retinol is bound to retinol-binding protein, which in turn

forms a complex with prealbumin. Accordingly, we find that B cells respond to retinol bound to its physiological serum carrier, retinol-binding protein. In conclusion, human B cells are critically dependent for optimal growth in cell culture on an external supply of retinol.

We thank Dr. E. Levi for performing SDS-PAGE gels and HPLC chromatography; Dr. T. Pettersson, Karolinska Institutet, Stockholm, Sweden, for a gift of RBP-conjugated beads; and B. Forbes for preparation of the manuscript.

Received for publication 12 September 1989 and in revised form 10 January 1990.

### References

1. Wolbach, S. B., and P. R. Howe. 1925. Tissue changes following deprivation of fat-soluble A vitamin. *J. Exp. Med.* 42:753.
2. David, C. Y., and J. L. Sell. 1983. Effect of retinol and retinoic acid nutrition on the immune system of chicks. *J. Nutr.* 113:1914.
3. Bieri, J. G., E. G. McDaniel, and W. E. Rogers. 1969. Surviving of germfree rats without vitamin A. *Science (Wash. DC)*. 163:574.
4. Dennert, G. 1984. Retinoids and the immune system: immunostimulation by vitamin A. In *The Retinoids*. Vol. 2. M. B. Sporn, A. B. Roberts, and D. S. Goodman, editors. Academic Press, New York. 373-390.
5. Amatruda, T. T., and H. P. Koeffler. 1986. Retinoids and cells of the hematopoietic system. In *Retinoids and Cell Differentiation*. M. I. Sherman, editor. CRC Press, Inc., Boca Raton, FL. 79-103.
6. Roberts, A. B., and M. B. Sporn. 1984. Cellular biology and biochemistry of the retinoids. In *The Retinoids*. Vol. 2. M. B. Sporn, A. B. Roberts, and D. S. Goodman, editors. Academic Press, New York. 209-286.
7. Buck, J., U. Hammerling, M. K. Hoffmann, E. Levi, and K. Welte. 1987. Purification and biochemical characterization of a human autocrine growth factor produced by Epstein-Barr virus-transformed B-cells. *J. Immunol.* 138:2923.
8. Blake, C. C. F., M. J. Geisow, and S. J. Oatley. 1978. Structure of prealbumin: secondary, tertiary and quaternary interaction determined by Fourier refinement at 1.8 Å. *J. Mol. Biol.* 121:339.
9. Goodman, D. S. 1984. Plasma retinol-binding protein. In *The Retinoids*. Vol. 2. M. B. Sporn, A. B. Roberts, and D. S. Goodman, editors. Academic Press, New York. 41-48.
10. Newcomer, M. E., T. A. Jones, J. Åqvist, J. Sundelin, U. Eriksson, L. Rask, and P. A. Peterson. 1984. The three-dimensional structure of retinol-binding protein. *EMBO (Eur. Mol. Biol. Organ.) J.* 3:1451.
11. Åqvist, J., P. Sandblom, T. A. Jones, M. E. Newcomer, W. F. van Gusteren, and O. Tapia. 1986. Molecular dynamics simulations of the holo and apo forms of retinol binding protein. *J. Mol. Biol.* 192:593.
12. Blazar, B. A., L. M. Sutton, and M. Strome. 1983. Self-stimulating growth factor production by B-cell lines derived from Burkitt's lymphomas and other lines transformed in vitro by Epstein-Barr virus. *Cancer Res.* 43:4562.
13. Heller, J. 1975. Characterization of bovine plasma retinol-binding protein and evidence for lack of binding between it and other bovine plasma proteins. *J. Biol. Chem.* 250:6549.
14. 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.
15. Kanai, M., A. Raz, and D. S. Goodman. 1968. Retinol-binding protein: the transport protein for vitamin A in human plasma. *J. Clin. Invest.* 47:2025.

16. DeRuyter, M. G., W. E. Lambert, and A. P. DeLeenheer. 1979. Retinoic acid: An endogenous compound of human blood. Unequivocal demonstration of endogenous retinoic acid in normal physiological conditions. *Anal. Biochem.* 98:402.
17. A. P. DeLeenheer, W. E. Lambert, and I. Claeys. 1982. All-trans retinoic acid: measurement of reference values in human serum by high performance liquid chromatography. *J. Lipid Res.* 23:1362.
18. Sporn, M. B., and A. B. Roberts. 1984. Biological methods for analysis and assay of retinoids. Relationship between structure and activity. In *The Retinoids*. Vol. 1. M. B. Sporn, A. B. Roberts, and D. S. Goodman, editors. Academic Press, New York. 235-279.
19. Slack, J. M. 1987. We have a morphogen. *Nature (Lond.)* 327:553.
20. Thaller, C., and G. Eichele. 1987. Identification and spatial distribution of retinoids in the developing chick limb bud. *Nature (Lond.)* 327:553.
21. Strickland, S., and V. Mahdave. 1978. The induction of differentiation in teratocarcinoma cells by retinoic acid. *Cell.* 15:393.
22. Breitman, T. R., S. E. Selonick, and S. J. Collins. 1980. Induction of differentiation of the human promyelocytic leukemia line (HL-60) by retinoic acid. *Proc. Natl. Acad. Sci. USA.* 77:2936.
23. Honma, Y., K. Takenaga, T. Kasukabe, and M. Hozumi. 1980. Induction of differentiation of cultured human promyelocytic leukemia cells by retinoids. *Biochem. Biophys. Res. Commun.* 95:507.
24. Fell, H. B. 1957. The effect of excess vitamin A on cultures of embryonic chicken skin explanted at different stages of differentiation. *Proc. R. Soc. Lond. B. Biol. Sci.* 146:242.
25. Petkovich, M., N. J. Brand, A. Krust, and P. Chambon. 1987. A human retinoic acid receptor which belongs to the family of nuclear receptors. *Nature (Lond.)* 330:444.
26. Brand, N., M. Petkovich, A. Krust, P. Chambon, H. de Th'e, A. Marchio, P. Tiollais, and A. Dejean. 1988. Identification of a second human retinoic acid receptor. *Nature (Lond.)* 332:850.
27. Benbrook, D., E. Lernhardt, and M. Pfahl. 1988. A new retinoic acid receptor identified from a hepatocellular carcinoma. *Nature (Lond.)* 333:669.
28. Mason, K. E. 1933. Differences in testes injury and repair after vitamin A deficiency, vitamin E deficiency and inanition. *Am. J. Anat.* 52:153.
29. Howell, J. McC., 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.
30. Gordon, J., S. C. Ley, M. D. Melamed, P. Aman, and N. C. Hughes-Jones. 1984. Soluble factor requirements for the autostimulatory growth of B lymphoblasts immortalized by Epstein-Barr virus. *J. Exp. Med.* 159:1554.