Title

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Journal

Journal of Experimental Medicine, 168(1)

ISSN

0022-1007

Authors

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et al.

Publication Date

1988-07-01

DOI

10.1084/jem.168.1.55

Peer reviewed

RETINOIC ACID INDUCES THE DIFFERENTIATION OF B CELL HYBRIDOMAS FROM PATIENTS WITH COMMON VARIABLE IMMUNODEFICIENCY

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Retinoids (vitamin A compounds) have been shown to profoundly affect cellular development in vitro (1). The differentiation-inducing activity of these compounds has been demonstrated with a variety of cell types including human promyelocytic leukemia (2), neuroblastoma (3), and embryonal carcinoma tumor cells (4). Antiproliferative effects of retinoids, uncoupled from cellular differentiation, have also been extensively reported (1, 5). Recently, retinoic acid (RA)[1] was identified as an endogeneous morphogen in chick limb buds (6), suggesting a physiologic role for retinoids in regulating certain "determinational" events during normal development. RA also displays immunomodulating properties, enhancing both cellular and humoral responses in rodents (7). In studying the effect of retinoic acid on the human immune response, Sidell and colleagues showed that RA enhanced the mitogenic responses of thymocytes to various stimuli (8) and increased the number of antibody-producing cells from tonsillar lymphocytes sensitized to sheep erythrocytes (9). In this latter humoral response, RA acted directly on the B cells to enhance antibody production, an effect that may relate to the differentiating properties of RA.

Common variable immunodeficiency (CVI) is an acquired syndrome associated with the inablility to produce a normal quantitative and qualitative antibody response (10). Patients have been described who primarily have had enhanced T or monocyte/macrophage suppressor cell activity (11), loss of helper T cell activity (12), B cell inability to secrete synthesized Ig, or even loss of pre-B cells and B cells in association with tumors of the thymus (13). The majority of patients with CVI, however, have normal or only somewhat reduced levels of B cells (as defined by surface Ig) which fail to differentiate to secrete Ig in vitro even when provided with the appropriate stimuli for terminal differentiation (14–16). Denis et al. (17) have previously identified such individuals with apparent intrinsic B cell defects and subse-

This work was supported by National Institutes of Health grants AI-15332 (CIRID at UCLA), CA-30515, AI-15251, and CA-12800. M. Gilly received support from National Research Scientist Award predoctoral training grant CA-09056. D. C. Adelman received postdoctoral support from U. S. Public Health Service grant CA-09120. Address correspondence to Neil Sidell, Ph.D., Dept. of Pathology, 18-170 Center for Health Sciences, UCLA School of Medicine, Los Angeles, CA 90024.

[1] *Abbreviations used in this paper:* CVI, common variable immunodeficiency; μm, membrane form of μ heavy chain mRNA; μs, secretory form of μ heavy chain mRNA; RA, retinoic acid; SAC, *Staphylococcus aureus* Cowan strain I; TRF, T cell-replacing factor.

quently established B cell human-human hybridoma lines from these patients, fusing in vitro-activated B lymphocytes with the parent line WIL2/729 HF (17). These CVI B cell hybridomas were shown to maintain the functional "defect" of the patient's B cells: Ig production was substantially lower in comparison to hybridomas generated with B cells of healthy donors and their ratio of membrane to secretory form of μ chain mRNA (μm/μs) was skewed toward the membrane form, characteristic of an immature B cell (18).

In the present study we have investigated the effects of RA on hybridomas established with B cells from patients with CVI or normal individuals. In assessing a number of parameters associated with their maturational stage and functional capabilities, we demonstrate that RA can selectively induce the differentiation of the CVI hybridomas. These findings have important implications for the use of retinoids to overcome maturational defects of B cells.

Materials and Methods

Cell Cultures. Human-human B cell hydridomas were established through fusion of normal or CVI PBL with WIL2/729 HF (17), a cloned hypoxanthine guanine phosphoribocyl transferase-negative ($HGPRT^-$) B lymphoblastoid cell line (obtained from Dr. R. Lundak, University of California at Riverside). The cloned hybrids are free of mycoplasma and have a modal chromosome number of 92 (tetraploid). Individual patient clones were selected for their ability to secrete maximum IgM (17). To maintain cell lines, cultures of $0.2\text{–}1.0 \times 10^6$ cells/ml were passaged twice weekly, resuspended 1:5 in fresh RPMI 1640 (M. A. Bioproducts, Walkersville, MD) supplemented with 10% FCS, 2 mM glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin (CRPMI).

Treatment of Cells with RA. All-*trans*-RA (Sigma Chemical Co., St. Louis, MO) was diluted in DMSO to a stock concentration of 2×10^{-2} M, aliquoted, and stored at −70°C. For each experiment, RA was diluted from the stock solution directly into the growth medium so that the final DMSO solvent concentration was <0.05% (vol/vol). This solvent concentration had no effect on any of the cell lines tested. Unless otherwise indicated, the starting cell concentration for assessing RA effects was 2×10^4/ml. Cells were counted both by microscopic inspection with a hemacytometer and by Coulter Counter (Coulter Electronics, Hialeah, FL). All handling of the RA was done under subdued light. [^{3}H]Thymidine incorporation was measured as previously described (8).

Measurement of IgM in Culture Supernatants. Cumulative IgM levels in the culture supernatants were measured by ELISA as reported (19). To estimate the secretion of IgM during 1 d, we calculated the difference between the levels for 2 successive days.

Surface Phenotype Analysis. Cell surface binding of fluorescence-conjugated mouse mAbs directed against B cell surface markers as listed in Table III *A* was accomplished by incubating 0.5×10^6 cells with saturating concentrations of antibody in 0.1 ml PBS/0.02% sodium azide for 30 min at 4°C in the dark. After washing the cells, flow cytometry was performed using an Epics C Flow Cytometer (Coulter Electronics) where at least 5,000 cells were collected and results were displayed as frequency distribution histograms of log fluorescence. Nonreacting antibodies were used as negative controls.

Biosynthetic Labeling Immunoprecipitation, and Autoradiography. 3×10^6 cells were washed two times in leucine-deficient CRPMI (Select-Amine, Gibco Laboratories, Grand Island, NY) and resuspended in 1 ml. To this was added an equal volume of pH-neutralized, leucine-deficient CRPMI containing 100 μCi/ml of 4, 5-[^{3}H]L-Leucine (ICN Pharmaceuticals, Inc., Cleveland, OH). After incubation for 3 h at 37°C, the cells were washed one time in a hypotonic salt solution (HSS), 0.01 M NaCl, 0.015 M $MgCl_2$, 0.01 Tris-HCl, pH 7.4, then they were pelleted and resuspended in cold HSS with 0.5% NP-40 (Sigma Chemical Co.). After 20 min at 4°C, the nuclei were pelleted and the lysate was cleared with an additional spin at 20,000 *g* for 30 min. For immunoprecipitation, the cleared lysates were incubated for 30

min with 5 μg/ml of mouse IgG monoclonal against human κ chain (Cappel Laboratories, Malvern, PA) or anti-μ chain purified from the ascites of BALB/c females injected with the hybridoma cell line HB57 (American Type Culture Collection, Rockville, MD). 0.5 ml of suspended Tachisorb (Calbiochem-Behring Corp., La Jolla, CA) was then added and the precipitates were collected as described (20). Precipitates were electrophoresed in SDS-10% PAGE gels (21) which were fixed, soaked in autofluor (National Diagnostics, Inc., Somerville, NJ), dried, and exposed to photographic film (Kodak X-Omat). Intensity of protein bands was quantitated by laser densitometry.

RNA Analyses. Cytoplasmic RNA was isolated from tissue culture cells using the NP-40 lysis method (22). RNA blotting was carried out essentially as described by Thomas (23) using the minifold II apparatus from Schleicher & Schuell, Inc., Keene, NH. Glyoxalated RNA samples (10 μg per slot) were affixed to nitrocellulose paper and hybridized with nick-translated probes specific for either κ, μ, or the 3'UT region of β actin. After removal of nonspecifically bound probe by high-stringency washes, the filters were exposed to x-ray film, and the resulting autoradiographs were analyzed by densitometry. The quantity of RNA immobilized for the different samples was standardized by comparison of β actin expression. The techniques used for the riboprobe S1 nuclease protection assay have been described in general outline by Quarless and Heinrich (24). Hind III-cleaved pGlus4DNA was used as a template for the in vitro T7 transcription reaction (see technical bulletin from Promega Biotech, Madison, WI). The composition of the reaction mixture was such that ~1/40 of the incorporated GTP residues were labeled with ^{32}P. The complementary RNA products of this reaction were electrophoresed over a 4% polyacrylamide, 7 M urea gel using a Tris-borate running buffer, and full-length probe transcripts (visualized by autoradiography) were eluted with 0.5 M NH_4OAc(pH 7.5), 10 mM $Mg(OAc)_2$, 0.1% SDS, and then were EtOH precipitated. The EtOH precipitate was washed once in 70% EtOH at room temperature and dried under vacuum. The dried probe pellet was then resuspended in hybridization buffer (see technical bulletin from Promega Biotech) to a concentration of 1,000 cpm/μl and stored at -20°C until being used. Cytoplasmic RNA samples (10 μg) were dried under vacuum at room temperature, then resuspended in 10 μl of the probe solution. The mixture was heat denatured for 15 min at 85°C, then quickly transferred to a 45°C hybridization bath for 18 h. After hybridization, 10 vol of S1 digestion buffer (30 mM NaOAc, pH 4.5, 100 mM NaCl, 2 mM ZnSO4, 2,500 U/ml S1 nuclease from Sigma Chemical Co.) were added, and the digestion was allowed to proceed for 1 h at 37°C. The S1 digestion was stopped by the addition of 3 vol of stopping solution (130 mM Tris HCl, pH 7.5, 17 mM EDTA, 200 mM NaCl, 1.3 SDS, 100 μg/ml proteinase K[BMB]), followed by a 30-min incubation at 37°C. The mixtures were then phenol extracted once, combined with 20 μg of carrier tRNA, and were EtOH precipitated at -20°C for 2 h. The resulting precipitate was washed once with 70% EtOH at room temperature, dried briefly, and resuspended in 80% formamide loading buffer and electrophoresed on a 4% polyacrylamide, 7 M urea gel using a Tris-Borate running buffer. After electrophoresis, the gel was soaked in 10% HAc, 20% MeOH for 20 min in order to leach excess urea, then it was dried and autoradiographed. Densitometric scanning and normalization to a unit probe length allowed for determination of the molar ratio of secreted to membrane form transcripts (μs/μm).

Plasmid DNAs. The 0.9 kb genomic Eco RI fragment encompassing Cu4 was ligated into the Eco RI site of the vector pGEM1 to create plasmid pGlus4. The orientation of the insert was chosen such that cleavage of pGlus4 with Hind III, and in vitro transcription from the plasmid-borne T7 promoter yields a complementary probe that can be used for the S1 analysis described above. Plasmid pGlus4 was also nick translated and used to probe RNA slot blots for μ. The plasmid pHuKgpt* (25) was nick translated and used as a κ-specific probe. Plasmid pHL 1216 (26) was nick translated and used as a source of probe for the 3' untranslated region of the human β-actin gene product.

Cytoplasmic Antibody Staining. Cells were washed three times in PBS with 0.02% sodium azide, cytocentrifuged onto glass microscope slides (1-2 × 10^5 cells/slide), then fixed in acetone for 10 min at room temperature. Staining was accomplished by incubating the slides with appropriate concentrations of mouse monoclonal (IgG_1) anti-human μ, anti-human light chain, or anti-KLH as a negative isotype control (Becton Dickinson & Co., Mountain

View, CA) and developing with a standard ABC kit (Vector Laboratories, Inc., Burlingame, CA) according to manufacturers' instructions.

Cell Cycle Analysis. Cells, grown in CRPMI in the absence or presence of RA for varying times, were washed and resuspended in a hypotonic sodium citrate buffer containing 20 μg/ml RNAse (Sigma Chemical Co.), 100 μg/ml propidium iodide (PI) (Calbiochem-Behring Corp.), and 3 μl/ml Triton-X (staining solution). The samples (10^6 cells/ml staining solution) were incubated for ½ to 1 h at 4°C in the dark and submitted to analysis immediately using an Epics C Cytometer (Coulter Electronics) equipped with an argon laser tuned to 488 nm. PI emission was collected with an appropriate filter combination by using a 610 LP filter in front of the red BMT. For each sample, at least 10,000 cells were collected and displayed as frequency distribution histograms of log red fluorescence. The data were stored on diskette and subsequently analyzed with the Paral program (parametric analysis program), a software package supplied by Coulter Electronics to compartmentalize the histograms into the components of the cell cycle.

Cultures of Fresh B Cells. Small resting B cells were isolated from the heparinized venous blood of CVI patients or normal donors by unit gravity sedimentation of lymphocytes depleted of SRBC-rosetting cells as described in detail (27). These B cells were cultured in CRPMI with various combinations of *Staphylococcus aureus* Cowan strain I (SAC), RA, and T cell-replacing factors (TRF, a crude 2-d supernatant of PWM-stimulated T cells [27]) at 5×10^5 cells/ml. After 7 d, the cumulative IgM in the culture supernatants were quantitated by ELISA.

Results

RA Enhances Ig Secretion from CVI Hybridomas. We previously derived the hybridomas used in this study by fusing an $HGPRT^-$ clone of the lymphoblastoid line, WIL2/729 HF, with in vitro-activated peripheral blood B cells from individuals with CVI (17). Hybridomas were also established with B cells from normal individuals and persons with selective IgA deficiency. These hybridoma clones (all IgM producers), were notable because they appeared to reflect the functional ability of the activated B cells of the donor fusion partner and because their phenotypes were different in comparing normals with CVI patients' hybridomas (17, 18). Clones derived from CVI B cells secreted lower levels of Ig than normals and the μs/μm mRNA ratio was 5–10 times lower than in those derived from normal B cells. In contrast, hybridomas derived from IgA-deficiency patients were similar to normals in their Ig secretion and μs/μm ratio (18). Thus, by the criteria of Ig secretion and μs/μm ratio, the CVI hybridomas presented a less differentiated phenotype than the normal clones or IgA deficiency clones. This occurred despite the deliberate selection of low IgM-secreting hybridomas from the pool of normal or IgA-deficiency hybridomas for comparison with the optimal IgM-secreting CVI hybridoma clones.

We evaluated the effects of RA on Ig production from hybridomas of four CVI patients, two IgA-deficiency patients, and one normal donor. 2×10^4 cells/ml were incubated in the absence and presence of 10^{-6} M RA for 5 d. On day 4 and 5 of culture, viable cell counts were obtained by trypan blue exclusion and cumulative IgM levels in the culture supernatant were assessed. IgM secretion on days 4–5 was then determined on a per cell basis by calculating the difference between the cumulative IgM levels on days 4 and 5 divided by the average number of cells over this time period. RA enhanced Ig secretion from four of four CVI hybridomas tested, with no significant enhancement from the normal or IgA-deficient hybridomas ($p < 0.001$) (Fig. 1). With all the CVI hybridomas, there was a 30–50% decrease in

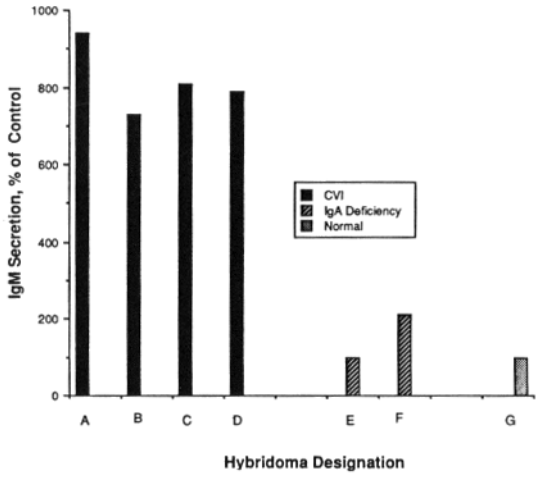

FIGURE 1. Increase in IgM secretion from RA-treated hybridomas. IgM secretion on a per cell basis on days 4–5 was calculated as the difference between the cumulative IgM levels on days 4 and 5 divided by the average number of cells over this time period. Results show the increased secretion from cells cultured in the presence of 10^{-6} M RA compared with that from cells cultured in the absence of RA (% control). Values of controls are as follows (in nanograms of IgM/ml/10^5 cells): *A*, 8.2; *B*, 3.8; *C*, 1.1; *D*, 0.8; *E*, 14.9; *F*, 4.9; *G*, 7.3.

the average number of cells counted compared with untreated cultures. Little or no difference in cell number was observed in the presence or absence of RA with the normal or IgA-deficient hybridomas. These results were confirmed by measuring [^{3}H]thymidine incorporation (data not shown). No differences were seen between the percent viability in control and RA-treated cultures.

Fig. 2 shows that the increase of IgM secretion by RA was dose dependent; significant enhancement was first detected at 10^{-9} M while the hybridoma responded equally well to RA concentrations between 10^{-5} M and 10^{-7} M RA. At 10^{-8} M RA, IgM secretion was still at least 80% of the maximal response. As seen in Fig. 3, augmented IgM secretion could be detected after 2 d of culture, with the greatest differential change occurring at day 3.

RA Induces De Novo Synthesis of IgM. Cells from the CVI hybridoma clone JK 32.1, grown in the absence or presence of RA for different time periods, were pulsed for 3 h with [^{3}H]leucine and the cells and supernatant were harvested. A portion of the cell lysates and supernatants were tested for incorporation of label into total protein while the remaining lysates and supernatants were immunoprecipitated, electrophoresed, and analyzed for in vitro μ and light chain synthesis by autoradiography. At 72 h, cells treated with 10^{-6} M RA demonstrated a 12-fold increase in μ chain synthesis and a 3-fold increase in κ chain production (Fig. 4.) Total protein

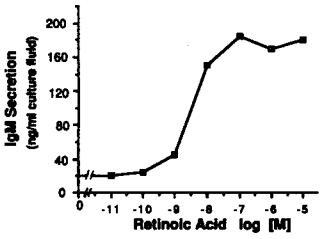

FIGURE 2. Dose-response of RA effects on IgM secretion from JK32.1 CVI hybridoma cells. IgM secretion into the supernatant was assessed on days 4–5 from cultures treated with the indicated concentrations of RA.

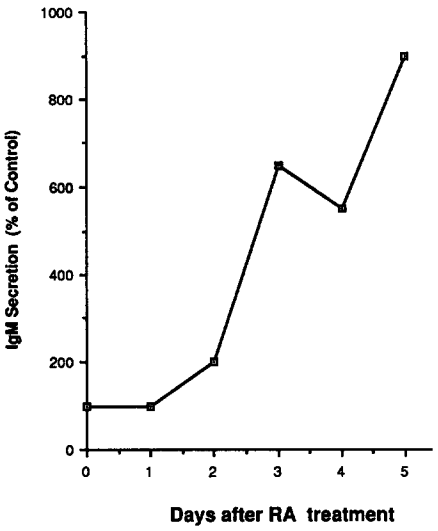

FIGURE 3. Time course of RA enhancement of IgM secretion from JK32.1 cells. IgM secretion on a per cell basis on each day was calculated as the increase in cumulative IgM level from the preceding day divided by the average number of cells over the same time period. Results show the increased secretion for that 24-h period from cells cultured in the presence of 10^{-6} M RA compared with that from cells cultured in the absence of RA (% control).

synthesis usually remained unchanged and never increased more than twofold in the presence of RA. We also examined the kinetics of μ chain production. Culturing in RA for as short as 8 h often produced detectable increases in μ chain synthesis. By 24 h, RA-treated cells showed a five fold increase in synthesis of μ chain (Table I), in contrast to the more delayed response (2 d) seen with actual IgM secretion.

To determine if these same events were reflected in the cells' content of μ chains, JK 32.1 cells were cultured for 72 h in the absence or presence of 10^{-6} M RA and then stained for cytoplasmic μ. In two experiments, ~50% of both the treated and untreated cells stained positively for cytoplasmic μ while the treated cells showed a greater than sevenfold increase in secreted IgM. Correspondingly, we observed a marked qualitative increase in the intensity of cytoplasmic μ staining in the treated cells, suggesting that the RA had induced a marked increase in the IgM production from lower level producing cells rather than inducing cells from a non-IgM to an IgM-producing state in this 72-h time period (Fig. 5). However, longer treatment periods with RA (>5 d) often showed an increase in the percentage of positively staining cells (data not shown).

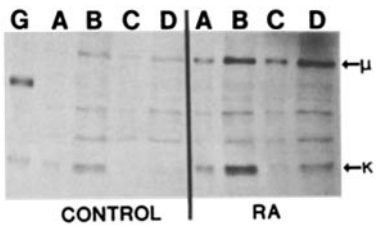

FIGURE 4. Increase in μ and κ chain synthesis in RA-treated JK32.1 cells. Biosynthetically labeled lysates from 3×10^6 cells that had been treated for 72 h with RA (10^{-6} M) or solvent control were immunoprecipitated with anti-μ (*C* and *D*) or anti-κ (*A* and *B*) and run on SDS-10% polyacrylamide gel at relative dilutions of 1:1 (*B* and *D*) or 1:4 (*A* and *C*) as detailed in Materials and Methods. ^{125}I-labeled human IgG (lane *G*) was used for orientation of the κ chain band. μ and κ chain levels were quantitated by densitometry from protein bands obtained by immunoprecipitation with anti-μ and anti-κ antibody, respectively, and showed a 12-fold increase in μ chain and a 3-fold increase in κ chain induced by RA. Background bands showed a two fold increase, indicating an enhancement by RA of total protein synthesis as well as specific increases in μ and κ chain production.

TABLE I

Kinetics of RA Enhancement of μ and κ Chain Synthesis in JK32.1 Cells

Treatment period	Increased synthesis of:*	
	κ chain	μ chain
h	%	
8	ND	1.8
24	2.3	4.2
72	3.0	5.0

Cells were cultured for the indicated time period in 10^{-6} M RA or solvent control, then pulse-labeled with [^{3}H]leucine for determining μ and κ chain levels as described in Materials and Methods.

* Values represent percent increase in the area of μ and κ chain protein bands (as determined by densitometric scanning of autoradiograms) from RA-treated cells relative to control cultures evaluated at the same time. Total TCA precipitable counts did not vary by >25% between RA-treated and control samples at any of the time points.

Cell Cycle Analysis. In Ig-secreting cells, >60% of Ig production occurs late in G_1 (28). In a variety of cell types, RA causes a shift in the distribution of cells within the cycle, significantly enhancing the percentage of cells in G_1. To assess whether a shift of CVI hybridoma cells into G_1 could account for the enhanced Ig production, supernatants from 3–5 d RA-treated and untreated JK 32.1 cells were assayed for levels of IgM, while cell nuclei from the same cultures were analyzed for DNA content by PI staining. The results shown in Fig. 6 from day 3 are representative. The proportion of cells in G_1 changed from 36 to 43% with RA treatment, while the concentration of IgM in the supernatant increased from 60 to 185 ng/ml. Thus, although small changes in cell cycle distribution were consistently observed in RA-treated cultures (up to 15% increase in the number of cells in G_1), this fact could not account for the marked enhancement in Ig secretion caused by RA.

RA Increases Total Ig mRNA and Shifts μ mRNA Processing to Production of the Secreted Form. Normal B cells stimulated to Ig secretion accumulate increased levels of heavy and light chain mRNA, and undergo a dramatic shift in the heavy chain RNA processing pathway, leading to the predominant production of secreted μ mRNA (29). We analyzed changes in the steady-state levels of μ and κ mRNA, and the ratio of the two forms of the heavy chain transcript (μs/μm) in cytoplasmic mRNA isolated from control and RA-treated JK 32.1 cells. RNA slot blot hybridization (23) was used to quantitate the steady-state levels of heavy and light chain mRNAs in cells treated with increasing concentrations of RA for 3 d. The results in Table II indicate that even very low concentrations of RA (10^{-8} M) enhanced the level of μ mRNA with minimal effects on the level of κ mRNA. The highest concentration of RA (10^{-5} M) led to increased levels of κ mRNA (4-fold), and an even more marked increase in the level of μ mRNA (15-fold). Thus, RA treatment of the CVI hybridoma clearly influenced the steady-state levels of both heavy and light chain mRNAs.

To address more specifically the nature of the species contributing to the pool of heavy chain mRNA, we used a nuclease S1 protection assay to measure the relative amounts of μs and μm mRNA. The scheme used to distinguish the two species of heavy chain transcripts and the autoradiographic results from the S1 analysis of RNAs derived from control and RA-treated CVI hybridoma cells are shown in Fig. 7. The

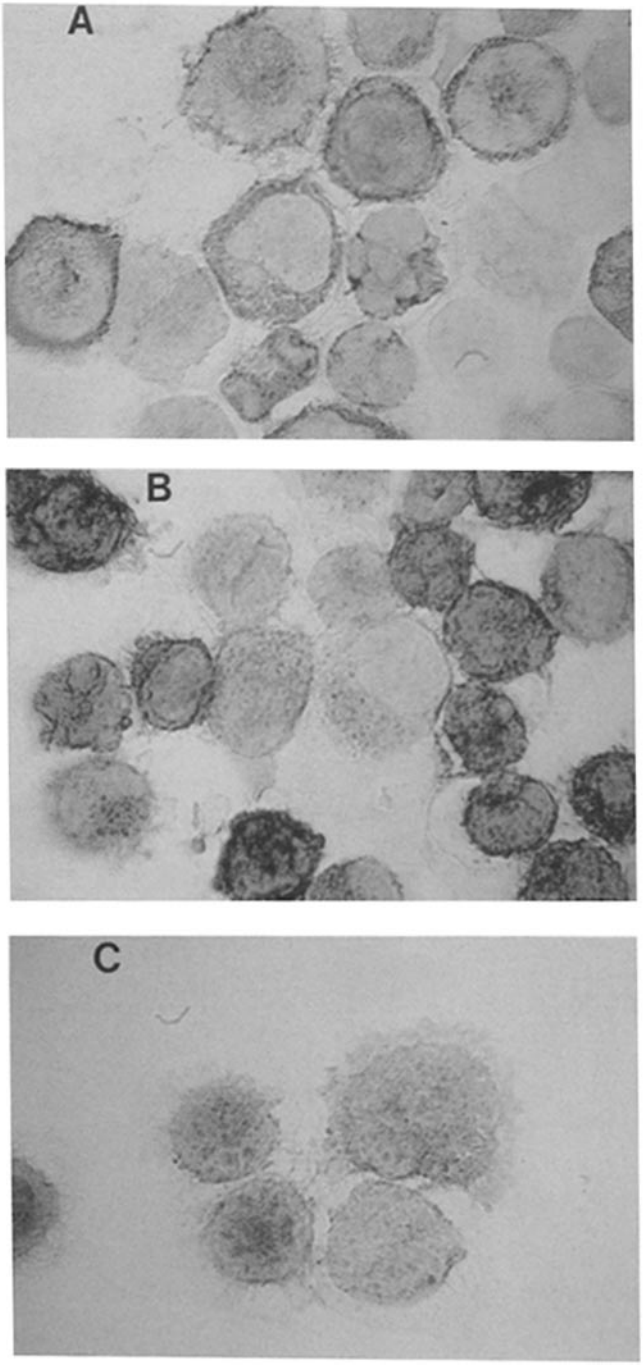

FIGURE 5. Immunoperoxidase staining for cytoplasmic μ in JK32.1 cells. Cells were cultured for 72 h in the absence (*A*) or presence of 10^{-6} M RA (*B*). Fixed cells were then incubated with a mouse monoclonal (IgG_1) anti-human μ heavy chain specific antibody and developed with a commercial ABC kit according to manufacturer's instructions. A nonreacting antibody (mouse anti-KLH) was used as a negative isotype control (*C*).

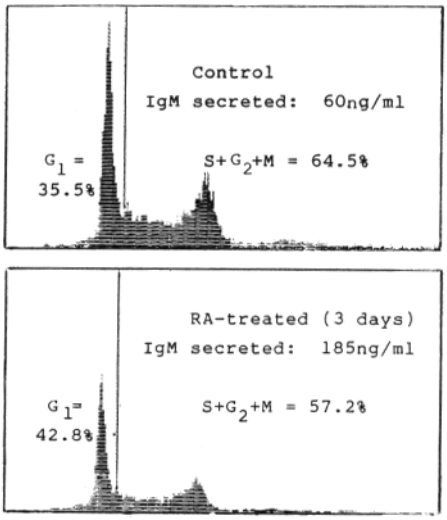

FIGURE 6. Cell-cycle profiles for JK32.1 hybridoma cells. Cells were treated for 3 d in 10^{-6} M RA or solvent control, stained with 0.1 mg/ml propidium iodide in 0.1% citrate + 0.02 mg/ml RNase on ice, and analyzed by flow cytometry. Cumulative IgM secreted into the supernatant of the 3-d cultures was also assessed.

data indicate that cells grown in media alone, or in the presence of DMSO alone demonstrate a slight preference for the production and accumulation of the μs mRNA (approximately two transcripts for every one μm transcript). Treatment of the hybridoma for 3 d with 10 nM RA shifted the μs/μm ratio to ~8:1. Increasing the RA concentration to 10 μM gave a μs/μm ratio of 11:1. Significantly, this same RA concentration range showed enhancement of IgM secretion in the culture supernatants (see Fig. 2). Hence, in vitro RA treatment of the CVI hybridoma leads to a marked alteration in the RNA processing pathway for μ heavy chain mRNA.

Analysis of Surface Phenotype Changes in RA-treated Cells. As described above, the

TABLE II

Effects of RA on Steady-State Levels of μ and κ Chain mRNA in JK 32.1 Hybridoma Cells

Treatment	Increased expression of: κ chain	μ chain
	%	
Control	1.0	1.0
DMSO	0.7	1.3
10^{-8} M RA	1.5	4.9
10^{-7} M RA	1.7	4.6
10^{-6} M RA	2.0	6.1
10^{-5} M RA	3.9	15.4

* Glyoxalated RNA samples (10 μg per slot) were affixed to nitrocellulose paper and hybridized with nick-translated probes specific for either κ, μ, or the 3'UT region of β actin using standard protocols (Materials and Methods). After removal of nonspecifically bound probe by high-stringency washes, the filters were exposed to x-ray film, and the resulting autoradiographs were analyzed by densitometry. The quantity of RNA immobilized for the different samples was standardized by comparison of β actin expression. Cells were cultured for 3 d.

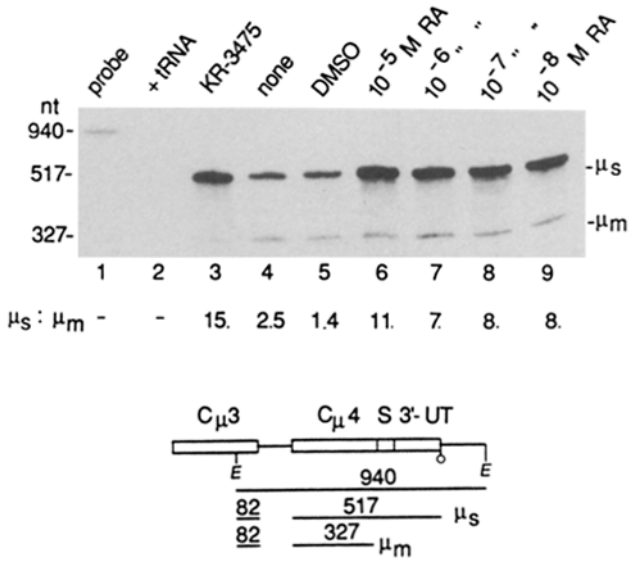

FIGURE 7. Quantitation of μs/μm ratios in RA-treated JK32.1 cells using the riboprobe S1 assay. Autoradiographic results from the riboprobe S1 assay are shown in the top portion of the figure. The full-length probe used for all subsequent hybridizations is shown in lane *1*. Lanes *2* and *3* are negative and positive controls, respectively. The cell line KR-3475 produces both forms of the heavy chain transcript, with a strong predominance of the secreted-form mRNA. Cytoplasmic RNA from untreated (lane *4*) and DMSO carrier-treated (lane *5*) JK32.1 cells was used to establish baseline ratios of the two mRNA species. Lanes *6–9* show the effect of a 3-d treatment of JK32.1 with various concentrations of RA. Quantitation of the μs/μm ratio was carried out using densitometry and normalized to unit probe length. The bottom part of the figure shows the strategy for detecting transcripts corresponding to mRNAs for the secreted and membrane forms of the μ heavy chain. The regions of the probes that are protected from S1 digestion after mRNA hybridization are pictured.

TABLE III

A. B Cell Distribution of Surface Markers

Surface marker	Distribution on B cells
IL-2-R (CD25)	Activated B cells
Leu-8	Resting B cells
Leu-17 (CD38)	Pre-B and immature B cell, reexpressed on plasma cells
B2 (CD21)	Resting B cells
PCA-1	Plasma cell blast

*B. Surface Phenotype of B Cell Hybridomas**

Hybridoma (fusion partner)	Surface marker (percent positive)				
	IL-2-R	Leu-8	Leu-17	B-2	PCA-1
WIL2/729 HF (parent line)	<5	57	12	<5	<5
729-PBL (normal)	16	8	14	<5	32
IE 25 (IgA deficiency)	15	13	51	28	20
IE 8.6 (IgA deficiency)	20	8	2	47	15
JK 32.1 (CVI)	<5	72	11	80	<5
RB 18 (CVI)	<5	71	21	29	<5
JP 6.12 (CVI)	<5	65	<5	62	ND

* Cells were removed from maintenance cultures (2–8 × 10^5 cell/ml in CRPMI), stained as described in Materials and Methods, and immediately analyzed in an EPICS C flow cytometer. Results are means of at least three separate tests. Percentages were standardized by setting cursor to yield 2% positives for the isotype control.

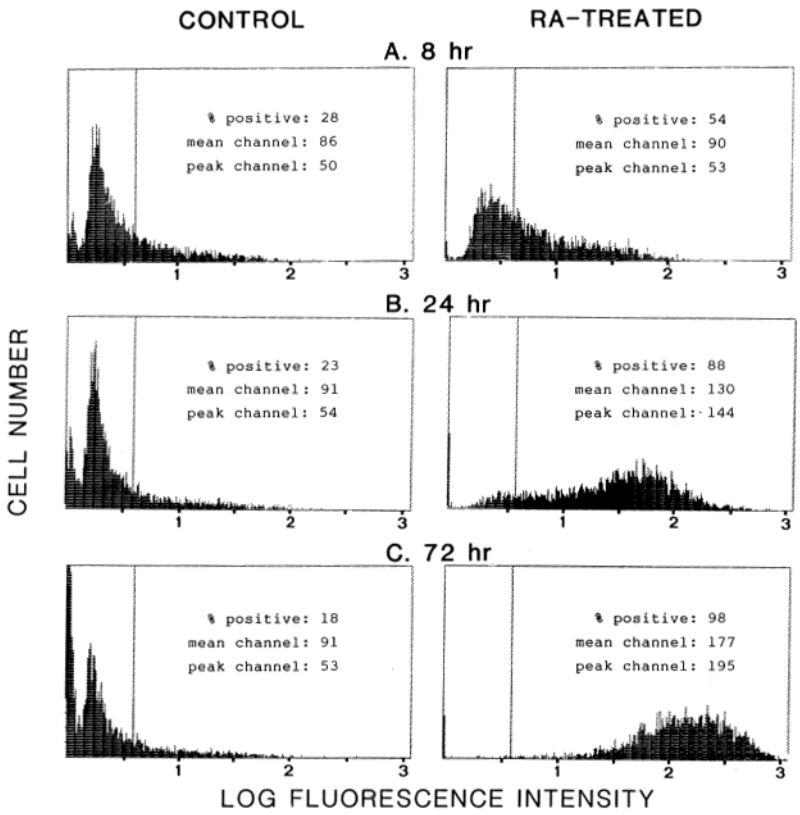

FIGURE 8. Time course for RA induction of Leu-17 (CD38) on JK32.1 cells. Cells were cultured in 10^{-6} M RA or solvent control and analyzed for expression of Leu-17 after 8 h (*A*), 24 h (*B*), and 72 h (*C*). The mean channel number of Leu-17$^+$ cells increased two fold after 72 h, representing a 10-fold increase in mean fluorescence intensity, while the percent of cells scoring positive increased from ~20% to 98%.

CVI hybridomas appear to maintain an immature character comparable to that of the peripheral blood B cells of the CVI patients; the cells secreted low levels of IgM, and the μs/μm ratio was ~2:1, characteristic of a resting B cell. The CVI and normal hybridomas were screened with five mAbs that define steps in B cell development (Table III). Notably, there are instances in which the phenotype of the hybridomas varies significantly from that of the WIL2/729 HF parent line, suggesting that the surface phenotype of the hybridomas reflects, in part, the B cell fusion partner. Furthermore, there are differences between the CVI hybridomas and those obtained using cells from healthy individuals or persons with selective IgA deficiency. These differences are most striking for the display of IL-2-R, Leu-8, and PCA-1 (Table III). Overall, the CVI hybridomas display a less mature B cell phenotype relative to their normal counterparts.

Since RA enhanced Ig production and shifted the μs/μm ratio, we examined the CVI hybridomas for changes in surface molecule display in response to RA treatment. Expression of Leu-17, a marker that appears late in B cell development on pre-plasma cells, increased both in percentage and intensity on RA-treated cells (Fig.

TABLE IV

RA Enhancement of Leu-17 Expression on B Cell Hybridomas

Cell line*	Control cultures			RA-treated cultures		
	Percent positive	Mean channel	Peak channel	Percent positive	Mean channel	Peak channel
WIL2/729 HF	18	71	45	22	75	42
729-PBL	14	79	45	89	136	154
IE 25.0	52	95	42	96	192	227
RB 18.C7	22	78	44	93	122	146
FG 7.0	43	84	48	97	134	135
FG 3.1	15	69	41	48	85	45
JL 6.4	31	96	45	92	116	122
JK 32.1	18	91	53	98	177	195
LB B9	45	88	44	91	126	129

Cells were cultured for 3 d in the absence or presence of 10^{-6} M RA. Initial cell density was 5×10^4 cell/ml. Staining and analyses were performed as described in Materials and Methods.

* WIL2/729 HF is the parental fusion partner for producing the hybridomas. 729-PBL is derived from a normal donor while IE 25.0 is a hybridoma from an IgA-deficiency patient. The other hybridoma lines are all derived from patients with CVI.

8). Unlike the changes in Ig production which appeared restricted to the CVI hybridomas, Leu-17 induction by RA occurred on all hybridoma lines tested, although not on the parent line WIL2/729 HF (Table IV). Changes in Leu-17 expression were rapid; significant differences were detectable by 8 h, but the maximal stimulation was present only after 72 h (Fig. 8). There was no consistent change induced by RA in the other surface markers shown in Table III.

Reversibility of RA-induced Changes. To test for the reversibility of the RA-induced effects on CVI hybridomas, we assessed Leu-17 expression and IgM production after the exposure of JK 32.1 cells to RA and subsequent withdrawal of the drug. Control or RA-treated (10^{-6} M) cells were initially cultured for 72 h, washed three times, then recultured with or without RA at a concentration of 2×10^5 cell/ml. Every 3–4 d, supernatants were assessed for IgM production, cells were stained for Leu-17

TABLE V

Reversibility of RA-induced Changes in JK 32.1 Cells

Days in culture	Leu-17 (percent positive)			IgM*		
	Untreated	RA1‡	RA2§	Untreated	RA1‡	RA2§
					ng/ml	
0	19	18	20	NT‖	NT	NT
3	16	97	97	24	280	280
7	18	98	97	26	263	282
14	31	58	93	26	144	571
17	24	39	98	9	16	457

* Cumulative concentration in culture supernatant from 3 d before to the day indicated.

‡ Cells were cultured in 10^{-6} M RA for 3 d, washed, then recultured without RA.

§ Cells were cultured continuously in the presence of 10^{-6} M RA.

‖ Not tested.

TABLE VI

Induction of IgM Synthesis from Fresh B Cells of Patients with CVI

Stimulus	IgM produced				
	Patient 115	Patient 113	Patient 125	Patient 111	Normal
			ng/ml		
None	0	0	0	0	0
SAC	9	0	NT*	NT	60
SAC + RA	41	12	NT	NT	30
TRF	0	2	0	0	100
SAC + TRF	122	8	26	18	110
SAC + TRF + RA	380	49	22	8	110

Small resting B cells from heparinized venous blood were isolated by unit gravity sedimentation (27). The cells were then stimulated for 7 d as indicated and the IgM in the culture supernatants was quantitated by isotype-specific ELISA. RA was used in 10^{-6} M, SAC was used at 0.001%, and TRF at 10% vol/vol. Results with the normal shown are representative of that obtained with cells from a total of five normal donors.

* Not tested.

expression, and the cells were recultured at the original concentration. As seen in Table V, after the initial 3-d treatment with RA, there was an 11-fold increase in supernatant IgM concentration over concurrent controls and an increase in Leu-17 expression from <20 to 98%. By 17 d, those cells that had been cultured constantly with RA had a 50-fold increase in their 14–17-d supernatant concentration of IgM over controls. In those cultures RA-treated for the initial 3 d only and then recultured without RA, IgM secretion and Leu-17 expression remained elevated for >2 wk but eventually returned to prestimulated levels after 17–21 d.

Effects of RA on IgM Secretion from Fresh B Cells from CVI Patients. In light of the demonstrated effects of RA on CVI hybridoma B cells, we have undertaken initial experiments to determine the effects of RA on fresh B cells obtained from CVI patients. In previous studies we have shown that these lymphocytes cannot be activated by TRF to produce antibody, but that Ig secretion can often be induced by combining TRF with SAC (30). As seen in Table VI, RA treatment of B cells from two of four CVI patients enhanced IgM secretion three to sixfold in response to TRF/SAC. In contrast, B cells from normal donors did not show similar effects induced by RA. This enhancing effect by RA with the fresh B lymphocytes from the two patients (Nos. 113 and 115) are analogous to the RA responsiveness of the CVI hybridomas constructed with cells from these same patients (JK 32.1 and JL 6.4, respectively). We do not have hybridoma lines derived from lymphocytes of patients 111 and 125. Although presently limited in scope, these initial experiments suggest that information gained on the ability of RA to modulate the maturation of the CVI hybridomas will be relevant also towards understanding the biology of this compound on fresh CVI B cells.

Discussion

This report demonstrates that RA induces the differentiation of human–human B cell hybridomas from individuals with CVI. These CVI hybridomas reflect the B cell defect of the fusion partner as indicated by low levels of IgM secretion, imma-

ture surface markers, and RNA processing resulting in preferential production of μm vs. μs mRNA. The result of RA treatment was a marked enhancement in the secretion of IgM per cell. This enhancement was not coupled to RA-induced alteration in the cell cycle and was seen with all of the CVI hybridomas but not with hybridomas from cells of normal individuals or those with selective IgA deficiency. In addition to enhancing Ig secretion, RA increased the *de novo* synthesis of the light and heavy Ig peptides (up to 4- and 15-fold, respectively). RA did not appear to affect the rate of secretion relative to synthesis since the percent of labeled Ig that was found in the supernatant versus the cell lysate was not altered (unpublished data).

Our results suggest that RA treatment of a cell bearing the CVI phenotype affects two aspects of Ig gene expression. First, the steady-state levels of the heavy and light chain mRNAs are increased. This increase is unlikely to result from an increased transcription rate since this parameter appears to be relatively invarient among cells representing very early and very late B lymphoid stages (31). Hence, RA may exert its effect on a little understood post-transcriptional mechanism which leads to an accumulation of Ig mRNA in RA-treated cells. Secondly, the processing of the heavy chain transcript to the secreted form is favored over the membrane form after RA treatment. This shift has been observed in the murine line 70Z/3 (29) and in freshly isolated murine B cells after stimulation by anti-μ and TRF (32). Our results demonstrate the first observed shift of this nature in human cells. Although the mechanism used by the cell to discriminate between the two RNA processing pathways is not understood at this time, the results confirm that RA affects a marked alteration in the differentiated state of the CVI hybridoma clones.

The differentiation effects of RA on the CVI hybridomas are further supported by our finding that RA substantially increased the surface expression of Leu-17 (CD38), a marker that reappears late in B cell development on pre-plasma cells (33). Similar to *de novo* Ig protein syntheses, increased Leu-17 expression was observable after only 8 h culture with RA. Both Leu-17 expression and IgM secretion remained elevated for long periods (>2 wk) after removal of RA. This finding is consistent with the long-term effects of RA in other cell systems where differentiation is induced (2–4), as opposed to the rapidly reversible growth inhibition (<48 h) that can be caused by RA in the absence of differentiation (1, 5). The mechanism of the slow reappearance of the pre-RA treatment phenotype of the CVI hybridomas is not known. It is possible that all the cells gradually return to the less differentiated state or, alternatively, that a small subset of RA-nonresponsive cells eventually regrow in the cultures. This latter possibility appears unlikely since after removing RA from the culture, both treated and untreated cells had comparable rates of DNA synthesis (unpublished observation), suggesting no obvious growth advantage for less differentiated cells. Studies using cloned populations of RA-responsive and nonresponsive cells will directly address this question.

An important issue when evaluating the data herein is whether the RA-responsive phenotype of the CVI hybridomas is derived from the CVI B cells or from the parent line. The evidence we have accumulated suggests that the changes reflect alterations in the development program of the CVI lymphocytes: (*a*) RA had no effect on parental WIL2/729 HF cells; (*b*) enhancement of IgM secretion was seen only with the CVI hybridomas and not with those constructed with cells from normal or IgA deficiency patients; (*c*) we observed similar RA enhancement of Ig secretion from

fresh peripheral B cells of two of four CVI patients but not from those of normal donors. In contrast, RA markedly enhanced Ig secretion from normal tonsil B lymphocytes (9, and unpublished data), cells that are known to show substantial differences in their maturational profile from their peripheral counterparts (34). Thus, the ability of B lymphocytes to be affected by RA might be dependent on the presence of cells in a certain stage of development that are overrepresented in the peripheral blood of CVI patients due to the apparent maturational block associated with the disease. If such is the case, it is possible that RA or other retinoids may play a role in alleviating this syndrome.

Summary

Human-human B cell hybridomas constructed from B lymphocytes of common variable immunodeficiency (CVI) patients and the nonsecreting cell line WIL2/729 HF consistently secrete low levels of Ig and appear to retain a defect characteristic of the CVI patient's B cells. We assessed the differentiative capacity of retinoic acid (RA) on these hybridomas, as well as on hybridomas constructed from normal B cells and from patients with selective IgA deficiency. RA at concentrations varying between 10^{-5} and 10^{-9} M augmented IgM secretion 4–20-fold from four of four CVI hybridomas tested, but did not affect Ig secretion from normal or IgA-deficiency hybridomas. In support of this elevated Ig secretion, RA enhanced the *de novo* synthesis of biosynthetically labeled light (κ) and heavy (μ) Ig (up to 4- and 15-fold, respectively) in the CVI hybridoma line JK32.1. The increase in IgM synthesis/secretion could not be accounted for by RA-induced alteration in the cell cycle. In inducing this increase in IgM production, RA was found to affect two aspects of Ig gene expression: (*a*) the steady-state levels of heavy and light chain mRNAs were enhanced, and (*b*) the processing of μ heavy chain transcripts to the secreted mRNA form became favored over the membrane mRNA form. We also show that expression of Leu-17 (CD38), a surface marker that is re-expressed in the late pre-plasma stage of B cell development, was increased by RA from <20% to >90% of the total cell population, with a concomitant 4–10-fold augmentation in the mean fluorescence intensity. Changes in both Leu-17 expression and *de novo* Ig synthesis were prominent by 24 h, but could be observed as early as 8 h after induction. Taken together, our study demonstrates that RA affects a marked alteration in the differentiated state of the CVI hybridoma clones. This finding suggests that retinoids can enhance the functional capabilities of B cells with defects in maturation and support further studies to evaluate their clinical potential in CVI.

We wish to thank Dr. P. Leder for the human κ clone pHuKgpt*, and Dr. W. Salser for the human β-actin clone pHL1216. We would also like to thank Aimee Nguyen and Alexis Brown for their technical assistance, Ingrid Schmid for performing the cell-cycle analysis, and Kathy Petersilie for the flow cytometric studies.

Received for publication 7 December 1987 and in revised form 1 March 1988.

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