

# Impaired Responsiveness to B Cell Growth Factor in a Patient With Common Variable Hypogammaglobulinemia

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Common variable hypogammaglobulinemia (CVH) is a clinical syndrome that includes a diverse group of patients with heterogeneous defects resulting in impaired B cell proliferation and terminal differentiation into mature plasma cells capable of normal immunoglobulin synthesis and secretion. In this study, we report our identification of a previously undescribed intrinsic B cell defect in a patient with CVH. This patient's B cells showed a marked impairment in hemolytic plaque-forming cell (HePFC) formation compared with control B cells (15 v 80 HePFCs per culture, respectively). In addition, this patient's B cells displayed decreased B cell colony formation compared with control B cells ( $5 \pm 2$  v  $93 \pm 8$ , respectively). When examined for their responsiveness to phytohemagglutinin-T cell conditioned media (PHA-TCM), the patient's B cells displayed impaired B cell proliferation compared with control B cells

(stimulation index [SI]  $1.3 \pm 0.20$  v  $26 \pm 1.4$  with 20% control PHA-TCM [vol/vol]). Impaired proliferation by the patient's B cells persisted with increasing concentrations of B cell growth factor (BCGF). Additionally, PHA-TCM prepared from the patient's T cells when compared with control PHA-TCM consistently showed less support for control B cell proliferation (SI  $1.27 \pm 0.21$  v  $26 \pm 1.4$ , respectively). In coculture studies of B cell proliferation and immunoglobulin synthesis, patient's T cells showed no evidence of an enhanced suppressive effect or decreased helper effect. This patient's immune defects involve, first, an intrinsic B cell defect characterized by an impaired responsiveness to BCGF's proliferation signal and, second, impaired production of BCGF by the patient's T cells.  
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**C**OMMON VARIABLE hypogammaglobulinemia (CVH) is a disorder characterized by severe hypogammaglobulinemia involving all immunoglobulin classes.<sup>1</sup> The described defects leading to the severe hypogammaglobulinemia in this disorder include specific abnormalities in lymphoid cell differentiation, in cell-cell interaction, and in antibody biosynthesis—all the steps required for the normal humoral immune response. CVH is not a single disorder but a clinical syndrome resulting from a variety of immunologic defects.<sup>2-4</sup> In some patients the abnormality appears limited to the process of terminal B cell differentiation into plasma cells capable of normal synthesis and secretion of immunoglobulin. These patients may have normal numbers of circulating B cells but develop few or no plasma cells in tissue sites.<sup>5</sup> This arrested B cell differentiation may be secondary to an intrinsic B cell defect<sup>6,7</sup> or to extrinsic factors known to be capable of modulating B cell development.<sup>8</sup> Examples of the latter have included the demonstration of enhanced suppressor T cell activity,<sup>8-10</sup> defective helper T cell activity,<sup>11</sup> and the presence of serum inhibitors<sup>12,13</sup> in individual patients with CVH.

Intrinsic defects in B cell function in CVH have been more difficult to assess. In vivo and in vitro attempts at stimulation of these B cells using antigen<sup>14</sup> or mitogen<sup>15</sup> usually have failed to induce their proliferation or differentiation to mature immunoglobulin-secreting plasma cells. The creation of human-human B cell hybridomas using in vitro stimulated lymphocytes from patients with CVH may now allow for further dissection of this disease at the B cell level.<sup>16</sup>

Recently, T cell-derived lymphokines such as B cell growth factor (BCGF) with specific effects on immune regulation of B cell proliferation and differentiation have been identified.<sup>17-20</sup> BCGF is a lymphokine capable of delivering a signal to activated B cells to commit to and maintain a proliferative state. Human B cells appear to require separate signals for proliferation and differentiation. Both of these signals appear to be provided by lymphokines secreted by T cells. The potential importance of lymphokines in the generation of a normal immune response has been strengthened by the recent demonstration of decreased interleukin 1 and interleukin 2 production in patients with systemic lupus

erythematosus, a disease characterized by disordered immune regulation.<sup>21</sup> Defective interleukin 2 production and defective responsiveness to interleukin 2 have been identified in some patients with primary immunodeficiency disease.<sup>22</sup>

We report here the study of a patient with CVH whose abnormality in immune function appears to be the result of an intrinsic B cell defect manifested by an impaired responsiveness to the lymphokine BCGF. In addition, this patient appeared to have an impairment in BCGF production. No evidence for an enhanced T cell suppressive effect was noted in this patient.

## MATERIALS AND METHODS

One 29-year-old female with CVH and multiple healthy, age- and sex-matched volunteer donors were studied. Informed consent was obtained from both the patient and the volunteers.

The patient, previously healthy, presented at age 26 with three years of recurrent respiratory tract infections. Severe hypogammaglobulinemia was found. No serologic or biopsy evidence of autoimmune or lymphoproliferative disease was identified. Her peripheral blood counts (including B and T lymphocyte numbers) and bone marrow cellular morphology were normal. Her clinical course has been complicated by several bouts of *Hemophilus influenzae* pneumonia and bacterial meningitis.

Peripheral blood mononuclear cells were isolated from venous blood by Ficoll-Hypaque. Monocyte depletion was done by adherence in tissue culture flasks for 45 minutes at 37 °C in 5% CO<sub>2</sub> and the nonadherent cells were harvested. Nonadherent cells were then

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*Supported in part by the Veterans Administration Merit Review and National Institutes of Health grant No. AI 18160. R.T.P. is a Research Associate at the Veterans Administration Medical Center, Minneapolis.*

*Submitted Sept 10, 1984; accepted Feb 11, 1985.*

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*0006-4971/85/6602-0020\$03.00/0*

allowed to rosette with 2-aminoethylisothiouonium bromide (AET)-sensitized sheep erythrocytes (SRBC) at 4 °C for two hours. Rosetted cells ( $\geq 96\%$  T cells) were separated from the nonrosetted B and null cells ( $\geq 90\%$  sIg+) by two successive Ficoll gradients. SRBCs were removed by hypotonic lysis with distilled water. The population of double nonrosetted cells was used as B cells in the following procedures. T cell contamination in this nonrosetted B cell-enriched population was  $< 1\%$  as determined by OKT3 reactivity.

Phytohemagglutinin (PHA)-T cell conditioned medium (PHA-TCM) was prepared by incubating T cells with 1% PHA in growth media at 37 °C with 5% CO<sub>2</sub> for three days. Supernatants were then collected, filtered, and stored at 4 °C.

**B cell colony assay.** The B cell colony assay was performed as described previously.<sup>23,24</sup> In brief, the initiating B cells were suspended to  $2 \times 10^5$ /mL in the presence of  $3 \times 10^5$ /mL irradiated T cells in  $\alpha$ -MEM with 10% fetal calf serum (FCS; GIBCO, Grand Island, NY), 20% PHA-TCM, and 0.8% methylcellulose. After vortexing, 0.1-mL aliquots were placed into 6-mm flat-bottomed microtiter wells (Linbro, Hamden, Conn). Plates were secured tightly and incubated at 37 °C with 5% CO<sub>2</sub> for five to seven days, and colonies were enumerated microscopically. All experiments were done in triplicate.

**B cell proliferation assay.** B cell proliferation was studied using  $5 \times 10^4$  B cells in 0.2 mL  $\alpha$ -MEM with 10% FCS plus 1% (vol/vol) staphylococcal protein A (SPA; Sigma Chemical Co, St Louis) in the presence of varying concentrations of human BCGF (Cellular Products, Buffalo, NY) or PHA-TCM in 6-mm flat-bottomed microtiter wells. BCGF was prepared as previously described.<sup>25-27</sup> The purification scheme yielded a product free of immune interferon, interleukin 1, and interleukin 2. This BCGF supports both the short-term proliferation of activated human B cells<sup>25,26</sup> as well as the proliferation of human B cells in long-term culture.<sup>27</sup> Cultures were incubated for three days at 37 °C with 5% CO<sub>2</sub> and pulsed with [<sup>3</sup>H]-thymidine (New England Nuclear, Boston) (1  $\mu$ Ci per well) over the last 16 to 20 hours. Cells were harvested using a MASH II cell harvester (MA Bioproducts, Walkersville, Md) in H<sub>2</sub>O and counted in a Packard Scintillation Spectrometer (Packard Instrument Co, Downers Grove, Ill). Stimulation index (SI) was determined by dividing the cpm incorporated by cells stimulated by SPA plus BCGF or PHA-TCM by the cpm incorporated by cells stimulated by SPA alone.

**Surface membrane analysis.** Pooled cells were washed three times in PBS with 2% bovine serum albumin (BSA) and 0.2% sodium azide. Cells were suspended in  $\alpha$ -MEM ( $2 \times 10^6$ /mL), and 0.05 mL of this cell suspension was mixed with 0.05 mL of a 1:100 dilution of murine monoclonal antibody. Antibodies used included OKT3, OKT4, and OKT8 (Orthoclone, Ortho Pharmaceutical Corp, Raritan, NJ). The cell suspension and monoclonal antibody were incubated for 45 minutes in an ice bath. Cells were then washed three times in cold  $\alpha$ -MEM and resuspended in 0.1 mL  $\alpha$ -MEM. To this cell suspension was added 0.025 mL of fluorescein isothiocyanate (FITC)-labeled goat antimouse globulin (Cappel Labs, Cochranville, Pa). This suspension was incubated for 45 minutes in an ice bath. Cells were then washed three times in cold  $\alpha$ -MEM, resuspended in a minimal volume, and observed immediately for immunofluorescence.

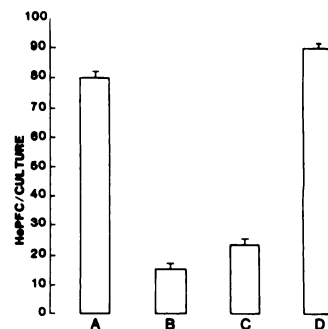
**Reverse hemolytic plaque assay.** For the generation of hemolytic plaque-forming cells,  $1 \times 10^6$  T cells and  $1 \times 10^6$  B cells were placed in 5 mL of  $\alpha$ -MEM and 10% FCS in 25-cm<sup>2</sup> tissue culture flasks (Costar, Cambridge, Mass) in 37 °C with 5% CO<sub>2</sub> for four to five days. Pokeweed mitogen (PWM) (Sigma) 10  $\mu$ g/mL was included during this culture period. A 0.025-mL mixture of packed SRBCs that had been complexed to SPA was mixed with 0.8 mL of 0.5% agarose, 0.025 mL antisera to human immunoglobulins with  $\mu$ ,

$\alpha$ , or  $\gamma$  heavy chain specificity (Cappel Labs), and 0.025 mL rabbit complement (Pelfreeze, Rogers, Ark) previously absorbed with SRBCs. To this, 0.1 mL of the four- to five-day-PWM-stimulated cell suspension was added, and 0.2 mL of the mixture was placed in a Petri dish and covered. The dishes were incubated for four hours at 37 °C, and the number of cells secreting specific immunoglobulins was identified by viewing the plates under an Olympus (New Hyde Park, NY) inverted microscope and identifying a zone of lysis around a single lymphocyte.

## RESULTS

The possibility that this patient's severe hypogammaglobulinemia was related to the presence of increased suppressor T cells was first examined. Both the patient's peripheral white blood cell (4,200/ $\mu$ L) and T cell (1,271/ $\mu$ L) counts were normal. The patient's peripheral blood T cells showed a decreased number of OKT4<sup>+</sup> cells (24%) and an increased number of OKT8<sup>+</sup> cells (46%). Normal values for OKT4 and OKT8 reactivity in our laboratory are  $51\% \pm 7\%$  and  $26\% \pm 5\%$ , respectively. Despite the increased number of T cells with suppressor cell phenotype, no evidence of enhanced suppression by the patient's T cells was demonstrable. Shown in Fig 1 is the effect of patient's T cells on autologous and allogeneic B cell immunoglobulin production in a reverse hemolytic plaque assay. Compared with control, the patient's B cells showed a marked impairment in plaque formation (Fig 1A and B) ( $P < .01$ ). The addition of control T cells to patient B cells did not significantly improve the patient's B cells ability to form plaques in vitro (Fig 1C). When patient T cells were mixed with control B cells, no evidence of an enhanced T cell-mediated suppressive effect was noted (Fig 1D). These findings suggest that this patient's severe hypogammaglobulinemia does not result from any increased suppressive effect mediated by her T cells, but from an intrinsic B cell defect.

Assays of B cell colony formation provided further evidence against the presence of an increased T-cell mediated

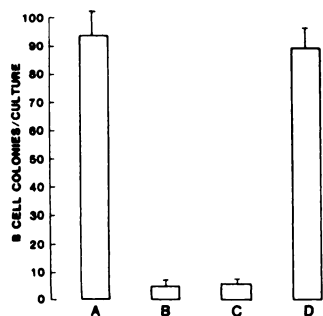


**Fig 1.** In vitro generation of hemolytic plaque-forming cells (HePFC) when autologous and allogeneic T and B cells from normal individuals and the patient with CVH were cultured with pokeweed mitogen as described in Materials and Methods. The following T cell and B cell combinations were cultured: (A)  $1 \times 10^6$  T cells and  $1 \times 10^6$  B cells from normal individuals; (B)  $1 \times 10^6$  T cells and  $1 \times 10^6$  B cells from the CVH patient; (C)  $1 \times 10^6$  T cells from normal individuals and  $1 \times 10^6$  B cells from the CVH patient; (D)  $1 \times 10^6$  T cells from the CVH patient and  $1 \times 10^6$  B cells from normal individuals. Data represent the mean  $\pm$  SEM of triplicate experiments.

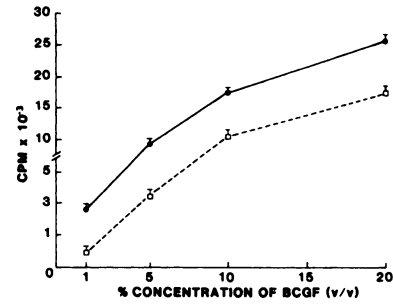
suppressive effect and confirmed an inherent B cell defect in this patient (Fig 2). The patient's B cell colony formation was markedly impaired compared with control B cell colony formation (mean  $\pm$  SEM,  $5 \pm 2$ ,  $\nu 93 \pm 8$ , respectively) (Fig 2A and B) ( $P < .01$ ). When control T cells were added, no enhancement in the patient's B cell colony formation was noted (Fig 2C). The addition of patient's T cells to control B cells did not significantly suppress control B cell colony formation (Fig 2D).

We next sought to further define the nature of the B cell defect present in this patient. We first studied the ability of this patient's B cells to respond to PHA-TCM in a B cell proliferation assay. After exposure to control PHA-TCM, there was a marked impairment in the proliferation capacity of this patient's B cells compared with control B cells (SI  $1.3 \pm 0.2 \nu 26 \pm 1.4$ , with 20% control PHA-TCM [vol/vol] [ $P < .01$ ]). The decreased responsiveness of this patient's B cells to control PHA-TCM remained consistent in our screening multiple lots of control PHA-TCM. This suggested the possibility that this patient's intrinsic B cell defect included an impaired responsiveness to BCGF. The ability of this patient's B cells to respond to purified BCGF was examined next (Fig 3). Over a wide range of purified BCGF concentrations, this patient's B cells showed a significant impairment in proliferative capacity compared with control B cells incubated with the same concentrations of BCGF ( $P < .01$ ). Neither control nor patient B cells showed significant proliferation in the presence of SPA 1% (vol/vol) or BCGF 10% (vol/vol) alone (data not shown).

As a control reagent in the B cell proliferation assay, patient PHA-TCM was prepared. Surprisingly, on multiple occasions, patient PHA-TCM preparations, when compared with control PHA-TCM preparations, consistently showed a marked impairment in their ability to support control B cell proliferation (SI  $1.27 \pm 0.21$ , 20% patient PHA-TCM [vol/vol]  $\nu 26 \pm 1.4$ , 20% control PHA-TCM [vol/vol], respectively [ $P < .01$ ]). These results suggest impaired BCGF production by this patient's T cells.



**Fig 2.** In vitro generation of B cell colonies when autologous and allogeneic T and B cells from normal individuals and the CVH patient were cultured as described in Materials and Methods. The following T cell and B cell combinations were cultured: (A) T cells and B cells from normal individuals; (B) T cells and B cells from the CVH patient; (C) T cells from normal individuals and B cells from the CVH patient; (D) T cells from the CVH patient and B cells from normal individuals. Data represent the mean  $\pm$  SEM of triplicate experiments.



**Fig 3.** SPA-stimulated B cell proliferation in the presence of varying concentrations of BCGF. Cultures ( $5 \times 10^4$  B cells per well) were incubated for three days with the addition of [ $^3$ H]-thymidine for the last 16 hours. Data represent the mean  $\pm$  SEM of triplicate experiments. Control B cells,  $\bullet$ — $\bullet$ ; CVH patient B cells,  $\square$ --- $\square$ .

DISCUSSION

CVH represents a heterogeneous group of disorders characterized by hypogammaglobulinemia that results from various defects in cell immune function.<sup>1-4</sup> These patients differ in the site of the block in differentiation of stem cells into mature immunoglobulin synthesizing and secreting plasma cells and in the pathogenesis of their defective immunoglobulin synthesis. A diversity of abnormalities in B cell function, T cell-B cell interaction and soluble mediators of B cell function have been implicated as the cause of the impaired B cell differentiation in various subgroups of patients with CVH. In this study, we describe another defect in B cell function in a patient with this disorder. Our patient's B cells display a marked impairment in responsiveness to the T cell lymphokine BCGF. In addition, there appears to be impaired production of BCGF in this patient.

The presence of an intrinsic defect in B cell proliferation was evident by the lack of formation of B cell colonies by this patient's B cells. This assay originally described by Izaguirre<sup>23</sup> and adapted by Perri and Kay<sup>24</sup> for the study of patients with chronic lymphocytic leukemia requires the presence of both irradiated T cells and PHA-TCM to support normal B cell colony formation. The defect in this patient's B cell colony formation was not improved by the addition of normal allogeneic T cells. The lack of improvement of this patient's B cell colony formation after the addition of normal T cells suggested the possibility that this patient's B cells were hyporesponsive to BCGF's proliferative signal and implied that defective T cell help by the patient's own T cells was not the apparent defect.

B cell proliferation has been shown to be a multiple step process,<sup>28</sup> initiated by activation of B cells. This activation process evidently is required for the increased expression of receptors for the T cell-derived lymphokine BCGF. Activated B cells expressing such receptors, when exposed to the second signal, BCGF, can then enter and maintain a proliferative state.

The inability of control PHA-TCM to stimulate proliferation in this patient's activated B cells further suggested that this patient's B cells are intrinsically unable to respond to BCGF. This was confirmed by the use of purified BCGF. The results described here do not distinguish BCGF resis-

tance due to diminished or absent surface membrane BCGF receptors or a postreceptor proliferative abnormality.

It is now known that human B cells differ in vivo in their state of activation and thus their responsiveness to BCGF.<sup>29</sup> Tonsillar B cells have been divided into two subpopulations simply on the basis of size. These two subpopulations of B cells differ in their sensitivity to the initial activation signal and the second proliferative signal delivered by BCGF. Larger tonsillar B cells could be directly stimulated by BCGF to proliferate without requiring an in vitro activation signal. The small tonsillar B cells required an initial activation signal before responding to the proliferation signal from BCGF. Pharmacologic agents are now beginning to be identified that have selective effects on the processes of B cell activation and proliferation that may prove useful in the future as tools for further dissection of abnormalities in various stages of human B cell activation, proliferation, and differentiation.<sup>30</sup>

The decreased responsiveness of this patient's B cells to BCGF might be expected to impair clonal expansion of B cell subpopulations when challenged with foreign antigens. This patient's immune defect appears complicated by impaired BCGF production by her T cells as well. Abnormalities in production of other T cell lymphokines (ie, interleukin 1,

interleukin 2) have been described recently in patients with systemic lupus erythematosus and primary immunodeficiency diseases.<sup>21,22</sup> Heterogeneity in defective T cell function resulting in decreased production of or responsiveness to interleukin 2 has been noted in children with Nezelof's T cell deficiency.<sup>22</sup>

In summary, the patient described in this study represents another disorder in immune regulation and function that is clinically manifested as CVH. This patient's immune defects involve, first, an intrinsic B cell defect characterized by an impaired responsiveness to the proliferation signal delivered by the T cell lymphokine BCGF and, second, impaired production of BCGF by the patient's T cells. No evidence of an enhanced suppressor T cell effect was noted in this patient. CVH remains a clinical syndrome that includes a diverse group of patients who have heterogeneous causes for their impaired capacity for B cell proliferation and terminal differentiation into mature plasma cells capable of normal immunoglobulin synthesis and secretion.

#### ACKNOWLEDGMENT

We especially thank Marti Dobson for her excellent technical assistance throughout this study.

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