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# CD40 Ligand Expression is Defective in a Subset of Patients with Common Variable Immunodeficiency.

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# CD40 ligand expression is defective in a subset of patients with common variable immunodeficiency

(gp39/primary immunodeficiency syndrome)

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**ABSTRACT** Common variable immunodeficiency (CVI) is characterized by hypogammaglobulinemia and recurrent bacterial infections due to failure of CVI B cells to differentiate *in vivo* into immunoglobulin-secreting plasma cells. We hypothesized that T-cell dysfunction resulting in abnormal contact-mediated B-cell activation may play a prominent role in the failure of CVI B cells to produce specific antibody. We have previously shown that B-cell proliferation and IgE production after stimulation with anti-CD40 and interleukin (IL) 4 were normal in 22 CVI patients evaluated, indicating that CVI B cells respond to signals delivered via CD40. Here we report that CD40 ligand (gp39) mRNA expression by activated lymphocytes from CVI patients ( $n = 31$ ) as a group was significantly depressed ( $P < 0.0001$ ) compared with normal controls ( $n = 32$ ). gp39 mRNA expression by activated lymphocytes from 13 CVI patients fell below the normal control range. T-cell surface expression of functional gp39 protein was correspondingly low in those patients with gp39 mRNA levels below normal control range and normal in patients with gp39 mRNA levels within normal control range. In CVI patients as a group, gp39 mRNA levels correlated with IL-2 mRNA levels ( $P < 0.002$ ,  $r = 0.6$ ) and production ( $P < 0.001$ ,  $r = 0.7$ ) but not with gene expression or production of other lymphokines evaluated, suggesting an as-yet-undetermined association between gp39 and IL-2 gene regulation. Of the 13 patients whose activated T cells exhibited gp39 mRNA expression below the normal control range, 2 had normal T-cell-derived lymphokine production, whereas the remaining 11 exhibited broader T-cell dysfunction, resulting in IL-2 deficiency, and in some patients deficient production of other lymphokines as well, reflecting a heterogeneity in the underlying mechanisms leading to depressed gp39 expression in these patients. The observation that both gene and surface expression of gp39 by activated T cells is depressed in a subgroup of CVI patients suggests that inefficient signaling via CD40 may be responsible, in part, for failure of B-cell differentiation in these patients.

Common variable immunodeficiency (CVI) is a heterogeneous group of disorders characterized by hypogammaglobulinemia, antibody deficiency, and recurrent bacterial infections (1). Most CVI patients have normal numbers of circulating T cells and surface immunoglobulin-positive B cells; however, CVI B cells fail to differentiate into immunoglobulin-secreting plasma cells *in vivo* (2–4). Consequently, CVI patients have reduced levels of serum immunoglobulin and respond abnormally to immunization with protein and polysaccharide antigens (5, 6). An intrinsic B-cell defect (3, 7, 8), excessive T-suppressor activity (9–11), or defective T/B-cell

interaction (12–15) has been postulated as responsible for the failure of CVI B-cell differentiation.

The finding that CVI B cells could proliferate and produce immunoglobulin if appropriately stimulated *in vitro* (13, 16) suggests that T-cell dysfunction resulting in depressed or absent contact-mediated B-cell activation may be of importance for the failure of CVI B cells to differentiate into immunoglobulin-producing plasma cells. It has been shown in both murine and human systems that signals delivered via the B-cell surface antigen CD40 play an important role in B-cell proliferation, differentiation, and isotype switching (17–19). Anti-CD40 monoclonal antibody (mAb) in synergy with interleukin (IL) 4 induces B-cell proliferation (20, 21) and IgE secretion (21–25) and in synergy with IL-10 induces B cells to produce IgA, IgG, and IgM (16, 26). The recent characterization (27–29) and cloning (30–32) of a 39-kDa protein (gp39) on activated human T cells that binds CD40 and transduces a B-cell activation signal provided the opportunity to investigate the expression of gp39, on both mRNA and protein levels, by activated T cells from patients with primary immunodeficiency syndromes. As a result of these investigations, we (33) and others (34–37) found that gp39 protein expressed by activated T cells from patients with X chromosome-linked hyper-IgM syndrome (HIM) is functionally defective and that the gene encoding gp39 is located at Xq2.6, the site where the gene responsible for HIM had been previously mapped by family linkage studies (38), suggesting that defective gp39 is the molecular defect directly responsible for the inability of HIM B cells to switch from IgM to other immunoglobulin isotypes. Further studies revealed that anti-CD40 mAb could drive HIM B cells (33) and CVI B cells (16) to proliferate *in vitro* and produce IgE if costimulated with IL-4. These findings suggest that defective gp39 expression by activated T cells may also play a role in the antibody deficiency observed in CVI patients. Using RNA blot analysis and CD40-immunoglobulin fusion protein binding experiments, we found that gp39 expression by activated T cells was depressed in CVI patients compared with normal controls, suggesting that suboptimal gp39-CD40 interaction may contribute to B-cell dysfunction in a subgroup of CVI patients.

## MATERIALS AND METHODS

**Patient Population.** A total of 31 patients, 20 males and 11 females (12–77 yr of age, mean = 38 yr, median = 37 yr), with well-documented CVI were included in the study. IgG, IgM,

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Abbreviations: CVI, common variable immunodeficiency; mAb, monoclonal antibody; IL, interleukin; HIM, X chromosome-linked hyper-IgM syndrome; PHA, phytohemagglutinin; PMA, phorbol 12-myristate 13-acetate.

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and IgA levels were less than age-matched normal control range in 31/31, 29/31, and 30/31 patients, respectively. All patients exhibited abnormal antibody responses to several recall antigens, including diphtheria/tetanus toxoid and pneumococcal polysaccharides. The immune response to the T-cell-dependent neoantigen bacteriophage  $\phi$ X174 had been evaluated in all 31 patients. Phage-specific antibody, of both IgM and IgG isotypes, was significantly depressed in all patients. The ability to switch from IgM to IgG during the secondary immune response to bacteriophage was completely absent or abnormal (<20% phage-specific IgG) in 21 patients and normal (>20% phage specific IgG) in 11 patients. Absolute lymphocyte count was normal in 18 patients and decreased (<1100) in 13 patients. CD4/CD8 ratio was normal in 22 patients and depressed (<0.75) in the remaining 9 patients. All but one patient received regular infusions of i.v. immunoglobulin at 2- to 4-week intervals. Each patient was studied at the maximum time interval after the last i.v. immunoglobulin infusion to avoid possible effects of i.v. immunoglobulin therapy on lymphocyte function. Healthy blood donors ( $n = 32$ ) served as controls and were studied in parallel with each patient.

**gp39 Gene Expression.** Peripheral blood was collected in preservative-free heparin, and peripheral blood mononuclear cells were isolated by Ficoll/Hypaque density gradient centrifugation. Peripheral blood mononuclear cells were cultured at a final concentration of  $5 \times 10^6$  cells per ml under standard conditions (37°C, 5% CO<sub>2</sub>) in RPMI 1640 medium (Sigma), containing 10% heat-inactivated fetal calf serum (HyClone), 1 mM L-glutamine, penicillin at 100 units/ml, and streptomycin at 100  $\mu$ g/ml (GIBCO) for 6 hr in the presence of phytohemagglutinin (PHA) (final concentration, 3  $\mu$ g/ml) (Wellcome) and phorbol 12-myristate 13-acetate (PMA; final concentration, 10 ng/ml) (Sigma). Total cellular RNA was isolated by the guanidinium thiocyanate/cesium chloride method (39), and RNA blot analysis was done as described (33). Human gp39 (30) and CD3  $\delta$ -chain (40, 41) <sup>32</sup>P-labeled cDNA hybridization probes were prepared by using a commercially available oligolabeling kit (Pharmacia).

mRNA levels were quantitated by densitometry and integration of autoradiograph bands (Bioimage Products version 4.1.1, Millipore). To correct for the amount of T-cell message on the blot, we divided the integrated OD for gp39 by the integrated OD for CD3 $\delta$ . For each blot, the corrected integrated OD for gp39 was divided by the median of the corrected integrated ODs of the normal controls. The generated value (with 1.0 representing the median of a normally distributed population) allowed comparison of normal controls and CVI patients from different blots.

**CD40-Immunoglobulin Binding Assays.** Peripheral blood mononuclear cells were isolated by centrifugation over lymphocyte separation medium (Organon Teknika-Cappel) and washed twice in RPMI 1640 medium. Cells ( $10^7$ ) were resuspended in 3 ml of RPMI 1640 medium/heat-inactivated 10% fetal calf serum, with or without PMA (10 ng/ml) plus ionomycin (1  $\mu$ g/ml) and incubated at 37°C in 5% CO<sub>2</sub>/95% air for 8 hr. Cells were subsequently washed, resuspended in RPMI 1640 medium/2% fetal calf serum/0.1% NaN<sub>3</sub> (staining medium) and plated at  $5 \times 10^5$  cells per well in a 96-well microtiter plate. Cells were stained for 1 hr on ice with anti-CD3 and anti-CD28 mAb (42), and anti-CD69 mAb (provided by S. M. Fu, University of Virginia) (43), at a final concentration of 10  $\mu$ g/ml and CD40-immunoglobulin fusion protein (27) at a final concentration of 25  $\mu$ g/ml. A murine-human chimeric antibody (cL6) (44) was used as isotype-matched control for CD40-immunoglobulin. Plates were centrifuged at 1000 rpm for 3 min. The medium was aspirated, and the cells were washed once with staining medium and resuspended in staining medium containing either fluorescein-conjugated goat anti-mouse or goat anti-human antibodies

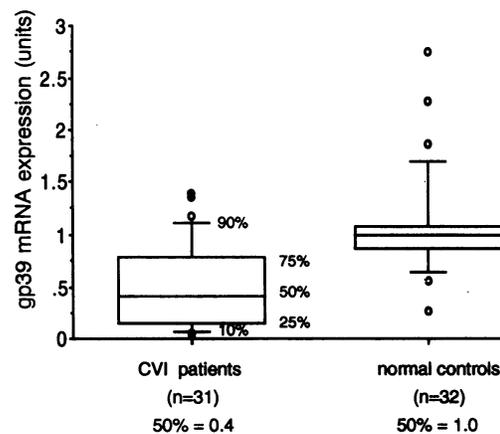


FIG. 1. Box plot illustration of gp39 mRNA expression, determined by RNA blot analysis, at 6 hr by PHA/PMA-activated lymphocytes from CVI patients and matched normal controls. gp39 expression by activated lymphocytes from CVI patients, as a group, is significantly less than by lymphocytes from normal controls ( $P < 0.0001$ ). %, Percentile.

(10  $\mu$ g/ml, Tago) and incubated on ice for 30 min. The cells were subsequently washed once with staining medium, and antibody or fusion protein binding was determined by flow cytometry (Epics C, Coulter).

**Statistical Analysis.** Statistical analysis, using Mann-Whitney, Spearman rank, and stepwise logistic regression tests, was done by using Statview IV (Abacus Concepts, Berkeley, CA) on a Macintosh IICx.

## RESULTS

**gp39 Gene Expression.** gp39 gene expression by PHA/PMA-activated lymphocytes, assessed by quantitative densitometry analysis of RNA blots, was found significantly diminished in CVI patients ( $n = 31$ ) as a group compared with normal controls ( $n = 32$ ) ( $P < 0.0001$ ) (Fig. 1). Activated lymphocytes from 13 of the 31 CVI patients expressed gp39 mRNA at levels less than the normal control range (<0.26 unit). In Fig. 2 we show RNA blots from representative patients whose activated lymphocytes expressed gp39 mRNA at levels within (Blot 1) and below (Blots 2 and 3) the normal control range.

**CD40-Immunoglobulin Binding.** To determine whether the quantity of gp39 mRNA expression correlates with membrane-associated gp39 protein, we evaluated gp39 surface expression by activated lymphocytes from five representa-

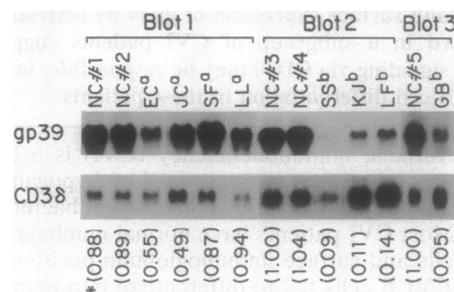


FIG. 2. RNA blot analysis of gp39 mRNA obtained from activated lymphocytes from representative normal controls (NC) and CVI patients with mRNA levels within (\*) and below (b) normal control range. RNA blots were probed for gp39 and CD3 $\delta$  chain, which was used to correct for the levels of T-cell mRNA in each sample. Corrected densitometry values (in units) for gp39 expression are shown below (\*). Differences in autoradiograph exposure time account for the differences in intensity of bands between CD3 $\delta$  chain and gp39 for each blot.

Table 1. Surface expression of functional gp39 protein by activated T cells

Patient	gp39 mRNA level, units	gp39, % +		CD3, % +		CD69, % +	
		Pt	NC	Pt	NC	Pt	NC
Normal range							
EC	0.552	24	32	71	82	54	70
JC	0.493	18	12	87	70	79	75
SF	0.868	22	18	95	87	79	82
LL	0.943	28	12	80	70	74	57
BJ	0.937	40	48	86	85	82	86
Below normal range							
LF	0.137	2	42	74	87	30	84
KJ	0.114	0	18	92	87	50	82
SS	0.089	3	42	63	87	70	84
RW	0.185	9	32	72	80	63	84
GB	0.051	0	40	53	89	17	85

Surface expression of functional gp39 protein by activated T cells is correspondingly normal in five representative CVI patients with gp39 mRNA levels within normal control range and low in five representative CVI patients with gp39 mRNA levels below normal control range ( $< 0.26$ ). Expression of CD3, found on all T cells, and CD69, expressed by activated T cells, is shown for comparison. % +, Percentage of cells with positive binding of the respective chimeric or mAb minus binding of the appropriate control (given in *Materials and Methods*). Pt, patient; NC, normal control.

tive patients selected from each of the two groups, those with gp39 mRNA levels within ( $n = 18$ ) and those below ( $n = 13$ ) the normal control range (Table 1). Surface expression of functional gp39 protein by *in vitro* activated T cells, determined by CD40-immunoglobulin fusion protein binding, correlated with gp39 message expression in that it was low in patients with depressed (less than normal control range) gp39 gene expression and normal in those with normal (within normal control range) gp39 gene expression. Expression of another T-cell activation marker, CD69, was variable (normal in some, depressed in others) in CVI patients whose activated T cells had decreased gp39 expression, whereas CD69 was normally expressed by activated T cells from all CVI patients studied who had normal gp39 expression (Table 1). One example each of CVI patients with either depressed (LF) or normal (BJ) gp39 surface expression, along with their matched normal control, is shown in Fig. 3. Considering our earlier observations that CVI B cells proliferate and differentiate normally to stimulation via CD40 (16), these results suggest that in some CVI patients a T-cell defect resulting in abnormal gp39 expression, rather than an intrinsic B-cell defect, may play an important role in B-cell dysfunction.

**Statistical Analysis Comparing gp39 Gene Expression with CVI Patient Characteristics and Activated T-Cell Lymphokine Production.** gp39 gene expression by activated T cells from CVI patients significantly correlated with CD4/CD8 ratio ( $P < 0.001$ ,  $r = 0.6$ ) (Fig. 4a) but not with absolute CD4 number ( $P = 0.7$ ) or absolute lymphocyte count ( $P = 0.09$ ). There was no correlation between gp39 gene expression and immunoglobulin levels at diagnosis, patient age, or sex. In prior studies we explored *in vitro* lymphokine production by PHA/PMA-activated lymphocytes from the same patient population (15). Comparison of gp39 gene expression with gene expression and production of the T-cell-derived lymphokines evaluated (IL-2, interferon  $\gamma$ , IL-4, IL-6, and IL-2 receptor) revealed that gp39 mRNA levels significantly correlated with IL-2 mRNA levels ( $P < 0.002$ ,  $r = 0.6$ ) (data not shown) and with IL-2 production ( $P < 0.001$ ,  $r = 0.7$ ) (Fig. 4b), determined by cytotoxic T-lymphocyte cell line bioassay but not with any of the other lymphokines evaluated. Stepwise regression analysis with gp39 mRNA level as the dependent variable and CD4/CD8 ratio and IL-2 production as the independent variables revealed that IL-2 production had the highest correlation (partial  $F$  ratio was significant at  $P < 0.05$ ). gp39 expression was less than normal control range in the CVI patients who exhibited deficiencies of other lymphokines (interferon  $\gamma$  or IL-4 or both) in addition to IL-2.

## DISCUSSION

The finding that CVI B cells respond *in vitro* to stimulation with anti-CD40 and IL-10 or IL-4 (16) and that T cells from a subgroup of CVI patients if stimulated *in vitro* fail to produce adequate amounts of lymphokines (15) suggested to us that T-cell dysfunction resulting in depressed or inadequate contact-mediated B-cell activation may play a role in the failure of CVI B cells to differentiate appropriately into immunoglobulin-secreting plasma cells. To test this hypothesis, we took advantage of the recent identification of a receptor-ligand pair, CD40/gp39, shown to be important in T cell-B cell communication. mAb (17, 18, 20) and ligand-binding (28, 30, 31) experiments have shown that gp39-CD40 interaction plays a central role in B-cell proliferation, differentiation, and isotype switching. Anti-CD40 mAb (21-25), gp39-expressing fibroblasts (28, 30, 31), or soluble gp39 construct (30) in the presence of IL-4 can induce B cells to proliferate and produce IgE. Anti-CD40 mAb (16, 26) and soluble gp39 construct (45) in the presence of IL-10 can drive

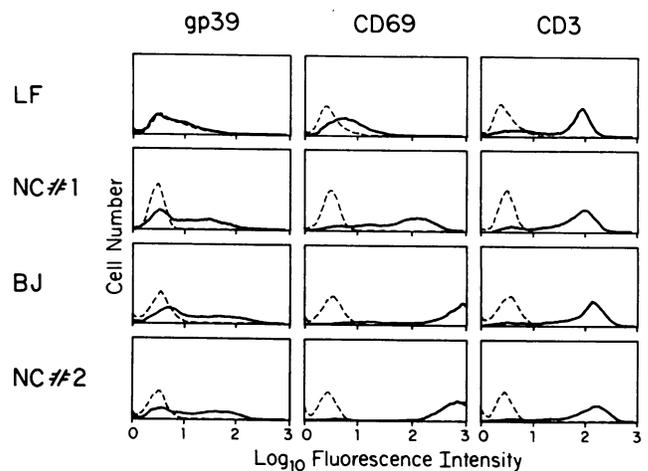


FIG. 3. gp39 surface expression by activated T cells is correspondingly decreased in a representative patient (LF) with depressed gp39 mRNA expression and normal in a representative patient (BJ) with normal gp39 mRNA expression. NC, matched normal control. Peripheral blood mononuclear cells were stimulated *in vitro* and stained, as described, with either CD40-immunoglobulin, CD69 mAb, or CD3 mAb (solid line) and a murine-human chimeric antibody (cL6) or goat anti-mouse antibody as controls for nonspecific binding (dashed line).

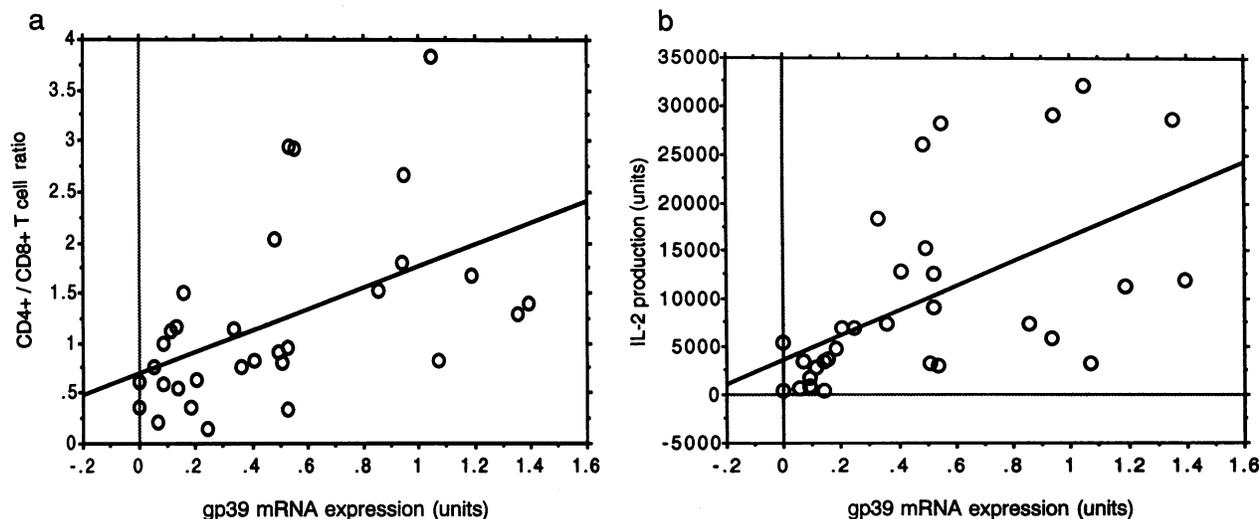


FIG. 4. (a) T-cell CD4/CD8 ratio significantly correlates with gp39 mRNA expression level, determined by RNA blot analysis, by PHA/PMA-activated lymphocytes from CVI patients ( $P < 0.001$ ,  $r = 0.6$ ). (b) PHA/PMA-activated CVI T-cell IL-2 production, measured by cytotoxic T-lymphocyte cell line bioassay, and level of gp39 mRNA expression, measured by RNA blot analysis, also correlate significantly ( $P < 0.001$ ,  $r = 0.7$ ). Stepwise logistic regression analysis revealed that IL-2 production correlated more highly with gp39 message expression ( $P < 0.05$ ) than with CD4/CD8 ratio.

committed B cells to differentiate into IgG-, IgA-, and IgM-producing cells. Because CVI B cells stimulated with anti-CD40 mAb in the presence of IL-4 proliferate and produce IgE normally (16), CD40 must be present and functional on CVI B cells. This observation also indicates that CVI B cells, similar to B cells from HIM patients (33), can mature into immunoglobulin-secreting cells if appropriate signals are provided, suggesting that B cells from most CVI patients are functionally normal. Several recent reports have shown that defective expression of functional gp39 by activated T cells is the molecular defect responsible for B-cell dysfunction in HIM (33–37). Considering the critical role of CD40–gp39 interaction in B-cell activation and differentiation, we examined in detail gp39 expression by activated lymphocytes from a large group of well-characterized CVI patients.

The levels of gp39 mRNA expressed by activated lymphocytes from CVI patients as a group were significantly lower than levels from normal control individuals and were below the normal control range in 42% of the patients studied. T-cell surface expression of functional gp39 protein, determined by binding of a soluble form of CD40 (CD40–immunoglobulin fusion protein), was correspondingly low in patients with reduced gp39 mRNA levels and normal in patients with normal gp39 mRNA levels. The nucleotide sequences of cDNAs encoding gp39 from two CVI patients, one with low and one with normal levels of gp39 mRNA expression (33), were identical to those reported for normal individuals (30, 31), indicating that CVI patients do not have a structurally defective gp39 protein, in contrast with HIM patients in which gp39 protein is structurally abnormal or absent (33–36). These data suggest that CVI patients with depressed gp39 expression by activated T cells, regardless of the underlying primary defect, may have abnormal *in vivo* antibody responses due to inefficient signaling via the B-cell surface receptor CD40.

CVI is a heterogeneous group of disorders that have in common a B-cell-differentiation defect, resulting in hypogammaglobulinemia and antibody deficiency. To explain the pathophysiologic basis for CVI, several mechanisms were proposed, including intrinsic B-cell abnormalities (3, 7, 8), the presence of T-suppressor cells (9–11), or abnormal T-cell-mediated B-cell help involving T-cell-derived lymphokines (12–15). The results of this study suggest that suboptimal

T-cell contact-mediated B-cell help via gp39–CD40 interaction, due to the depressed expression of gp39 by activated T cells, may contribute to the failure of B-cell differentiation and abnormal *in vivo* antibody responses in at least a subgroup of CVI patients. gp39–CD40 interaction is critical for isotype switching and clonal expansion, as demonstrated by its absence in patients with HIM. When immunized with the T-dependent antigen bacteriophage  $\phi$ X174, affected males with HIM have depressed antiphage antibody titer during both the primary and secondary responses and fail to switch from IgM to IgG isotype (45). The CVI patients included in this study all had significantly depressed production of antiphage antibody during the primary and secondary responses as well but differed in their ability to switch from IgM to IgG isotype. Most patients, 10 of 13 and 11 of 18 with depressed and normal gp39 expression, respectively, were unable to appropriately switch from IgM to IgG isotype during the secondary response. Potentially, a number of factors may be contributing to the observed B-cell dysfunction in these patients, including suboptimal signaling via gp39–CD40, an intrinsic B-cell defect or inadequate T-cell-derived costimulatory signals.

A number of different underlying molecular defects may be responsible for depressed gp39 expression by activated CVI T cells. Abnormally low gp39 expression may result from defective transcription or message stability of gp39, which, depending on the extent of the primary defect, may affect expression of one or more lymphokine(s) or other activation molecules as well. The hypothesis of variable T-cell defects in a subgroup of CVI patients is supported by the finding that activated lymphocytes from most CVI patients evaluated had depressed production of one or more T-cell-derived lymphokines (15). Further evidence for a broader T-cell defect in some patients is supported by the observations that decreased IL-2 production significantly correlated with depressed gp39 expression, that all patients with multiple lymphokine deficiency exhibited depressed gp39 expression as well, and that in some patients with poor gp39 surface expression, expression of another activation marker, CD69, was also decreased. The association between IL-2 and gp39 gene expression suggests that these two genes may share transcription regulation factor(s) that may be dysfunctional in a subset of CVI patients. Because CD4/CD8 ratio also correlated, but was not as predictive as IL-2 production as

determined by stepwise regression, with level of gp39 message expression, it follows that both may be regulated in such a way that they are predominately produced by CD4<sup>+</sup> T cells.

Our CVI patient population can be divided into two groups based on T-cell function assessed by lymphokine production and CD40 ligand expression. In our studies, 74% (23/31 patients) of the patients exhibit a T-cell defect, whereas the remaining 26% (8/31 patients) do not. Patients with T-cell dysfunction can be further subdivided into those with a broader defect ( $n = 11$ ), resulting in depressed gp39 expression and variable lymphokine deficiency and those with a more selective defect of either CD40 ligand expression ( $n = 2$ ) or deficiency of one particular lymphokine ( $n = 10$ ). We postulate that CVI arises from a number of different molecular aberrations that may include intrinsic B-cell abnormalities, broader defects of T- and/or B-cell activation, and more selective T-cell defects, resulting in abnormal expression of a particular surface molecule or lymphokine leading to dysfunctional B-cell proliferation, maturation, and/or differentiation. Further characterization of T- and B-cell function in these patients will eventually lead to the elucidation of the primary defects and point to additional diagnostic and therapeutic interventions for these patients, as well as contribute to our understanding of the workings of the immune system.

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