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Research Article

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Activated B Cells from Patients with Common Variable Immunodeficiency Proliferate and Synthesize Immunoglobulin

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Abstract

Most patients with common variable immunodeficiency (CVI) have normal numbers of circulating B cells but low concentrations of serum Ig. To determine if the hypogammaglobulinemia is caused by an intrinsic B cell defect, we studied B cell function of 22 CVI patients. Cultured B cells from all CVI patients underwent normal proliferation and synthesized normal quantities of IgE in the presence of anti-CD40 and IL-4. If cultured with anti-CD40 and IL-10, four patterns of Ig isotype synthesis were observed. Six CVI patients produced normal amounts of IgM, IgG, and IgA. Four patients produced normal quantities of IgM and IgG. Of the remaining 12 patients who failed to synthesize IgG and IgA, 8 produced normal and 4 synthesized decreased amounts of IgM. Analysis of the IgG subclasses produced by 10 patients with IgG-secreting B cells revealed that IgG_4 was the most affected subclass, followed by IgG_2; synthesis of IgG_3 and IgG_1 remained normal. Similarly, in the six IgA producing patients, IgA_2 was more often affected than IgA_1. The hierarchy of Ig isotype and subclass synthesis corresponds to Ig heavy chain constant region gene location on chromosome 14. Thus, circulating B cells of CVI patients are committed to synthesize one or more Ig isotypes or subclasses, and under proper conditions can proliferate, mature into Ig-secreting cells, and undergo class switch to IgE. (*J. Clin. Invest.* 1993. 92:1282–1287.) Key words: hypogammaglobulinemia • B cells • anti-CD40 mAb • interleukin

Introduction

Common variable immunodeficiency (CVI)[1] is a heterogeneous group of disorders characterized by defective antibody production. Diagnostic criteria include low levels of serum IgG and IgA and, in most cases, low levels of IgM (1). Although this syndrome is the most frequently observed primary immunodeficiency disease, the molecular and cellular defects responsible for this disorder are poorly understood. Most reports indicate that the majority of CVI-patients have normal numbers of circulating B cells (1, 2), implying that hypogammaglobulinemia is caused by a failure of B cells to mature into immunoglobulin (Ig) secreting cells. This failure of B cell maturation in CVI may be caused by a lack of appropriate T cell help or by an intrinsic B cell defect. To differentiate these possibilities, a number of investigators have co-cultured B and T cell populations from CVI patients and normal controls in various combinations, using a pokeweed-mitogen-driven system (3–6). Results of these studies are difficult to interpret since the abnormalities observed may have been directly due to allogeneic effects. The availability of new reagents has allowed us to directly induce B cells to proliferate and differentiate into Ig-secreting cells bypassing the need for T lymphocytes.

Using purified B cells, Clark and Ledbetter found that co-stimulation with anti-μ antibody and a mAb recognizing the B cell surface molecule CD40 promotes direct activation and proliferation of B cells (7). This stimulatory effect is increased by the presence in the culture system of murine L cells that are stably expressing human Fc receptors (CDw32 L cells) (8). If combined with anti-CD40, particular cytokines were shown to enhance B cell proliferation and to induce B cell differentiation and Ig synthesis. For instance, co-stimulation with IL-4 strongly induces anti-CD40 activated B cells to proliferate and to switch to IgE synthesis (8). Long-term cultures of human B cells have been initiated and maintained by the combination of anti-CD40 mAb and IL-4 (9). Whereas the Ig isotype produced by B cells stimulated with anti-CD40 mAb and IL-4 is mostly IgE, B cells stimulated with anti-CD40 mAb and IL-10 synthesize IgG, IgA, and IgM (10). Since these newly developed culture systems do not require the presence of T cells, they provide a useful experimental tool to evaluate B cell function in patients with CVI and to gain new insight into the cause of their hypogammaglobulinemia.

Methods

Patients. All 22 patients with CVI (12 males, 10 females, ages 13–75 yr) selected for the study had low or undetectable levels of serum IgG, IgA, and IgM, with the exception of two patients, one with normal IgA and one with normal IgM concentration. The arithmetic mean serum IgG level of CVI patients was 99 mg/dl (range: 0–390). Mean IgA level of 21 patients with abnormal IgA was 0.6 mg/dl (range: 0–9), and mean IgM level of 21 patients with abnormal IgM was 13 mg/dl (range: 0–39). Serum IgE was detectable in none of the patient sera (< 0.7 ng/ml). Serum Ig concentrations were measured before treatment of intravenous immunoglobulin was initiated. CD20(+) B cells were present in normal numbers in all patients. 16 healthy adult volunteers were studied simultaneously and served as normal controls.

Reagents. Purified anti-CD40 mAb (G28-5 (IgG_1)) was kindly provided by Dr. E. Clark, University of Washington, Seattle, WA. Purified human rIL-4 and rIL-10 were obtained from DNAX Research Institute, Palo Alto, CA. The CDw32/FcγRII-transfected Ltk^- cell line (CDw32 L cells) (11) was obtained through American Type Culture Collection, Rockville, MD.

Cell preparations. Peripheral blood mononuclear cells were prepared by the Ficoll-Hypaque gradient method (Winthrop Pharmaceu-

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1. *Abbreviations used in this paper:* CVI, common variable immunodeficiency.

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ticals, New York, NY). T cells were removed from mononuclear cells by twice rosetting with aminoethylisothiuronium bromide (AET) (Sigma Chemical Co., St. Louis, MO) treated sheep red blood cells. Natural killer cells and monocytes were removed by the treatment with 5 mM L-leucine methyl ester hydrochloride (Sigma Chemical Co.) in serum-free RPMI 1640 as described (12). The B cell–enriched populations obtained consisted of more than 90% B cells ($CD20^+$) and less than 1% T cells ($CD3^+$).

Stimulation of B cells with anti-CD40 mAb and cytokines. To induce B cell proliferation, 2.5×10^4 purified B cells were cultured for 4 d in 96-well round bottom microculture plates at a final volume of 200 μl of RPMI 1640 supplemented with 10% FCS (Hyclone, Logan, UT), 2 mM glutamine, 50 U/ml penicillin, and 50 μg/ml of streptomycin (complete medium) with anti-CD40 mAb G28-5 (1 μg/ml) and IL-4 (100 U/ml) followed by a 16-h pulse with 1 μCi [^{3}H]thymidine. To induce Ig production, 2.5×10^4 purified B cells per well were cultured in 96-well round bottom microculture plates in 200 μl of complete medium in the presence of 2.5×10^4 irradiated (7,500 rad) CDw32 L cells, mAb G28-5 (final concentration 1 μg/ml), IL-4 (100 U/ml) or IL-10 (10 ng/ml). After 12 d of culture, supernatants were collected and tested for Ig levels.

Immunoglobulin determination. The concentrations of IgG, IgA, IgM, IgE, IgG subclasses, and IgA subclasses in the sera of CVI patients or in the supernatants of cultured cells were measured by ELISA. For IgG, IgM, IgA, and IgE determination (12), 96-well microculture plates were coated with either goat anti-human IgG, IgA, or IgM (Cappel Laboratories, West Chester, PA) or a mixture of equal amounts of two mouse IgG_1 monoclonal anti-human IgE antibodies (CIA-E-4.15 and CIA-E-7.12, kindly provided by Dr. Andrew Saxon, University of California, Los Angeles, CA) in appropriate dilution and kept overnight at 4°C. After blocking the wells with PBS containing 1% BSA, serially diluted test samples or standards were added to each well and cultured at 37°C for 2 h. Purified human IgG, IgA, and IgM standards were purchased from Kent Laboratory (Kent, WA). IgE standard was purchased from Pharmacia AB, Uppsala, Sweden. After washing, secondary antibodies were added and cultured for 2 h at 37°C. Secondary antibodies used were as follows: alkaline phosphatase conjugated goat anti-human IgG (Cappel Laboratories), alkaline phosphatase conjugated goat anti-human IgA (Sigma Chemical Co.), alkaline phosphatase conjugated goat anti-human IgM (Sigma Chemical Co.), or biotin conjugated goat anti-human IgE (Kirkegaard and Perry Laboratories, Gaithersburg, MD). For IgE measurement, alkaline phosphatase conjugated extravidin (Sigma Chemical Co.) was also used. Substrate solution containing *p*-nitrophenyl phosphate disodium (Sigma Chemical Co.) was prepared at a concentration of 1 mg/ml in carbonate buffer, pH 9.8, with 10 mM $MgCl_26H_2O$. To measure IgG subclasses, 96-well microculture plates were coated with subclass specific mAbs as follows: HP6069 and HP 6001 (at equal volumes) for IgG_1, HP6002 and HP6014 for IgG_2, HP6050 and HP6047 for IgG_3, and HP6023 and HP6025 for IgG_4. These mAbs were purchased from Calbiochem-Novabiochem Corp., La Jolla, CA. After blocking the wells with PBS containing 1% BSA, serially diluted culture supernatants or standards (World Health Organization standards 67/97 obtained from Centers for Disease Control, Atlanta, GA) were incubated for 4 h at 37°C. After washing, peroxidase conjugated goat anti-human IgG (Boehringer Mannheim Biochemicals, Indianapolis, IN) was added to the wells. After 2-h incubation and washing, citrate buffer containing 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (Sigma Chemical Co.) and H_2O_2 (Parke-Davis, Morris Plains, NJ) was added. For IgA subclass measurements, microculture plates were coated with mAbs A1c and HP 6103 for IgA_1, and with 14-3-26 and HP6109 for IgA_2. These monoclonal anti-human IgA subclass specific antibodies were kindly provided by Dr. M. E. Conley, University of Tennesse, Memphis, TN, and the late Dr. C. B. Reimer, Centers for Disease Control, Atlanta, GA. Human IgA_1 and IgA_2 standard were purchased from Calbiochem-Novabiochem Corp. For secondary antibody, alkaline phosphatase conjugated goat anti-human IgA (Cappel Laboratories) was used. The optical density was determined with an automated ELISA plate reader (Beckman Instruments, Inc., Fullerton, CA).

Results

B cell proliferation. Purified B cells from all CVI patients (n = 22) and normal controls (n = 16) showed increased [^{3}H]-thymidine uptake if cultured with anti-CD40 and IL-4 (Fig. 1). The geometric mean thymidine uptake observed in stimulated normal B cells was 8,090 cpm (95% confidence interval, 1,324–49,431 cpm). [^{3}H]Thymidine uptake observed in stimulated CVI-B cells was similar to controls (P = 0.96, t test) with a geometric mean of 7,726 cpm (range: 1,428–41,347).

IgE production. As shown in Fig. 2, purified B cells from all normal subjects (n = 16) produced IgE if cultured for 12 d in the presence of anti-CD40 mAb and IL-4 (geometric mean IgE = 4,236 pg/ml, 95% confidence interval, 104–171,001 pg/ml). Using the same culture system, B cells from all CVI patients (n = 22) produced similar amounts of IgE (geometric mean = 3,147 pg/ml, range, 248–29,888 pg/ml) (P = 0.26, t test). Without stimulation, B cells from CVI patients failed to produce detectable levels of IgE (< 100 pg/ml), while B cells from 11 of 16 normal controls produced detectable IgE.

Production of IgG, IgA, and IgM with anti-CD40 and IL-10. In the absence of in vitro stimulation, B cells from 7 of 22 CVI patients produced detectable levels of IgM, 1 patient produced detectable levels of IgA, and 1 patient produced detectable levels of IgG. In contrast, unstimulated B cells from most normal subjects produced detectable levels of IgA, IgG, and IgM (Fig. 3).

If stimulated with anti-CD40 and IL-10, B cells from all normal subjects showed a marked increase in the synthesis of IgG, IgA, and IgM. Individual values, geometric means, and 95% confidence intervals are indicated in Fig. 3.

B cells from all CVI patients studied (n = 22), if cultured with anti-CD40 and IL-10, produced at least detectable levels of IgM. Based on the isotypes synthesized in vitro by stimulated B cells, the 22 CVI patients were divided into 4 groups. Stimulated B cells from patients in group A (n = 6) produced IgA, IgG, and IgM at normal concentrations (range: 170–3,538 ng/ml (IgA), 142–1,053 ng/ml (IgG), 845–29,867 ng/ml (IgM)). In group B (n = 4), the concentrations of IgG and IgM synthesized by cultured B cells were normal (range: 111–283 ng/ml (IgG), 340–2,279 ng/ml (IgM)), but IgA levels were below 2 SD of the normal. B cells from group C (n = 8) pro-

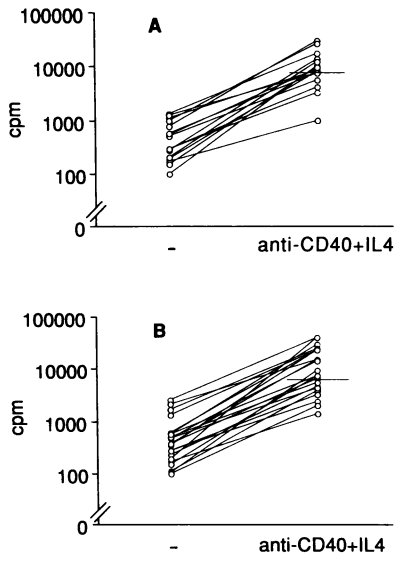

Figure 1. Proliferative responses of B cells from (*A*) normal controls (n = 16) and (*B*) CVI patients (n = 22). Purified B cells (5×10^4) were cultured in triplicates with or without mAb G28-5 (1 μg/ml) and IL-4 (100 U/ml) for 4 d. After a 16-h [^{3}H]thymidine pulse, proliferation was estimated and expressed as cpm. Results are means of triplicates. Horizontal bars indicate geometric mean of each group.

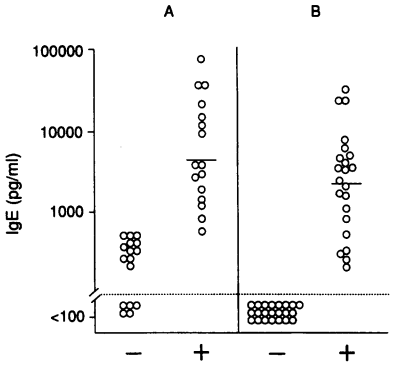

Figure 2. In vitro IgE production by B cells from (*A*) normal controls (n = 16) and (*B*) CVI patients (n = 22). Purified B cells (5 × 10^4) were cultured in triplicates with (+) or without (−) mAb G28-5 (1 μg/ml) and IL-4 (100 U/ml) on irradiated (7,500 rad) CDw32 L cells (5 × 10^3). Supernatants were collected on day 12 and IgE levels were measured by ELISA. Horizontal bars indicate geometric mean of each group. Dotted line indicates lower limits of detection (100 pg/ml).

duced normal amounts of IgM (range: 248–7,219 ng/ml); but IgG and IgA concentrations were below detectable levels (< 40 ng/ml). In group D (n = 4), only IgM at levels below 2 SD of normal controls (range: 62–109 ng/ml) was detectable.

In vitro Ig synthesis could frequently be induced in B cells derived from CVI patients who had undetectable serum Ig. Of the six CVI patients with B cells that produced normal amounts of IgA in vitro, five did not have measurable concentrations of serum IgA (< 5 mg/dl). Of the 10 CVI patients producing IgG in vitro, 3 had undetectable levels of serum IgG (< 5 mg/dl). Of 18 patients producing IgM at normal concentration, 3 had undetectable serum IgM (< 5 mg/dl). No significant correlation was found between serum Ig concentration and in vitro Ig production.

IgG and IgA subclasses. IgG subclasses were determined in culture supernatants from normal controls and from CVI patients whose isolated B cells produced IgG in response to anti-CD40 and IL-10 (group A and group B). As shown in Fig. 4, IgG_4 production was affected more often than IgG_2 production, and group B was more affected than group A. IgG_1 and IgG_3 production by patients from group A and B was not different from the normal controls. B cells from all patients in group B (4/4) produced decreased amounts of IgG_2 and IgG_4. B cells from three of six patients in group A showed decreased IgG_4 production, and only one showed decreased IgG_2 production, who also had decreased IgG_4 production.

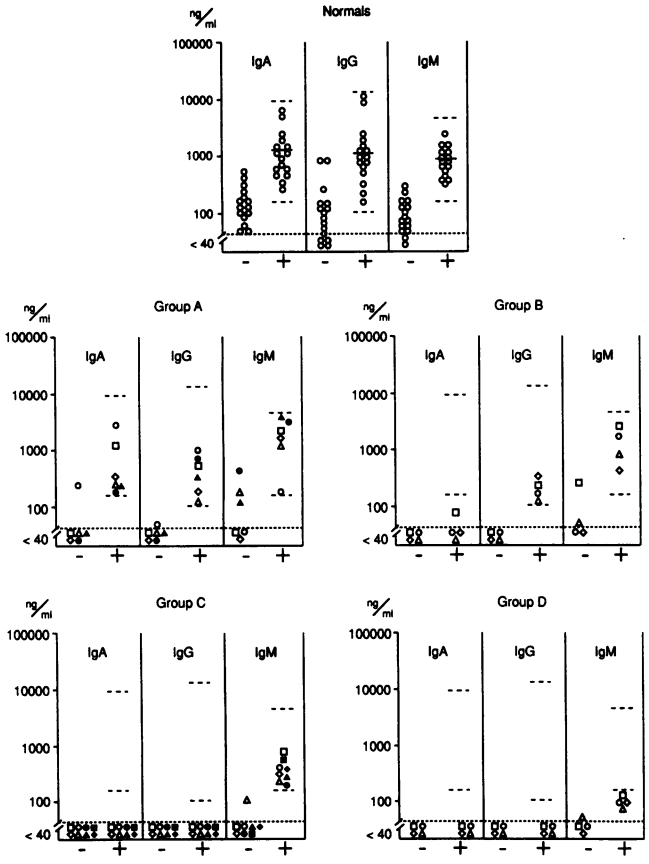

Figure 3. In vitro IgA, IgG, and IgM production by B cells from normal controls (n = 16) and CVI patients (Group *A–D*). B cells (5 × 10^4) were cultured with (+) or without (−) mAb G28-5 (1 μg/ml) and IL-10 (10 ng/ml) on irradiated (7,500 rad) CDw32 L cells (5 × 10^3). Supernatants were collected on day 12 and IgA, IgG, and IgM were measured by ELISA. Solid lines and dashed lines indicate geometric mean and 95% confidence intervals, respectively, determined for normal controls. Dotted line indicates lower limits of detection (40 ng/ml). CVI patients were pooled into 4 groups (*A–D*) based on the Ig isotypes synthesized in vitro by stimulated B cells.

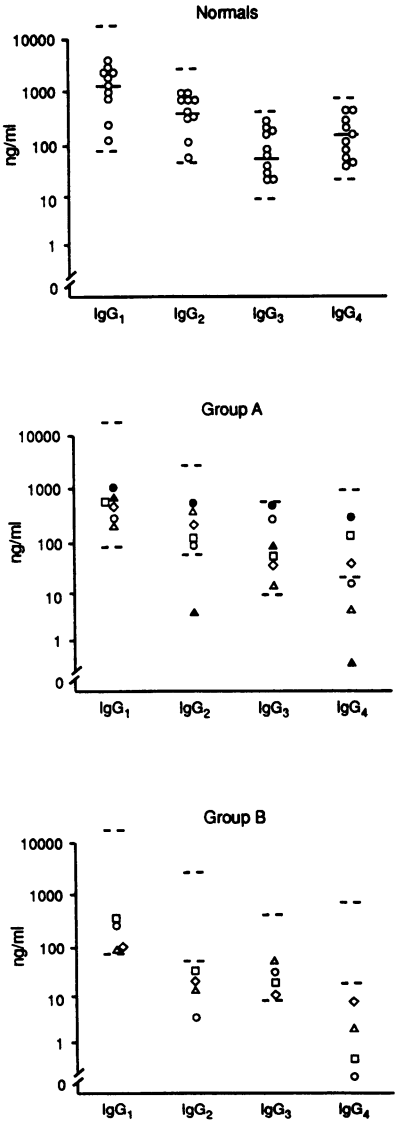

Figure 4. In vitro IgG subclass production by B cells from normal controls (n = 9) and CVI patients in group A and B (n = 10). B cells were cultured as described in the legend for Fig. 3. IgG subclasses were measured by ELISA. Solid lines and dashed lines indicate geometric mean and 95% confidence intervals, respectively, determined for normal controls.

IgA subclasses were determined in the supernatant of B cell cultures obtained from patients classified as group A (Fig. 5). IgA_2 production was below 2 SD of normal controls in two of the six patients, while IgA_1 production was normal in all six patients, including one patient who had decreased IgG_2 production. Of the two patients with low IgA_2 production, one had decreased IgG_2 and IgG_4 production, and one had normal IgG_2 and decreased IgG_4 production. With one exception (a patient with low IgG_4 but normal IgA_2 production), the extent of Ig isotype/subclass abnormalities observed in CVI patients corresponds with the order of Ig heavy chain constant region gene location on chromosome 14.

Discussion

In this study, we selected two schemes to assess peripheral blood B cell function in CVI patients, who, although hypogammaglobulinemic, had normal numbers of circulating B cells. B cell proliferation and IgE synthesis was induced by co-culture with anti-CD40 and IL-4, and immunoglobulin (IgG, IgA, and IgM) synthesis was initiated by exposing B cells in vitro to anti-CD40 and IL-10 in the presence of CDw32 L cells. These combinations are known to provide the most effective stimulation for B cell growth and differentiation (8, 10) in the absence of T cells.

Proliferation of CVI-B cells in response to stimulation with anti-CD40 and IL-4 was comparable to B cells from normal controls, suggesting that CVI-B cells receiving appropriate signals will respond with polyclonal proliferation. The same combination of reagents induced B cells to synthesize amounts of IgE comparable to that synthesized by normal control B cells in vitro. Stimulation with anti-CD40 and IL-4 is known to induce class switch to IgE in noncommitted B cells (9). Since none of the CVI patients had detectable serum IgE or produced IgE without stimulation in vitro, it is likely that the exposure of B cells to anti-CD40 and IL-4 resulted in heavy chain switching and secretion of IgE.

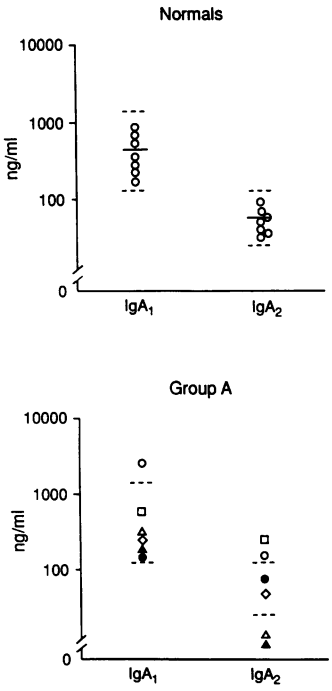

Figure 5. In vitro IgA subclass production by B cells from normal controls (n = 6) and CVI patients from group A (n = 6). B cells were cultured as described in the legend for Fig. 3. IgA subclass concentrations were determined by ELISA. Solid lines and dashed lines indicate geometric mean and 95% confidence intervals, respectively, determined for normal controls. Symbols indicating individual patients correspond to those used in Fig. 4.

Addition of IL-10 to anti-CD40-activated B cells induced the in vitro synthesis of IgM by cells from all CVI patients. Four different patterns of Ig isotype synthesis could be identified (Fig. 3, Table I). Group A produced normal amounts of IgM, IgG, and IgA, group B produced normal amounts of IgM and IgG, and group C secreted IgM at normal concentration but failed to synthesize detectable IgA and IgG. In group D, B cells were limited to the production of low concentration of IgM. These findings suggest a hierarchy in the Ig isotypes secreted by CVI-B cells under our experimental conditions. IgA synthesis is more likely affected than the synthesis of IgG; IgM is affected the least and produced by B cells from all CVI patients. This hierarchy represents the order of heavy-chain constant region genes on chromosome 14. The abnormalities in IgG and IgA subclass production further support this observation (Figs. 4 and 5). IgG_3 and IgG_1 synthesis was conserved, and IgG_2 and IgG_4 synthesis was frequently deficient, reflecting the location of $\gamma 3$ and $\gamma 1$ "upstream" and $\gamma 2$ and $\gamma 4$ "downstream". IgA_2 was more frequently affected than IgA_1, which corresponds to the finding that α_1 is located upstream of α_2.

The most likely explanation for the defective isotype production is a lack of circulating B cells committed to the isotypes. Patients within group A have circulating B cells already switched in vivo to IgA, IgG, and IgM; those in group B have circulating B cells committed to IgG and IgM; and patients in groups C and D have circulating B cells committed to IgM only. This explanation is consistent with the notion that stimulation with anti-CD40 and IL-10 does not induce switching but drives already committed B cells to Ig secreting cells (13). Unresponsiveness to IL-10 by CVI-B cell is not a likely explanation, since all CVI-B cells produced at least IgM, following stimulation with anti-CD40 and IL-10.

Whether the lack of committed B cells to certain isotypes and subclasses is due to an intrinsic B cell defect or to a primary T cell abnormality cannot be determined with certainty. However, the observation that B cells from all CVI patients studied produced amounts of IgE comparable to normal B cells if cultured in the presence of anti-CD40 and IL-4, supports the hypothesis that CVI-B cells are capable of class switching if an appropriate signal is provided. The recent observation that low molecular weight B cell growth factor, which is produced by lectin-activated T cells, can induce B cells from patients with immunodeficiency to secrete IgG and IgA in vitro (14) further supports our hypothesis. The demonstration that T lymphocytes from a subset of CVI patients, if optimally stimulated in vitro, produced decreased amounts of lymphokines (15–17) suggests that the B cell dysfunction observed in our CVI patients may be due to abnormal in vivo production of lymphokines necessary to initiate the process of heavy chain switching. Examination of cytokines involved in the induction of class switching will further clarify if a primary T cell defect plays a major role in the pathogenesis of CVI.

B cell proliferation in CVI has been examined by other investigators and often found to be defective. Of a group of 15 CVI patients, Saiki et al. found 5 whose B cells failed to proliferate in response to SAC or anti-μ antibody (18). Similarly, Saxon et al. reported that 3 of 14 CVI patients had depressed proliferative responses to SAC, to anti-μ antibody combined with BCGF or to PMA (19). Consistent with our results, Bryant and co-workers demonstrated normal proliferative re-

Table I. Summary of the In Vitro Function of B Cells from CVI Patients

CVI Group	Numbers (%)	Proliferation*	IgE*	IgM‡	IgG‡	IgA‡
Group A	6 (27.3)	+	+	+	+	+
Group B	4 (18.2)	+	+	+	+	−
Group C	8 (36.3)	+	+	+	−	−
Group D	4 (18.2)	+	+	±	−	−

+, normal levels; ±, decreased levels; −, undetectable levels.
* B cells were stimulated with anti-CD40 and IL-4, and proliferation and in vitro IgE synthesis was determined.
‡ B cells were stimulated with anti-CD40 and IL-10, and in vitro Ig synthesis was measured.

sponses of B cells from CVI patients stimulated with anti-μ and IL-2 (20). Abnormal in vitro Ig synthesis by CVI-B cells was reported by several investigators. Franz et al. observed that B cells from one third (26/78) of CVI patients produced normal amounts of IgM and IgG, one third produced only IgM at low concentration, and one third failed to synthesize either IgM or IgG, when cultured in the presence of anti-μ and IL-2. This abnormality could not be reversed by direct activation of protein kinase C with PMA and ionophore and simultaneous exposure to IL-2 or IL-4 (21). Saxon and co-worker reported that using T cell–replacing factor and PMA to induce CVI-B cells into Ig-secreting cells, B cells from 14 of 17 CVI patients failed to respond (19). In a study by Mayer et al., only 11 of 25 CVI patients generated Ig plaque-forming cells. 5 of the 11 responders generated IgG-secreting plaque-forming cells and only 1 CVI patient had IgA-secreting plaque-forming cells if B cells were stimulated with differentiation factor obtained from T cell hybridomas (22). The observation of Pasterelli et al. that PBL of six of eight CVI patients failed to produce measurable IgE if cultured in the presence of IL-4 (23) may well be due to abnormal T cell function.

The discrepancies between our results and those of others can be explained by the more effective stimulation of B cells with anti-CD40 and IL-4 or IL-10 in the presence of CDw32 L cells. Alternatively, since CVI is a heterogeneous group, the defect may vary within the study populations. However, since 20 of 22 patients had low or undetectable levels of serum Ig, it is not likely that our patient population represented a type of CVI that was less affected than that of other groups.

The observation that B cells from CVI patients including those with undetectable serum Ig produced normal levels of one or more isotypes if cultured with anti-CD40 and IL-10 suggests that CVI-B cells have the ability to mature into Ig secreting cells if appropriate signals are provided. We hypothesized that the profound hypogammaglobulinemia characteristic for CVI patients may, in part, be due to defective expression of the CD40 ligand on T cells (24) and/or defective production of IL-10. The observation by Ishida et al. that treatment of normal mice with neutralizing anti-IL-10 mAb results in decreased serum concentration of IgM and IgA (25, 26) supports this hypothesis. With the proper reagents available, this hypothesis can now be tested.

Acknowledgments

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