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FAILURE OF SECRETION OF A HUMAN B CELL DIFFERENTIATION FACTOR BY PATIENTS WITH COMMON VARIABLE IMMUNODEFICIENCY

by

Kasim Kazbay

A dissertation submitted to the graduate Faculty in Biomedical Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

1993

This manuscript has been read and accepted for the Graduate Faculty in Biomedical Sciences in satisfaction of dissertation requirement for the degree of Doctor of Philosophy.

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ABSTRACT

Selective Cytokine Secretion Defect in Patients with Common Variable Immunodeficieny

By

Kasim Kazbay

Advisor : Lloyd Mayer

Common variable immunodeficiency is a heterogeneous disorder characterized by absent, low, or ineffective antibody secretion *in vivo.* To date, a number of *in vitro* defects have been described, including diminished circulating B cells, intrinsic B cell defects, absence of T cell help, and excessive T cell suppression. In this study B cells from a majority of patients (84%) responded *in vitro* to isolated B cell differentiation factors (BCDF) with normal or near normal Ig secretion. These data suggest that the defect in these patients may reside in the T cell rather than the B cell. We activated CVI and normal control T cells with various stimuli including PHA, anti-CD3 or anti-CD2 mAbs and measured T cell proliferation and cytokine secretion. Despite normal proliferative

responses to all stimuli, BCDF secretion was absent in 72 % of patients. Interestingly, all patients who failed to secrete BCDF, were capable of responding to BCDF *in vitro* by normal or even enhanced Ig secretion. BCDF deficiency existed alone or in concert with defects in IL2 and/or IL6 secretion. These findings suggest that defects in T cell activation resulting in abnormal secretion of potent BCDFs may underlie the immunodeficiency in CVI.

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INTRODUCTION

To understand immunodeficiency diseases, and especially the main subject of this thesis, common variable immunodeficiency (CVI), it is important to comprehend the normal development and function of the cells belonging to the immune system.

The immune system employs two broad classes of cells that specifically interact with antigens: B and T cells. B cells are the precursors of antibody secreting cells in the immune system. T cells consist of an array of different subpopulations that are involved in effector functions such as cytolysis and provision of help or suppression to other cells of the immune system.

In the introduction, I will first review the differentiation of T and B cells as it relates to a normal immune response, then identify defects in that process which may contribute to the development of immunodeficiencies and CVI. In the results section, I will first present a paper which we have submitted for publication defining defects in cytokine secretion by CVI patients' T lymphocytes. The data suggest that such defects are a major factor in the development of this disease. Then I will present the normal in vitro

response of T and B cells to different stimuli, cytokine secretion profiles of T cells and our attempts to by-pass the defects by IL2 treatment in vivo and by combination of stimuli in vitro in CVI patients.

LYMPHOCYTE DEVELOPMENT:

Lymphocytes, along with all the other blood elements, are derived from a population of primordial hematopoetic precursors, which are first detectable in the anterior end of vertebrate embryo (Butcher, 1989). Partially differentiated stem cells, committed to lymphoid differentiation, are first found in the yolk sac, then in the liver, but eventually lodge along with their pluripotent hematopoetic precursors in the bone marrow. The bone marrow contains the microenviroment necessary for hematopoesis and B cell development (Ford, 1968).

The functions of the thymus include the maturation and/or positive or negative selection of antigen specific T cells, and release of such cells into the periphery. During T cell maturation genes encoding the TCR γ and δ, or β and α genes rearrange and then are expressed on the T cell surface as receptor heterodimers.

These changes accompany the expression of CD4 and CD8 molecules. Monoclonal antibodies specific for CD4 and CD8 molecules are invaluable tools for studying T cell functions because, except in the thymus, the expression of these markers is mutually exclusive. Thus mature T cells express either CD4 or CD8 markers. Although the TCR repertoire of CD4 and CD8 cells appears to be very similar, they show different patterns of MHC restriction and functional properties. CD8 cells are generally restricted by class I molecules, whereas CD4 cells are class II restricted.

These surface markers also indicate the functional phenotype of the T cells. Earlier studies conducted in 1970s revealed that activated T cells can influence the growth and differentiation of other cells. Most of the T cells with helper functions express CD4+, whereas most of the T cells with suppressor or cytotoxic functions express CD8 molecules on their surface.

The other arm of the antigen specific immune system consists of B lymphocytes (denoting their origin in the bone marrow of adult mammals or the bursa of Fabricius in birds). Like T cells, B lymphocytes originate from pluripotent stem cells through multistep differentiation stages, beginning in pro-B cells with D-J joining in the immunoglobulin heavy chain genes, pre-B cells where μ chain but not the light chain are rearranged, virgin B cells with

surface IgM, mature B cells with surface IgM and IgD expression and finally antibody-producing plasma cells (Yancopoulos, 1986).

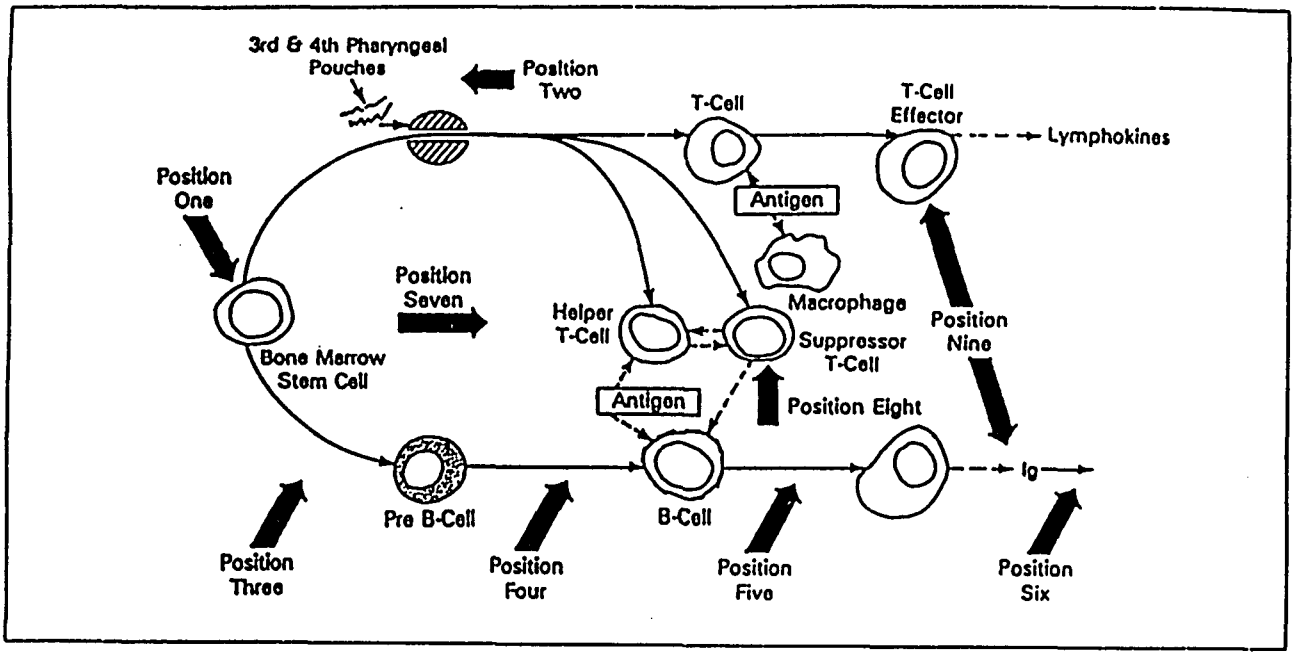

Figure 1 : Model of events in cellular maturation and cellular interactions required for a normal immune response. The arrows indicate defects in various immunodeficiency states.
Position 1: Failure in both B and T cell development, e.g. SCID. *Position 2:* Failure of development of the thymus, e.g. DiGeorge syndrome. *Position 3:* Failure of development of stem cells into pre-B cells, e.g. agammaglobulinemia. *Position 4:* Failure of maturation of pre B cells into peripheral B cells, e.g. X-linked agammaglobulinemia, *Position 5:* Failure of maturation of B cells into plasma cells. *Position 6:* Hypercatabolism of Ig. *Position 7:* Reduced helper T cells. *Position 8:* Increase in suppressor T cell activity. *Position 9:* Excessive loss of Ig and lymphocytes, e.g intestinal lymphangiectasia. CVI can be due to a defect in three positions at five, seven and eight in the figure, i.e. intrinsic B cell defect, reduced T helper activity, or increased T suppressor activity.

T AND B CELL COLLABORATION IN ANTIBODY RESPONSES :

Antigen-dependent activation of B cells into antibody secreting cells requires the presence of helper T cells. This was first demonstrated by adoptive transfer experiments; the transfer of neither thymocytes (T), nor bone marrow cells derived B cells alone into X-radiated recipient mice restored an antibody response against sheep red blood cells (SRBC). However, cotransfer of both T and B cells resulted in high anti-SRBC antibody responses (Nossal et al, 1968). T cells can modulate B cell differentiation either by the cytokines that they secrete or by cell:cell or cognate interactions.

a- Cytokines that play role in B cell differentiation :

In the mid 1970s the existence of T cell derived helper factors promoting B cell differentiation and antibody secretion as recognized. It was shown that anti-Ig crosslinking in the presence of T cell-conditioned medium could induce Ig secretion in B cells, indicating that antigen non-specific factors derived from T cells induced proliferation and Ig secretion of activated B cells (Kishimoto et al, 1975).

Further analysis of such supernatants in conjunction with the isolation and purification of lymphokines has allowed further definition of distinct B cell growth and differentiation factors. In 1976, T cell conditioned medium was found to include T cell growth factor activity (TCGF) which was later designated interleukin 2 (IL2) (Morgan et al, 1976). Further studies indicated that there were several other factors beside IL2 which may affect B cell proliferation and differentiation coordinately or separately. These factors can be categorized in three groups:

(a) factor(s) for the activation of resting B cells,

(b) factor(s) for the proliferation of activated B cells and

(c) factor(s) for the final differentiation of activated B cells into high-rate Ig-secreting cells.

IL4 is an example of a factor in the first group (Paul, 1987), IL5 the second, and IL6 the last group (Kishimoto, 1985). Recently, a novel factor was developed in this laboratory, called 446-BCDF. Different from previously described interleukins, it was shown to have potent B cell differentiation activity in pre-activated B cells. It is another example of the third group of factors (Sherris,1989).

The following are factors which have already been shown to affect B cell differentiation.

i- IL1 :

The discovery of IL1 was based on the ability of a macrophage product to act as a co-mitogen for thymocytes and lymphocytes (Durum, 1986). IL1 is now known to be a product of many cell types, not just macrophages.

IL1 affects B cells at two stages. In pre-B cells, IL1 induces maturation. In response to IL1, pre-B cells, which contain cytoplasmic Ig-μ chains, synthesize Ig light chains and express complete Ig on the plasma membrane (Giri, 1984).

At later stages, B cells can again respond to IL1. During antigen activation IL1 synergizes with other T cell helper factors, such as IL2, resulting in increased B cell proliferation and Ig production. Moreover indirect effects of IL1 on B cells could occur through the regulation of synthesis of secondary cytokines, as IL1 also augments production of IL2, IL4, IL5 and IL6, which control various stages of B cell activation.

ii- IL2 :

The first T-cell interleukin to be described, interleukin 2, was originally called T cell growth factor (Gillis, 1978). Use of HPLC

made it possible to purify the protein, and confirmed that IL2 is a protein of 15 to 19 kD (variation due to variable glycosylation). The availability of recombinant human and murine IL2 fostered a great deal of experimentation and confirmed the ability of this molecule to manifest a variety of immunostimulatory effects in vitro. Indeed, IL2 has been shown to enhance T cell proliferation and the generation of cytolytic T cells, induction of natural killer cell activity and in vitro induction of proliferation and Ig synthesis by activated B lymphocytes.

On the basis of these experimental findings, IL2 has been in clinical trials for some time used as an immunostimulatory agent for the treatment of variety of malignancies. IL2 therapy alone or in concert with some form of adoptive immunotherapy manifests an improved response in terms of enhanced remission rates in several previously untreatable malignancies, particularly renal cell carcinoma and malignant melanoma.

In vitro experiments also indicated that IL2 can greatly enhance Ig secretion from antigen, anti-IgM, or SAC activated B lymphocytes. From these observations the rationale for its use in the treatment of CVI where Ig secretion is defective was developed. IV and subcutaneous IL2 administration in CVI is still under

investigation in our clinic and preliminary results relating to the effects on B cells by this treatment will be discussed later.

Some of the initial enthusiasm for the use of IL2 as an antineoplastic agent was tempered by the relatively toxic side effects profile that result from IL2 therapy, such as capillary leak syndrome or hypotension.

iii- IL4 :

As a T cell derived cytokine, IL4 resembles IL2 in some aspects. Initially referred to as B cell growth factor (BCGF or BSF-1), early assays for this cytokine revolved around the ability of supernatants to enhance the proliferation of anti-IgM activated B lymphocytes. Confirmation that a novel T cell derived growth factor was in fact responsible for proliferation of activated B lymphocytes came from William Paul's laboratory in which a monoclonal antibody capable neutralizing its activity was developed (Howard et al, 1982). Soon after the development of the antibody, same group was able to purify the activity. It was found to be a 20 kD protein both in human and mouse, secreted by both T and mast cells, and responsible for a wide variety of effects in different cell types.

Besides being a growth factor for activated B lymphocytes, IL4 is also been found to upregulate Class II MHC and enhance the production of certain immunoglobulin isotypes (IgG1 and IgE) from activated B lymphocytes. Furthermore, it has also found to induce FcεRII receptor (CD23) expression on B lymphocytes, which has been characterized as a phenotypic marker for early B cell activation.

In addition, IL4 was found to synergize with IL2 in terms of enhancing the in vitro and in vivo killing of syngeneic tumor cells. In these experiments it was found that far lower doses of IL2 were needed to promote generation of tumoricidal activity, resulting not only in a beneficial anti-tumor effect, but also in the reduction of IL2 induced toxicity.

iv- IL5 :

Human BCGF distinct from IL4 was initially identified in the supernatants of a T cell hybridoma clone (Okada, 1983). Initially named BCGF-II due to its similarities to IL4, it was later named IL5 following its cloning (Kinashi, 1986). Interestingly, this molecule was found to be identical with a molecule described with eosinophil differentiation factor activity causing the rapid in vitro proliferation of eosinophils. In B cells, aside from its ability to induce proliferation, IL5 can induce B cell differentiation to IgA

secreting plasma cells, as well. It can also synergize with IL4 for the induction of IgE synthesis. IgE synthesis and overproduction of eosinophils typifies many immune responses to parasitic infections.

v- IL6 :

In addition to cytokines that promote proliferation of mitogen- or antigen-activated B lymphocytes, investigators had long been interested in purifying factors that affect B cell differentiation. Independently discovered for its antiviral activity as IFN-β2, hybridoma and plasmacytoma growth activities, hepatocyte stimulating activities and B cell stimulating activities, this cytokine was defined according to each activity, IL6 was eventually cloned by Kishimoto and his co-workers (Hirano, 1986) and found to be homologous with all of these other factors. It is the prototype of pleiotropic cytokines and falls in the family of proinflammatory cytokines including IL1 and TNF.

The 184 amino acid secreted protein has a predicted 21 kD molecular weight with the actual measured size of 23 to 32 kD depending on glycosylation, phosphorylation and sulphation.

IL6 can induce Ig production in EBV-transformed cell lines and in normal mouse B cells activated with pokeweed mitogen (PWM) in

the presence of irradiated T cells. Anti-IL6 antibodies can inhibit Ig secretion without significant effect on cell proliferation.

Several cell types including macrophages, T lymphocytes, fibroblasts, myeloma cells and a number of transformed cell lines can produce IL6. Monocytes were found to produce IL6 in the absence of any apparent stimulus in the culture medium, and the peak of IL6 could be achieved 5 hour following the culture initiation. The production of IL6 by T cells is dependent on monocytes, and peaks around 48 hours. It is thought that monocyte derived IL6 can activate T cells to secrete IL6 and IL2 and subsequently induce B cells to secrete Ig (Garman, 1987).

vi- IL10 :

Recently an important regulatory cytokine, cytokine-synthesis inhibitory factor, or IL10 has been cloned and characterized. This cytokine is secreted by the TH2 subset of mouse T cell clones. It has been shown to suppress IL2 and γIFN production by TH1 T cells, but enhances IL4 and IL5 production which trigger predominantly a humoral response. The fact that IL10 secretion by TH2 cells suppresses cytokine production by TH1 cells confers upon this cytokine a central role in regulating humoral and cell mediated responses.

IL10 can also directly affect B cells by enhancing proliferation and Ig secretion induced by IL4. It also upregulates MHC-Class II and FcγRI (CD64) expression on B cells.

vii- 446-BCDF :

Recent studies in our laboratory indicated the existence of a novel B cell differentiation factor secreted by T cells when they were stimulated with an anti-CD3γ chain mAb (mAb446), this cytokine was called 446-BCDF (Sherris, 1989). 446-BCDF is a potent stimulator of Ig synthesis in *Staphylococcus aureus* Cowan I (SAC) activated B lymphocytes.

This factor is different from previously described B cell differentiating factors by its chemical and functional characteristics. First, mAb-446 stimulated T cell supernatants depleted of IL2, IL4 and IL6 are still able to stimulate B cells to secrete Ig. Second, recently we have been able to generate mAbs against this factor which are able to block 446-BCDF activity, without affecting the activities of other cytokines. mAb-929 is one of these mAbs. We have been able to purify 446-BCDF from mAb-929 affinity columns although not in sufficient quantity to obtain an N-terminal sequence.

Cognate Interactions between T and B Cells Inducing Ig Secretion :

Beside the role of secreted factors derived mainly from activated T cells, recent data indicate that cognate interactions between T and B cells are also very important for B cell differentiation, especially for Ig isotype class switching (Mayer, 1987). It has recently been demonstrated that a defect in the CD40-ligand, gp39, may be the cause of defective class switching, which occurs in the X-linked hyper-IgM syndrome (DiSanto, 1993; Aruffo, 1993).

These findings indicate that binding to surface Ig on B cells is not sufficient to drive B cells to proliferate, or differentiate to plasma cells. T cells are involved in the control of every step of this process.

PRIMARY IMMUNODEFICIENCIES :

Any defect in the differentiation pathways of T or B cells, or communication between these cells may result in an immunodeficiency state depending on the nature and site of the

defect. Figure 1 illustrates the possible sites that can be defective leading to the development of specific immunodeficiencies.

Clinically immunodeficiency syndromes are characterized by an unusual susceptibility to infection and sometimes, autoimmune phenomena, allergy, anemia, thrombocytopenia, malabsorption, diarrhea, arthritis and lymphoreticular malignancies.

The types of infection often provide the first clue to the nature of the immunological defect. In general, patients with impaired humoral immunity have increased incidence of recurrent infections with high grade encapsulated bacterial pathogens, such as *Pneumococcus* or *Hemophilus influenzae*, leading to chronic sinopulmonary infections and meningitis. Abnormalities of T cells and thus cell-mediated immunity are predisposed to infection with a wider variety of agents, including viruses, particularly herpes simplex, varicella zoster, and cytomegalovirus; fungi, especially candida; and parasitic organisms, including the protozoan *Pneumocystis carinii*. Patients with defects in both T- and B-cells experience infections with all types of microorganisms.

DEFECTS IN THE HUMORAL IMMUNE SYSTEM :

Patients with hypo- or agammaglobulinemia may have defects at varying levels along the pathway of maturation from stem cells to Ig secreting plasma cells (Figure 1). For example, patients with severe combined immunodeficiency disease (SCID) do not have demonstrable pre-B cells; patients with X-linked agammaglobulinemia of Bruton usually have pre-B cells but cannot produce mature B cells; and most patients with CVI can produce B cells, but these cells do not differentiate into immunoglobulin producing plasma cells.

COMMON VARIABLE IMMUNODEFICIENCY :

CVI is a heterogeneous group of disorders having in common hypogammaglobulinemia, decreased ability to produce antibodies following antigenic challenge, an increased incidence of infections, and a tendency to develop autoimmune and lymphoproliferative diseases (Waldman, 1988). This term includes a number of syndromes with different pathophysiological defects. The patients in this category have a disease that is variable in time of onset as well as in clinical manifestations and immunological patterns.

CVI is associated with a high incidence of infections involving highly virulent encapsulated extracellular bacterial pathogens. Virtually all of the patients have a high incidence of acute and

chronic sinusitis, otitis media, and recurrent pneumonia, which may results in bronchiectasis. Respiratory failure is the principal cause of death. Patients also develop gastrointestinal abnormalities with diarrhea, giardiasis, nodular lymphoid hyperplasia and malabsorption.

A high proportion of the patients with CVI have associated with hematological disorders, especially autoimmune ones. Between 20-50 % of the patients are significantly anemic. The frequency of malignancy, especially lymphoid neoplasms are also higher than in the general population. Gastrointestinal abnormalities are also common in CVI. A sprue-like syndrome, characterized by steatorrhea, malabsorption of folate and vitamin B-12 and lactose intolerance has been reported in 5-10% of these patients.

The heterogeneity of clinical and immunological parameters in affected individuals suggests that CVI is not a single entity but rather consequence of more than one defect of the immune system. However, the key to the puzzle in most patients should be to understand the reason for the failure of B cells to undergo terminal plasma cell differentiation. A number of hypotheses have been proposed to explain the nature of this defect. Early studies suggested that the absence of antibody secretion was due to specific intrinsic B cell defects, either failure of B cells to mature *in vivo*

(Cooper, 1971; Ambrus, 1991), or failure to secrete assembled Ig (Ciccimara, 1976). Alternatively T cell defects may cause reduced Ig secretion either via active T cell suppression (Waldmann, 1974; Hoeger,1991; Durham,1987; Mayumi, 1989), or inactive or absent T cell help (Sneller, 1990; Geha, 1991; Spicket,1990) which would account for the failure of terminal B cell differentiation. Studies from our laboratory as well as others have indicated that the latter would be the case in the majority of the patients (Stohl,1988). Therefore, isolated CVI B cells may be incapable of responding to conventional B cell differentiation stimuli because they have not attained the appropriate responsive stage of maturation. This B cell immaturity is reflected in the predominance of IgM production when CVI B cells are successfully differentiated *in vitro.*

Using 446-BCDF as a model system we assessed the ability of patients with CVI to respond and secrete 446-BCDF. In our studies we analyzed thirty-two patients with an established diagnosis of CVI. Each patient has been studied on several occasions (at least twice and in some cases more than ten times) with reproducible results. Male to female ratio was 15+17 (0.88). The average age of the patients was 41.2 $\pm$15.3 ranging from 14 to 67 at the time of the study.

Recurrent infectious disorders including upper respiratory tract infections, sinusitis, otitis, pneumonia, empyema and urinary tract infections were the most common symptoms (66 %). Infectious problems were followed by disorders with autoimmune components like arthritis, juvenile rheumatoid arthritis, Crohn's disease, asthma, eczema, pulmonary fibrosis, hemolytic anemia, primary biliary cirrhosis (38 %). Gastrointestinal disturbances including diarrhea, sprue, malabsorption were also common (22 %). Hematologic problems such as anemia, Hodgkin's disease, neutropenia, pernicious anemia were also observed (19 %).

Serum IgG levels of the patients ranged from 594 mg/dl to undetectable (normal range 800-1800 mg/dl). Serum IgA levels were low in most of the patients with low normal levels in 3 of them. IgA concentrations of patients sera ranged from 202 mg/100ml to undetectable where 90-450 mg/ 100 ml accepted as normal range. Similarly serum IgM levels ranged from above normal to low or undetectable where in the majority of the patients the latter was the case.

T cell numbers were within the normal range (65-90%) in most of the patients. Data on B cell percentage was available in 25 of the patients (Table 1). Out of these 25 patients only 5 patients had low (1-0%) B cells compared to controls (2-15%). Whereas two patients

had high B cell percentage (30%). Remainder of the patients B cell were in the normal range (18 out of 25 = 72%).

In the following section I would like to present an article which we have recently submitted for publication. The data demonstrate near normal function of CVI B lymphocytes in vitro when an appropriate signal is provided, but defective cytokine secretion by same patients T cells indicating that the defects intrinsic to T cells rather then B cells are more likely be the underlying the cause of this disease. Following this paper I will present results of further experiments to demonstrate T cell response to CD3 stimulation, our attempts to bypass secretion defects in CVI patients T cells using combinations of several stimuli in vitro and IL2 in vivo.

FAILURE OF SECRETION OF A HUMAN B CELL DIFFERENTIATION FACTOR BY PATIENTS WITH COMMON VARIABLE IMMUNODEFICIENCY [1]

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Key Words: B Cell differentiation, Common variable immunodeficiency, Cytokines, T cell activation, B lymphocyte.

INTRODUCTION

CVI is a heterogeneous group of disorders having in common hypogammaglobulinemia, decreased ability to produce antibodies following antigenic challenge, an increased incidence of infections, and a tendency to develop autoimmune and lymphoproliferative diseases (1). The etiology and pathogenesis of this disorder is largely unknown, and the pathophysiologic mechanisms resulting in B cell dysfunction can be many fold.

The heterogeneity reflects the multiple pathogenetic mechanisms which have been proposed to explain the defects seen in this disorder. Early studies suggested that the absence of antibody secretion was due to specific intrinsic B cell defects (2,3), either failure of B cells to mature *in vivo* or failure to secrete assembled Ig (4). More recently, however, a number of groups have identified T cell defects [i.e. active T cell suppression (5,6,7), inactive or absent T cell help (8,9,10)] which would account for the failure of terminal B cell differentiation. Studies from our laboratory as well as others have suggested that the lack of appropriate T cell signals *in vivo* result in functional immaturity of B cells *in vivo* and *in vitro* (11). Therefore, isolated CVI B cells may be incapable of responding to conventional B cell differentiation stimuli because they have not attained the appropriate responsive stage of maturation. This B cell

immaturity is reflected in the predominance of IgM production when CVI B cells are successfully differentiated *in vitro.*

In vivo maturation of T and B cells is largely mediated by cytokines. Recent studies have suggested that cytokine defects do exist in CVI (8,12). Sneller and Strober documented that T cells from 4 CVI patients failed to make IL2, γ-IFN, IL4 mRNA upon stimulation with PHA (8). Part of the defect was reversible by co-culture with exogenous IL2. Geha and his colleagues reported two patients with specific cytokine defects that could account for the inability of their B cells to make Ig (13). Lastly, we have recently reported five CVI patients who received a long acting IL2 preparation, PEG-IL2, for a period of 12 weeks. After this therapy, B cells from these patients were capable of normal *in vitro* differentiation in response to both T dependent and independent stimuli (14), but more importantly, we could detect antigen specific responses of the IgG class. These data strongly suggest that defects in cytokine production by T cells may result in B cell immaturity *in vivo*, and resultant hypogammaglobulinemia found *in vivo.*

The process of B cell differentiation is complex and the cytokines regulating this process are incompletely understood. Both IL-4 and IL-6, potent differentiation factors in the murine system, are poor differentiation stimuli in man. IL-2 can augment Ig

secretion by activated B cells, but it acts more in a synergistic than primary fashion (15). Our group has described a novel human B cell differentiation factor, 446-BCDF, derived from anti-CD3 stimulated peripheral blood T cells (16). This 32kd, pI 6.0 cytokine, distinct from IL-2, IL-4 and IL-6, is a potent stimulator of Ig secretion from *Staphylococcus aureus* Cowan I (SAC) activated peripheral blood B cells. Using uncharacterized B cell differentiation factors derived from human T cell hybridomas, we had previously documented that CVI B cells could achieve normal or near normal levels of Ig secretion in greater than 50% of patients (17). In these studies we identified a series of patients who were supra-normal responders to BCDFs and who displayed evidence for increased receptor density for BCDF. These data suggested that a subgroup of CVI patients might reflect a defect in BCDF secretion coincident with enhanced BCDF sensitivity manifest by increased receptor expression. In order to address this more directly and determine whether BCDF secretion defects were confined to a small subgroup or reflective of a more global cytokine secretion defect, we assessed the ability of CVI B cells to respond to 446-BCDF and CVI T cells to secrete this cytokine. We found that all but two CVI B cells differentiated normally in response to 446-BCDF and that the majority of CVI T cells failed to secrete 446-BCDF. This defect was cytokine specific in some patients, in that B cell growth factor secretion by the same T cells was comparable to that seen in control T cells. In other

patients more global defects of IL2 and/or IL6 secretion were also detected. These data further support the concept that defects in cytokine secretion by CVI T cells result in the B cell defects seen in this disorder.

METHODS

Patients

Thirty-two patients satisfying the WHO criteria for CVI were recruited from the Primary Immunodeficiency Clinic at the Mount Sinai Hospital. The average age of the patients were 41.2 ± 15.7 and there was no significant gender difference. Characteristics of these patients including serum Ig levels and phenotypic lymphocyte markers are depicted in Table I. Several patients were analyzed on multiple occasions with reproducible results. Twenty seven age and sex matched controls were obtained from laboratory volunteers and leukocyte concentrate packs from designated donors in the Mount Sinai Blood Bank.

Cell Separation and Culture :

Heparinized blood samples were diluted 1:3 with PBS, and separated by Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, NJ) density gradient centrifugation at 400g for 30 minutes. T and non-T cells were further separated by rosetting 2-4 hours at 4°C with neuraminidase-treated sheep red blood cells (17). Non-T cells were enriched for B cells by depleting monocytes by plastic adherence for 1 hour at 37°C. T cells were more than 95% CD3

positive with less than 1% CD20+ cells and 1-4% CD14+ cells. B cells more than 70% CD20+ with 10-15% CD14+ cells. Resultant T and B cells were cultured in RPMI-1640 (GIBCO, Grand Island, NY) containing 2mM glutamine (GIBCO), 100 U/ml Penicillin/Streptomycin, and 10% heat inactivated fetal calf serum (FCS) (GIBCO), henceforth termed culture medium (CM).

T Cell Proliferation:

T cell growth was measured by culturing 10^5 PBMC per well in 100 µl of CM in triplicate microwell cultures (Falcon Labware, Lincoln Park, NJ). The cells were stimulated with either 1µg/ml PHA (GIBCO), anti-CD2 antibody (mAb-340, kind gift of W. Stohl), or anti-CD3 antibody (mAb-446 and mAb-454) from the supernatants of the hybridomas secreting these respective antibodies. mAb at a final concentration of 0.1 µg/ml was found to give maximum stimulation of normal cells and was used at this concentration throughout the studies. [Both anti-CD3 antibodies, mAb-446 and mAb-454, were previously shown to induce normal T cell proliferation (16)]. In preliminary studies, stimulated PBMC cultures were pulsed every 24 hours from 24 to 144 hours. Optimum growth (i.e. highest counts in stimulated and lowest background counts in unstimulated wells) was observed at 48 hours. Thereafter all stimulated cultures were pulsed at 48 hours with 1 µCi ^{3}H-Thymidine (ICN Radiochemicals, Irvine, CA) per well for 6 hours. The cells were then harvested onto

filter mats and bound thymidine was counted in a Beta-counter (Beckman, LS 3801, Somerset, NJ), and averages of the triplicate cultures determined.

In Vitro B Cell Maturation :

Proliferation :

Monocyte-depleted B lymphocytes (10^5/well) were cultured in triplicate in 96-U bottom microtiter plates in 100 µl CM in the presence or absence of either 0.001% v/v fixed *Staphylococcus aureus* Cowan I strain organisms (SAC) (Calbiochem, La Jolla, CA) and IL2 (20 U/ml) (Boehringer-Mannheim, Germany), SAC and LMW-BCGF (5% v/v) (Cellular Products, Buffalo, NY), 10% v/v mAb-446 stimulated T cell supernatants, or medium alone (background control) for 72 hours. The triplicates were pulsed with ^3H-Thymidine (1 µCi) for 10 hours, and thymidine incorporation measured as discussed above.

Differentiation :

10^6 B cells from CVI or normal donors were cultured for 8 days in 1.5 ml CM in the presence of either SAC+IL2, SAC+mAb-446-stimulated-T cell supernatant, or medium alone. At the end of this period, cell free supernatants were collected and tested for total Ig secretion using an ELISA assay as previously described (16)

Generation of T Cell Supernatants and Cytokine Assays:

PBMC, cultured in 24-well culture plates (Linbro, Flow Laboratories, McLean, VA) at a concentration of 10^6 cells per well in 1.5 ml CM, were stimulated with either PHA (1µg/ml), mAb-446 (0.1 µg/ml), or medium alone. Cell free supernatants were collected at 14 hours for measurement of IL2 , and at 48 hours for measurement of BCGF, 446-BCDF and IL6. mAb-446 stimulated cultures contained no IL2 (Data is not shown). Supernatants were either tested immediately, or kept frozen at -20°C until tested.

Assessment of Cytokine Secretion Profiles :

BCGF Secretion:

10^5 monocyte depleted normal B cells were cultured in U-bottom microtiter plates in the presence of PHA, 446 or unstimulated T cell supernatants (10% v/v) derived from CVI or normal donors for 72 hours. At the end of this period, B cell proliferation was measured by ^{3}H-Thymidine incorporation as described above. Stimulated cultures were compared with the wells that contain B cells alone (background control), or with B cells stimulated with SAC+IL2 as a positive control. Stimulation index was determined by the ratio of CPM experiment / CPM medium control.

446-BCDF Secretion :

BCDF activity within the stimulated T cell supernatants was measured using normal B cells as the responder population. 10^6 B cells were cultured in 1.5 ml CM in macrowells in the presence of PHA, mAb-446 stimulated, or unstimulated CVI or normal T cell supernatants (10% v/v). The cultures were kept at 37°C, in 5% CO_2 humidified incubators for 8 days, after which cell free supernatants were collected. SAC+IL2 or partially purified 446-BCDF were used as positive controls. Unstimulated B cells in CM were used as background controls. Secreted Ig is measured in the B cell cultures using the ELISA assay described previously and represented as secreted Ig or stimulation index as described above. A stimulation index of 2 was considered the lower limit of normal based on our previous studies (16).

IL-2 Assay :

The murine cytolytic T cell line, CTLL, was utilized to measure IL2 as previously described (18). rIL2 (Boehringer-Manheim) was used as a standard in each assay. Varying dilutions of T cell supernatant were tested for each sample. A concentration of more than 2.3 units/ml was considered as the lower limit of normal based on the distribution of the control group. Specificity of the response was defined by addition of anti-IL2 mAbs DMS-2 and DMS-3.

IL-6 Assay:

The murine B cell hybridoma line B9 was used as an indicator cell for IL6 as previously described (19). rIL6 (kind gift of Dr. Edward Siden) was used to generate a standard curve in each assay. A concentration of 6000 units/ml was considered as the lower limit based on the distribution of the control group. The specificity of the response was defined by inhibition with a rabbit polyclonal anti-IL6 Ab (Amgen, Thousand Oaks, CA).

RESULTS

T Cell Proliferation :

Responses of CVI T cells to mitogen and anti-CD3 stimulation are comparable to controls.

Figure 2 demonstrates the distribution of PBMC responses to PHA, anti-CD3 and anti-CD2 stimulation in normal and CVI patients. As seen in each group there is a wide variation in response to each stimulus. While there was a trend towards a lesser response to PHA in our patient group, no difference in response was seen with either anti-CD3 or anti-CD2 stimulation. Importantly, the distribution in responses in CVI was comparable to controls and did not show any clear low or high responding group. The absence of observed differences would also argue against contaminating suppressor cell (T cell or monocyte) populations.

Response of CVI B cells to growth and differentiation factors :

Ambrus et al had previously reported that while CVI B cells proliferate normally in response to LMW-BCGF, a subset of patients

fail to proliferate in response to HMW-BCGF (3). We assessed the ability of our patients' B cells to be activated by LMW-BCGF and by a distinct B cell growth factor, 446-BCGF, which activates resting B cells (16). Only 6/24 failed to proliferate in response to LMW-BCGF (Figure 3), consistent with the findings of Ambrus et al, and only 3 patients failed to proliferate in response to 446-BCGF. These nonresponder patients may represent true intrinsic B cell defects. The difference did not achieve significance comparing CVI patients to controls. As alluded to on Figure 2 the absence of a difference between controls and patients speaks against the presence of suppressor cells. Although proliferation is not an absolute prerequisite for differentiation, these data suggest that CVI B cells are apparently responsive to conventional growth stimuli and that their failure to differentiate *in vivo* does not reflect a defect in B cell growth.

Similar results were observed when CVI B cells were tested for their ability to differentiate in the presence of 446-BCDF. The data in Figure 4 are depicted in two forms; Figure 4A represents the absolute total Ig secretion by CVI and control B cells in response to 446-BCDF. Stimulated Ig secretion was generally lower in patients when compared to controls and this is reflected in an overall lower mean Ig concentration. However, spontaneous secretion of Ig was also significantly lower in patients than controls (Figure 4 legend),

so we chose to express the data as fold increase (stimulation index) in Ig secretion (Figure 4B). When analyzed in this manner, the mean of the response of CVI B cells to 446-BCDF was actually higher than controls suggesting a normal to increased response (although this did not achieve statistical significance). In fact two patient populations emerged from this analysis; one without an appreciable response to BCDF (intrinsic B cell defect) and those with a normal response (? T cell defect). The former group was composed of only 4 patients, a clear minority, with 84% of patients falling into the latter group. Even in the control group, 4 B cell preparations failed to secrete Ig in response to 446-BCDF. Since previous studies have demonstrated clear immaturity of CVI B cells with predominance of IgM secretion upon stimulation, we measured IgG and IgM in these cultures. 446-BCDF is a polyclonal BCDF but is potent stimulator IgG secretion in contrast to PWM. As seen in figure 5, CVI B cells from 91 % of the patients secreted IgG (absolute antibody concentration) and 18 % secreted greater amounts than that seen in the control group. Interestingly IgM secretion was low which is probably more reflective of 446-BCDF stimulation rather than any intrinsic B cell property. In this analysis we were able to define BCDF hyper responders as previously described in 4/23 patients. Thus consistent with our previous reports, the overwhelming majority of CVI B cell preparations can respond normally to appropriate differentiation stimuli with a mature isotype secretion pattern.

Having documented that both total Ig and IgG secretion of the patients as response to 446-BCDF were comparable to the normals, we next tested their ability to secrete specific antibodies. For this purpose we measured tetanus toxoid specific in vitro IgG secretion, since all the patients were immunized for tetanus infections as a routine prophylaxis. As seen in figure 6, only 3 patients failed to secrete tetanus toxoid specific IgG (7%). Remaining of the patients were either able to secrete it constitutively (15%) or as response to 446-BCDF stimulation. Interestingly, of the five controls, none of them secreted without stimulation and 2 of them actually did not secrete TT specific antibody even when they were stimulated with 446-BCDF. However, we had no information regarding blood bank donors' vaccination state as we had for the patients, therefore we do not know whether the controls were supposed to respond.

Secretion of cytokines by CVI and normal T cells :

Given these findings, one plausible scenario is that, in CVI, T cells fail to secrete appropriate cytokines to allow for terminal differentiation. Therefore T cells were stimulated with either PHA, anti-CD3 mAb, or medium alone for 14-48h, and cell free supernatants were screened for their ability to stimulate normal B

cell growth and differentiation, as well as, for IL2 and IL6 secretion.

As seen in Figure 7 no BCGF activity was detected in either PHA stimulated or unstimulated T cell supernatants from either patients or controls. The lack of BCGF in PHA stimulated cultures may reflect kinetics of secretion (more than 48h) or a lower concentration of PHA from that previously used. However, significant BCGF activity was detected in mAb-446 stimulated T cell supernatants, and BCGF secretion in the CVI-TCS was comparable to that of controls.

However, there was a clear difference when BCDF activity was measured in these same supernatants. Two groups emerged from this analysis. T cells from 23/32 (72%) patients failed to secrete BCDF whereas only 2/27 control TCS had no detectable BCDF activity (Figure 8). These findings were consistent in each patient retested at multiple time points. The lack of BCDF secretion was not reflective of therapy, antibiotic use, infection etc. Interestingly, there was a correlation between lack of BCDF secretion and a heightened response of the patients B cells to 446-BCDF. This was best illustrated by analyzing single patients (Table 2). Two patients and a normal control were tested in parallel. Patient LMC's T and B cells proliferated normally to anti-CD3 mAb-446, and LMW-BCGF and

446-BCGF, respectively. However, her response to BCDF was markedly increased over background (694.1 fold increase) and greater than the control (33.4-fold increase). mAb-446-stimulated LMC T cells secreted low levels of BCGF, low-normal levels of IL2 with PHA (1.61 U/ml), markedly diminished IL6, and no BCDF. This profile of hyper-response to 446-BCDF and decreased cytokine secretion was reproduced on the two occasions when that patient was tested The dichotomy between the defects in the secretion of cytokines and normal B cell responses suggests that the defect in this patient lies within the T cell. On the other hand, patient CV had normal secretion of all the cytokines tested including BCDF, but her B cells did not respond to BCDF at all, implying that her defect is more likely intrinsic to the B cell.

We next elected to measure other T cell derived cytokines to more adequately assess the selectivity of the 446-BCDF secretion defect. IL6, a B cell differentiation factor in mouse although its effect in human is less clear, has been reported to be increased in the serum of patients with CVI. This increase has been proposed as a compensatory mechanism for the lack of differentiation of B cells (20). When compared to normals, anti-CD3 stimulated IL6 secretion was lower in 14 out of 32 patients (44%), and higher in only 4 patients (13%) (Figure 9). IL6 was compared with secreted BCDF production (Figure 10). From this analysis IL6-low patients

appeared to be a sub-group of 446-BCDF deficient patients, since IL6 deficiency was seen only in the patients who were already deficient in 446-BCDF. There were no patients with normal BCDF and low IL6 secretion (empty upper right quadrant of Figure 9). The patients who had elevated IL6 secretion (4/32) had decreased BCDF production. However, since IL6 can be generated by both T cells and monocytes, our experiments do not address from which cells the IL6 originates.

Lastly, since IL2 is a critical cytokine for T cell growth and differentiation, and IL2 production defects by CVI T cells have been previously documented (8,21,22), we measured its production after PHA stimulation. We have previously shown that mAb 446 stimulated T cells fail to generate IL2 mRNA or protein, therefore its absence in these supernatants would not be informative. As seen in Figure 11, 66 % of CVI T cells failed to secrete IL2 in response to PHA, despite normal proliferative responses to PHA. However, there was no clear correlation between the IL2 secretion defect and lack of BCDF secretion. There were patients who secreted IL2 but failed to secrete BCDF, and vice versa, suggesting that these cytokines are not coordinately regulated.

INITIALS	AGE	SEX	SYMPTOMS AND SIGNS	IgG*	IgA*	IgM*	T%°	B%°	T4/T8°
TL	20	M	Recurrent pneumonia,septic arthritis age3	594	68	13	75	7	ND
SK	56	M	Pneumonia, empyema, history of hemolytic anemia	45	7	3	88	9	1
PK	48	F	Arthritis, sinusitis	576	80	100	75	ND	2
CC	40	M	Recurrrent sinusitis, otitis	245	0	0	ND	ND	ND
MAL	42	F	Malabsorption,neutropenia,hemolytic anemia	8	5	0	90	0	1.45
LE	14	M	Recurrent sinusitis, JRA, s/p Hodgkin's disease	486	0	27	50	30	1.65
MC	35	M	Recurrent pneumonia	66	15	24	79	0	3.02
UMC	65	F	Recurrent URI	320	94	121	71	13	3.7
CT	27	F	Pneumonia	48	7	6	78	5	2
CV	56	F	Duedenal ulcer, diarhea	540	23	36	91	12	1.59
NK	35	M	Asthma, bronchiectasis, Kleinfelter's syndrome	356	8.4	18.9	ND	ND	ND
UH	26	F	Diarrhea, nodular lymphoid hyperplasia	245	10	63	75	11	1.7
JC	66	F	Pernicious anemia, malabsorption	110	3	45	70	15	3.38
MJ	38	M	Hodgkins, primary biliary cirrhosis	253	22	131	80	5	0.78
RH	33	M	Small airways disease	171	202	217	65	9	1.4
DM	36	F	Sinusitis, pelvic abcesses	351	8	12	81	4	0.75
HW	48	M	Recurrent sinusitis, Crohn's disease	196	155	26	39	1	0.8
VK	35	M	Bronchiectasis	195	15	26	78	2	2
BR	47	F	Recurrent sinusitis	275	55	90	70	ND	1.6
MK	41	M	Neutropenia	225	0	113	90	5	0.6
RC	50	M	Chronic lung disease, anti-IgA antibody, s/p splenectomy	554	0	28	58	9	0.63
LF	51	F	Pneumonia, history of renal failure	269	72	130	83	8	1.14
CB	15	F	Recurrent pneumonia, diarrhea	90	0	400	ND	ND	ND
AN	67	M	Bronchiectasis	244	3	4	87	1	ND
LC	25	F	Recurrent URI, pneumonia, otitis	180	8	19	85	10	1.8
AM	55	F	Asthma, eczema, restrictive lung disease	0	0	0	38	5	1.5
DL	28	F	Pulmonary fibrosis, granulamatous hepatitis	246	10	46	65	5	ND
AS	66	F	Recurrent sinusitis	371	20	15	ND	ND	ND
JM	20	F	Muco-cutaneous candidiasis, bronchiectasis, malabsorption	402	38	110	61	30	0.72
VDS	57	M	History of hepatitis	145	7	30	64	14	0.45
LG	49	F	Asthma, arthritis, cardiomyopathy, pneumonia	140	75	35	64	1	0.54
MS	29	M	Sprue	435	59	115	ND	ND	ND

TABLE 1: Patients and their clinical presentation. Each of the 32 patients have been studied at least twice during their regular clinical visits. (Male/Female=15/17, average age=41.2 ± 15.3).

* Immunoglobulin levels (mg/dl) at the time of diagnosis, prior to treatment with IVGG. Normal range for IgG=800-1800 mg/dl; IgA=90-450 mg/dl; IgM=80-350 mg/dl.

° Lymphocyte populations are detected by staining with anti-CD3 mAb for T cells and anti-CD20 for B cells, anti-CD4 for helper T cells, anti-CD8 for suppressor/cytotoxic T cells. Normal range for T cells (CD3)=65-90%; B cells (CD20)=2-15%; T4/T8 (CD4/CD8)= 1.0-3.0.

ND : Not done.

ASSAY	STIMULUS	LMC	CV	CONTROL
T Cell Proliferation (S.I.)	*PHA*	401.2	285.5	57.5
	Anti-CD3	188.1	154.4	44.2
	Anti-CD2	113.3	12.7	27.6
B Cell Proliferation (S.I.)	*LMW-BCGF*	6	ND	7.4
	446-TCS	4.1	ND	11.3
B Differentiation (S.I.)	*446-BCDF*	694.1	0.8	33.4
BCGF Secretion (S.I.)	*PHA*	2.3	2.6	0.8
	mAb-446	1.6	6.9	4.8
	Unstim.	0.9	1.7	0.8
BCDF Secretion (S.I.)	*mAb-446*	0.6	11.4	12
IL2 Secretion (U/ml)	*PHA*	1.61	3.57	4.17
IL6 Secretion (U/ml)	*mAb-446*	831	9146	15009

Table 2 : Functional profiles of patients and control. Two patients (LMC and CV) represent example of selective T and B cell defects, respectively. A normal control that was tested together with these patients with the data shown in the third column for comparison. (N.D.=Not determined; S.I.= Stimulation Index) Each assay has been defined in methods.

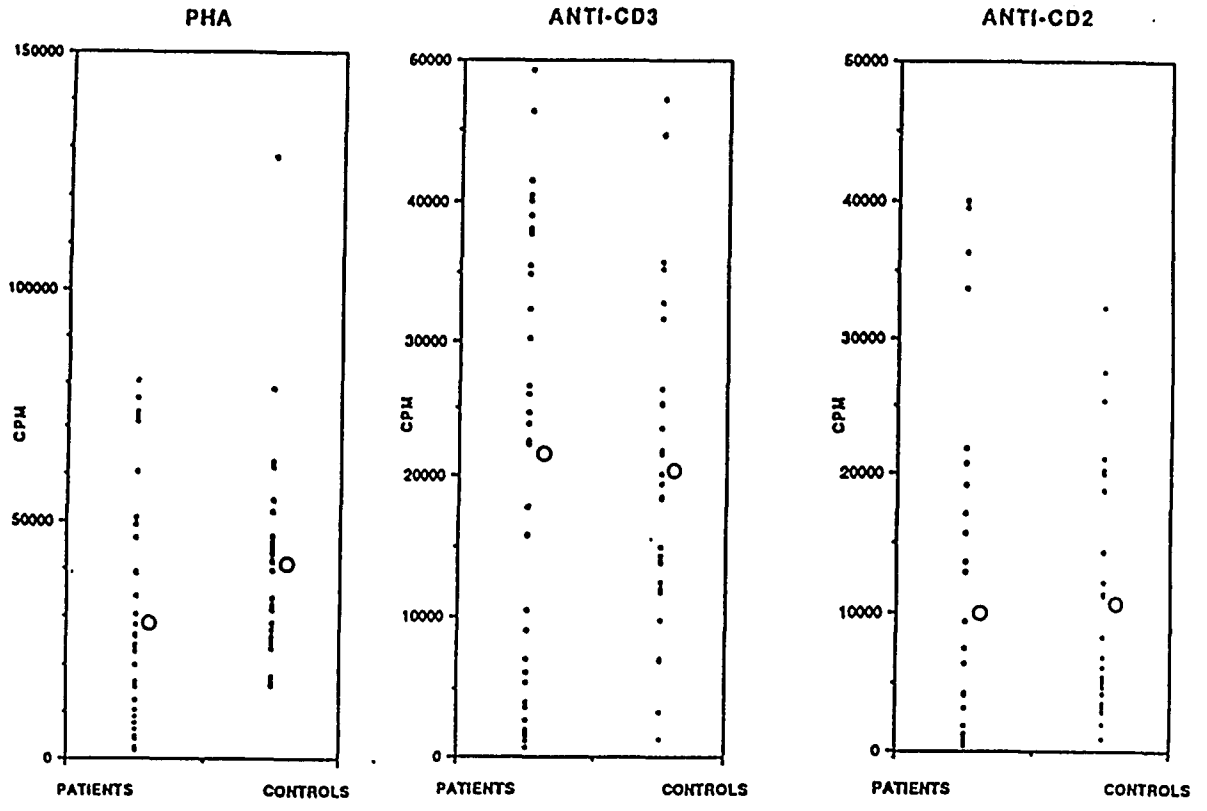

Figure 2: T cell proliferation as response to PHA (1 µg/ml), anti-CD3 (0.1 µg/ml) or anti-CD2 (10 µg/ml) stimulation. PBMC were stimulated for 48h and pulsed with ^{3}H-Thymidine for 6 h. Individual patients (▪) or controls (▪). The mean of the population is represented by the open circle (O). Each patient point represents experiments performed at least twice.

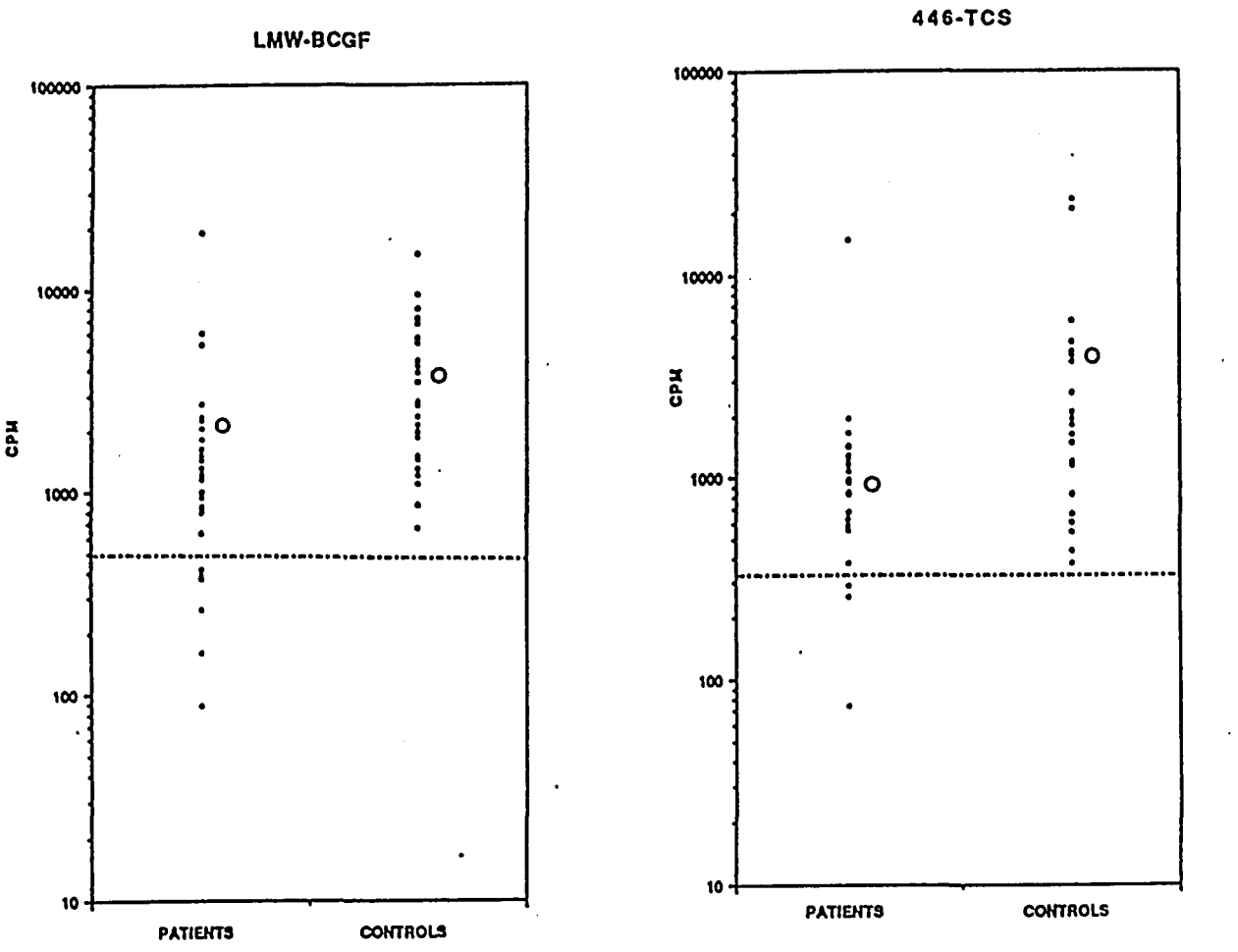

Figure 3: B cell proliferation in response to LMW-BCGF or 446-TCS. B cells were stimulated and pulsed as described in methods. Although the mean (O) was lower for the patients, this did not achieve statistical significance. Lower limits of the normal were 500 cpm for LMW-BCGF response and 300 cpm for 446-TCS as determined by ±2 SD in the normal control group. Background B cell proliferation for controls and patients was always less than 100 cpm. Each patient point represents the results of at least 2 experiments.

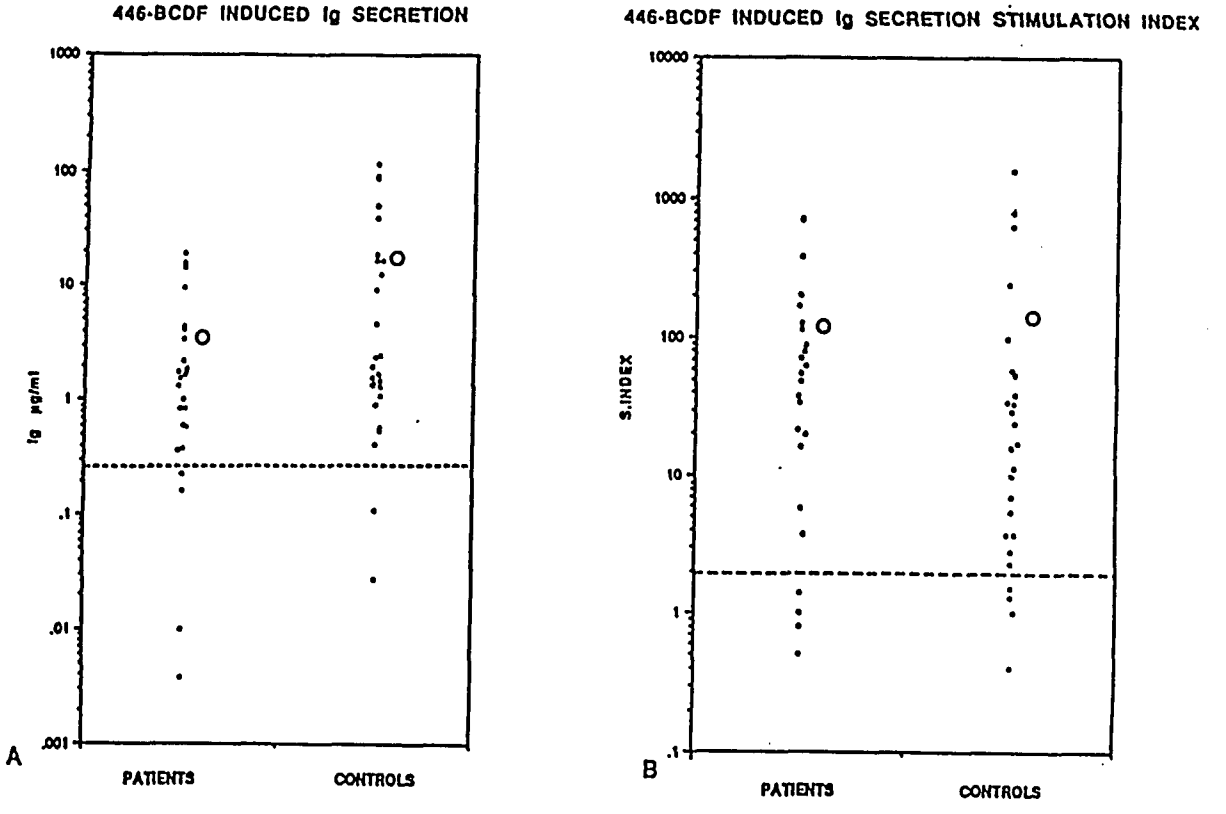

Figure 4: B cell differentiation measured as 446-BCDF induced Ig secretion. The average absolute Ig secretion (**O**) was lower in the patient group (not statistically significant). However, when the stimulation indices (446-BCDF induced Ig secretion/baseline Ig secretion) were computed this difference disappeared as baseline Ig secretion was much lower in the patient group. The lower limit of response was (dotted lines) chosen as the 95th percentile of the normals for Ig secretion and a minimum of two-fold increase over baseline Ig secretion for stimulation index. Each point represents at least 2 experiments per patient.

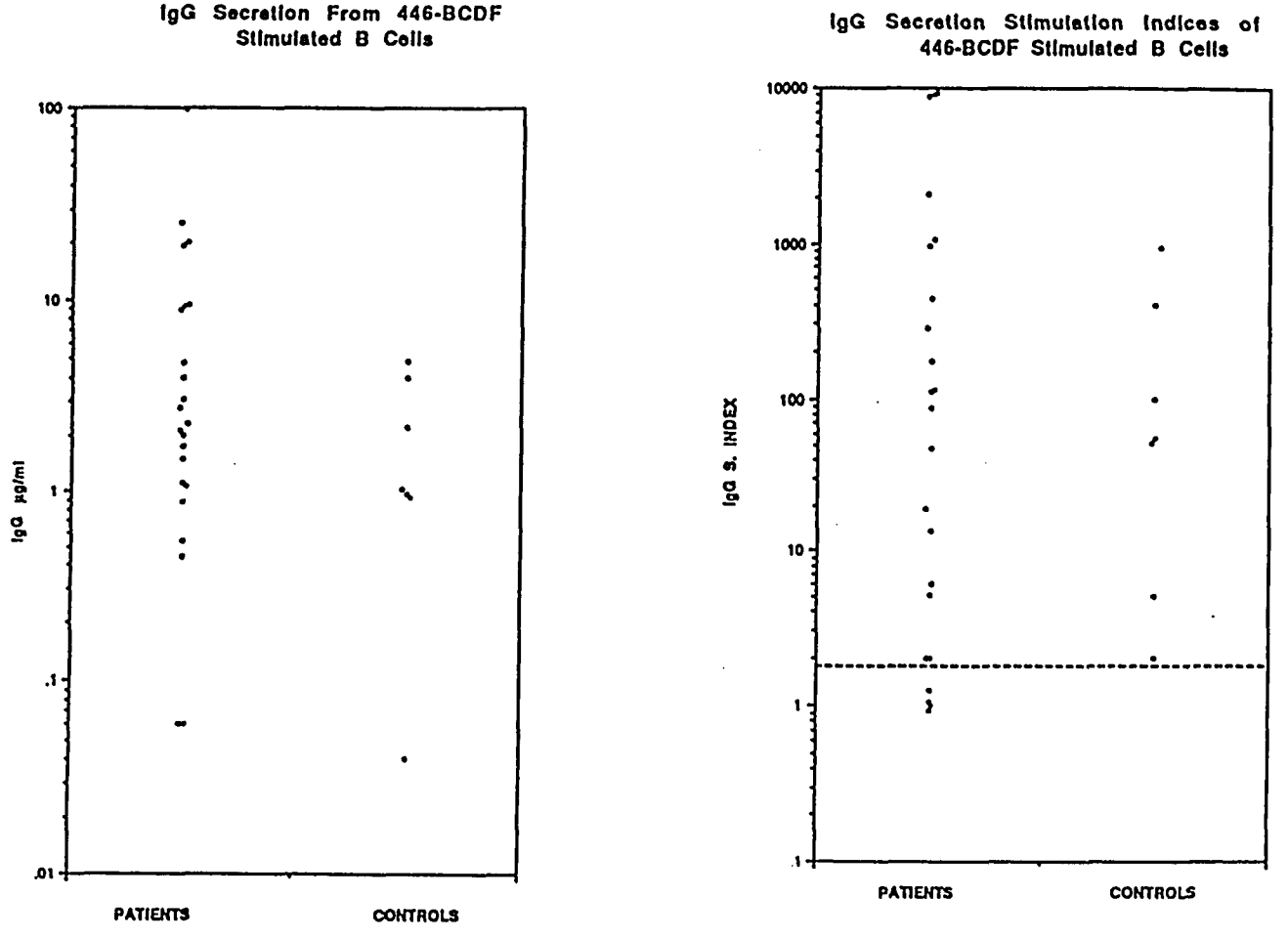

Figure 5: IgG secretion by 446-BCDF induced B lymphocytes. Both absolute amount of IgG secreted and stimulation indices (446-BCDF induced IgG secretion / baseline IgG secretion) were comparable to the controls. The lower limit for the stimulation index (dotted lines) was chosen as the lowest limit of the controls.

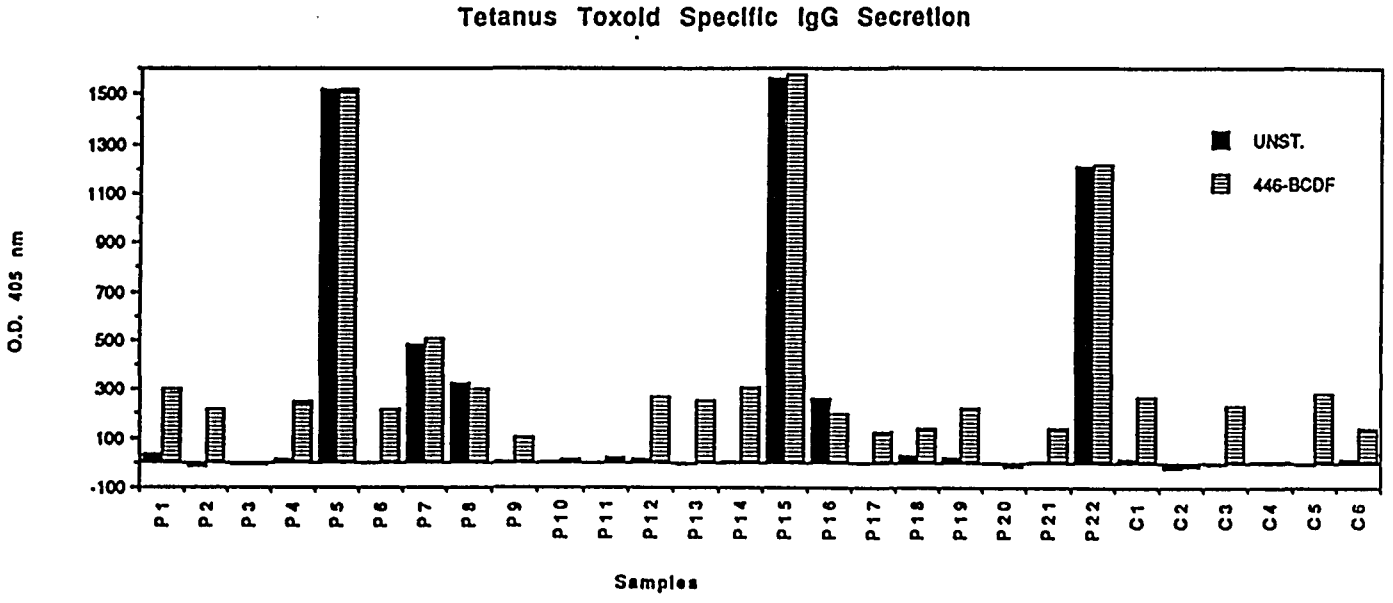

Figure 6 : Tetanus toxoid specific IgG secretion. 446-BCDF stimulated and unstimulated B cell supernatants ability to bind TT covered ELISA plates were measured. Since no standard was available for TT specific IgG, O.D. values of the specific binding (O.D. of the test serum-O.D. of the culture medium) at 405 nm are expressed.

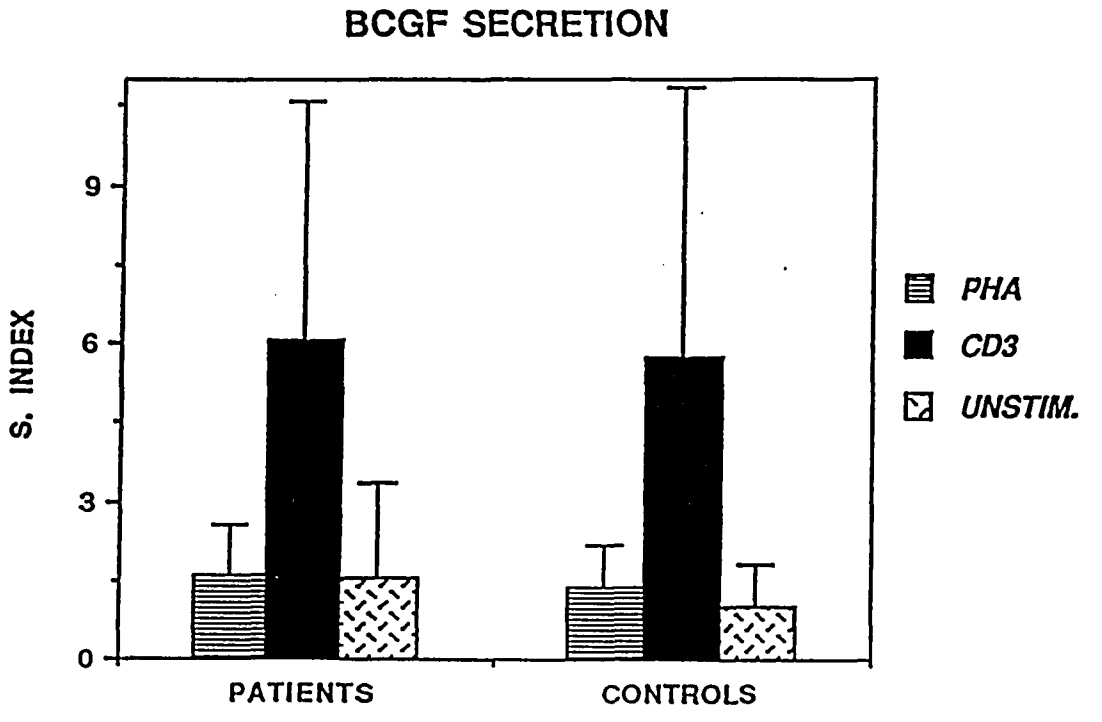

Figure 7 : BCGF secretion by stimulated PBMC. PBMC are stimulated for 48h with either PHA, mAb-446, or medium alone, and BCGF activity of the supernatants (10% vol/vol) measured using proliferation of normal B cells as the indicator cell population. No difference was observed between patients and controls, and no activity was observed in either PHA-stimulated or unstimulated supernatants.

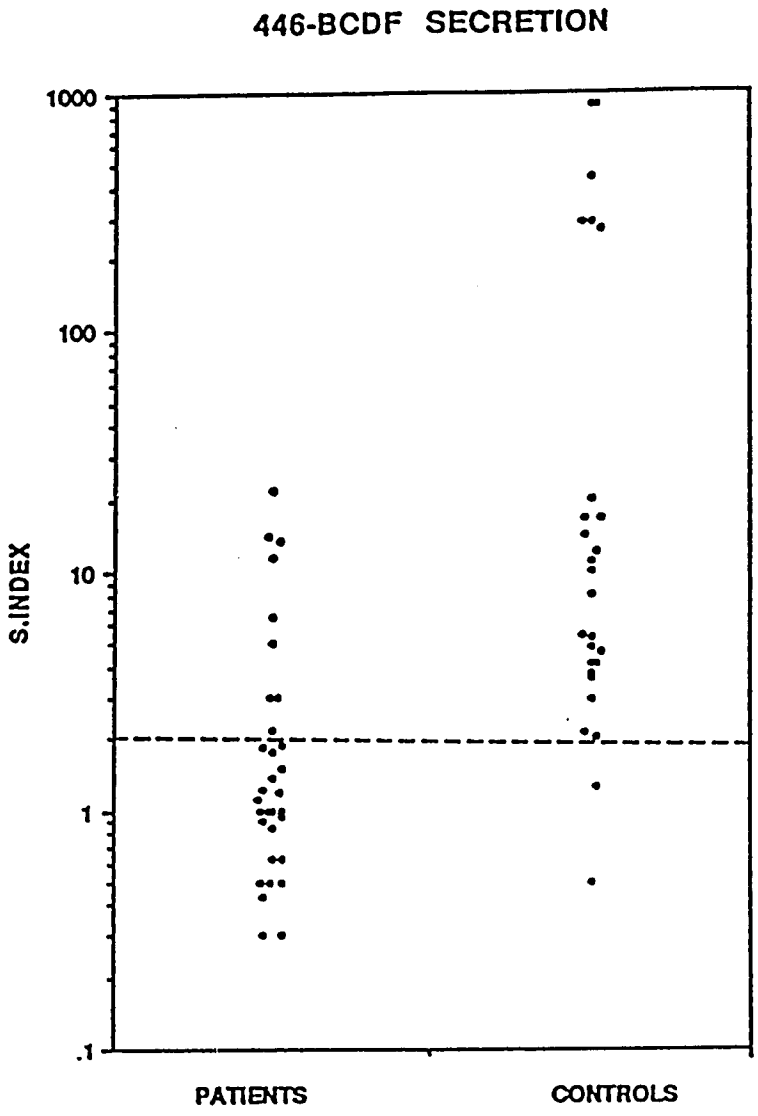

Figure 8: 446-BCDF secretion by patient and control T cells. 48h supernatants of mAb-446 stimulated PBMC were added to normal SAC-activated B cells with Ig secretion measured as described in methods. As described in Figure 3A, two-fold (dotted line) increase in Ig secretion was considered normal 446-BCDF secretion. No BCDF activity is noted in unstimulated cultures.

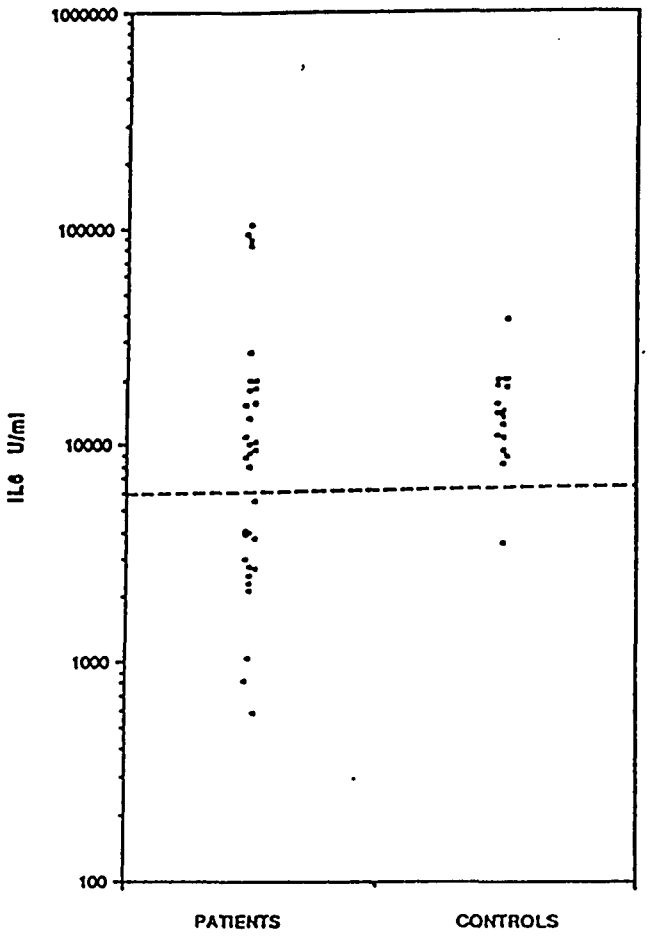

Figure 9 : IL6 Secretion by anti-CD3 stimulated PBMC. The TCS described in figures 4 and 5 were tested for the presence of IL6 using the B9 cell line as the indicator line. Each point represents at least 2 supernatants per patient. The lower limit was determined to be 6000 U/ml (dotted line) (95th-percentile of the normals). Specificity of this assay was determined with an anti-IL6 polyclonal antibody.

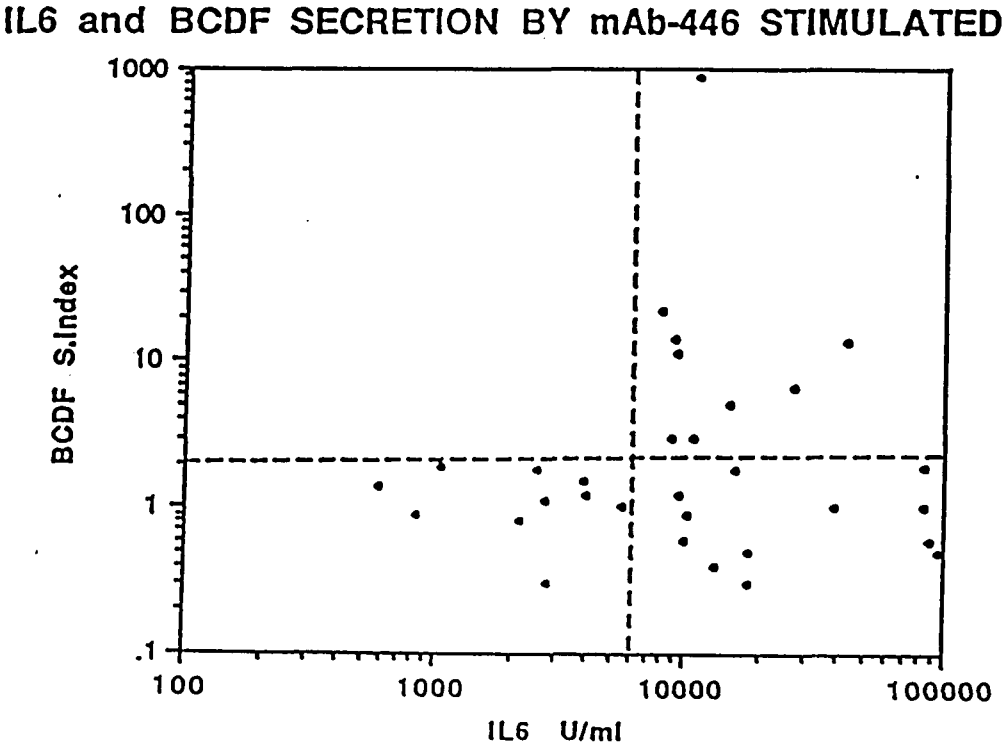

Figure 10 : Comparison of IL6 vs BCDF secretion. A compilation of figures 5 and 6 comparing IL6 (B9 assay) and BCDF secretion in individual patients. The lower limits of normal have been previously defined. Each point represents at least two supernatants per patient without variation.

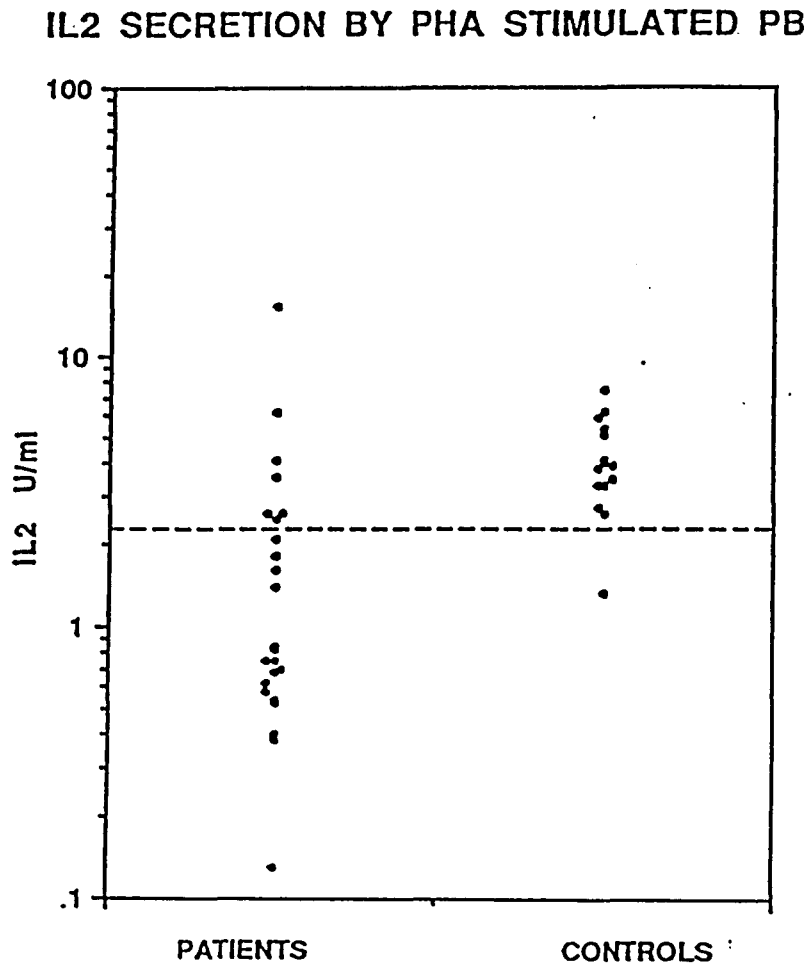

Figure 11 : IL2 Secretion by patient and control PBMC. PBMC were stimulated with PHA for 14h and supernatants were analyzed for IL2 activity using the CTLL line. The lower limit (dotted line) (95th-percentile) was determined by the distribution in the controls tested. Specificity of the assay was determined by inhibition with specific anti-IL2 antibody.

DISCUSSION :

Hypogammaglobulinemia can be the result of a number of defects in the pathway of B cell maturation. Until recently these defects were thought to reside entirely within the B cell (3,4,10). Yet several recent studies have called this concept into question. While conventional differentiation stimuli (mitogens, EBV) were incapable of promoting normal Ig secretion, single cytokines or the combination of cytokines and cognate interactions were capable of restoring normal or near normal Ig secretion by CVI B cells (11,12,23). These findings, coupled with the observations that cytokine secretion defects were detected in isolated cases, strongly suggested a defect in helper T cell function, potentially a specific defect in differentiation factor secretion. The results from our current study support and extend this hypothesis. We demonstrate that B cells from 84% of CVI patients respond normally to a novel B cell differentiation factor, 446-BCDF and in fact secrete IgG, a more mature isotype. The finding of induction of IgG synthesis by CVI B cells is the strongest evidence to date to support the hypothesis that, unless given the appropriate signal one cannot dismiss the lack of B cell response as an intrinsic defect. More important finding was the same patients' ability to secrete tetanus toxoid specific antibodies. These are clear indications for the normal differentiation capabilities of B cells from CVI patients as they can

secrete specific and non-specific IgG which reflects a more differentiated phenotype than IgM secretion. This is an important observation considering IgG and specific Ig secretion defects are the main problems in CVI patients. This observation coupled with the finding of a defect in 446-BCDF secretion by anti-CD3 stimulated CVI T cells (23/32 = 72%) provides strong evidence that T cell dysfunction is the underlying defect in the majority of CVI patients.

This is best illustrated by analyzing individual patients. One patient (LMC in Table 2) whose B cells responded extremely well to 446-BCDF failed to generate any BCDF when her T cells were stimulated with anti-CD3 mAb-446. In contrast several patients who failed to differentiate in the presence of 446-BCDF secreted normal amounts of this cytokine (CV in Table 2). These latter patients may represent true intrinsic B cell defects. There were no other distinguishing clinical or laboratory features that could help to identify either patient subgroup (Table 1).

The hierarchy of cytokine defects also provided insight into the regulation of cytokine secretion. While there were a subset of patients who demonstrated global cytokine secretion defects (IL2, 446-BCDF, IL6) comparable to those described by others (8, 9, 12, 13, 20, 21, 22) (for IL2, IL4 and γIFN), there were also distinct profiles of cytokine secretion defects. IL6, which has been shown to

be an effective B cell differentiation factor in mouse and in the presence of IL2 in man (26,27,28), is reportedly elevated in the serum of patients with CVI (20). Our data suggest that IL6 deficiency is a subset of BCDF deficiency. The majority of BCDF deficient patients did secrete normal amounts of IL6, but no IL6 deficient patients secreted BCDF. Therefore it appears that BCDF is independent of IL6 secretion and IL6 may not correlate well with Ig production. In contrast, no correlation was seen between BCDF and IL2 secretion. The comparison is problematic since IL2 production could only be measured in PHA stimulated supernatants and BCDF only in anti-CD3 stimulated supernatants. Still early IL2 production may be a critical event for later cytokine secretion. Alternatively, defects in early cytokine production may reflect more global defects in the T cell. Thus far, in limited studies, we have been unable to correct the 446-BCDF secretion defect *in vitro* using costimulation (anti-CD2, anti-CD28) or cytokines (IL1,IL2, γIFN). Interestingly, however, in a recent study (14), we were able to restore normal PWM driven T helper activity following intravenous treatment with a long acting form of IL2 and, concomitantly restore B cell differentiation capabilities *in vitro* to both T dependent (PWM) and T independent (BCDF) stimuli. We have yet to clearly document that such therapy restores the cytokine secretion defects described in the current study. Taken together these findings support an intrinsic cytokine secretion defect as the major pathogenetic mechanism in CVI.

The finding that some CVI B cells are capable of normal Ig secretion speaks to the capacity for partial restoration *in vitro*. The second issue relates to the nature of the defect in the T cell. Our system utilizes an antigen surrogate, anti-CD3 crosslinking, to polyclonally activate T cells. Many groups have described clear defects in Ag specific T cell activation, in some cases noting it as the earliest defect expressed in this disease. If signalling through CD3 is defective, then secondary signals triggered in the membrane and cytosol would not be translated into induction of cytokine secretion. Thus far we have not been able to consistently document a defect in this signalling process although Ca++ flux is reduced in response to mAb-446 in a number of patients (Kazbay, Pizzimenti, Mayer in manuscript). Lastly these studies do not address the potential role of the antigen presenting cell in this process and other defined co-stimulatory molecules such as CD40/gp39 which are defective in other immunodeficiency states (29, 30).

These findings lay the groundwork for future therapies. If, in fact, 446-BCDF can restore normal IgG and more importantly specific IgG secretion *in vitro*, it may serve as a more physiological therapeutic replacement compared with IVGG. Rather than relying on passive immunity via protective antibody, the patient would generate his own directed immune response. Tailoring therapy to

specific defects in cytokine production may render these patients more capable of generating normal immune responses.

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ADDITIONAL STUDIES

Our data indicate that, in CVI T cells are defective in secretion of factors inducing B cells to differentiate into Ig secreting plasma cells. We also demonstrated that B cells from same patient could be driven to perform within the normal range both for proliferation and Ig secretion when appropriate signals are provided in vitro. The question remains as to what the defect in CVI represents- either a failure of appropriate signalling within the T cell or some downstream event. In order to dissect this scenarios in this section I would like to analyze normal T cell responses to anti-CD3 stimulation.

1- T CELL RESPONSES TO ANTI-CD3 STIMULATION :

a) Proliferation :

In order to define defects in CVI, we first wanted to establish the optimal conditions for the induction of proliferation in response to anti-CD3 and the kinetics of this response. Normal T cells showed maximal proliferation when stimulated with anti-CD3 monoclonal antibodies at a concentration of 0.1 µg/ml. Furthermore proliferation was observed only when the mAbs were crosslinked

either with a secondary antibody against mouse Ig, or in the presence of antigen presenting cells. To achieve a more physiological state we used autologous antigen presenting cells as crosslinkers which would also allow for the availability secondary signals.

Under these conditions cells showed a significant proliferation to PHA, CD3 or CD2 stimulation initiating at 24 hours. Proliferation was maximal around 48 hours and declined thereafter (Figure 12).

Kinetics of the stimulation were not different in the patients. Initially patients' PBL stimulated for 48, 72, 96 and 120 hours. Similar to controls optimum proliferation observed at the 48th hour and 48 hour stimulation indices used thereafter. Figure 13 compares stimulation kinetics of three patients and two controls in response to anti-CD3 stimulation.

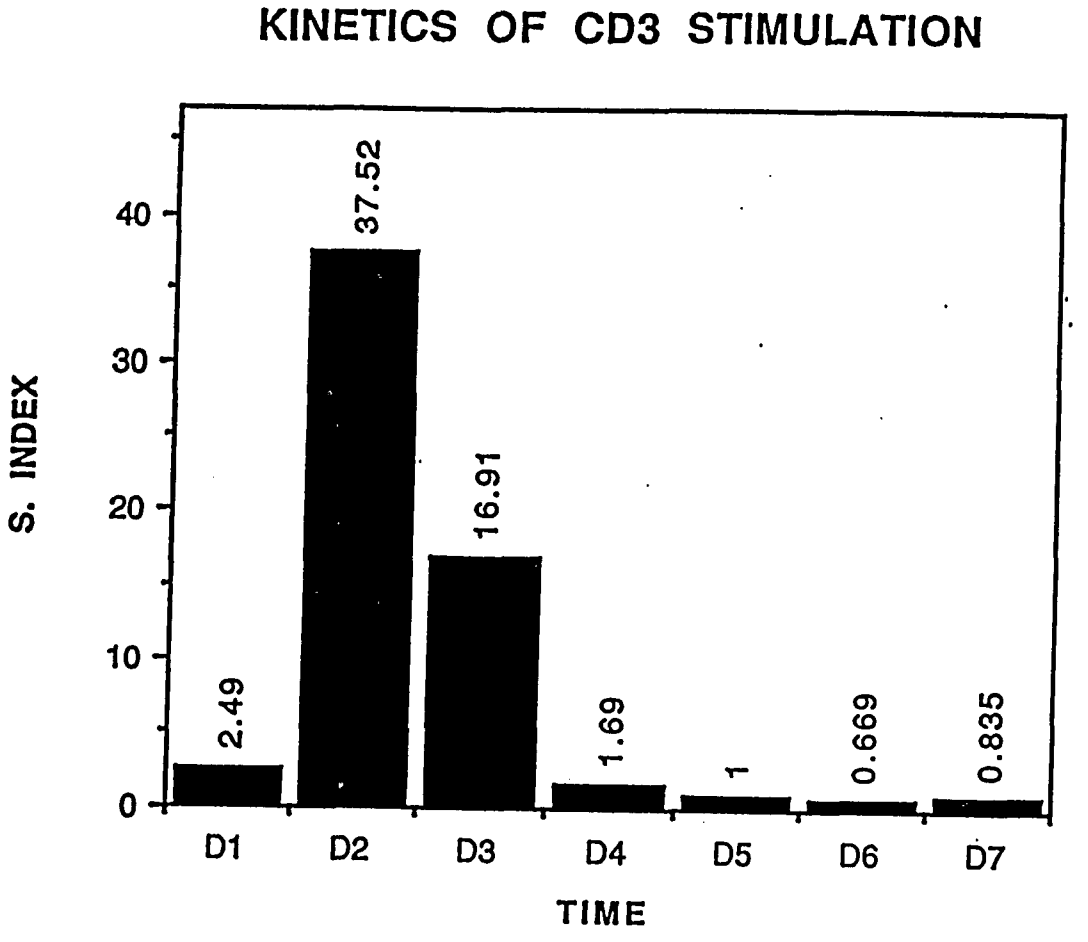


Figure 12 : Proliferation kinetics of anti-CD3- mAb-446 induced T cell proliferation. 10^5 freshly isolated PBLs from a normal donor were stimulated with 0.1µg/ml of mAb-446. Triplicate cultures were pulsed for 6H at 24 H intervals and 3H-Thymidine incorporation was compared to unstimulated cultures. Stimulation index = CPM of anti-CD3 stimulated wells ÷ CPM of unstimulated wells.

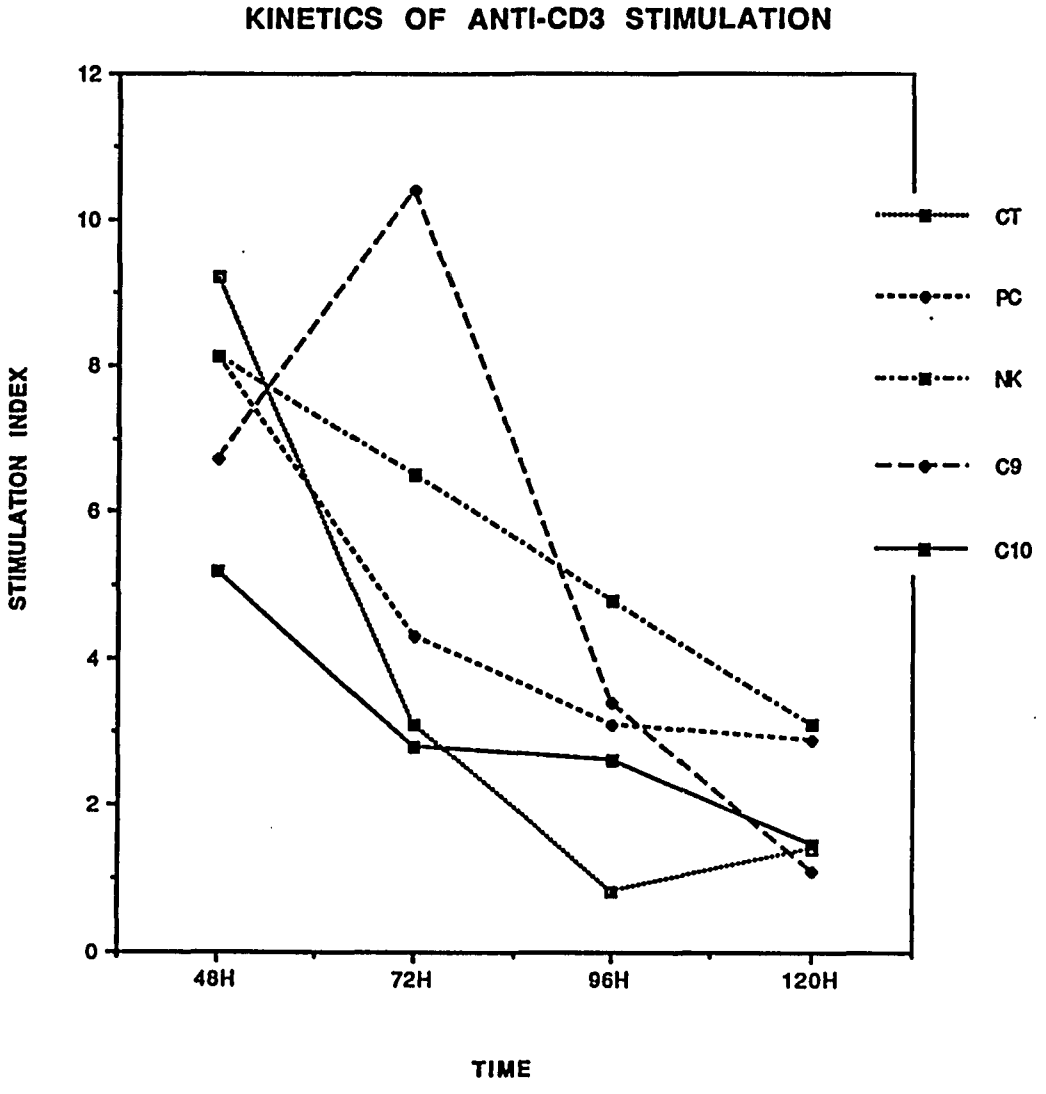


Figure 13 : Comparison of the stimulation kinetics for anti-CD3 mAb-446 induced T cell proliferation. Three patients and two controls are demonstrated.

b) Early signal transduction events in T cell activation :

Preliminary data had suggested that different T cell activating signals like PHA, or two different anti-CD3 mAbs can induce different cytokine secretion profiles. We wanted to determine whether these effects were mediated by different intracellular signaling pathways. A number of intracellular biochemical changes occur within the first seconds to minutes after stimulation of T cells. These include an increase in the cytoplasmic free Ca++, protein phosphorylation, pH changes and changes in the cyclic nucleotides (cAMP or cGMP).

In this part of the study we wanted to address whether the difference in the cytokine secretion profiles following T cell stimulation could be attributed to the difference in the intracellular signalling. We initially analyzed Ca++ flux and phosphorylation of intracellular proteins following either PHA, the anti-CD3γ mAb (mAb-446) or or the anti-CD3ε mAb (mAb-454) stimulation.

i- Ca++ Flux :

The kinetics and the magnitude of the calcium flux after PHA, mAb-446, mAb-454 and anti-CD2 (mAb-340) stimulation are measured using a calcium sensitive fluorescent dye, Indo-1

(Molecular Probes, Eugene, OR). Briefly, $5x10^6$ PBLs were washed several times in Ca++ free medium and loaded with Indo-1 for 15 minutes. Five minutes before stimulation of the cells, the tubes were warmed to 37°C. Then the cells were placed in a fluorimeter. After achieving a stable baseline of fluorescence, the cells were stimulated with either PHA, mAb-446, mAb-454, or mAb-340. The ratio of O.D. 490/405 was then measured for 4 minutes by a spectro-fluorometer. At the end of this period the cells were lysed with Triton-X to release all intracellular Ca++ and Indo-1 to measure the level of Indo-1 loaded into the cells. This was followed by the addition of EGTA to chelate free Ca++ and ascertain the background fluorescence.

The magnitude and the kinetics of the Ca++ flux following PHA, mAb-446 and mAb-454 stimulation of the T cells is depicted in Figure 13. All stimuli that induce proliferation in unprimed T cells, did induce a significant Ca++ flux. PHA stimulation induce the sharpest and fastest increase in intracellular Ca++ concentration, followed by mAb-446. mAb-454 induced Ca++ flux was somewhat slower and lower in amplitude.

Ca++ flux induced by the anti-CD2 mAb-340 was similar to that seen with mAb-446 induced flux. IL2, even though it induced proliferation in activated cells, didn't induce a Ca++ flux either in

activated, or in fresh T cells. Similarly, anti-CD28 (mAb-9.3) which modifies cytokine secretion by CD3 stimulated T cells did not induce Ca++ flux itself, and did not modify anti-CD3 induced flux when they were used together.

Figure 14 shows that there is a clear difference in the Ca++ flux induced by either PHA, mAb-446 or mAb-454. However, one cannot say that the difference in Ca++ flux between mAb-446 and mAb-454 is responsible for the differences in their cytokine secretion profiles.

Furthermore, we did not observe any difference between the Ca++ flux profiles of a number of patients and controls that we have studied. Therefore, we cannot postulate that the difference in the cytokine secretion profiles between patients and controls is due to the difference in the intracellular signal transduction event using Ca++ as mediator. Figure 15 shows the kinetics of the Ca++ flux of T cells stimulated with anti-CD3 mAb-446 in three patients and two controls.

A

B

C

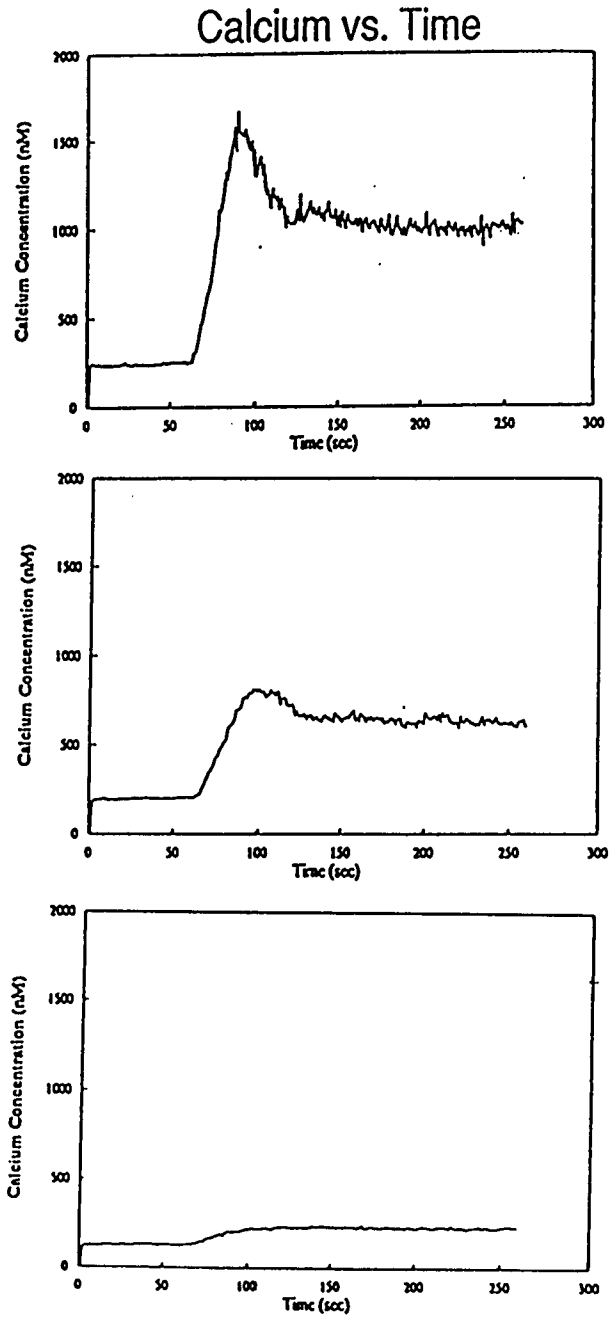


Figure 13: Ca++ flux induced by PHA (A) and anti-CD3 monoclonal antibodies, mAb-446 (B) and mAb-454 (C).

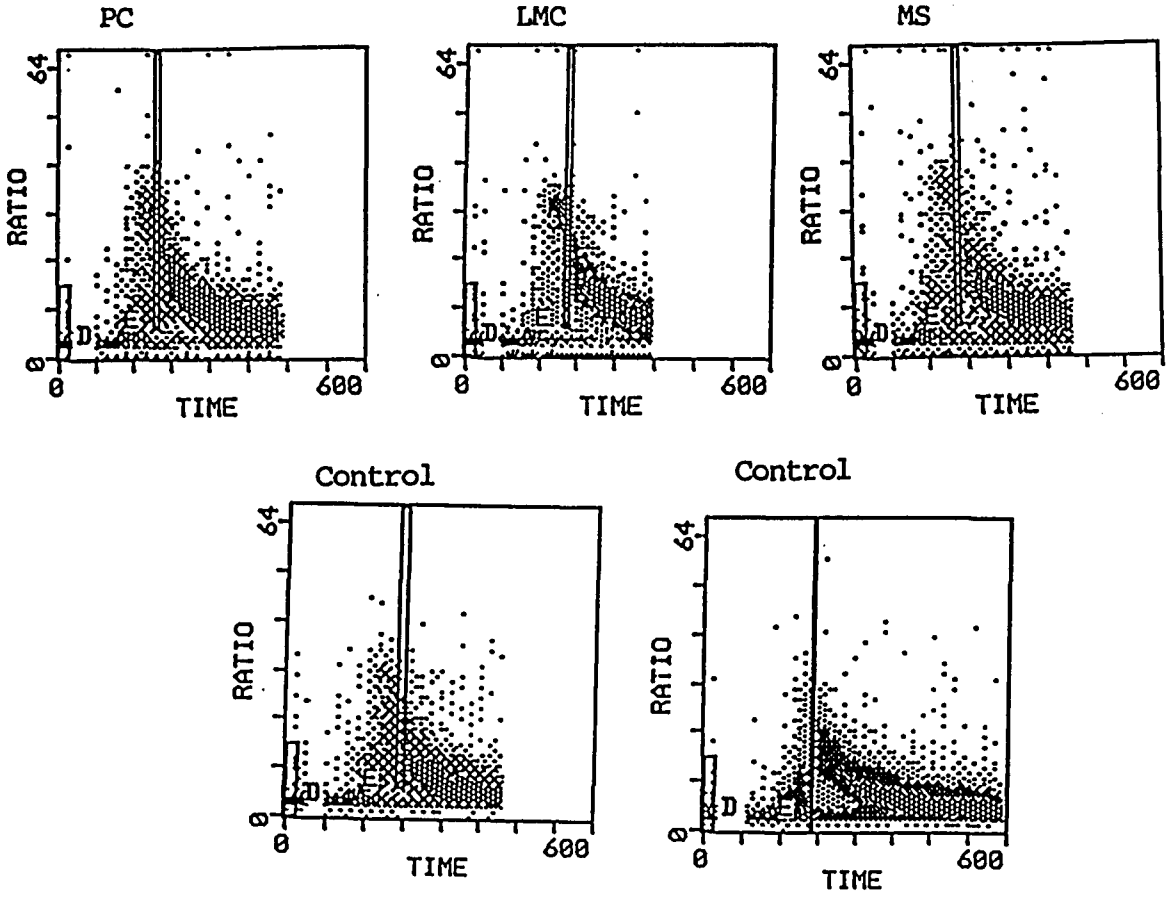


Figure 15 : Kinetics of anti-CD3 mAb-446 induced Ca++ flux. Comparison of three patients and two controls.

ii- Phosphorylation Studies :

Beside Ca++ flux, the other early event in T cell activation is the phosphorylation of number of intracellular proteins. CD3 ζ chain, as well as p56-lck (via CD4/8), p59-fyn and p70-zap have all been shown to be phosphorylated when T cells are stimulated. We wanted to determine whether the protein phosphorylation pattern would differ if T cells were stimulated by different mAbs, and whether these findings would explain the cytokine secretion differences seen after T cell activation with these mAbs.

In order to determine the phosphorylation patterns of T cells stimulated with PHA or anti-CD3 mAbs, free phosphorus was removed by washing in saline, and the cells were starved of phosphorus by incubating them for two hours in phosphorus free medium. Afterward 30 million T cells were loaded with ^{32}P-inorganic phosphorus and cells were stimulated with PHA or with mAbs. After stimulation cells were lysed at 1 and 5 minutes with mild detergent containing 1mM Na orthovanidate and EDTA to inhibit dephosphorylation of proteins by phosphatases present in the cells. The crude cell lysates or anti-phosphtyrosine precipitated samples were denatured by boiling for 5 minutes in the presence of 10% SDS. The samples were then run overnight at 20 mV on a 10%

polyacrylamide gel. Commasie blue stained gels were then dried and exposed to X-ray film for autoradiography.

Interestingly, phosphorylation induced by PHA did indeed differ from anti-CD3 induced stimulation. However, we did not observe any major differences in the phosphorylation patterns induced by mAbs 446 and 454. Figure 16 shows that there are a fairly large number of proteins that are phosphorylated after anti-CD3 stimulation. However, since we did not observe significant differences in the phosphorylation induced by mAb-446 and mAb-454, it seems unlikely that the difference in the cytokine secretion profiles is the end result of differences in kinase activity. However we did not assess the kinetics of phosphorylation, nor did we measure specific phosphorylation of known signalling molecules. Therefore while preliminary evidence suggest that no differences exist further evaluation is warranted.

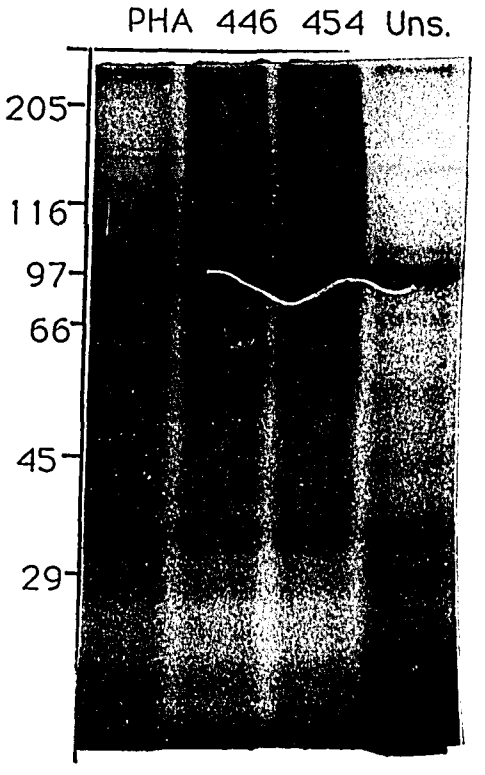


Figure 16 : Phosphorylation of intracellular proteins following PHA, and anti-CD3 stimulation.

c) Cytokine Secretion Profiles of T Cells Stimulated with Different Activators :

Studies in the last 20 years have clearly demonstrated that T cells play a crucial role in B cell maturation, both in an antigen specific and nonspecific fashion. Antigen specific regulation of Ig secretion requires cognate interactions between T and B cells, while non-specific induction of Ig secretion is mediated by cytokines secreted by T cells.

The importance of cognate interactions has been demonstrated in earlier hapten-carrier induced Ig secretion models (Mitchison, 1971). In our experimental systems we tested the cognate interaction between T and B cells by culturing them together in the presence of mAb-446, where Fc receptors on B cell serves as the crosslinker of the anti-CD3-mAbs and T cells are activated via CD3. Even in this system cytokines secreted may have contributed in Ig secretion.

T cells may also regulate Ig secretion in an antigen non-specific manner by the cytokines that they secrete. As described earlier a number of T cell secreted factors have already been shown to induce B cell proliferation and differentiation, including IL2, IL4, IL6, several B cell growth factors (BCGFs) and 446-BCDF.

We measured cytokine secretion profiles of PHA, mAb-446, mAb-454 stimulated T cells.

Supernatants were generated by culturing PBMC in 24-well culture plates (Linbro, Flow Laboratories, McLean, VA) at a concentration of 10^6 cells per well in 1.5 ml CM. Cells were stimulated with either PHA (1µg/ml), mAb-446 (0.1 µg/ml), or medium alone. Cell free supernatants were collected at 14 hours for measurement of IL2 , and at 48 hours for measurement of BCGF, 446-BCDF and IL6. Supernatants were either tested immediately, or kept frozen at -20°C until tested.

i- IL2 Secretion :

Under physiological conditions the most important source of IL2 is the T cell stimulated via its TCR-CD3 complex with specific antigens presented via autologous antigen presenting cells.

For our in vitro stimulation experiments, we first wanted to define the optimal conditions and time points to maximize IL2 secreted into the culture supernatants. For these experiments we stimulated both pure T cell populations and PBMCs with different stimuli, alone or in combination. Pure T cells did not secrete any cytokine, proliferate to any stimulus in the absence of APCs.

In the presence of APCs, the cells were able to secrete IL2 when they were stimulated with PHA or anti-CD3 and anti-CD28 used together. However, IL2 was utilized as well as secreted by activated T cells. The maximum concentration of IL2 in the supernatants could be detected between 12-to-18 hours after stimulation. Thereafter IL2 levels declined abruptly and became undetectable within 48 hours, very possibly due to enhanced IL2-R expression by T cells and utilization of this cytokine. IL2 is a very stable molecule in culture medium even at 37°C. Figure 17 documents the concentration of IL2 in the 18 and 48 hour supernatants of PBLs stimulated with different stimuli.

The murine cytolytic T cell line, CTLL, was utilized to measure IL2 as previously described (Gillis, 1978). rIL2 (Boehringer-Manheim) was used as a standard in each assay. In each experiment a standard curve was generated using two-fold serial dilutions of the rIL2. Several dilutions of test supernatants starting from 10% v/v is used in each experiment. 2,000 CTLL cells/well in 100 µl of culture medium in 96 well culture plates were cultured for 24 hours. At the end of this period 1 µCi 3H-Thymidine was added for 6 hours. Cells were then harvested onto filter mats, and incorporated thymidine was counted using a Beckman LS 3801 β emission counter (Somerset, NJ).

As can be seen in figure 17, anti-CD3 mAb-446 stimulation does not induce any significant IL2 secretion by T cells in any time point, whereas PHA alone is sufficient (first six columns). However, combining any of these stimuli with anti-CD28 stimulation significantly increases IL2 secretion. CD28 alone does not induce the secretion of IL2 nor the proliferation of T cells (next set of six columns). CD2 stimulation alone or in combination with CD3 had no effect on IL2 secretion, and even decreased the PHA induced IL2 secretion (third set of six columns). γIFN had no effect on IL2 secretion whatsoever. Addition of external IL2 (20 U/ml) demonstrated that activated T cells use the IL2 as the IL2 content decreases with time, and the decrease was most significant in anti-CD3 stimulated cultures where IL2-receptor expression is upregulated and no detectable IL2 is secreted (last set of six columns).

Based on these data, in order to assess IL2 secretion by CVI patients, we used 14 hour supernatants of PHA stimulated PBMC supernatants and compared these results with the controls. In a limited number of experiments, where patients were defective in IL2 secretion induced by PHA stimulation, we also tested whether anti-CD28 or anti-CD3 co-stimulation would restore this secretory defect.

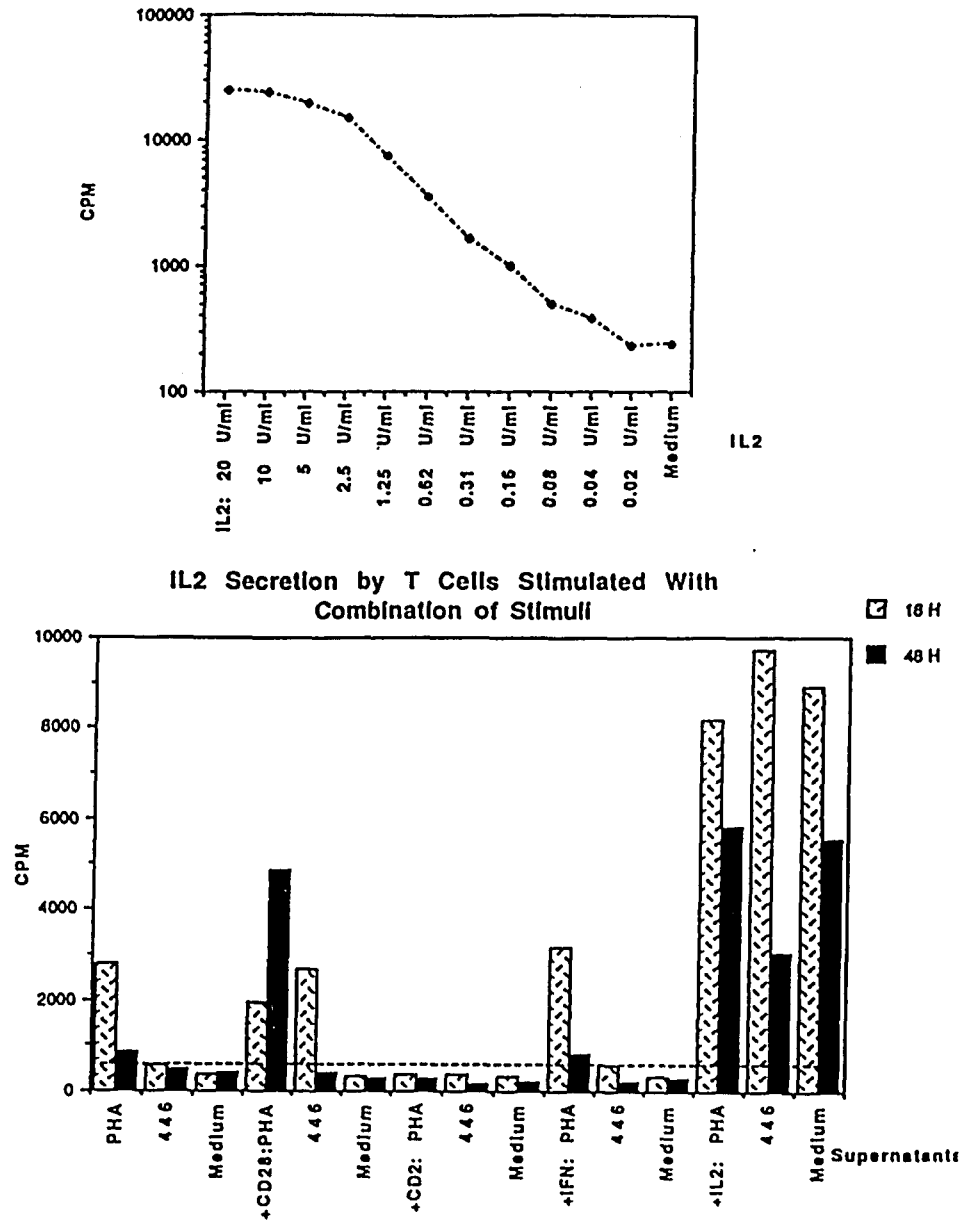


Figure 17 : IL2 Secretion by normal peripheral blood mononuclear cells. The cell free supernatants were collected at 18 and 48 hours. Proliferation of the CTLL cell line in the presence of 10% supernatants (v/v) was measured as 3H-Thymidine incorporation as described in methods.

ii) IL4 Secretion :

In our experiments we wanted to address whether some of the activities of our mAb-446 stimulated T cell supernatants could be attributed to IL4 secretion. In order to test this possibility 18, 48 and 96 h T cell supernatants stimulated with PHA, mAb-446 alone or in combination with anti-CD28 were tested for their IL4 activity using the IL4 sensitive Ramos cell line (ATCC, Rockville, MD).

Similar to our observations with IL2 secretion we observed that IL4 was not secreted when T cells were stimulated only with anti-CD3. However, even though CD28 alone did not induce IL4 secretion, anti-CD3 and anti-CD28 together induced significant IL4 secretion (Figure 18). The secretion was an early event as it was observed in the 18 hour supernatants. In contrast to IL2, IL4 did not decrease in supernatants after 18 hours, and was detected at comparable levels at 48 and 96 hours.

A CD23+ B cell line sensitive to IL4, Ramos cells, was used as an indicator cell line. Ramos cells upregulate CD23 expression on their surface when they are cultured in the presence of IL4. 10% culture supernatants were added to cultures of Ramos cells for 18 hours. CD23 expression was then measured by the fluorescence of cells stained with the anti-CD23 mAb (Blast-2) and FITC conjugated

goat-anti-mouse antibodies. Class I and class II MHC expression was also measured as a positive control for staining. IgG isotype control was used to establish negative background staining. rIL4 in different concentrations was used to obtain a standard curve of CD23 expression in response IL4. Fluoresence intensity of the staining was measured by using a flow cytometer (EPICS-Profile II, Coulter, Hialeah, Florida).

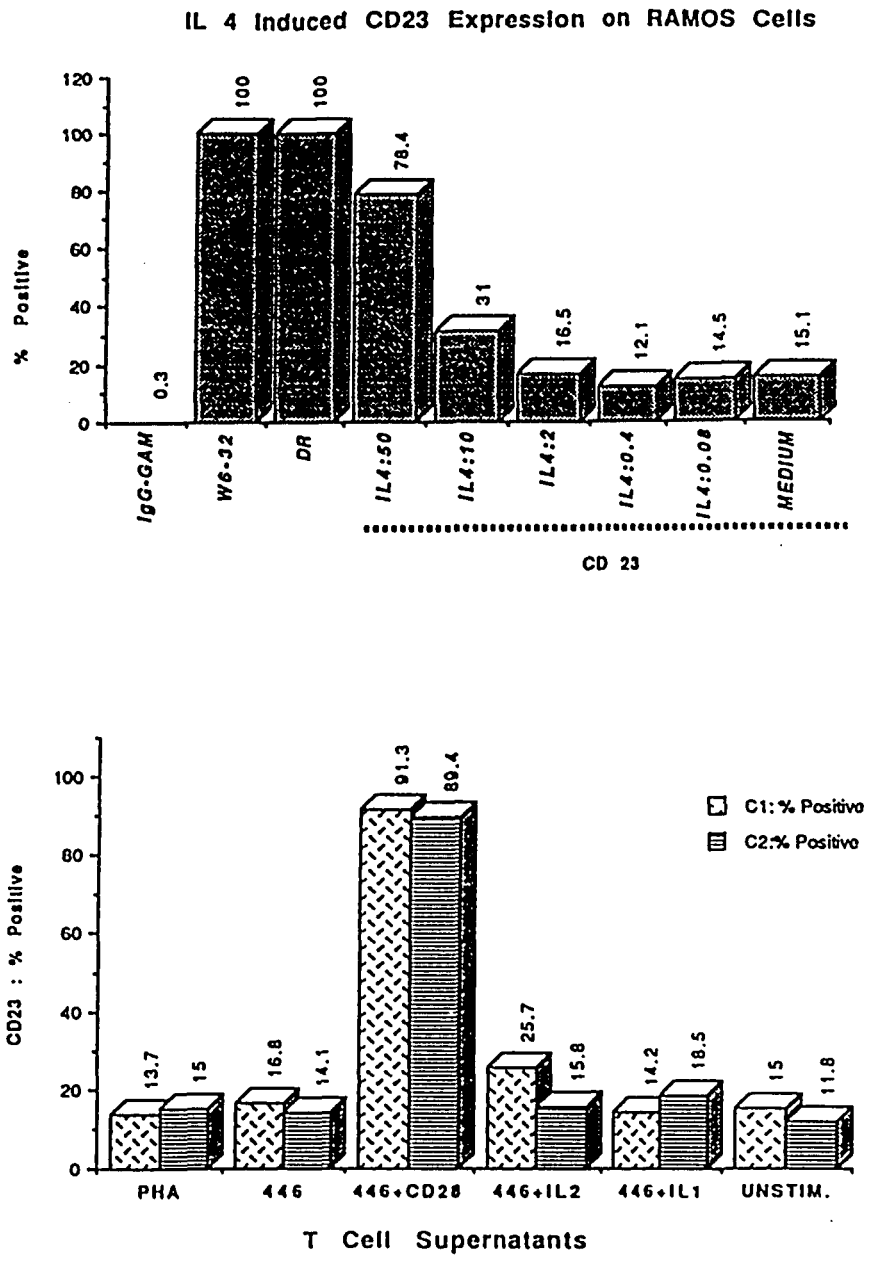


Figure 18 : IL4 secretion by mAb-446 stimulated T lymphocytes. Two normal subjects (C1 and C2) are presented here. Supernatants were collected at 48h after stimulation. 10% supernatants (v/v) were added the cultures of the Ramos cell line for 18h. Ramos cells were then stained with the anti-CD23 mAb (Blast-2) and FACS analysis was conducted as previously described.

iii) IL 6 Secretion :

In addition to lymphokines that promote proliferation of activated B lymphocytes, investigators have long been involved in attempts to purify factors that affect B cell differentiation. Kishimoto and his co-workers from Osaka, Japan characterized one such molecule that enhanced Ig secretion from a B cell lymphoma line which they called BSF-2 (Kishimoto, 1985; Maraguchi 1988). Sequencing of this protein revealed that it was the same factor previously described as IFN-β2, as well as many other functions (addressed earlier). Furthermore it has been shown that many cell types, especially monocytes can secrete this cytokine. Nonetheless even though it has ability to stimulate Ig secretion in activated murine B cells, its activity in human B cells is somewhat limited.

In our experiments with SAC activated human B lymphocytes, several lines of evidence indicated that IL6 in the cell supernatants may not play as crucial a role in the Ig secretion seen. First, all the supernatants from normal donors tested, whether mAb-446 stimulated or not, expressed very high levels of IL6 activity as measured by the induction of the proliferation of the IL6 dependent cell line B9. The IL6 content of the PHA stimulated supernatants was as high as mAb-446 stimulated cells, and nearly as high in the

unstimulated T cell supernatants (both of which did not have BCDF activity).

Second, in a different set of experiments, activated post anergic T cell cultures deprived of any APCs were able to proliferate and secrete 446-BCDF in response to mAb-446 without the requirement for APCs, but could not secrete any IL6. This also indicates that the source of IL6 is mainly from APCs in the culture, rather than T cells itself. Third, there were several CVI patients who were able to secrete normal amounts of IL6, but their supernatants did not contain any BCDF activity.

Figure 19 shows the IL6 content of the 48 h PBL culture supernatants from 4 CVI patients and two controls. Purified T cells (accessory cells depleted) did not secrete any IL6 (Data are not shown). However stimulating T cells in the presence of accessory cells with PHA or either CD3 mAbs did not increase the IL6 content of the supernatants above the unstimulated culture levels.

The murine B cell hybridoma line B9 was used as an indicator cell for the measurements of IL6 as previously described (Nordan, 1986). rIL6 (kind gift of Dr. Edward Siden) was used to generate a standard curve in each assay. Briefly, 5000 B9 cells per well were cultured in the U-bottom 96-well culture plates. Two-fold dilutions

of standard IL6 starting from 100 U/ml in first 12 triplicates are used to generate a standard curve. Six five-fold dilutions of culture supernatants starting from 10% v/v were used to test IL6 content. B9 cells were cultured for 3 days in this conditions. 1 μCi 3H-Thymidine was added to wells at least 12 hours and wells were harvested onto filter mats. Thymidine incorporation was measured in a β-counter (Beckman, LS-3801).

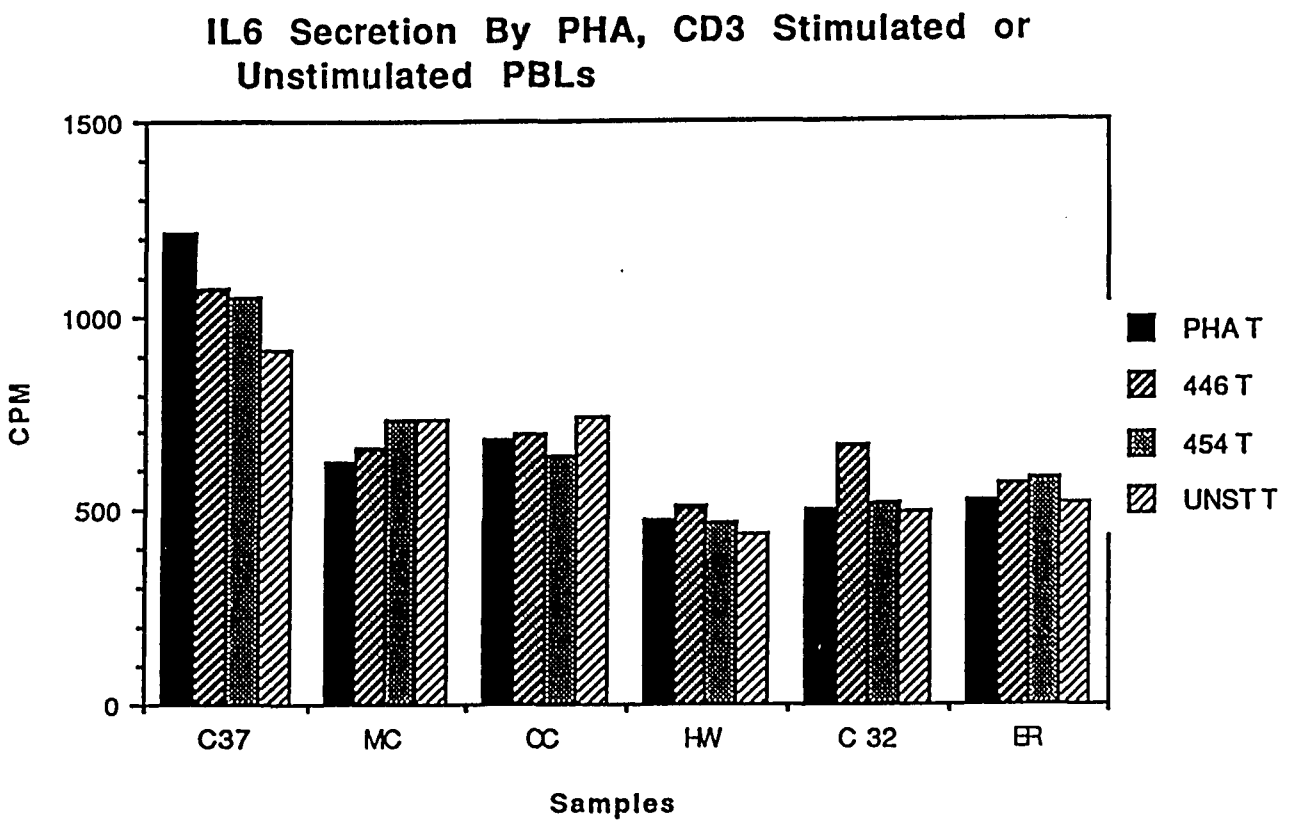


Figure 19 : IL6 secretion by 48 h PBL cultures. PBL were stimulated either with PHA, or anti-CD3 antibodies mAb-446 and mAb-454 and compared to unstimulated cultures. 10% supernatants (v/v) were added to 5,000 B9 cells and these cells were cultured for 2 days at the end of which they were pulsed with 3H-Thymidine.

iv) 446-BCDF Secretion :

As our laboratory has previously demonstrated that stimulation of peripheral blood T lymphocytes with the anti-CD3 γ chain specific mAb-446 induces secretion of a B cell differentiation factor distinct from previously demonstrated factors that affect Ig secretion (Sherris, 1989). This factor can be found as early as 18 hours after mAb-446 stimulation and the height of activity is observed at 48 hours.

This 32 kD, pI 6 protein is found predominantly in T cell supernatants stimulated with anti-CD3γ mAb-446. Similar activity may also can be observed in the anti-CD3ε mAb-454 stimulated T cell supernatants, even though the activity is significantly lower compared to mAb-446 stimulation. PHA or anti CD2 stimulation of T cells does not induce its secretion, and CD28 does not augment the level of secretion, and may even decrease it in some cases.

446-BCDF activity within the stimulated T cell supernatants was measured using normal B cells as the responder population. 10^6 B cells were cultured in 1.5 ml CM in macrowells in the presence of mAb-446 stimulated T cell supernatants (10% v/v). The cultures were kept at 37°C, in 5% CO_2 humidified incubators for 8 days, after which cell free supernatants were collected. SAC+IL2 or partially

purified 446-BCDF were used as positive controls. Unstimulated B cells in CM were used as background controls. The Ig content of these supernatants were then measured using an ELISA assay for total Ig.

Briefly, ELISA plates (NUNC, Immunoplate Maxisorp, Denmark) were coated with 65µl of 6µg/ml unconjugated goat-anti-human Ig (TAGO, Burlingame, CA) in Tris pH 8.4 buffer overnight at 4°C, followed by blocking with 200 µl of PBS containing 1% bovine serum albumin, and kept at 4°C until use (up to 10 days). After washing with Tween-20 (0.01%) PBS, 65 µl of test supernatants or serial dilutions of standard concentrations of human Ig were added to the wells. After 1 hour incubation period at 37°C, plates were washed and 65 µl of alkaline phosphatase conjugated goat-anti-human antibodies (TAGO) were added for another one hour incubation period. Finally, 120 µl of of alkaline phosphatase substrate (0.5 µg/ml), p-nitrophenyl phosphate disodium (Sigma, St.Louis, MO), in substrate buffer were added to the plates and incubated at 22°C to observe characteristic change in color. The optical density of the wells were measured at 405 nm (Genetic Systems Microplate Reader), and the average of the triplicate wells were calculated. The Ig concentrations were calculated by using polynomial regression analyses comparing the observed values to standard known concentrations of Ig.

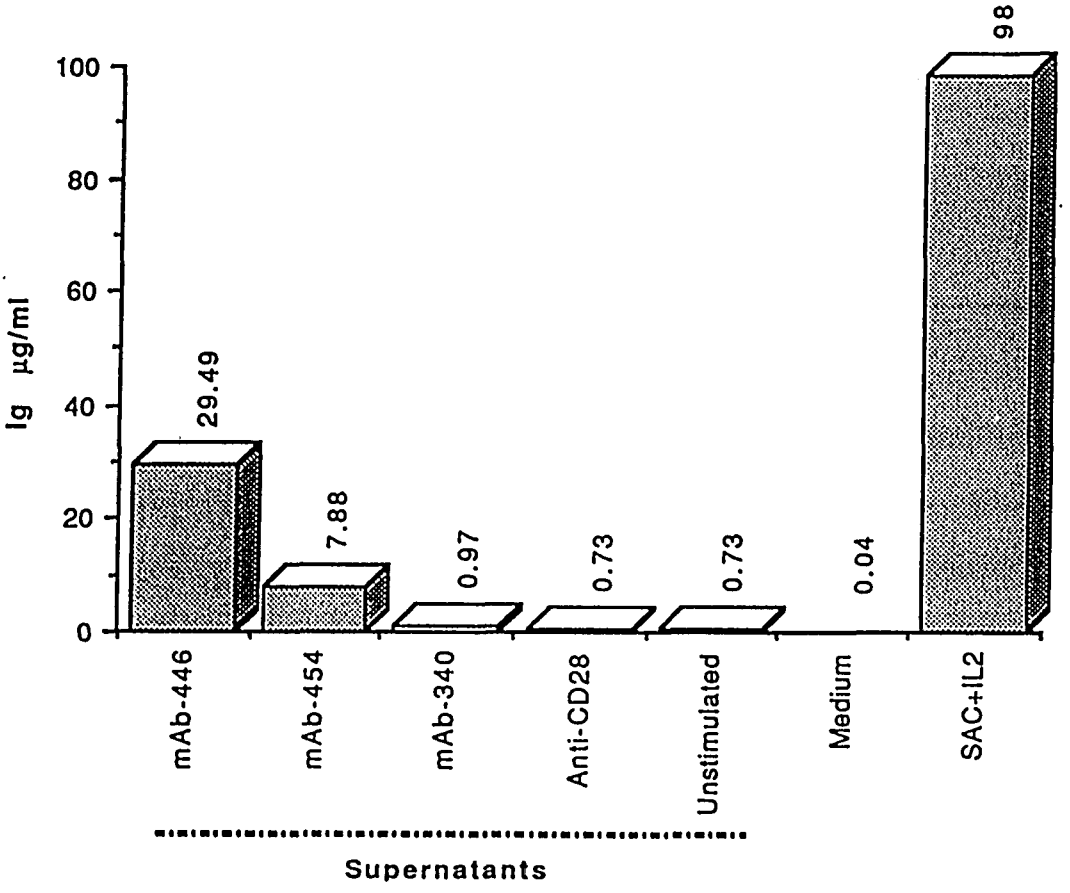


Figure 20 : Ig secretion by normal B lymphocytes cultured in the presence of 10% v/v supernatants of PBLs stimulated with anti-CD3 mAbs-446 and 454, anti-CD2 mAb-340, anti-CD28 or unstimulated PBL supernatants. The last two columns represent Ig secretion from unstimulated (negative control), and SAC (0.001% v/v) + IL2 (10 U/ml) stimulated (positive control) B lymphocytes.

v) BCGF Activity of mAb-446 and mAb-454 Stimulated PBL Supernatants :

Anti-CD3 stimulated PBL supernatants can also induce B cell proliferation. This BCGF activity was not due to IL2 or IL4, since 48 hour supernatants of mAb-446 and mAb-454 stimulated PBL supernatants do not contain either cytokine. The BCGF activity of the supernatants were equally potent in both mAb-446 and -454 activated cultures. In contrast PHA stimulated and unstimulated PBL supernatants did not have any BCGF activity. Even though we have evaluated a number of controls and CVI patients for their BCGF secretion abilities, we did not conduct further studies to characterize factor(s) which may induce B cell growth.

In order to measure BCGF activity in the supernatants, 10^5 monocyte depleted normal B cells were cultured in U-bottom microtiter plates in the presence of PHA, mAb-446, mAb-454 or unstimulated T cell supernatants (10% v/v) derived from CVI or normal donors for 72 hours. At the end of this period, B cell proliferation was measured by ^{3}H-Thymidine incorporation as described above. Stimulated cultures were compared with the wells that contain B cells alone (background control), or with B cells stimulated with SAC+IL2 as a positive control. Stimulation index was determined by the ratio of CPM experiment / CPM medium control.

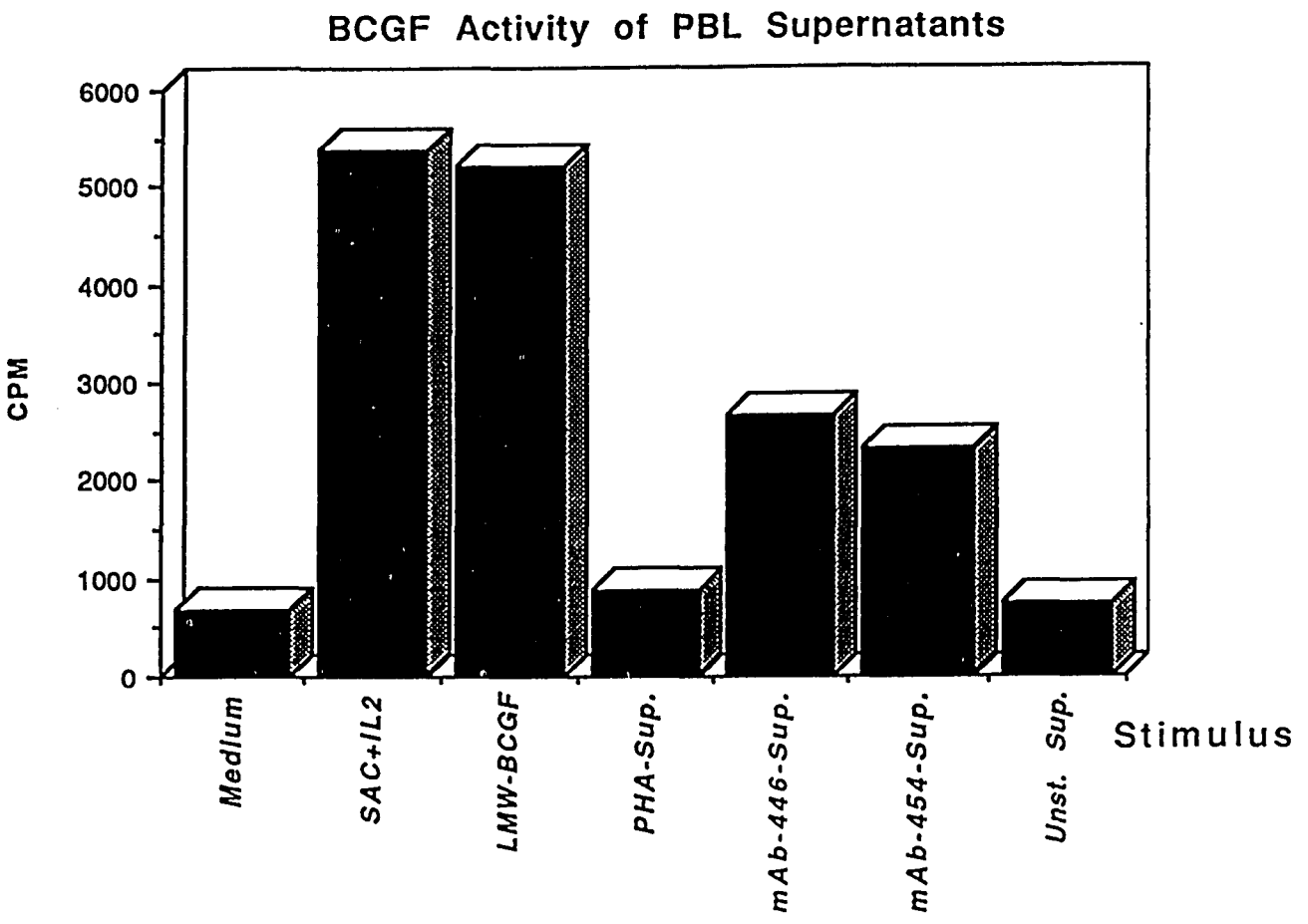


Figure 21 : BCGF activity of PBL supernatants which are stimulated with PHA, mAb-446, mAb-454 or unstimulated. Supernatants were collected at 48 hours. 10% v/v supernatants were added to normal B cell cultures (10^5 B cells in 100 µl of culture medium). Cultures were pulsed with 3H-Thymidine at the 60th hour and harvested 12 hours later. First three columns are negative (medium) or positive [SAC+IL2 or LMW-BCGF (Cellular Products, Buffalo, NY)] controls.

d) Cognate Interaction Between CD3 Activated T Cells and B Cells :

T cells may also influence Ig secretion by B cells by cognate cell to cell interactions. For example, recent observations in the patients with X-linked hypogammaglobulinemia with hyper-IgM syndrome indicated importance of such interactions (Mayer, 1987; Aruffo, 1993; DiSanto, 1993). Former paper indicated that T and B cell interaction is defective in this disorder, since patients' B cells were able to secrete Ig and switch Ig isotype class *in vitro* when they were co-cultured with normal T cells. Latter two papers showed the mutations on the T cells' gp-39 surface protein which prevents its interaction with CD40 molecule of B cells.

In our experiments we have tested such interactions using several pathways. First we have demonstrated that when we stimulate T cells with anti-CD3 antibodies in the presence of the B cells and maintain cultures for extended periods of time (up to eight days), B cells start to secrete Ig. While, Ig secretion by B cells may be partly due to secreted T cell factors, cognate interactions may play a role, since B cells in this system were not pre-activated with SAC. Without pre-activation, B cells do not respond to most cytokines.

Figure 22 demonstrates two experiments with the PBL of different donors. Even though the background level of Ig secretion and the absolute concentration of Ig differs greatly between individuals the trends are similar in response to the stimulus. mAb-446 was a potent inducer of Ig secretion in both experiments. This effect did not change significantly when anti-CD2 mAb was added to mAb-446. There was a modest inhibition when γIFN was added. IL1 on the other hand augmented Ig secretion. Most interestingly, anti-CD28 and IL2 blocked the mAb-446 effect almost totally. None of the factors other than IL2 had a direct effect on Ig secretion by B cells when they are used alone (data not shown). The negative effect of the anti-CD28 and IL2 on mAb-446 induced T cell helper effects on B cells can be explained as a modification of T helper functions more through a TH1 phenotype, rather than a TH2 phenotype which might provide more help for a humoral response.

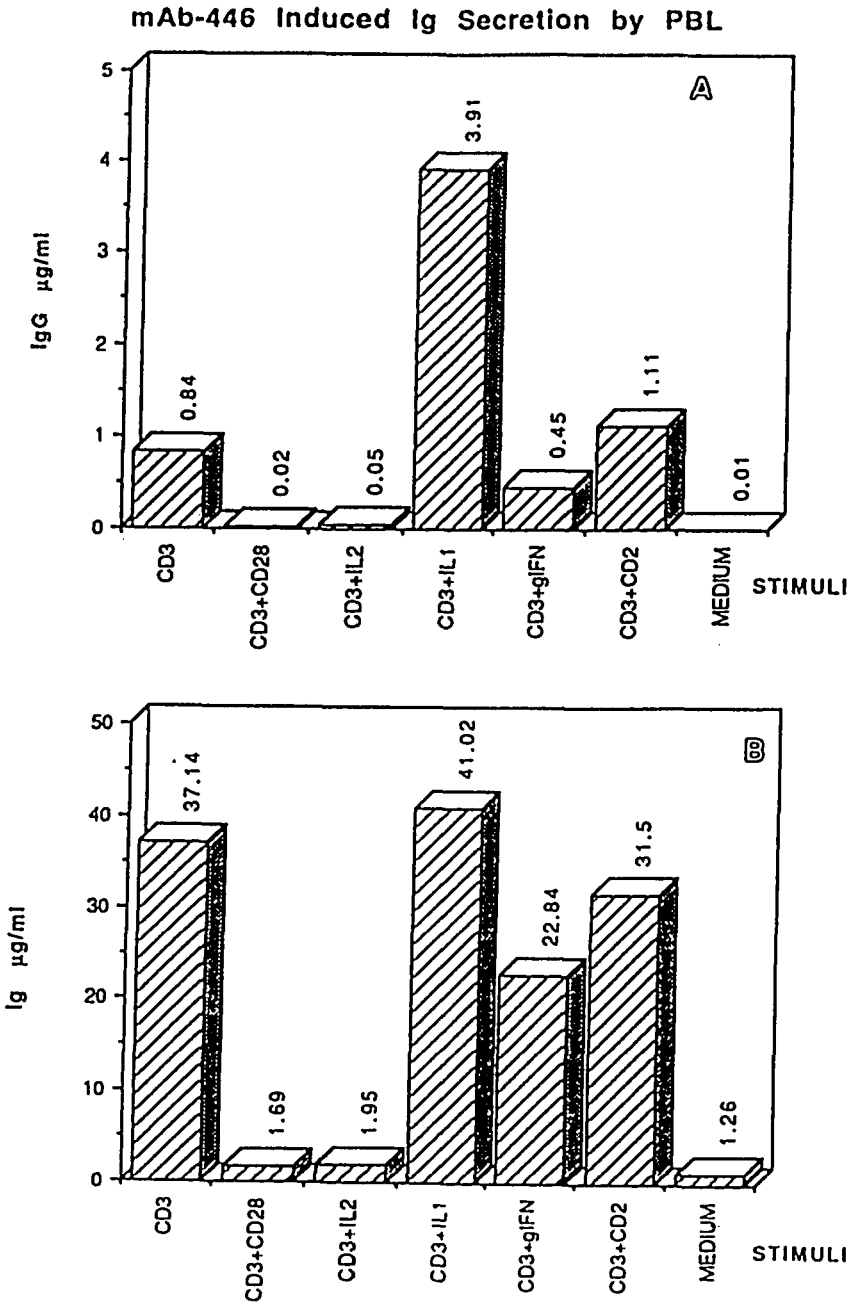


Figure 22 : Cognate interaction between mAb-446 stimulated T cells and un-primed B cells. One million PBL in 1 ml of culture medium were stimulated with indicated mAbs and Ig secretion was measured 8 days later. A and B represents two separate experiments.

2- THE CYTOKINE SECRETION PROFILE RELATES TO THE INITIAL ACTIVATION STIMULUS

Several reports have indirectly suggested that cytokine secretion by T cells may be altered by the initial activation signal. Our laboratory have previously reported that peripheral blood T cell stimulation with an anti-CD3ε chain mAb (mAb-454) induces IL2 secretion but no B cell differentiation factor (BCDF) activity, while anti-CD3γ chain stimulation (mAb-446) induces BCDF but no IL-2 secretion (Sherris, 1989). Both mAbs were equally effective in inducing proliferation of T cells and secretion of BCGF, IL-4 and γIFN.

In order to determine whether the dichotomy between BCDF and IL-2 secretion was due to activation of distinct T cell subpopulations or differential activation of the same T cell, we generated T cell clones from mAb-446 and 454 stimulated T cells by limiting dilution and then restimulated them with either mAb 454 or 446 and assessed their cytokine secretion profiles. The data generated strongly support the concept that the mechanism of T cell activation dictates the cytokine secretion profile. These findings may have significant implications with regard to the concept of Th1/Th2 clones.

a) Generation of Clones:

10 T cells per well were cultured in 100µl of CM in the presence of X-radiated (3000 rad) autologous non-T cells in the presence of either mAb-446 or mAb-454. Cultures were fed every other day with fresh medium containing anti-CD3 antibodies and 10 U/ml IL2, and previously frozen X-radiated autologous APCs. After the generation of visible clones in microwells, cells were transferred to macrowells and further growth was supported.

Since stimulation with anti-CD3 renders T cells anergic to restimulation with CD3, cells were washed and rested in the absence anti-CD3 and external IL2 four days before restimulation.

b) Dichotomy in the cytokine secretion profiles of mAb-446 and mAb-454 stimulated T Cells:

We have previously shown that stimulation of peripheral blood T cells with mAb-446 which recognizes the γ chain of the CD3 complex induces T cells to secrete a novel B cell differentiation factor (446-BCDF), whereas stimulating them with mAb-454, which is against the e chain of CD3 complex induces them to secrete IL2 (Figure 22), but not 446-BCDF (Figure 20). Both of them were equally

potent in the induction of B cell proliferation, and secretion of IL4, IL6 and BCGF (not shown).

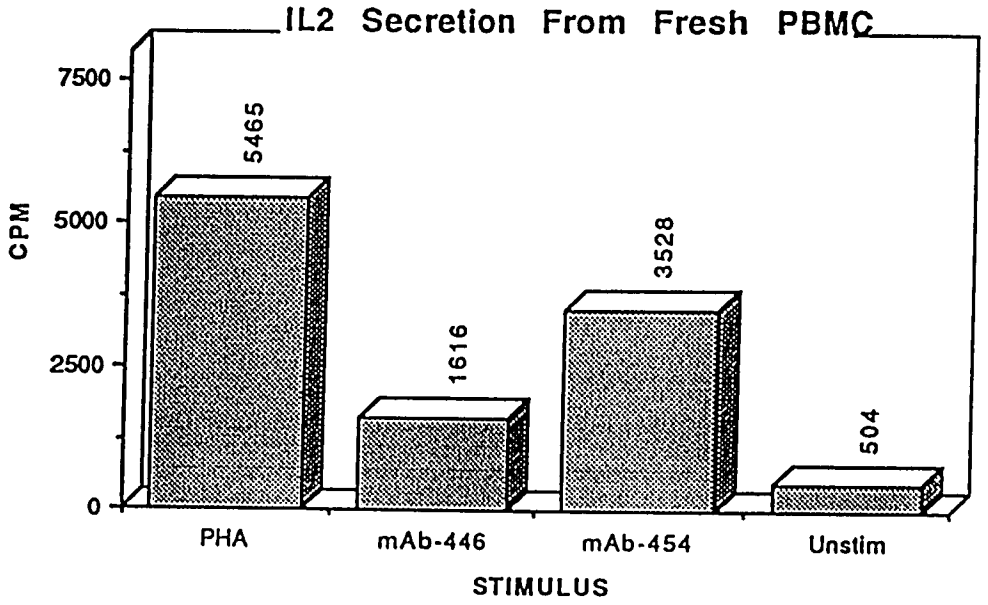


Figure 23 : IL2 secretion by PHA, mAb 446, mAb 454 or unstimulated fresh T cells. Culture supernatants were collected at 14 h and 10% v/v supernatants were added to CTLL cultures for 24 h. Proliferation of the CTLL cell line was measured by pulsing cells with 3H-Thymidine for 6 h.

c) T Cell Clones Maintain Their Cytokine Secretion Response to mAb-446 and mAb-454 Stimulation Regardless of Their Initial Stimulation:

Regardless of their primary stimulation, anti-CD3 derived T cell clones were able to proliferate (Figure 24) and flux Ca++ (data not shown) in response to either mAb and secrete BCGF comparable to fresh cells (Figure 25).

However, the dichotomy between IL2 and 446-BCDF secretion was maintained even after clonal expansion. Clones, regardless of their primary stimulus secreted more BCDF when they were stimulated with mAb-446 (Figure 26), and IL2 when they were stimulated with mAb-454 (Figure 27). Nonetheless, 446-clones secreted more 446-BCDF then 454-clones when they were stimulated with mAb-446, but they were not fixed in their phenotype.

These data strongly support the concept that the mechanism of T cell activation dictates the cytokine secretion profile. These findings may have significant implications with regard to the concept of Th1/Th2 clones.

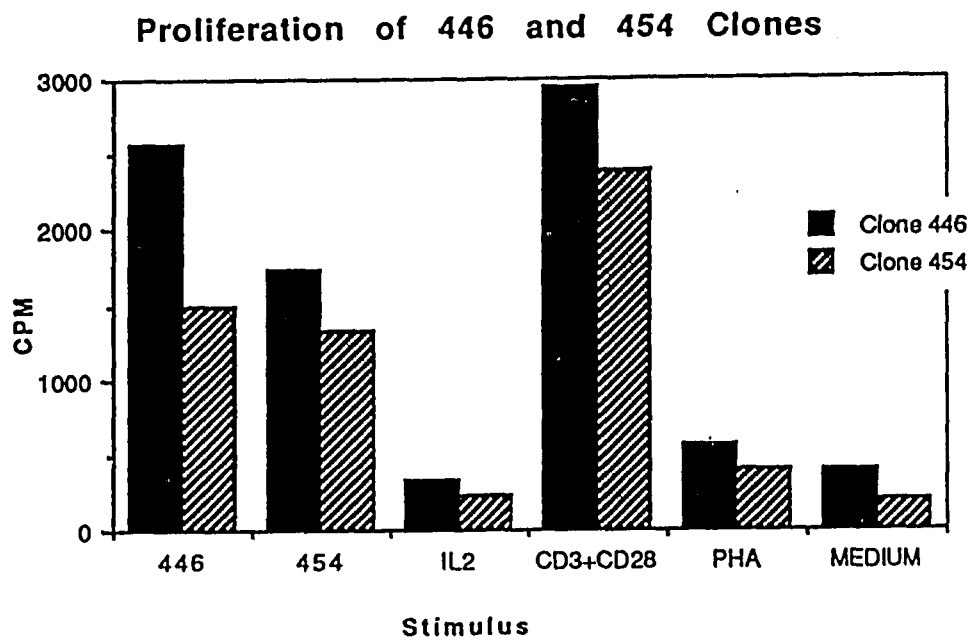


Figure 24 : Proliferation of mAb-446 and mAb-454 generated T cell clones. $5x10^4$ T cells were stimulated for 24 h in the presence of X-radiated autologous APC.

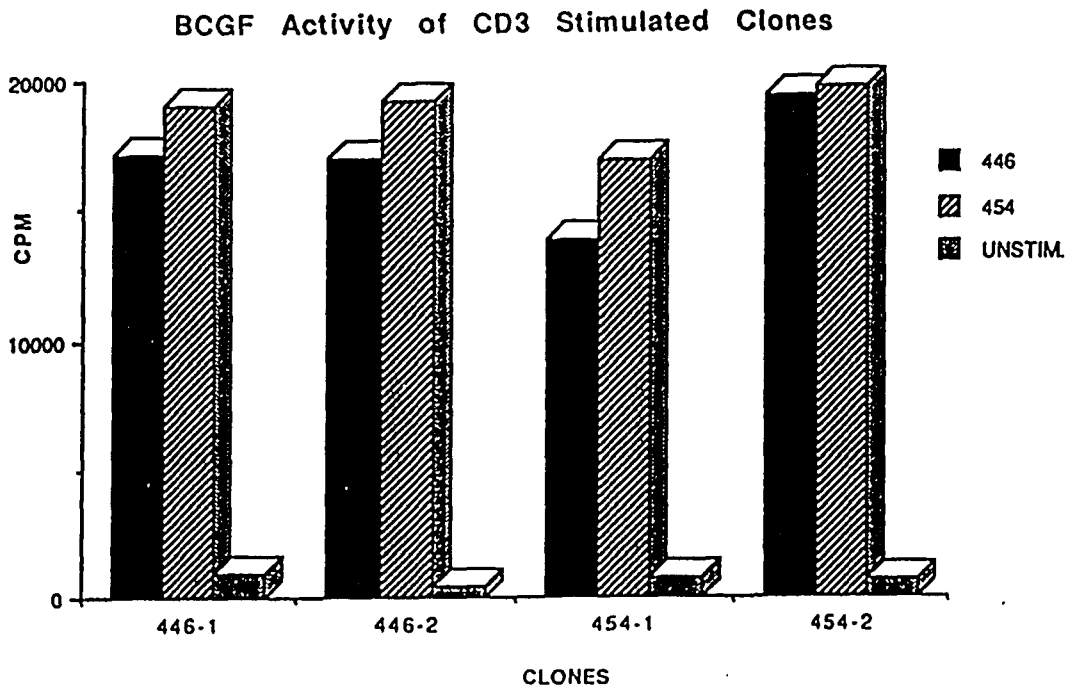


Figure 25 : BCGF activity of supernatants from mAb-446 and mAb-454 stimulated T cell clones. Supernatants we collected at 48 h. 10% v/v supernatants were added on 10^5 normal B cell in 100µl of CM. B cell proliferation was measured 3 days later by ^{3}H-Thymidine incorporation.

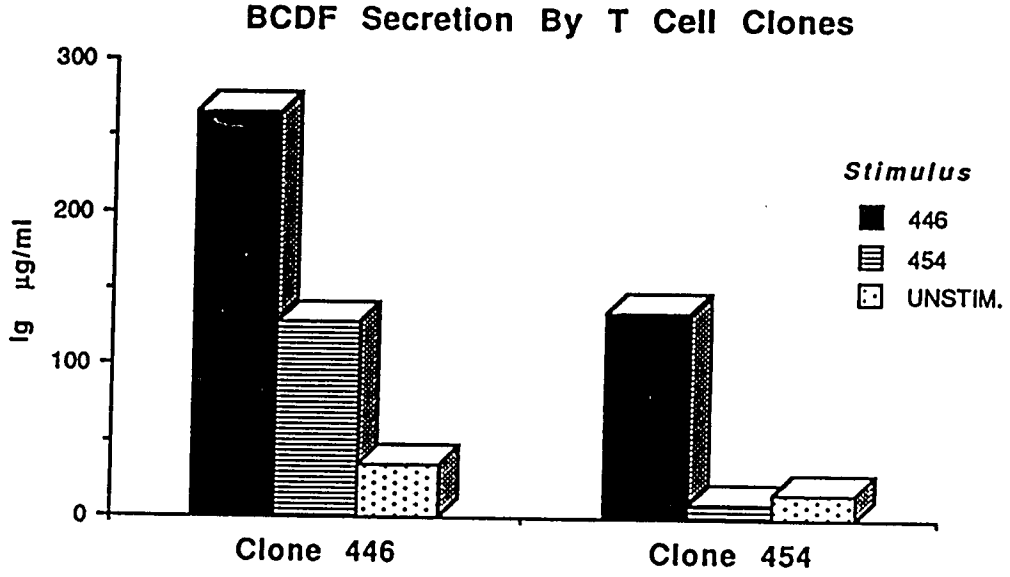


Figure 26 : BCDF activity of supernatants from 446 and 454 clones. Supernatants were collected at 48 h from mAb-446 or -454 stimulated clones. BCDF activity was measured as Ig secretion from SAC activated normal B lymphocytes in the presence of 10% v/v test supernatants.

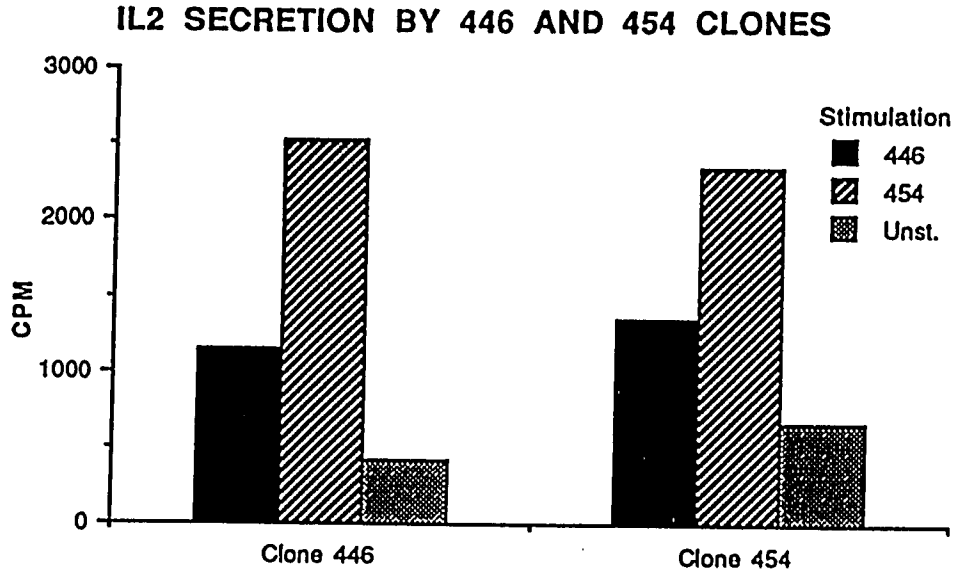


Figure 27 : IL2 secretion by 446 and 454 clones. Clones were stimulated with mAb-446 and mAb-454 for 14 h and supernatants were collected. IL2 content of the supernatants were tested by the proliferation of IL2 dependent CTLL cell line.

3- BY-PASSING CYTOKINE SECRETION DEFECTS :

Having demonstrated that a major factor in the development of CVI relates to the cytokine secretion profiles of T cells, i.e that IL2 and 446-BCDF secretion were defective in most patients, we next tried to reverse these defects in vitro in 5 patients using a variety of stimuli.

As we showed previously mAb-446 alone is not a good stimulator of IL2 secretion. However, combining mAb-446 with anti-CD28 stimulation can induce significant IL2 secretion in normal subjects (Figure 17). This attempt failed to induce IL2 secretion in all 5 patients tested (Figure 28 demonstrates one patient as an example).

Unlike IL2 secretion, mAb-446 alone can induce 446-BCDF secretion by T cells. No costimulus tested has been shown to increase 446-BCDF secretion. Nonetheless, we tested whether combining anti-CD28, anti-CD2, IL2 or γIFN would induce 446-BCDF secretion in BCDF defective CVI patients. Similar to the findings with IL2 secretion, combinations of stimuli all failed to induce 446-BCDF secretion in CVI patients (Figure 29).

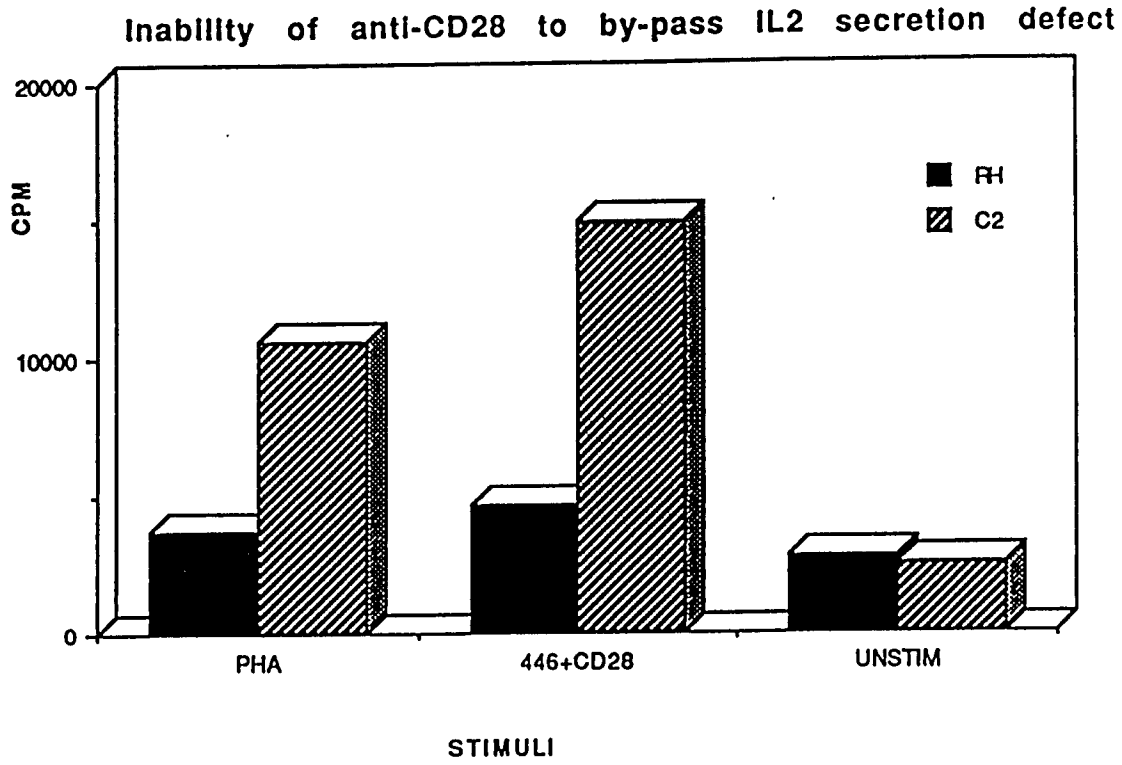


Figure 28 : Inability of anti-CD28 co-stimulation to by-pass IL2 secretion defect. Patient and control PBMC were stimulated with mAb-446 and anti-CD28 for 14 h and supernatants from these cultures were tested for IL2 contents using CTLL cells.

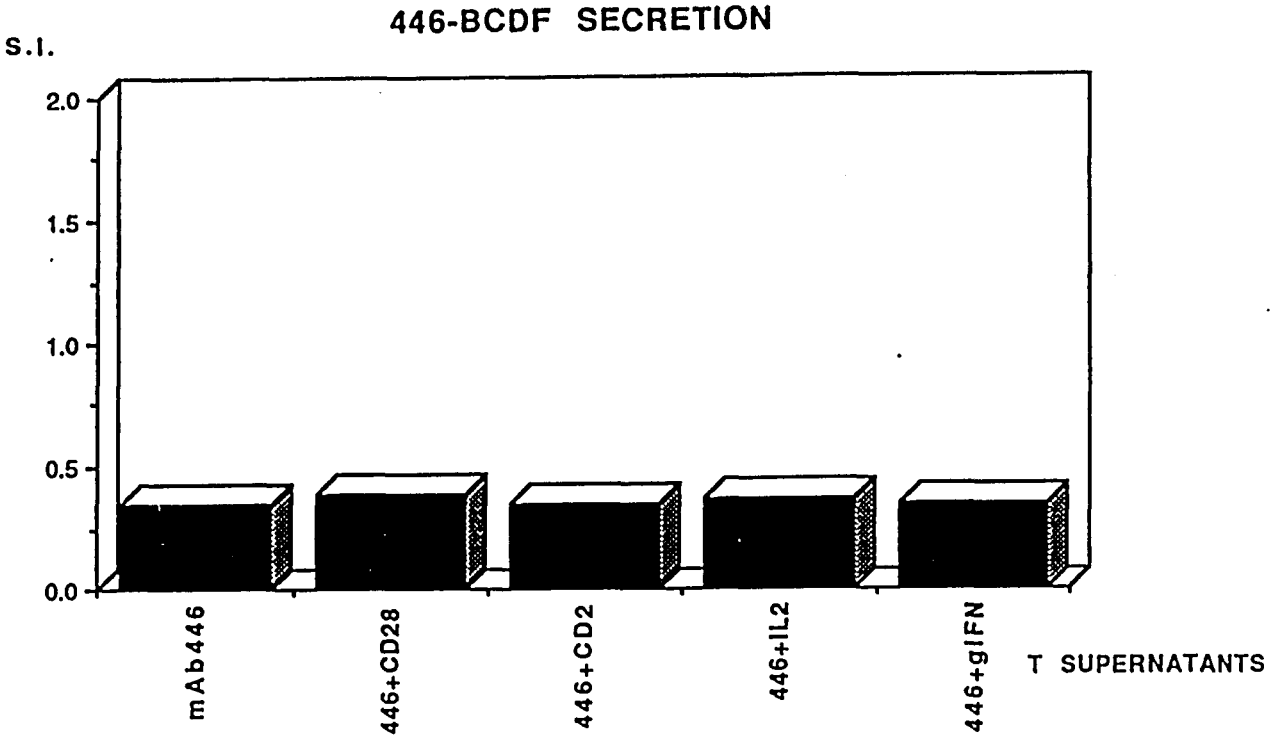


Figure 29 : PBMC from a patient who does not secrete 446-BCDF were stimulated with several factors together with mAb-446. None of the factors were able to restore 446-BCDF secretion defect. S.I. = Ig secretion from normal B cells in the presence of test supernatants / Background Ig secretion from same cells.

4- Ca++ FLUX IN CVI PATIENTS IS NORMAL IN RESPONSE TO mAb-446:

As discussed earlier, mAb-446 is a strong stimulus for Ca++ flux when compared to the other anti-CD3 mAb-454. In a small group of patients we tested whether the defect in 446-BCDF secretion in CVI patients related to a difference in mAb-446 induced Ca++ flux.

Six patients 446-BCDF deficient patients and 8 controls were tested and, as seen in Figure 30, they did not differ in the Ca++ flux induced by mAb-446. From these data it seems unlikely that the intracellular signalling pathway that induces Ca++ flux is defective in CVI patients. This finding is also reflected in the T cell proliferation data presented earlier. CVI patients' T cells were able to proliferate within the normal range in response to PHA and anti-CD3 stimulation.

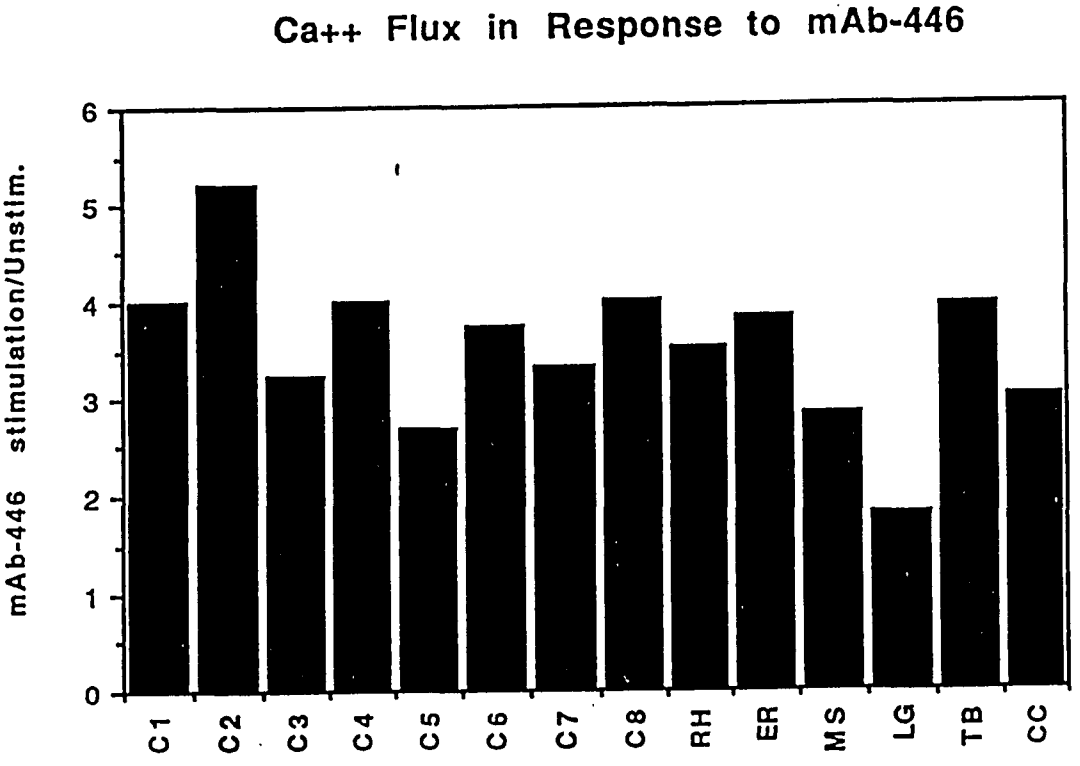


Figure 30 : Ca++ flux induced by mAb-446. Isolated indo-1 loaded PB T cells were stimulated with mAb-446 and crosslinked with goat anti-mouse Ig antibodies and analyzed by flow cytometry. The O.D. ratios (490nm/405nm) of unstimulated versus mAb-446 stimulated T cells are presented.

5- IMMUNOLOGIC EFFECTS OF PEG-IL2 TREATMENT ON CVI PATIENTS :

The question remains as to which is the critical cytokine defect in CVI patients. Clearly IL-2 has been shown to be a critical factor in T cell growth and differentiation and would be an excellent candidate as a primary defect. The presence of IL-2 in the local environment can stimulate the production of other cytokines, but the maturation of T cells to potent effector cells may require longer periods of time than just the 48h in vitro cultures which we had performed.

IL2 is not only a potent stimulator of T cells, but also can modulate other cellular components of immune system. Its stimulatory effects have been shown to result in tumor Ag recognition and rejection by T cells. This effect has already been documented in the treatment of renal cell carcinomas and malignant melanomas, giving promising results with up to 10% complete remission and 30% partial remission.

The use of IL2 in the treatment of CVI is based on two observations. First, its direct effect on Ig secretion in vitro by B cells which have been activated by SAC or anti-IgM. Second, and

possibly more important, it can activate T cells and modulate their helper functions.

However, IL2 has very short life time in the body (t1/2 approximately 3-4 minutes) and treatment with large doses required to achieve levels in the tissue may induce a number of undesired side effects including severe hypotension, hemorrhage and capillary leak syndrome. These problems limit its use in large I.V. doses. Conjugating IL2 with three to five molecules of PEG-5000 (Cetus, Chiron Corp., Emeryville, CA) can extend its half life up to 20-fold with no loss in its biological activity, and permit its use in lower doses with longer intervals.

For our initial PEG-IL2 treatment trial in CVI, five patients were chosen with hypogammaglobulinemia, lack of T helper activity for Ig secretion and an enhanced in vitro proliferative response to PEG-IL2. Although all the patients had common in vitro defects (decreased in vitro T helper activity) they were heterogeneous with regard to their clinical presentation, serum Ig levels and B cell numbers (Table 3).

PAT.	AGE	SEX	SYMPTOMS AND SIGNS	IgG*	IgA*	IgM*	T%†	B%†	T4/T8†
SK	56	M	Pneumonia, empyema, history of hemolytic anemia	45	7	3	88	9	1
CB	15	F	Recurrent pneumonia, diarrhea	90	0	400	85	1	0.19
AN	67	M	Bronchiectasis	244	3	4	40	1	0.63
JM	20	F	Muco-cutaneous candidiasis, bronchiectasis, malabsorption	402	38	110	61	30	0.72
LG	49	F	Asthma, arthritis, cardiomyopathy, pneumonia	140	75	35	64	1	0.54

Table 3 : CVI patients that participated in the PEG-IL2 trial.

(*) Immunoglobulin levels at the time of diagnosis, prior to treatment with IVIG. Normal range for IgG= 800-1800 mg/dl, IgA=90-450 mg/dl, IgM=80-350 mg/dl.

(†) Normal range for T cells (CD3)=65-90%, B cells (CD20)= 2-15%, CD4/CD8= 1-3.

The patients were given PEG-IL2 weekly by subcutaneous injection in a 12 week study. PEG-IL2 was given in doses of 50,000 IU/m^2 body surface for weeks 1-4; 150,000 IU/m^2 for weeks 5-8; and 250,000 IU/m^2 for weeks 9-12. All patients were also treated with intravenous immunoglobulin (IVIG) at 3-4 week intervals 300-400 mg/kg, respectively. For *in vitro* studies blood was drawn prior to administration of PEG-IL2 and IVIG.

There was heterogeneity observed in the in vitro improvement by patients during the treatment. Considering the small size of the treatment group and the heterogeneity of the in vitro experimental results, it is difficult to make generalizations regarding responses to PEG-IL-2 from these five patients to all CVI patients.

Nonetheless improvement in in vitro function was observed in 4 patients and in one patient the parameters remained unaltered during the study. One patient who was evaluated most extensively is presented here and compared to the other three patients. This is contrasted to the one patient who did not benefit from PEG-IL2 treatment.

a) Patient L.G. :

Patient L.G. is a 49 year old female with the history of recurrent pneumonia, asthma and arthritis. She started PEG-IL2 treatment on 3/7/92 and she continue treatment at present. She has not complained of any side effects of the PEG-IL2 treatment. Her blood was drawn at regular intervals, at least once every four weeks before the administration of IL2 or IVIG.

The *in vitro* studies conducted with her PBMC included T cell proliferation; secretion of IL2, IL6, 446-BCDF, BCGF; B cell proliferation and differentiation to SAC+IL2 and SAC+446-BCDF; and helper assays using either her T cells with normal B cells, or her B cells with normal T cells in the presence of PWM.

i) T and B Cell Proliferation :

T cell proliferation in response to PHA and anti-CD3 stimulation by patient LG was within the normal range (5 to 100 fold increase in 3H-Thymidine incorporation) throughout the study with wide variations (Figure 31). In contrast she did not respond to anti-CD2 stimulation, the significance of which has not been clearly addressed.

Her B cell proliferation in response to SAC+IL2 or LMW-BCGF was within the normal range and did not change during the study (data not shown).

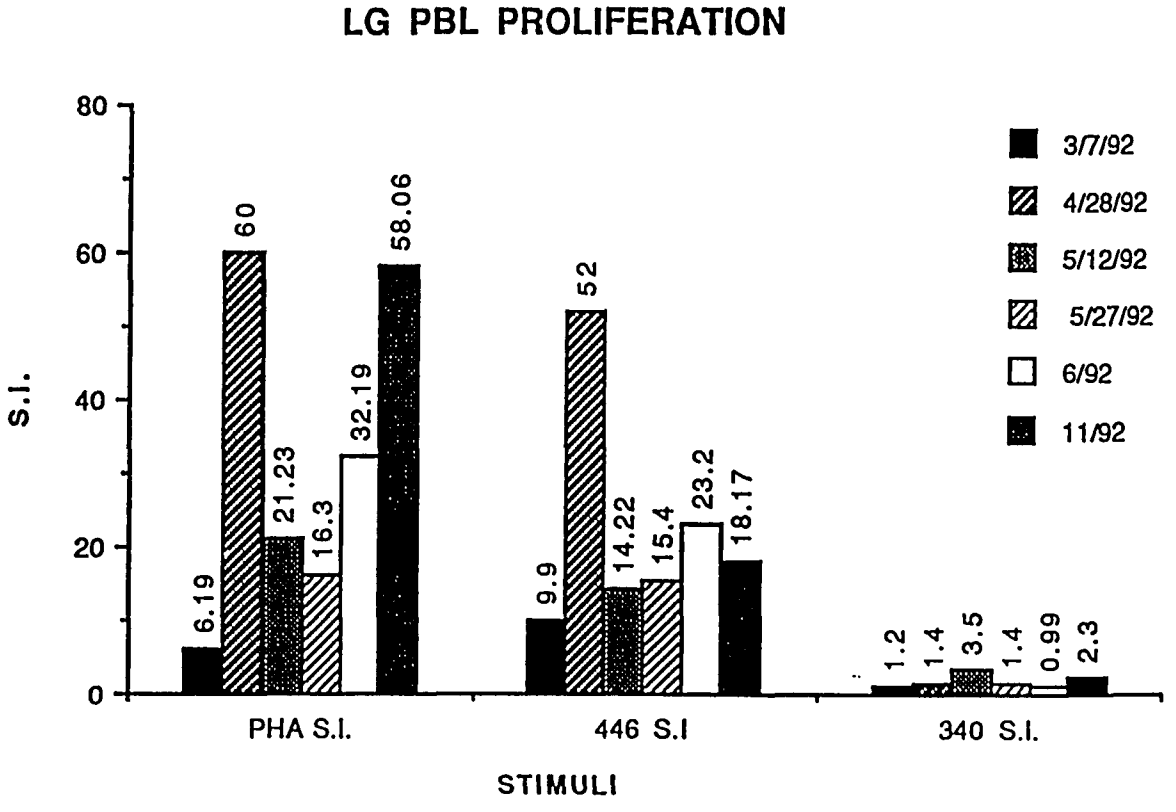


Figure 31 : Proliferation of LG's T cells in response to PHA, anti-CD3 (446), anti-CD2 (340) stimulation. Stimulation Index = CPM of stimulated wells ÷ CPM of the unstimulated wells.

ii) Improvement in the Cytokine Secretion by T Cells :

At the beginning of the study LG was a normal secretor of IL2, but IL6 secretion was reduced and she did not secrete 446-BCDF. During the course of the treatment her IL6 secretion normalized early on and remained within the normal range for the rest of the study (Figure 32).

However, probably the most dramatic improvement was in the secretion of 446-BCDF. She was a non-secretor for a long time. After she committed to the therapy, T cells from the patient could be activated to secrete 446-BCDF and the secretion kept increasing significantly thereafter (Figure 33).

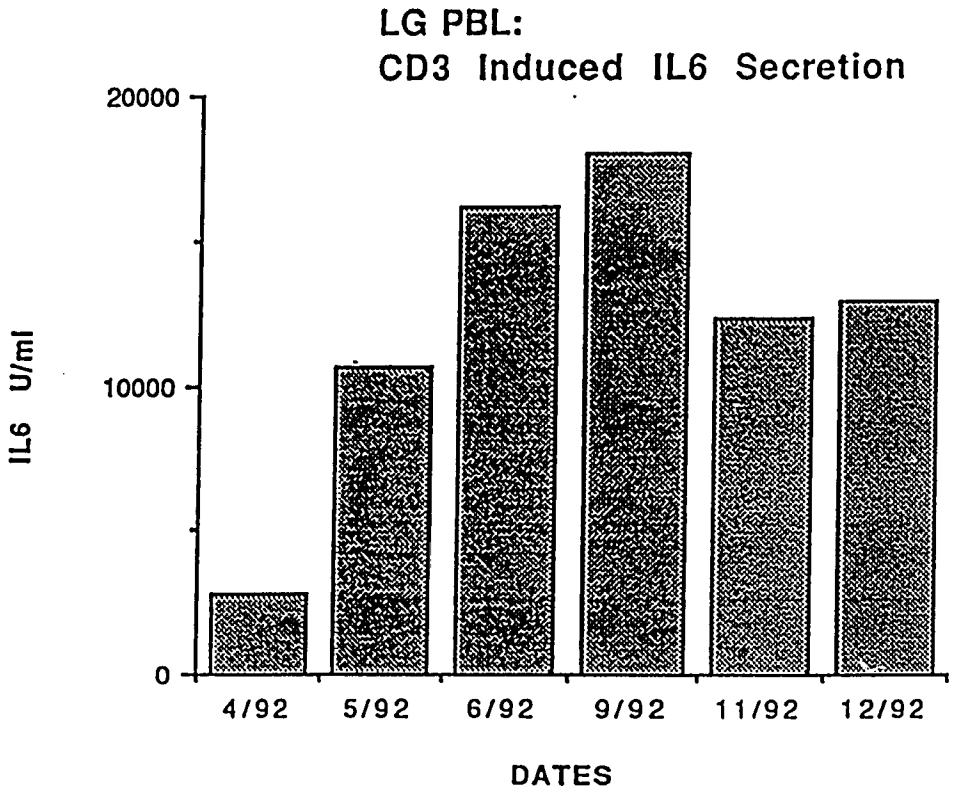


Figure 32 : IL6 secretion of anti-CD3 stimulated PBMC during the PEG-IL2 treatment. The cells were stimulated with mAb-446 and the IL6 activity of the 48 hour supernatants was measured using IL6 dependent cell line B9.

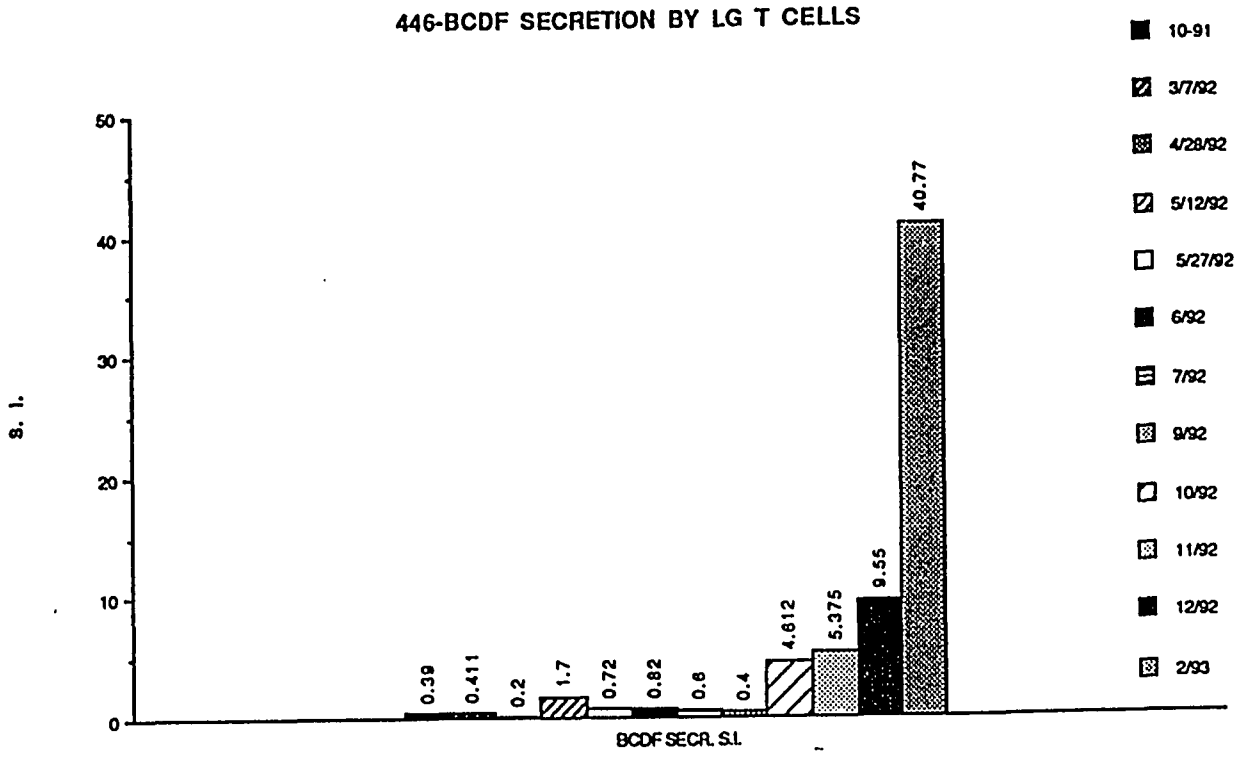


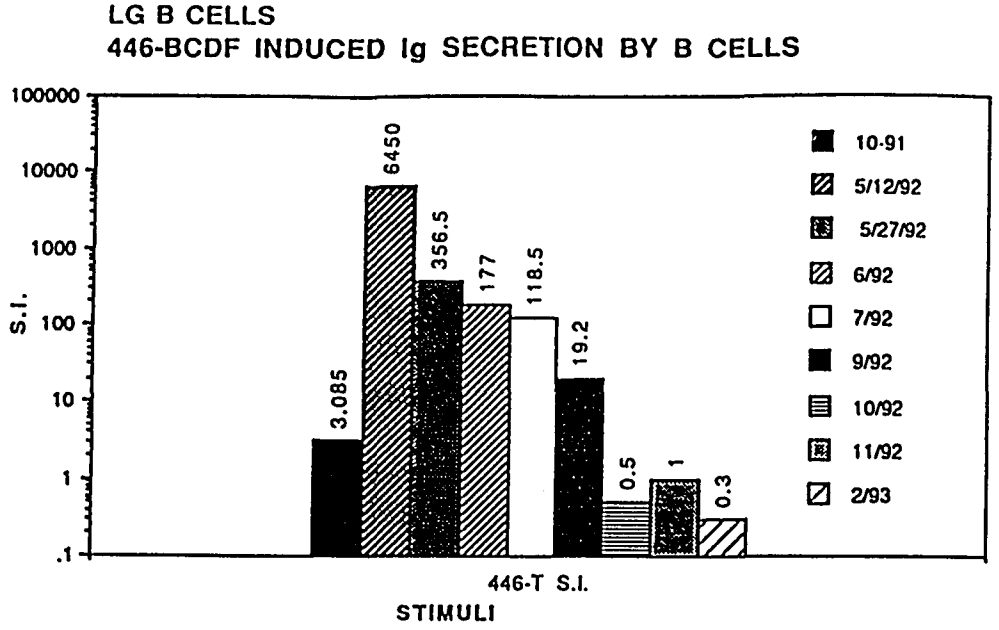
Figure 33 : Improvement in the 446-BCDF secretion during PEG-IL2 treatment. 48 hour supernatants of mAb-446 stimulated PBMC were collected and 446-BCDF activity of the supernatants measured using normal peripheral blood B lymphocytes. Stimulation index = Ig secretion from B cells cultured with patient's supernatants ÷ Ig secretion by unstimulated B cells.

iii) Changes in B cell differentiation in response to IL2 and 446 BCDF secretion :

During the PEG-IL2 treatment not only was the cytokine secretion profile by T cells changed, but also some significant changes were observed in the response of the B cells to differentiation signals. At the beginning of the study LG's B cells were not able to secrete Ig in response to SAC+IL2 stimulation. However, LG B cells were hyper-responsive to 446-BCDF with thousands-to-hundred fold increases in Ig secretion. This may reflect an over-expression of the receptor for 446-BCDF in the setting where the factor is not secreted or possibly reflect an immature state of the B cells that are incapable of responding to IL2.

Nonetheless, during the study, parallel to the improvement in 446-BCDF secretion, patient's B cell responses to 446-BCDF gradually decreased while they became responsive to SAC+IL2 (Figure 34 A and B), supporting our hypothesis.

A



B

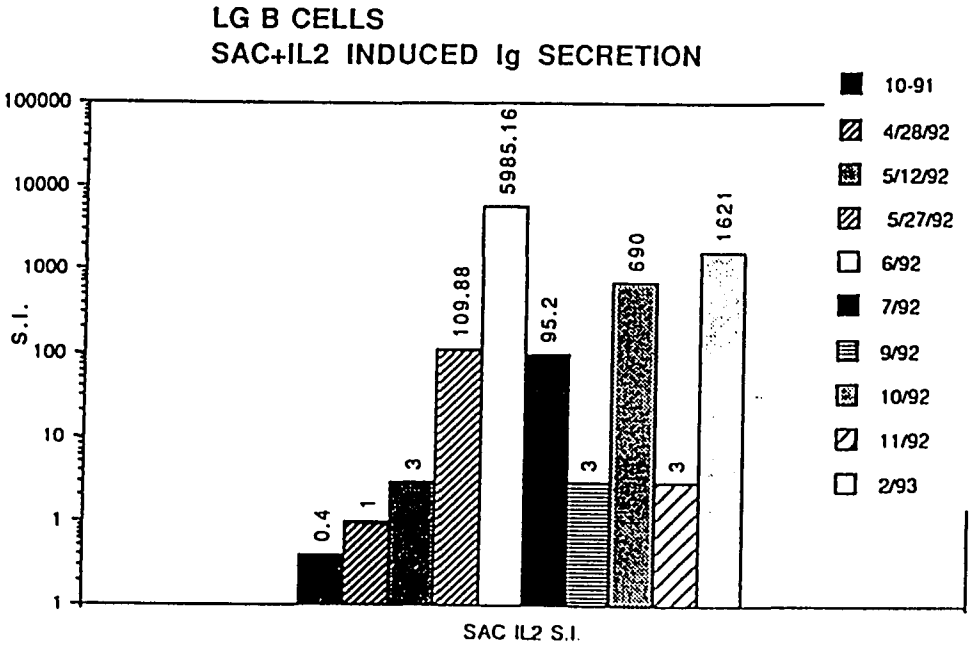


Figure 34 : Changes in B cell differentiation in response to SAC + 446-BCDF or SAC + IL2 stimulation. Stimulation index = Ig secretion from stimulated wells ÷ Ig secretion from unstimulated wells.

iv) Improvement in T - B Cell Interaction :

Unlike SAC + 446-BCDF induced B cell differentiation, PWM induced B cell differentiation requires cell-to-cell cognate interaction between T and B cells.

Prior with treatment PEG-IL2, PBMC from patient were not able to differentiate in the presence of PWM. After treatment, there was an improvement in PWM induced B cell differentiation and Ig secretion. However, the improvement was not sustained in contrast to seen with 446-BCDF secretion. Nonetheless, an increase in Ig secretion observed in 6 out of 7 experiments, albeit with significant variation between experiments (Figure 35).

A similar trend of improvement in T cell help was also observed when patients T cells were cultured with the normal B cells and stimulated with PWM (data not shown). Although there was marked variability, these observations provide evidence that T cell helper function were affected by PEG-IL2 treatment.

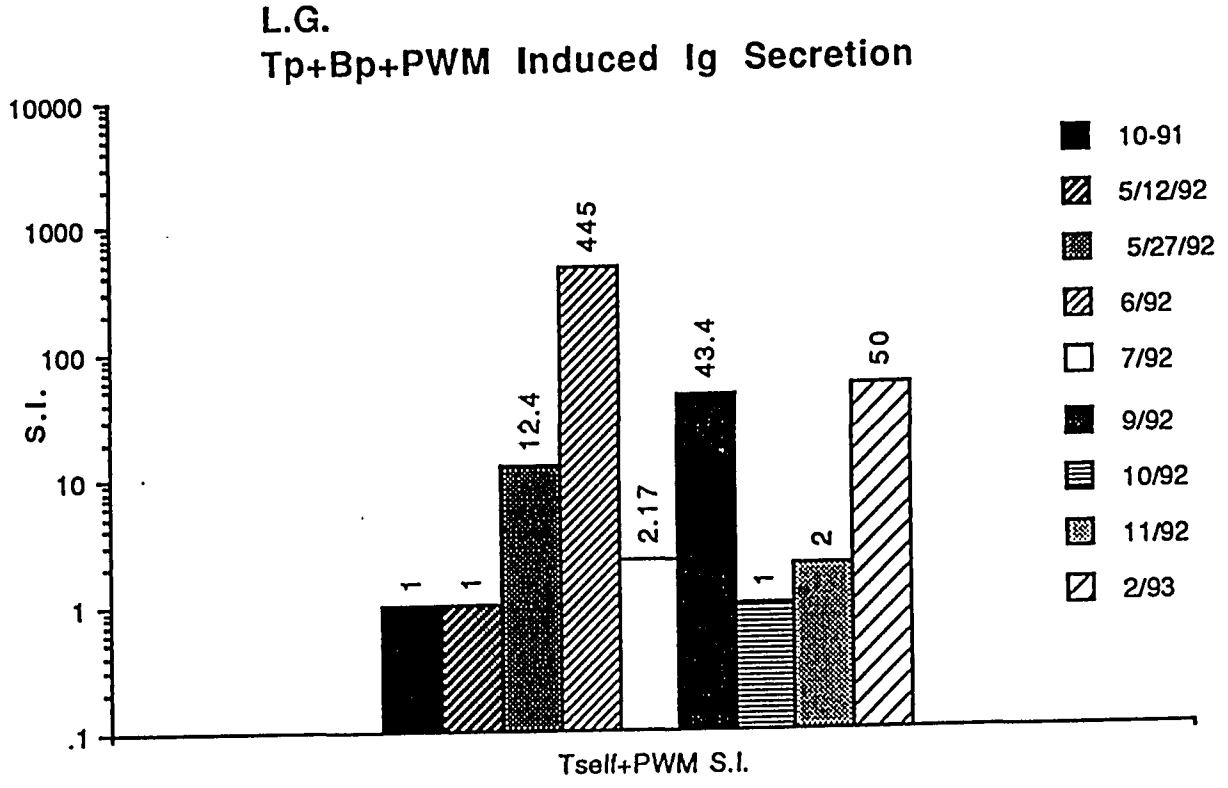


Figure 35 : Improvement in Ig secretion with PWM induced Ig secretion. Patients T cells were mixed with patients autologous B cells in a 2 : 1 ratio and stimulated with 1% PWM for 8 days. Stimulation Index = PWM induced Ig secretion + Ig secretion from unstimulated cultures.

b) Response to PEG-IL2 by Other Patients :

Out of five patients treated, three patients demonstrated improvement in their in vitro parameters similar to those observed in patient LG. Only patient CB, remained unchanged during treatment (see below).

Overall all patients' T cells proliferated normally to PHA and anti-CD3 stimulation and remained within the normal range throughout the study. Two patients (LG and CB) were unresponsive to the CD2 stimulation and this remained unchanged during treatment.

B cell proliferation in response to SAC+IL2 or LMW-BCGF was within the normal range in all patients and did not change with the treatment.

Ig secretion in response to SAC + IL2 was low in all four patients and remained low during PEG-IL2 treatment except improvement seen in LG.

B cell differentiation in response to 446-BCDF was either normal or hyper-responsive in all four patients. This responsiveness diminished during treatment except in CB where response remained unchanged in a hyper-responsive state. IL2 and IL6 secretion were

either remained in the normal range or normalized during the study in 4 out of five patients.

i) Patient CB :

CB is a 16 year old girl with a clinical presentation resembling the hyper-IgM syndrome rather than CVI. She had very low serum IgG, no detectable IgA, but above normal levels of IgM (440 mg/dl).

She had normal T cell proliferation to PHA and anti-CD3 stimulation but, similar to LG, she did not respond to anti-CD2 stimulation. Her B cell proliferation was normal in response to SAC+IL2 stimulation. However, she never secreted Ig in response to SAC+IL2 during the 15 months that she was followed. She was however hyper-responsive to 446-BCDF stimulation. Her T cells never secreted 446-BCDF, but IL2 and IL6 secretion was within the normal range. Her B cells failed to secrete Ig when stimulated with PWM either in the presence of autologous or normal allogeneic T cells. In contrast to the other 4 patients, no change in any parameter seen with PEG-IL2 therapy.

Therefore the overall trend observed during PEG-IL2 treatment was an improvement in T cell helper functions and a decrease in the response of B cells to 446-BCDF. These observations further support

the hypothesis that the main problem in CVI is a defect in cytokine secretion by T cells. This in turn leads to BCDF-receptor upregulation and functional immaturity of the B cells. If T cell helper functions can be improved via an exogenous stimulus such as IL2, this may lead the normalization of T cell help and subsequently a diminution in B cell hyperresponsiveness.

DISCUSSION

Since the discovery of that T and B cell interactions regulate antibody responses, the mechanisms underlying this regulatory process has been one of the central issues in immunology. In the early 1970s the existence of T cell-derived helper factors that promoted B cell proliferation and differentiation was recognized. Production of these factors could be induced by activation of T cells with mitogens, phorbol esters, or stimulation via the TcR.

Following these early discoveries, the literature was replete with reports about different factors, each named for its biological activity and given its own acronym. It was not until cytokines were purified and cloned that the vast array of factors generated in different biological systems could be shown to represent the activities of a limited number of cytokines.

It is now more clear that the pleiotropy (each lymphokine has a number of different effects on different cells), redundancy (more than one lymphokine may act in a similar way in a given cell), synergy and antagonism between the cytokines secreted by activated T cells may create a network of events in B cell differentiation.

To date a number of cytokines have been shown to play a role in B cell proliferation and differentiation. Among these factors IL1 induces B cell differentiation and clonal expansion. Similarly, IL2 can induce both proliferation and Ig secretion by activated B cells. IL4 was first identified as B Cell Growth Factor 1 (BCGF-I) by virtue of its ability to induce B cell proliferation. It can also induce B cell differentiation and a preferential switch to IgE and IgG1 in mouse. This switch can be blocked by IFNγ. IL6 was also recognized as a B cell differentiation factor (BCDF) in murine systems and can function as a differentiation factor in human B cell lines and in primary B cells in the presence of IL-2. Even though IL7 is not a T cell derived factor, it can induce the differentiation of progenitor B cells in the bone marrow. IL10 can affect B cell differentiation by the induction of TH2 type T cells allowing for IL4, IL5 and IL6 secretion by these cells. TGFβ on the other hand can induce switching to IgA.

It is very clear that Ig secretion by B cells is under strict control in every step of the B cell differentiation process, and a network of events can modulate the magnitude and the nature of the B cell response. This network is likely to become increasingly complex in the future as new cytokines are discovered and cognate interactions are more clearly defined.

From this complex picture, we can have a better understanding of patients with hypogammaglobulinemia. The defect in Ig secretion can result from a number of defects in the pathway of B cell maturation. We can group these defects into four categories :

1- Defects intrinsic to B cells .

2- Defects in T helper functions.

3- Inhibition of Ig secretion by suppressor cells or factors.

4- Defects in the antigen presenting cells causing a failure in the stimulation of helper T cells.

In this thesis we attempted to define the nature and site of defects in CVI using in vitro stimulation assays. The availability of a novel B cell differentiation factor helped us to address these questions. This factor, designated 446-BCDF because of its secretion from anti-CD3 mAb-446 stimulated T cells, is a very potent cytokine that can induce a 2 to 100 fold increase in Ig secretion by activated normal B lymphocytes.

The hierarchy of cytokine defects which we observed in CVI patients also provided insight into the regulation of cytokine secretion. While there were a subset of patients who demonstrated global cytokine secretion defects (IL2, 446-BCDF, IL6) comparable to those described by others (Sneller, 1990; Geha, 1991; Pastorelli, 1989; Adelman, 1990; Cruger, 1984; Ramp, 1991) (for IL2, IL4 and

γIFN), there were also distinct profiles of cytokine secretion defects. IL6, which has been shown to be an effective B cell differentiation factor in mouse and in the presence of IL2 in man (Hirano, 1985; Emilie, 1988; Maraguchi, 1988), is reportedly elevated in the serum of patients with CVI (Adelman, 1990). Our data suggest that IL6 deficiency is a subset of BCDF deficiency. The majority of BCDF deficient patients did secrete normal amounts of IL6, but no IL6 deficient patients secreted BCDF. Therefore it appears that BCDF is independent of IL6 secretion and IL6 may not correlate well with Ig production.

Similarly, although somewhat less clear, a correlation was also seen between 446-BCDF and IL2 secretion as a dual secretion defect was observed in the majority of the patients, while a selective IL2 secretion defect was observed in three patients. The comparison is problematic since IL2 production could only be measured in PHA stimulated supernatants and BCDF only in anti-CD3 stimulated supernatants. Furthermore the IL2 secretion kinetics were different than 446-BCDF secretion kinetics. Only 18h supernatants of PHA stimulated cultures were studied as determined from control studies. Still early IL2 production may be a critical event for later cytokine secretion. Alternatively, defects in early cytokine production may reflect more global defects in the T cell. Thus far, in limited studies, we have been unable to correct the 446-

BCDF secretion defect *in vitro* using costimulation (anti-CD2, anti-CD28) or cytokines (IL1,IL2, γIFN). Interestingly, however, in a recent study (Cunningham-Rundles, 1992), we were able to restore normal PWM driven T helper activity following intravenous treatment with a long acting form of IL2 and, concomitantly restore B cell differentiation capabilities *in vitro* to both T dependent (PWM) and T independent (BCDF) stimuli. Furthermore, we have evidence in one patient that such therapy restores the cytokine secretion defects (BCDF) that we have described. Taken together these findings support an intrinsic cytokine secretion defect as the major pathogenetic mechanism in CVI.

The second issue relates to the nature of the defect in the T cell. Our system utilizes an antigen surrogate, anti-CD3 crosslinking, to polyclonally activate T cells. Many groups have described clear defects in Ag specific T cell activation in CVI patients, in some cases noting it as the earliest defect expressed in this disease. If signalling through CD3 is defective, then secondary signals triggered in the membrane and cytosol would not be translated into the induction of cytokine secretion. From the studies described in this thesis it appears that the proximal events are intact (i.e Ca++ flux) and that proliferation in response to anti-CD3 is normal. Thus far we have not been able to utilize the defect in cytokine secretion to define the biochemial pathways which dictate

BCDF secretion. In this regard, these studies do not address the potential role of the antigen presenting cell in this process and other defined co-stimulatory molecules such as CD40/gp39 which are defective in other immunodeficiency states (DiSanto, 1993; Aruffo, 1993).

In one set of experiments we tried to define these potential co-stimulatory pathways in an attempt to by-pass the T cell secretion defect in 5 BCDF deficient patients. We stimulated their T cells with a number of different co-stimuli which have been shown to induce or modulate T cell stimulation or cytokine secretion: anti-CD28, anti-CD2, IL2, IL1 and γIFN alone or in combination with anti-CD3 stimulation.

None of these agents were able to stimulate 446-BCDF secretion by themselves. We have shown that CD28 co-stimulation of normal T cells with anti-CD3 may greatly enhance IL2 secretion and can induce IL4 secretion. Interestingly, in spite of CD28's positive regulatory role in IL2 and IL4 secretion, its effect on 446-BCDF secretion was inhibitory. CD3/CD28 co-stimulation of 446-BCDF deficient patients' T cells did not result in any change in their cytokine secretion profile.

Similarly, CD2, IL2, IL1 and γIFN co-stimulation did not by-pass the 446-BCDF secretion defect in the patients. This may relate to an intrinsic defect in the patients T cells that cannot be by-passed or may reflect the fact that the combination of stimuli used in this study were not appropriate.

We were able to bypass the BCDF secretion defect in one patient using an in vivo agent, PEG-IL2 and show slight improvement in another 3 patients. Weekly sub-cutaneous PEG-IL2 treatment of patients gave promising results in both clinical and laboratory parameters. In four out of five patients 446-BCDF secretion improved during treatment. The improvement in BCDF secretion, however, was observed only later in the course of treatment after we had already documented improvement in in vitro responses to B cell stimuli and IL2 and IL6 secretion. Significant changes in laboratory parameters could only be observed several months after the treatment was started. It is still not clear which cell population was targeted in the cascade of events that leads to Ig secretion. Almost all cell populations in the immune system can be modulated by IL2 treatment including professional antigen presenting cells like monocytes and macrophages, as well as T and B cells themselves.

As we have discussed earlier, CVI is not a disease but rather a group of diseases having in common the laboratory finding of

hypogammaglobulinemia as well as a number of other related problems. In this study we have shown that in most cases the defects are in the T cells failing to support B cell differentiation.

These findings lay the groundwork for future therapies. If, in fact, 446-BCDF can restore normal IgG and more importantly specific IgG secretion in the majority of the patients *in vitro*, it may serve as a more physiological therapeutic replacement than IVGG. Rather than relying on passive immunity via monthly administrations of pooled protective antibodies, the patients would produce their own specific immunoglobulins. Tailoring therapy to specific defects in cytokine production may render these patients more capable of generating normal immune responses.

Studies to isolate 446-BCDF are already underway in our laboratory. We have been able to generate monoclonal antibodies that can specifically bind to and inhibit its action *in vitro*. However, 446-BCDF appears to be a very potent factor secreted at low protein concentrations making it extremely hard to obtain sufficient quantities of protein to sequence. Once the protein is purified, and cloned it will be more readily available so that studies defining its mechanism of action will be easier. Quite possibly it will find its place in the treatment of CVI with T helper defects as well as other conditions.

SIGNIFICANCE

CVI is a relatively common disorder affecting approximately half a million people nationwide. Even though hypogammaglobulinemia is the laboratory parameter shared by all patients, the cause of this disorder may differ between patients. The defect can be intrinsic to B cells with Ig secretion defects or extrinsic to the B cell where factors inducing B cells to secrete Ig are lacking.

Current treatment of CVI is the correction of the low serum Ig levels by IV immunoglobulin administration pooled from multiple donors. Obviously the ideal approach would be correction of the actual defect, allowing patients' B cells to secrete specific Ig as needed in response to foreign antigens.

In this study we have shown that the main defect is likely to reside within the T cells, failing to supply help to B cells, rather than being intrinsic to B cells themselves. We have demonstrated that a novel B cell differentiation factor, 446-BCDF, is a deficient factor in the majority of the patients. This defect was accompanied by IL2 or IL6 secretion defects in some patients, indicating a global

secretion defect; whereas 446-BCDF secretion was selectively defective in the remaining patients.

Most importantly, our in vitro experiments indicate that patients' B cells can respond to 446-BCDF by a significant increase in the secretion of IgM and IgG, as well as, specific IgG antibodies against tetanus toxoid. Knowing that a single or multiple cytokine secretion defect is potentially the cause of the disease in most patients, may open new avenues for future therapies. Finding the selective factors that patients need may allow us the tailor their treatment, to drive their own B cells to differentiate and secrete Ig as needed.

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