

13-Cis retinoic acid enhances in vivo B-lymphocyte differentiation in patients with common variable immunodeficiency

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Retinoic acid (RA) has been demonstrated to drive both phenotypic and functional in vitro differentiation of B cell hybridomas from patients with common variable immunodeficiency (CVI) who manifest an "intrinsic" defect in terminal B cell differentiation (J Exp Med 1988;168:55-71). Therefore, we conducted an open trial to determine the effects of oral 13-cis RA (0.5 mg/kg/day: 12 weeks receiving and 12 weeks without drug) on in vivo B cell differentiation in subjects with CVI. At various times before, during, and after drug administration, patients' B cells were tested for changes in cell-surface phenotype and in vitro immunoglobulin production in response to recombinant cytokines. Before 13-cis RA, all patients had decreased Leu-8 coexpression on CD20⁺ cells. Seven of eight subjects demonstrated "normalization" of this phenotype after 8 to 16 weeks of 13-cis RA administration. Patients whose B cells demonstrated more than normal CD20 display also had a fall toward normal in this parameter. These effects persisted for 6 to 12 weeks after drug was stopped. It appears that 13-cis RA drives B cells of patients with CVI to express a more differentiated cell-surface phenotype and may promote functional differentiation in some patients. (J ALLERGY CLIN IMMUNOL 1991;88:705-12.)

Key words: 13-Cis retinoic acid, common variable immunodeficiency, B-lymphocyte differentiation, Leu-8

CVI is the most common serious disorder of humoral immunity in humans. It is characterized by hypogammaglobulinemia that results in recurrent bacterial infections. Even with optimal care, including

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Abbreviations used

CVI:	Common variable immunodeficiency
Ag:	Antigen
Ab:	Antibody
DT:	Diphtheria toxoid
IVIg:	Intravenous immunoglobulin
RA:	Retinoic acid
TT:	Tetanus toxoid
UCLA:	University of California-Los Angeles
PBMC:	Peripheral blood mononuclear cell
IL:	Interleukin
rIL:	Recombinant interleukin
PWM:	Pokeweed mitogen

high-dose IVIg replacement, these patients may suffer from increased bacterial infections because immunoglobulin infusion essentially replaces only IgG. A wide variety of immunologic abnormalities have been associated with the low immunoglobulin levels observed in patients with this syndrome, including defective B cell differentiation, decreased helper T cell function, excessive suppressor T cell activity, or ab-

normal Ag processing and presentation by macrophages.¹⁻³ We and other investigators have demonstrated that most patients' B cells characteristically demonstrate a functional inability to differentiate to high-rate immunoglobulin secretion despite normal responses to activation and proliferation signals.⁴

Retinoids may play an important role in immune function, as evidenced by epidemiologic studies that have demonstrated that there is an increase in the severity and frequency of bacterial infections in patients with vitamin A deficiency,⁵ and animal data that suggest that retinoids may provide a protective effect against infections with *Listeria*, *Pseudomonas*, and *Candida*.^{6,7} Sidell et al.⁸ have demonstrated that RA enhances human humoral responses by inducing Ab-producing cells from human tonsillar lymphocytes sensitized to sheep erythrocytes.

We have previously studied the in vitro effects of RA on B-lymphocyte differentiation with human B cell hybridomas constructed from B-lymphocytes from individuals with CVI and from normal individuals.⁹ We demonstrated that RA induced a tenfold to 15-fold increase in IgM production by cell hybridomas from patients with CVI B without increasing total protein synthesis and promoted the expression of a more differentiated cell-surface phenotype. Importantly, RA increased the steady-state levels of heavy and light chain mRNA, and processing of μ heavy chain mRNA transcripts was shifted to the secreted form. Such changes were not observed with normal B hybridoma cells. These findings demonstrated that RA specifically enhanced CVI B cell differentiation.⁹

In this article, we describe the results of an initial trial in which the effects of 13-cis RA administered in vivo to patients with CVI were examined. The objective was to determine whether the in vivo administration of retinoids to patients with CVI resulted in increased B cell maturation. Maturation was assessed by measuring (1) immunoglobulin levels in vivo, (2) changes in the abnormal B cell phenotype observed in CVI, (3) the ability of small resting B cells to respond to in vitro signals of differentiation, and (4) in vivo Ab responses to immunization with TT/DT.

MATERIAL AND METHODS

Patients and study design

Ten patients with CVI, seven men and three women, who were regularly followed at the UCLA Center for Health Sciences were enrolled. Nine of the 10 patients were receiving IVIg (200 to 400 mg/kg/mo), and one patient (No. 132) was receiving twice monthly intramuscular immunoglobulin. All patients were free of any serious infections at

the time of enrollment. One patient (No. 128) continued to smoke approximately 10 cigarettes daily throughout the study. All female participants were either past the age of fecundity, surgically sterilized, or were using an approved and effective means of contraception. Informed consent was obtained from all subjects, and the study was approved by the UCLA Institutional Review Board.

The trial was an opened, self-controlled, crossover trial. Patients were followed for 16 weeks with oral 13-cis RA, 0.5 mg/kg/day, administered in divided doses twice daily, and for 16 weeks after the drug was stopped. Complete blood count, and/or routine blood chemistries (serum electrolytes, blood urea nitrogen, creatinine, albumin, phosphorus, liver function chemistries, cholesterol, and triglycerides) were obtained on weeks 0, 2, 4, 8, 12, 16, 20, 24, 28, and 32 to monitor for potential drug-associated toxicity. Quantitative IgG, IgM, IgA, and IgE levels in serum and phenotypic expression of developmentally regulated cell-surface markers were determined on weeks 0, 4, 8, 12, 16, 20, 24, 28, and 32. Functional responses of small or large B-lymphocytes to recombinant cytokines ex vivo were determined on weeks 0, 16, and 32. Specific IgM and IgG responses to TT/DT inoculation administered week 12 were determined on weeks 12 (baseline), 13 (IgM response), and 16 (IgG response).

Preparation of mononuclear cell fractions

PBMCs were obtained by whole-blood separation on Sepacell-MN (Sepratech Corp., Oklahoma City, Okla.) and washed three times in Eagle's minimum essential medium, Whittaker Bioproducts, Inc., Walkersville, Md., containing 1% fetal calf serum (Hyclone, Sterile Systems, Inc., Logan, Utah). B-enriched cells were obtained by removing T cells from PBMCs with the aminoethylisothiuronium bromide-treated sheep red blood cell rosette-depletion technique with a 60% Percoll (lot No. NF04276, Pharmacia AB, Uppsala, Sweden) density-gradient modification of the procedure as previously described.¹⁰ Purified B cells were derived by monocyte depletion of B-enriched cells to Falcon 1029 Petri dishes (Becton Dickinson Labware, Oxnard, Calif.) in the presence of serum. B-lymphocytes were additionally fractionated by density centrifugation at 1280 g for 15 minutes on a discontinuous 30%, 50%, 70% Percoll gradient. Large, low-density B-lymphocytes (activated in vivo) were harvested from the 30% to 50% interface, and small, high-density (unactivated, resting) B-lymphocytes were harvested from the 50% to 70% interface.

Analysis of cell-surface phenotype

Lymphocyte subsets were quantified with two-color flow cytometry, according to the method of Giorgi et al.¹¹ In this study, a phycoerythrin-conjugated monoclonal antibody directed against CD20 (Leu-16, Becton Dickinson Immunocytometry Systems, Mountain View, Calif.) was used as a pan-B cell marker. Subsets of B cells were identified with the following fluorescein isothiocyanate-labeled mono-

clonal antibodies: anti-CD10, Leu-8, anti-CD19, anti-CD25, antiTransferrin receptor, anti-CD5, anti-CD38 (all from Becton Dickinson Immunocytometry Systems), and purified (unlabeled) anti- μ and anti- δ (human heavy immunoglobulin chains, Coulter Immunology, Hialeah, Fla.). Fluorescein isothiocyanate-conjugated reagents were incubated simultaneously with the phycoerythrin-conjugated CD20, whereas for those B cell subsets identified by unlabeled purified Ab, a multistep staining procedure was used, and analysis of cell-surface phenotype was performed on an EPICS C (Division of Coulter Electronics, Hialeah, Fla.) flow cytometer that was calibrated daily to standardize fluorescence intensity, as previously described.⁴

Assessment of response to TT/DT immunization

The ability of the patients to generate B cells spontaneously producing IgG or IgM Abs (lymphoblastoid B cells) following in vivo immunization with TT and DT was assessed.^{12,13} Such responses are absent in patients with CVI.¹⁴ Each patient was administered a TT/DT immunization after 12 weeks of 13-cis RA. PBMCs were obtained before, 1, and 3 weeks after immunization and cultured at 37° C in a 5% CO₂ humidified incubator in duplicate 12 by 75 mm polystyrene sterile culture tubes (Costar Corp., Cambridge, Mass.) for 7 days in either complete RPMI with 1% PWM or in serum-free medium (HL-1, Ventrex Laboratories, Inc., Portland, Maine) in the presence of 0.5 U of rIL-6. Specific IgM or IgG anti-TT and anti-DT Abs were detected by ELISA with a modification of the technique described by Saxon et al.¹⁵ in which the second Ab is an alkaline phosphatase-conjugated antihuman IgG or IgM.

Assessment of in vitro IgG, IgM, and IgA production

Unfractionated, low- and high-density B-lymphocyte populations (5×10^4 cells per well in triplicate round-bottom 200 μ l microtiter wells, Nunclon Delta, InterMed Nunc, Roskilde, Denmark) were cultured in complete RPMI containing 10% fetal calf serum (lot No. 5121103, Irvine Scientific, Santa Ana, Calif.), 50 U/ml of penicillin, 50 μ g/ml of streptomycin, 2 mmol/L of L-glutamine, and either cycloheximide, 5% vol/vol (lot No. 48C-0090, Sigma Chemical Co., St. Louis, Mo.), allogeneic irradiated (3000 rad) T-lymphocytes plus PWM (lot No. 12N3094, Gibco, Grand Island, N.Y.), 1% vol/vol, IL-2 (lot No. 705-1, Amgen, Thousand Oaks, Calif.), 200 U/ml, and/or IL-6 (kindly provided by T. Hirano, MD, and T. Kishimoto, MD, PhD), 0.5 U/ml, in a 5% CO₂ humidified incubator at 37° C. Supernatants were collected on day 7, and cumulative IgG, IgM, and IgA Ab levels in cultured supernatants were measured by ELISA, as previously reported.¹⁶

Statistical analysis of data

To evaluate the effect of retinoid administration on cell phenotypes, flow cytometry measurements at weeks 8, 16,

24, and 32 were expressed as ratios to the baseline value taken at week 0. This was done for both the percentage of B cells displaying a given surface marker as well as for the mean channel fluorescence. For each subject, the resulting values were plotted against week, and a line connecting the medians at each time point was drawn to indicate changes over time. The numbers on the vertical axis represent the actual ratios to baseline measurements (i.e., a value of 2 indicates a twofold increase in the measurement over the baseline value). The numbers are not equally spaced; they are on a logarithmic scale so that complementary ratios, such as $\frac{1}{2}$ and 2 would plot equidistant from the baseline. An additional advantage with this scale is that a constant percentage increase per week will plot as a straight line. Tests for changes from baseline (i.e., a ratio different from 1) were done by first taking the logarithm of the ratios and then with the Wilcoxon's signed-rank test for paired data. This transformation is used to achieve symmetry of the distribution of the measurements that is a necessary condition for applying this test.¹⁷ That patients with CVI exhibit a less mature surface phenotype⁴ and that retinoids are known to promote cellular differentiation⁹ was strong a priori evidence to believe that 13-cis RA should lead to a rise in Leu-8 and a fall in CD20 display; therefore, *p* values for one-sided alternatives were appropriately applied.

RESULTS

Clinical outcome

Patients' characteristics and quantitative immunoglobulin levels before replacement therapy are summarized in Table I. Of the 10 patients who were enrolled, eight subjects completed the study without development of any serious drug-associated toxicity. Patients 151 and 153 were unable to complete the study because of RA-related side effects of generalized erythroderma or oral stomatitis that rapidly resolved on cessation of the drug. Reported side effects of the therapy were similar to side effects observed in patients with cystic acne treated with 13-cis RA, especially side effects related to drying of the skin and mucous membranes. None of the subjects developed functional liver abnormalities while they were taking the drug. By 4 to 8 weeks, six of the eight patients reported a general decrease in the severity of chronic symptoms (i.e., upper or lower respiratory symptoms, arthralgias, etc.) and increased sense of well being. Patients 115, 152, 118, 148, 125, and 143 reported that they felt better during the treatment phase and perceived a benefit from therapy that lasted 4 to 8 weeks after the drug was stopped. Patients 115 and 143 reported continued subjective benefit for 16 weeks after drug treatment (through completion of the study). Although this was a self-controlled, crossover trial, the study design was neither placebo controlled

TABLE I. Characteristics of immunodeficient patients studied

Patient ID no.	Sex	Age (yr)	Diagnosis	Serum Ig levels before therapy*		
				IgG	IgM	IgA
115	M	50	CVI	7	17	22
118	F	34	CVI	130	12	7
125	M	59	CVI	65	5	3
128	F	37	CVI	75	28	7
132	M	50	CVI	61	1	2
143	M	60	CVI	90	24	20
148	M	28	CVI	85	10	5
151	M	40	CVI	73	15	<1
152	F	34	CVI	198	35	0
153	M	31	CVI	292†	<7	<7
Normal range (UCLA clinical laboratory)‡				706-1868	76-436	52-337

ID, Identification; Ig, immunoglobulin.

*Values reported in milligrams per deciliter.

†Values obtained with intramuscular immunoglobulin (100 mg/kg/month).

‡Normal range from 5th to 95th percentile.

nor blinded, and thus, the reported subjective responses must be interpreted with caution. None of the patients demonstrated significant quantitative changes in serum IgA, IgM, or IgE levels during the course of the study.

Cell-surface phenotype of patients' B-lymphocytes demonstrates maturation toward normal

All patients had detectable B-lymphocytes as determined by the presence of the CD20 Ag and membrane IgM. B cell percentages and absolute numbers were similar to those previously reported.⁴ With CD20 as a pan-B cell marker, two-color flow cytometry was used to assess the effects of RA on the expression of developmentally regulated cell-surface markers, including CD20.

In seven of the eight individuals who completed the study, Leu-8 expression on CD20⁺ B cells was evaluated before, during, and after administration of 13-cis RA. In Fig. 1 are found plots of the changes from baseline and the median ratios for the Leu-8 mean channel fluorescence on B cells. Increases from baseline in mean channel fluorescence for Leu-8 were not significant at 8 weeks, but by week 16, the rise was clearly evident with seven of seven subjects demonstrating an increase in mean channel fluorescence ($p = 0.008$). This trend continued at 24 ($p = 0.031$) and 32 weeks ($p = 0.031$). A similar upward trend from 8 weeks was observed for changes in the percent of Leu-8 on B cells. In one subject (patient 143), technical difficulties resulted in the loss of the baseline Leu-8/CD20 value, although the 8-week measure-

ment demonstrated a bright CD20, dim Leu-8 phenotype typical of that that we have previously reported in patients with CVI. During the following 8 weeks, this patient demonstrated an increase in both percent and mean channel Leu-8 expression on B cells. These data suggest that the effect of RA on alteration of B cell surface markers may not be noticeable after 8 weeks of therapy, but at 16 weeks and for at least 8 weeks after RA was stopped, there is a significant rise in the expression of Leu-8 on B cells.

These flow data also indicate that with prolonged administration of 13-cis RA, the very bright CD20⁺ cell population observed in patients with CVI shifts toward a more normal B cell phenotype. In Fig. 2, CD20 mean channel fluorescence on B cells plotted as ratios to baseline, with medians indicated for weeks 8, 16, 24, and 32, are illustrated. There are no changes overall at 8 weeks and an insignificant drop at 16 weeks. At week 24, however, there was a significant decrease ($p = 0.016$). This continued to week 32, in which all five of five subjects demonstrated decreased CD20 display compared to that of baseline ($p = 0.031$).

Response to immunization with TT/DT

To determine if RA treatment would allow patients to generate specific Ab-producing B cells in vivo (lymphoblastoid B cells), patients were immunized with TT/DT after 12 weeks of 13-cis RA therapy. PBMCs were obtained at 0, 1, and 3 weeks after immunization and cultured for 7 days either alone, with PWM, or with 1 U/ml of IL-6. The supernatants were assayed for specific Ab to TT or DT. There were no consistent

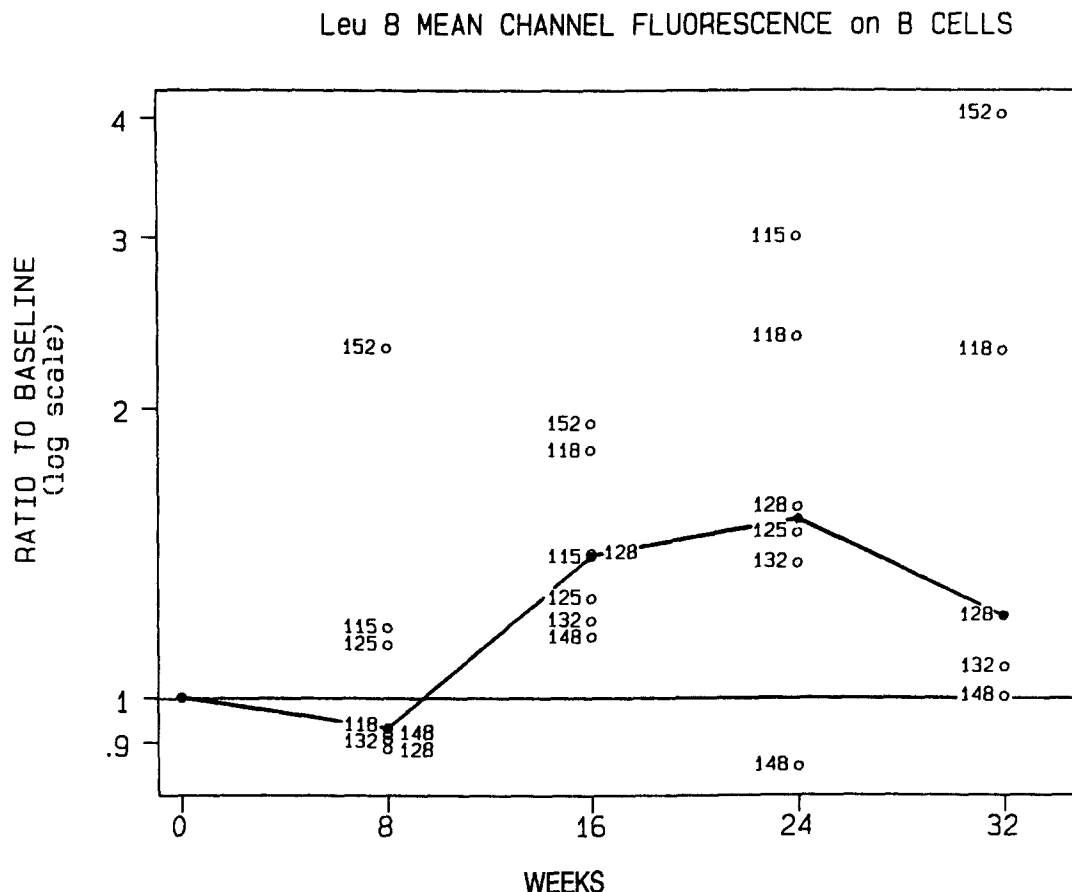


FIG. 1. Ratios (on a log scale) indicating changes from baseline (at week 0) for Leu-8 mean channel fluorescence on CD20⁺ B cells from subjects with CVI at weeks 8 (N = 7), 16 (N = 7), 24 (N = 6), and 32 (N = 5). Each subject is plotted with the patient's identifying number. The baseline ratio at 1 indicates no change. The change is statistically significant at weeks 16 ($p = 0.008$), 24 ($p = 0.031$), and 32 ($p = 0.031$).

responses following immunization, either at 1 or 3 weeks. Isolated responses were observed in subjects 115 and 118. At 1 week after immunization, PBMCs from subject 115 revealed a measurable increase in Ab when they were stimulated with PWM, displaying a large increase in TT-specific IgG and relatively small increases in TT-specific IgM and DT-specific IgG and IgM. Although the absolute values are too small to reach statistical significance, Ab production to DT immunization in patient 118 appeared to increase over the 4-week period when PBMCs were stimulated in culture with PWM. Alterations in in vitro Ab production by unfractionated large, low-density, or small, high-density B cells to PWM, or cloned IL-2, IL-4, and/or IL-6 were not detected (data not presented).

DISCUSSION

The development of an in vivo pharmacologic approach to overcome the defects in CVI is appealing not only from a cost perspective but also particularly

because of the possibility of providing patients with a more normal spectrum of isotypes than is provided by replacement immunoglobulin therapy. We and other investigators have established that most patients with CVI have B cells that demonstrate in vitro an inability to make the transition from the proliferative to the fully differentiated phase of development.^{4, 18-20} We have also determined that RA promotes the development of a more differentiated phenotype associated with functional differentiation by B-lymphocyte hybridoma cell lines from patients with CVI.⁹ This finding is consistent with numerous studies demonstrating the ability of retinoids to affect the differentiation of a variety of cell types²¹ as well as enhancing both cellular and humoral responses in rodents.⁵ In in vitro human systems, modulation of cellular and humoral responses by retinoids have also been reported.^{8, 22-24} Human humoral responses by tonsillar lymphocytes to sheep red blood cells⁸ and proliferation by human thymocytes to a variety of

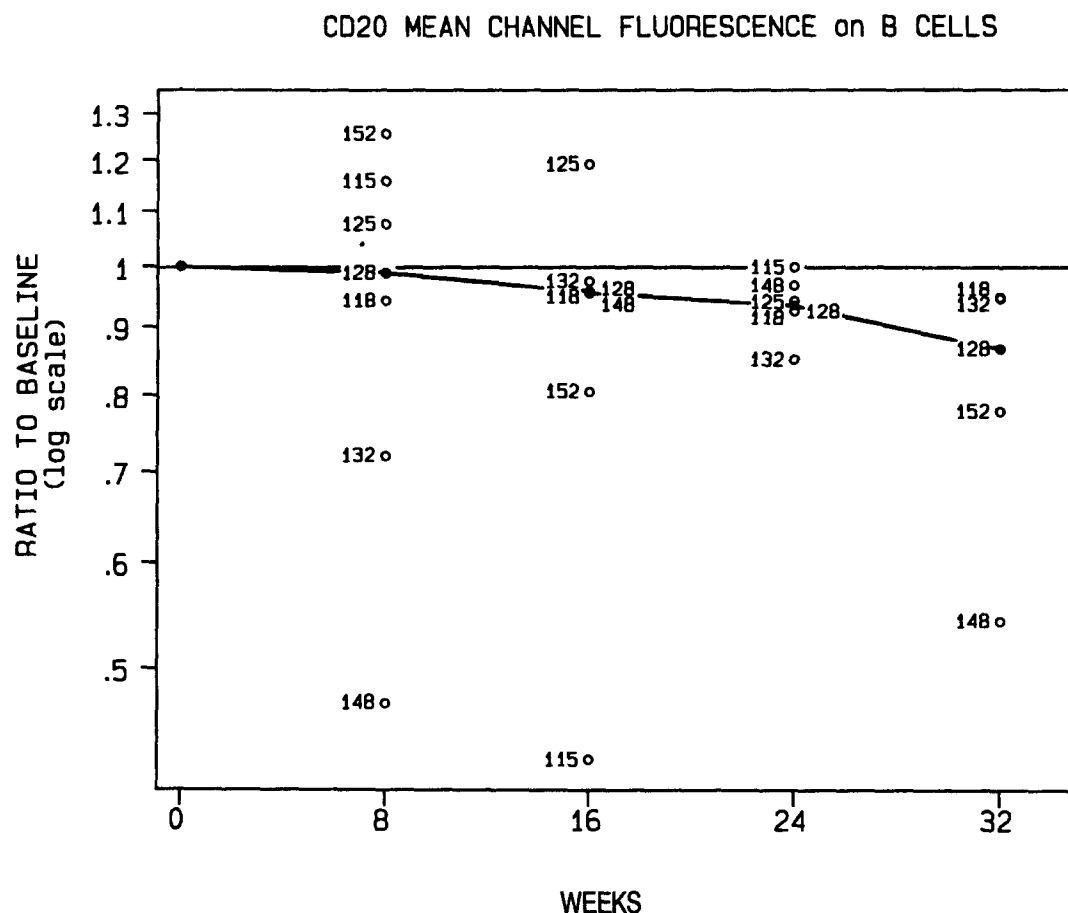


FIG. 2. Ratios (on a log scale) indicating changes from baseline (at week 0) for CD20 mean channel fluorescence on B cells at weeks 8 (N = 7), 16 (N = 7), 24 (N = 7), and 32 (N = 4). Each subject is plotted with the patient's identifying number. The reference line drawn at 1 indicates no change. The change is statistically significant at weeks 24 ($p = 0.016$) and 32 ($p = 0.031$).

stimuli²² are also enhanced by RA. Recently, enhancement of antikeyhole limpet hemocyanin responses have been demonstrated in patients with acne receiving 13-cis RA therapy,²⁵ a finding that represents the first demonstration in humans that in vivo administration of retinoids can modulate Ag-specific immune responses.

In the present study, we provide direct evidence that the in vivo administration of 13-cis RA induces circulating peripheral B cells from patients with CVI to express a more differentiated phenotype. Seven of eight patients demonstrated normalization of Leu-8/CD20 expression, and three subjects concomitantly manifested a partial return of functional Ab production to exogenously administered Ag (TT and DT). The low level of Leu-8 expression as well as the brighter than normal expression of CD20 on B cells from patients with CVI appears to be a stable phenotype. Several patients enrolled in this trial had participated in a study 3 years earlier in which the diminished Leu-8 and bright CD20 expression was first observed.⁴

Leu-8 is a cell-surface adhesion molecule and has recently been identified as a human homologue of the mouse Mel-14 lymph node homing receptor²⁶ that directly mediates adhesion of lymphocytes to high endothelial venules in Peyer's patch lymph nodes.²⁷ The system of cell-surface adhesion molecule interactions appears to regulate physical interactions of lymphocyte subpopulations within lymphoid organs, facilitating appropriate functional responses of B cells to antigenic stimulation within the lymph node.²⁸ In this regard it is noteworthy that cells exhibiting a dim Leu-8 and very bright CD20 phenotype are characteristic of germinal center B cells, the function of which is to undergo clonal expansion and affinity maturation rather than terminal differentiation. Furthermore, it has now been demonstrated that germinal center B cells have both a functional and phenotypic profile similar to that which we have described for the circulating B cells in CVI. Thus, we believe that the population of dim Leu-8 and bright CD20 circulating B cells observed in patients with CVI represents a

functionally immature population of B cells. Moreover, circulating B cells from patients with CVI also demonstrate the VH gene utilization pattern characteristic of immature germinal center cells.²⁹ Whether the B cells that appear in the circulation of subjects with CVI after 13-cis RA therapy also demonstrate a change toward normal in their VH gene use remains to be determined.

The generation of specific Abs to TT and DT in three patients is remarkable since they previously had been unable to mount an Ab response to glycoprotein immunization.¹⁴ However, PBMCs from the patients with CVI still did not demonstrate increases in total IgG, IgM, or IgA production when these were stimulated in vitro with PWM plus irradiated T cells, or cloned IL-2, IL-4, and/or IL-6. These results suggest two nonmutually exclusive interpretations: (1) in vivo administration of 13-cis RA does not promote a "generalized" differentiation effect on the vast majority of unstimulated B cells or (2) the level of differentiation induced by the retinoid on most of the B cells from patients with CVI is not sufficient to allow them to make large quantities of immunoglobulin. With regard to the first point, it is possible that the retinoid, by itself, is ineffective on B cells in the resting state but must be present during the stimulation process to drive differentiation. This finding is supported by previous studies that demonstrate that RA can increase the immunocompetence of certain populations of "activated" but not resting human T cells.²⁴ Furthermore, we have previously demonstrated that the continuous presence of RA during in vitro stimulation of fresh B cells from patients with CVI does result in increased immunoglobulin secretion.⁹

With regard to the second point, we previously observed relatively rapid in vitro functional responses of B cells from patients with CVI to 13-cis RA⁹ and therefore anticipated that 16 weeks of therapy would be sufficient to induce similar changes in vivo. However, it is important to note that in this study, the effects of 13-cis RA on in vivo B cell phenotypic differentiation (normalization of the Leu-8/CD20 display) only began to be demonstrable at 12 to 16 weeks of treatment, at which point the drug was stopped as per protocol. Moreover, in most patients, the normalization of B cell phenotype appeared to persist up to 16 weeks after the retinoid was stopped. It is interesting that in the treatment of acne with 13-cis RA, effects are usually first noted 3 weeks to 2 months after initiation of therapy, and clinical benefits persist or even improve after the drug is stopped, implying permanent correction of the disease.³⁰ Together, these observations suggest that initial cellular maturation driven by RA is the development of true, phenotyp-

ically mature B cells as evidenced by the changes in surface molecule display of Leu-8/CD20. However, the ability to functionally differentiate to Ab-secreting plasma cells, as measured by specific Ab response, was only marginally achieved; these results suggest that longer exposures in vivo to 13-cis RA will be needed to drive functional differentiation of B cell from patients with CVI.

In the subjects with CVI, the relatively long lag period until the effects on B cells were noted may reflect the slow recruitment of in vivo antigenically stimulated B cells that can be acted on by 13-cis RA. Alternatively, it is possible that the retinoid only acts on B-lymphocytes at early stages in their in vivo development, in which case it would take weeks to months to recruit cells from the bone marrow through the germinal center stage and out into the peripheral system. In any case, the long carry-over effect observed after the drug is stopped suggests that a permanent differentiation process has occurred, and apparent reversibility may only depend on B cell clearance through maturation and cell death. These observations lead us to expect an even greater specific Ab response and more complete overall normalization of the humoral immunity in patients with CVI treated with 13-cis RA for longer periods in an ongoing study.

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