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Characterization and signal transduction pathways of a novel human B cell differentiation factor

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Characterization and Signal Transduction Pathways of A Novel Human B Cell Differentiation Factor

by

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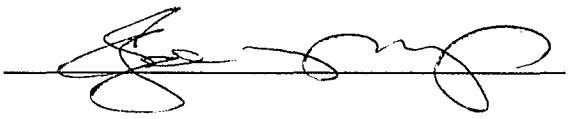

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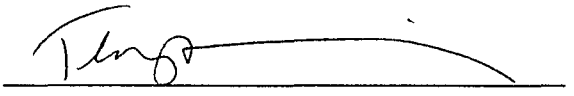

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ABSTRACT

Characterization and Signal Transduction Pathways of A Novel Human B cell Differentiation Factor

By

RUOQING HUANG

Advisor: Professor Lloyd Mayer

We have recently identified a novel human B cell differentiation factor, 446-BCDF derived from anti-CD3 stimulated peripheral blood (PB) T cells. This novel cytokine induces a more than 5 fold increase in immunoglobulin secretion by both SAC activated PB B cells and tonsil B cells. 446-BCDF has been partially purified and has an apparent molecular weight of 32 Kd and a pI of 6 which are distinct from the molecular weight of IL2, IL4, IL10, IL13, and the pI of IL6. Bioassays show that this partially purified 446-BCDF does not contain detectable IL2, IL4, and IL6 activity and 446-BCDF activity can not be blocked by antibodies against these cytokines or their receptors. 446-BCDF induces IgM, IgG, IgA but no IgE secretion, and can be specifically inhibited by a monoclonal antibody 929. With this partially purified cytokine, we initiated studies to investigate signaling pathways involved in 446-BCDF mediated B cell differentiation. a cAMP analogue (Dibutyryl cAMP), an adenylate cyclase stimulator (forskolin), and phosphodiesterase inhibitors (aminophylline and IBMX) all inhibited Ig secretion induced by 446-BCDF. Direct measurement of cAMP levels demonstrated that 446-BCDF induced a reduction of intracellular cAMP in a time dependent

manner. This reduction may be due to the stimulation of adenylate cyclase via a Gi linked receptor since a potent Gi protein inhibitor, pertussis toxin, was able to prevent the 446-BCDF induced decrease in intracellular cAMP and inhibit Ig secretion. Angiotensin II (AT II) also induced a reduction in intracellular cAMP via a Gi linked receptor and stimulated Ig secretion by SAC activated B cells. Ig secretion induced by AT II was significantly enhanced by 446-BCDF, suggesting that in addition to inducing a decrease in intracellular cAMP 446-BCDF may also deliver other signals to induce Ig secretion. This hypothesis was supported by the observation that 446-BCDF stimulated B cells to produce IP_3 and flux Ca^{++}. The latter event was critical to Ig secretion since a calcium chelator, BAPTA, was able to block Ca^{++} flux and Ig secretion induced by 446-BCDF without affecting cell viability. PKC activity was not detected using a specific PKC assay system. 446-BCDF activity was not inhibited by PKC and serine/threonine kinase inhibitors. However, 446-BCDF activity was inhibited by a tyrosine kinase inhibitor, genistein. Taken together, these data suggest that the reduction in intracellular cAMP may an important signal to trigger human B cell Ig secretion which is a Ca^{++} dependent event. Tyrosine kinase may also play a role in B cell differentiation.

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INTRODUCTION

B Cell Development

B cells develop from a pluripotent stem cell which can give rise to all hematopoietic cells (Brouet et al., 1983). Although several functional properties have been ascribed to the B cell, its major function remains the production of specific antibody (Ab) against a specific antigen (Ag). According to genetic rearrangements, cell surface phenotype and function, the development of the B cell can be divided into several stages: pro-B (DJ rearrangement), pre-B (VDJ rearrangement), immature B (light chain gene rearrangement), mature B ($sIgM^+$), as well as the terminal plasma cell (Ab secreting) stage (cIg^+). Once the light chain gene has undergone rearrangement and a functional Ig molecule is produced the B cell expresses surface (s) IgM and is responsive to exogenous Ag. It is at this stage (and thereafter) where Ag become critical for B cell activation and response to cytokines (Cooper et al., 1987, Levitt et al., 1987). Mature resting B cells, activated by antigens (Ags) or mitogens, lose surface IgD as they gain receptors for soluble growth and differentiation factors (cytokines) produced by activated T cells and monocytes

Mature resting B cells circulate in the blood and go into the secondary lymphoid organs where antigens are trapped. Antigen specific B cells can capture and process the antigen there (Lanzavecchia et al., 1990). Activated T cells present in secondary lymphoid tissues can induce Ag-bearing B cells to migrate into B cell follicles by T cell derived cytokines (Clark et al., 1994). In the follicle,

B cells interact with follicular dendritic cells, leading to the formation of germinal centers where the immunoglobulin variable (V) region genes of B cells undergo somatic mutation to increase their affinity for Ag (Moller et al., 1992). This process occurs after antigenic stimulation and during germinal center development. B cells with high affinity receptor for antigen are then selected. Here too, immunoglobulin class switch from IgM to other isotypes occurs. This event requires the presence of antigen activated B cells, helper T cells and follicular dendritic cells. Eventually, germinal center B cells mature into either memory or plasma cells (Clark et al., 1994).

B Cell Growth and Differentiation

In an immune response, B cells need to be activated to perform their immune function. The activation of B cells leads to two distinct phases: proliferation and differentiation. In response to B cell growth factor (BCGF), B cells undergo clonal expansion. During this proliferative event a subpopulation of B cells differentiates further into Ig-forming cells in the presence of BCDF, while others give rise to memory B cells which can be called upon to differentiate when rechallenged with Ag. Mitogens can substitute for Ag, crosslinking sIg or other less well defined surface molecules (Levitt et al., 1987). In human, fixed Staphylococcus aureus Cowan I strain organisms (SAC) is a potent crosslinker of Ig and at low concentrations (0.001%) promote B cell activation (Saiki et al., 1981). SAC has also been shown to induce tyrosine phosphorylation which is an obligatory early signal in human B cell proliferation (Roifman et al., 1991). Other mitogens, such as PWM and LPS, may extensively crosslink

other surface receptors promoting growth and differentiation of the B cell.

The differentiation of resting B cells into Ig-secreting cells is a highly regulated event. The interaction between B and T cells through cognate and noncognate (cytokine) mechanisms results in the activation and differentiation of B cells. Many receptor-ligand pairs mediating the effects of T cells on B cell function have been well characterized (Clark et al., 1994). Some cell surface adhesion molecules are likely to affect the migration and adhesion of lymphocytes, others bind to their ligands to allow T and B cells to sense the state of their partner's activation (Clark et al., 1994). The discovery of CD40 and its ligand gp39 has created new insights into the activation of B cells by helper T cells. CD40 is a 45-50 Kd membrane glycoprotein expressed on pre-B cells, mature B cells, follicular dendritic cells, and some carcinoma cells (Clark et al., 1994, Noelle et al., 1992). CD40 ligand (CD40L) is expressed on activated $CD4^+$ T cells (Armitage et al., 1992). Both CD40 and the CD40 ligand have recently been cloned and expressed. A panel of monoclonal antibodies against CD40 have also been generated. In the human system, an anti-CD40 mAb has been shown to induce B cell proliferation, suggesting that CD40 may be an important triggering molecule for B cells (Noelle et al., 1992). Recombinant murine CD40 ligand is directly mitogenic for both murine and human B cells and is a potent inducer of IgE production by B cells cocultured with IL4 (Armitage et al., 1992). Similarly, human CD40 ligand is also directly mitogenic for purified tonsil B cells. The proliferation is significantly

enhanced by IL2, IL4, or IL10 (Armitage et al., 1993). Although CD40 ligand alone does not enhance Ig secretion, the addition of IL2 or IL10 to the cultures has been shown to stimulate IgM, IgG, and IgA secretion by human B cells, and, in the presence of IL4, induces the production of human IgE (Armitage et al., 1993). Recent data demonstrate that B cells cannot proliferate or produce Ig in response to T cell signals when the CD40-CD40L interaction is blocked in vitro with soluble CD40 or anti-CD40L (Clark et al., 1994, Armitage et al., 1992). These data suggest that CD40 is an important stimulatory signal for B cell proliferation, and a costimulatory signal for B cell Ig secretion.

Cytokines which act on B Cells

While cognate T-B interaction appears to be critical to B cell responses, noncognate factors (cytokines) clearly have a major role. Over the past few years, rapid progress has been made in the definition, purification, and molecular cloning of polypeptide growth factors that affect B cells, elucidating the roles that each of these cytokines play in B cell development. With a few exceptions, the activities of homologous cytokines in mouse and man are quite similar. It is clear, however, that none of the B cell stimulatory factors described act exclusively on the B cell lineage. Most cytokines are pleiotropic with a partially overlapping range of activities. Furthermore, cytokines can either act on distinct stages of B cell maturation or have unique effects at different stages.

The predominant cytokines which regulate B cell proliferation and differentiation include IL2, IL4, IL6, IL10, IL13, IFN-γ, IL1, BCGF and BCDF. Both human and murine IL2 are glycoproteins with a molecular weight of about 18 Kd. Produced by Th0 and Th1 cells, IL2 has been found to be a potent synergistic signal for both B cell growth and differentiation (Nakagawa et al., 1987). IL2 can synergize with IFN-γ in mouse (Nakagawa et al., 1986), as well as IL4 and IL6 (Tohma et al., 1991) promoting B cell proliferation and differentiation, respectively. Mitogen-stimulated B cell growth and differentiation may also be regulated by IL2.

IL4 is a 20 Kd glycoprotein produced by activated T cells. Murine IL4 was originally described as the primary BCGF (BCGF I) but also plays an important role in the regulation of IgE and IgG1 production with specific decreases in IgG_{2b} and IgG_3 production in cultures of LPS-stimulated murine B cells (Tohma et al., 1991). In contrast, the role of IL4 in human B cell differentiation has been controversial. IL4 induces proliferation of human B cells but inhibits mitogen induced B cell differentiation (Berridge et al., 1985). However, there are recent reports of IL4 inducing IgE production after co-stimulation with anti-CD40 antibody (Zhang et al., 1991; Banchereau et al., 1991). The combination of IL4 and IL6 induces Ig secretion by human B cells stimulated by fixed activated T cells, but neither IL4 nor IL6 alone have this function (Tohma et al., 1991). Although one report showed that human IL4 induced SAC-activated tonsillar B cells to produce IgG and IgM (DeFrance et al., 1988), our studies and other reports have demonstrated that human IL4 has inhibitory

effect on Ig secretion by peripheral blood B cells. In addition to the effect on Ig secretion and B cell growth, IL4 can upregulate the expression of the Fc receptor for IgE (CD23) on B cells and monocytes (Vercelli et al., 1988).

Similar mouse/human dichotomies exist for IL5. In mouse, IL5 (45-50 Kd) promotes B cell growth when costimulated with dextran sulfate (Swain et al., 1982) and stimulates IgA and IgM production in normal B cells and B cell lines (Harriman et al., 1988; Murakami et al., 1988). Recent studies have also suggested that the induction of polyclonal Ig secretion by the recombinant CD40 ligand requires IL4 and IL5 although optimal Ag-specific antibody formation requires IL2 (Grabstein et al., 1993). In contrast to mouse IL5, the effect of human IL5 (45Kd) on human B cells is quite controversial. The majority of studies to date show no detectable activity of human IL5 on human B cells (Clutterbuck et al., 1987; Simonsson et al., 1991; Bende et al., 1992) although one report showed that recombinant human IL5 induced SAC-activated human B cells to secrete IgM (Bertolini et al., 1993).

IL6 is a pleiotropic cytokine originally identified as B cell stimulatory factor 2 (BSF-2) by its ability to induce Ig production by an Epstein Barr virus (EBV) transformed B cell line, CESS (Hirano et al., 1986). Both murine and human IL6 are quite heterogeneous in function and size with molecular weights ranging from 19 to 64 Kd due to the glycosylation, sulfation, and phosphorylation (May et al., 1988). IL6 has been demonstrated to act on PWM-stimulated human

tonsil B cells to produce IgM, IgG, and IgA (Bertolini et al., 1990). However, IL6 may only be a costimulator of B cell differentiation, since IL6 alone does not induce Ig secretion by preactivated B cells (Tohma et al., 1991). While having no proliferative effect on normal B cells, IL6 has been shown to possess hybridoma growth factor activity for the murine B cell hybridoma line, B9 (Nordan et al., 1986), providing an extremely sensitive and specific bioassay for IL6.

IL10, originally identified by virtue of its ability to inhibit cytokine synthesis in Th1 clones, has now been shown to possess a wide range of activities on a number of cell types. IL10 is produced by Th2 clones, certain B cell subsets, and LPS-activated human monocytes (Malefyt et al., 1991, Benjamin et al., 1992). Both human and mouse IL10 genes have been cloned and expressed. Recombinant IL10 has a molecular weight of 18.5 kd in both species. Human IL10 has been demonstrated to be a potent growth and differentiation factor for activated human tonsil B cells, stimulating DNA replication and inducing increased viability of B cells (Rousset et al., 1992), which is similar to the effects of IL4. However, IL10 dose not induce CD23 expression and IgE secretion (Rousset et al., 1992, Go et al., 1990), suggesting that the effects of IL10 on human B cells are not due to induction of IL4 production. IL10 induces activated human tonsil B cells to secrete large amounts of IgG, IgA and IgM, and the combination of IL10 and IL4 results in the secretion of the four Ig isotypes (Rousset et al., 1992).

A newly described cytokine, IL13, has been cloned and characterized. Human IL13 is produced by different human T cell subsets. Both $CD4^+$ and $CD8^+$ T cell clones synthesize IL13 in response to antigen specific or polyclonal stimuli (Zurawski et al., 1994). Both murine and human IL13 is predominantly secreted as an unglycosylated species of about 10 kd although minor glycosylated species of higher molecular weight are also secreted (Zurawski et al., 1994). Like IL4, IL13 upregulates the expression of CD23 on monocyte and B cells (Defrance et al., 1994; Mckenzie et al., 1993). Like IL4 and IL10, IL13 enhances the proliferative response of human B cells preactivated by anti-IgM or through ligation of surface CD40 (Cocks et al., 1993). IL13 alone does not induce Ig secretion in human tonsil B cells, however, it induces IgM, IgG, but no IgA secretion when cocultured with an activated $CD4^+$ T cell clone or human CD40 ligand (Mckenzie et., 1993; Cocks et al., 1993), suggesting that IL13 is only a costimulatory factor in human B cell differentiation. In addition to IgM and IgG, considerable levels of IgE are synthesized (Mckenzie et al., 1994, Cocks et al., 1993), indicating that IL13, like IL4, acts as a switch factor directing synthesis of IgE, an isotype not observed with IL10. IL13 is less potent than IL4 in inducing IgE production and has no additive or synergistic effects with IL4 on induction of IgG4 or IgE synthesis, suggesting that both cytokines use common signal pathways for the induction of these Ig isotypes (Mckenzie et al., 1994, Cocks et al., 1993).

Additional B cell stimulatory factors in man which have been less well characterized include human IL1-α and IL1-β which may act as

cofactors during B cell activation. Human γ-IFN was reported to act in concert with human IL4 to induce proliferation of anti-IgM stimulated B cells. However, γ-IFN can also antagonize IL4 induced IgE production, class II Ag and FcεRII (CD23) expression in both human and murine B cells (Coffman et al., 1990), and inhibit IL6 and IL2 induced Ig secretion by mitogen preactivated human tonsillar B cells, suggesting a more negative regulatory role. Two human cytokines which modulate B cell growth have been isolated and do not yet have clear murine equivalents. High molecular weight BCGF (HMW-BCGF, 50-60 Kd) (Ambrus et al., 1993) preferentially promotes the proliferation of larger, preactivated B cells. In contrast, low MW BCGF (12-15 Kd) (Maizel et al., 1987) acts on small resting B cells (Coffman et al., 1990).

We have recently utilized a novel system to identify a new human BCDF, termed 446-BCDF. One anti-CD3 mAb, mAb 446, stimulates T cells to secrete IL4, IL6, BCGF, and 446-BCDF (Sherris et al., 1989). Superose 12 permeation chromatography and chromatofocusing of 446-BCDF reveals that this new cytokine has an apparent M_r. of 32 Kd and a pI of 6, in contrast to the reported pI of IL6 (5-5.1) and the M_r of IL2, IL4, IL10, and IL13. This cytokine can be separated from IL6 and other cytokines by fractionation on a DEAE anion exchange column. Furthermore, the activity of this cytokine can not be inhibited by antibodie angainst IL2, IL4, IL6 and their receptors. 446-BCDF acts on SAC-activated B cells and tonsil B cells inducing a more than 5 fold increase in Ig secretion. The effects are polyclonal, stimulating IgG, IgA and IgM production. However, this cytokine

does not have apparent effects on T cells and does not possess BCGF activity. These data suggest that 446-BCDF is a unique potent polyclonal human BCDF regulating terminal B cell differentiation. Furthermore, the potency of this cytokine is underscored by its ability to induce near normal Ig production by B cells from patients with common variable immunodeficiency (Kasbay, Cunningham-Rundles, Mayer submitted).

Although the precise mechanisms definding how cytokines regulate B cell function are not completely understood, the initial step that allows a cytokine to exert its effect is to activate a receptor complex, generally composed of a receptor (binding unit), a signal transducer, an effector subunit that generates intracellular signals, and a regulatory subunit (Miyagima et al., 1992). The effect of a cytokine on B cells, as in the effect of a hormone or a growth factor on many cell types, is exerted by conversion of extracellular signal(s) into intracellular signal(s). Understanding the intracellular signal transduction pathways will help us to better understand the mechanisms by which a cytokine functions.

Overview of general Signal Transduction Pathways

Two classical signal pathways, the cAMP pathway and the phosphoinositide (PI) pathway, have been well studied in many cell systems. Both pathways share some similar biochemical components and both involve activation of distinct protein kinases. In both pathways, receptors on the cell surface are associated with a transducing protein which is activated when the receptor binds to

its specific ligand. The transducing protein in both pathways has been shown to be a member of a highly conserved family of G proteins. The interaction of a G protein with an amplifying second messenger system produces mediators initiating a series of important biochemical events affecting a variety of cellular functions.

G proteins and cAMP pathway

G proteins are a class of heterotrimeric, membrane-associated proteins that serve as intermediaries between receptors and effectors, where the effector can be either an enzyme or an ion channel. The α subunit binds GTP and is capable of hydrolyzing GTP to GDP. Structurally different α subunits distinguish the various members of G protein family. All members of the G protein family share common β and γ subunits (Weiss et al., 1988). Two types of G proteins have been known to be involved in the generation of cAMP, a critical intracellular second messenger (Berridge et al., 1985). The stimulatory protein Gs activates adenylate cyclase (AC) when an external signal binds to its receptor, Rs. The activated AC thereby hydrolyzes ATP to generate cAMP. The activity of Gs is ended by hydrolysis of bound GTP and AC activity is thus turned off. The GTPase activity can be inhibited by cholera toxin (CTX) through ADP-ribosylation of the α subunit so that the cell produces cAMP continually (Weiss et al., 1988). The other type of G protein, Gi, inhibits AC, preventing the generation of cAMP. Pertussis toxin (PTX) blocks the inhibition of AC by ADP-ribosylation of the α subunit of Gi (Weiss et al., 1988). The intracellular cAMP level is also

regulated by cyclic nucleotide phosphodiesterases (PDEs) which specifically or nonspecifically hydrolyze cAMP into 5'-AMP (Beavo et al., 1982, Prigent et al., 1990). 5'-AMP is the target of 5'-nucleotidase resulting in the generation of adenosine and free phosphate. Several cyclic nucleotide PDE isozymes have been identified (Beavo et al., 1982). Some enzymes effectively hydrolyze both cAMP and cGMP, while others hydrolyze either cAMP or cGMP (Beavo et al., 1982). In general, PDEs which hydrolyze cAMP can be roughly classified as Ca^{++}/calmodulin-dependent or independent PDEs. More than 90% of the PDE activity in lymphocytes is cytosolic (Prigent et al., 1990).

The regulation of the intracellular cAMP level is also a complex event. As discussed above, Gs and Gi regulate the generation of intracellular cAMP, PDEs hydrolyze cAMP into 5'-AMP which is further hydrolyzed by 5'- nucleotidase (5'-NT) to adenosine and free phosphate. Adenosine can be released from cells, crossing the plasma membrane in both directions by a specific nucleoside transporter or by simple diffusion (Stilest et al., 1992, Plagemann et al., 1981). The released adenosine can then act on adenosine receptors on cell surface, causing many important physiological responses. Adenosine receptors (ARs) are expressed on a wide variety of cell types, coupled to different G proteins to alter the activity of second messenger systems. AR1 has been shown to be coupled to Gi, inhibiting adenylate cyclase activity, or coupled to Go and Gz to regulate K^+ and Ca^{++} channels, phospholipase C (PLC) and phospholipase A_2 (PLA_2) activity. On the other hand, AR2 is only

known to couple to Gs, stimulating adenylate cyclase to increase intracellular cAMP level (Stilest et al., 1992, Plagemann et al., 1981, Londos et al., 1980). The presence of AR1 on human lymphocytes is still controversial. Marone et at showed that although adenosine did not directly induce a decrease in intracellular cAMP it inhibited the increase in intracellular cAMP induced by forskolin in human lymphocytes, presumedly by activation of AR1 (Marone et al., 1985). In contrast, the absence of detectable AR1 on human lymphocytes has also been reported (Schultz et al., 1988). The most consistent data has been that human lymphocytes do possess AR2 (Kontny et al., 1992; Nordstedt et al., 1989; Schultz et al., 1988). The activation of AR2 on human lymphocytes increases the intracellular cAMP level.

Intracellular cAMP is an important second messenger as it can function in a number of regulatory roles. In one such pathway, cAMP activates PKA. In general, in the absence of cAMP, PKA is an inactive, asymmetric tetramer containing two regulatory (R) subunits and two catalytic (C) subunits. Binding of cAMP to R subunits alters its affinity for the catalytic subunits and promotes dissociation of the tetramer into a dimer of R subunits and two active monomeric C subunits (Taylor et al., 1990) which can now phosphorylate and activate other cellular proteins to regulate other cellular functions. The regulation of specific gene expression by PKA has been well-documented in non-lymphocyte systems (Buchler et al., 1988, Dash et al., 1991). PKA has been known to activate Ca^{++}/calmodulin-dependent kinase II (CAM kinase) which in turn phosphorylates the

cAMP response element-binding protein (CREB), an important transcriptional activator, increasing gene expression (Dash et al., 1991). However, the physiological substrates of PKA in lymphocytes remain mostly unknown (Perlmutter et al., 1993). In general, treatment of lymphocytes with agents that increase intracellular cAMP levels inhibits their activation. This blockade occurs early in the anti-receptor stimulation pathway because membrane phospholipid turnover is blocked, which could result from direct phosphorylation and inhibition of PLC activity (Park et al., 1992, Kim et al., 1989) or through inhibition of a protein tyrosine kinase (Klausner et al., 1987). Thus it appears that PKA has a negative effect during lymphocyte stimulation (Perlmutter et al., 1993).

Phospholipid metabolism and Calcium flux

In the PI signal pathway, the agonist-receptor interaction may potentially couple with at least two distinct G proteins, G_p and G_A, which activate phospholipase C (PLC) and phospholipase A_2 (PLA_2), respectively (Burch et al., 1986). PLC hydrolyzes phosphoinositol bisphosphate (PIP_2) to generate two important products, inositol triphosphate (IP_3) and diacylglycerol (DAG). The former releases Ca^{++} from the endoplasmic reticulum, the latter activates Ca^{++}-dependent PKC. The extracellular signal is thus transformed into two important intracellular second messengers. PLC can also hydrolyze phosphatidylcholine to generate phosphocholine (PC) and DAG (Besterman et al., 1986) which may activate Ca^{++}-independent PKC (Harris et al., 1985). Unlike PLC, PLA_2 hydrolyzes phosphatidylcholine to generate arachidonic acid which, like some

other unsaturated fatty acids, activates Ca^{++}-independent PKC. This activation pathway would not generate IP3 and consequentially intracellular Ca^{++} release. These phenomena have been observed in cytokine mediated PKC translocation. IL2 and IL3 induces PKC translocation (Farrar et al., 1985a; 1985b), however, IL2 and IL3 do not induce hydrolysis of inositol phospholipid (Whetton et al., 1988, Morla et al., 1988). In contrast, no IP3 generation but intracellular Ca^{++} flux have been observed in an anti-μ stimulated B lymphoma cell line (Monroe et al., 1989). Apparently, alternative signal pathways may also exist. The second messenger Ca^{++} can activate Ca^{++}-dependent kinases and other enzymes. Both Ca^{++}-dependent kinases and PKC have been known to phosphorylate important proteins which regulate cellular functions (Dash et al., 1991, Nishzuka et al., 1986).

Tyrosine kinase and serine/threonine kinase

One of the early biochemical changes after interaction of various cells with cytokines, mitogens and surface receptor crosslinkers is the phosphorylation of protein, particularly, phosphorylation of tyrosine residues by tyrosine kinases (PTKs). The requirement of PTK activity for signal transduction has been well established for a variety of growth factor receptors (Ullich et al., 1990). Binding of a ligand induces autophosphorylation of the receptor itself as well as PLC, PI-3 kinase, GTPase activating protein (GAP) (Ullich et al., 1990, Cantley et al., 1991). These signaling proteins are physically associated with the growth factor receptors through a SH2 domain which is present in tyrosine kinases. Therefore, a single growth factor causes

activation of multiple signal transduction pathways. Many cytokines such as IL2, IL4, IL6 induce such protein tyrosine phosphorylation. However, none of the receptors for these cytokine has a PTK (Miyajima et al., 1992). It thus appears that there is an intracytoplsmic PTK closely associated with these receptors. This has been shown in IL2Rβ which is associated with a PTK, $p56^{lck}$ (Hatakeyama et al., 1991). The role of p56lck in IL2 mediated signal transduction, however, is not yet clear. The association of intracytoplasmic PTK with other cell surface molecules was also observed. In T cells, CD4 and CD8 molecules are physically associated with $p56^{lck}$. (Ravichandran et al., 1994). Crosslinking of these molecules activates the tyrosine kinase activity of $p56^{lck}$ which phosphorylates numerous intracellular substrates (Rudd et al., 1989) and enhances IL2 production (Ravichandran et al., 1994). The importance of PTK in B cell function has been well documented. Crosslinking of B cell surface Ig activates protein tyrosine kinases through the subunits of B cell antigen receptor complex, Igα (mb-1 gene product) and Igβ (B29 gene product) in both human and murine B cells (Kim et al., 1993; Clark et al., 1992). The resultant protein tyrosine phosphorylation is important for oncogene expression and B cell proliferation (Roifman et al., 1991). The cytoplasmic PTK, Bruton's tyrosine kinase (BTK), has been shown to be critical for B cell development. Loss of BTK activity results in the human immunodeficiency, X-linked agammaglobulin, characterized by a failure to produce B cells (Smith et al., 1994). In mouse, the mutation of BTK gene also results in X-linked immnuodeficency disease (XID) (Rawlings et al., 1993). Although B cells are present in murine XID

they respond abnormally to activating signals, leading to a decrease in Ig production. All these data suggest that PTK may be important in B cell normal development and function.

Most protein kinases characterized thus far are specific either for tyrosine or serine/threonine residues. The involvement of serine/threonine kinase in signal transduction has been well described. The most well characterized serine/threonine kinase is Raf, an oncogene product (Rapp, 1991). The activation of Raf is common to many receptor systems and is regulated by protein kinase C (Sozeri et al., 1992) and tyrosine kinase (Thompson et al., 1991). Raf has been reported to be involved in signal transduction mediated by IL1, tumor necrosis factor (TNF) (Saklatvala et al., 1992), IL2 (Maslinski et al., 1993) and transforming growth factor beta (TGF-β) (Massague et al., 1992). Crosslinking surface Ig activates Raf in human B cells, which is responsible for B cell proliferation (Tamaki et al., 1992). Raf has also been known to play a role in the induction of kappa B site-dependent gene expression (Finco et al., 1993) and IL2 production (Owaki et al., 1993). Other serine/threonine kinases such as DNA-activated protein kinase (DNA-PK) and mitogen-activated protein (MAP) kinase are known to be important in coordinating signal transduction pathways and nuclear events (Lees-Miller et al., 1991; Daeipour et al., 1993).

Signal Transduction Pathways in B Cells

Recent studies have addressed signal transduction pathways in B cell activation and proliferation (Cambier et al., 1987; Gold et al., 1990).

B cell proliferation after anti-IgM crosslinking is associated with turnover of inositol phospholipids, Ca^{++} mobilization and PKC translocation, which is similar to that seen in T cell proliferation (Cambier et al., 1987). Crosslinking membrane Ig in both mature splenic B cells and an immature B cell line BCL-1 stimulates phosphorylation of tyrosine residues, a reaction which has been implicated as a key regulator of B cell growth (Gold et al., 1990). The involvement of PTK in PI turnover has also been suggested. Padeh et al. demonstrated that crosslinking of the antigen receptor on human B cells activated PLC to generate IP3 and DAG, causing Ca^{++} mobilization and PKC translocation, and increased tyrosine phosphorylation of specific substrates. Inhibition of PTK activity blocked anti-IgM induced tyrosine phosphorylation of six proteins, B cell proliferation, oncogene expression and phospholipid hydrolysis, indicating that a PTK acted as an essential link between the B cell antigen receptor and PLC (Padeh et al., 1991). However, in different cell types PTK appears to play a different role. It has been shown that anti-IgM mediated growth inhibition of a human B lymphoma cell line was independent of PI turnover, Ca^{++} mobilization and PKC translocation, but involved tyrosine phosphorylation, suggesting that PTK activation was a critical component of the inhibitory response (Beckwith et al., 1991). In addition to PTK and PI turnover, crosslinking surface Ig on human leukemic B cells and tonsillar B cells induced a rapid activation of a serine/threonine kinase, Raf. An inhibitor of serine/threonine, H7, inhibited the proliferation as well as Raf phosphorylation in response to the proliferative signal of anti-Ig (Tamaki et al., 1992). These data suggest that

serine/threonine kinase may also be involved in signal transduction mediated by surface Ig on human B cells.

Although extensively studied, most lymphokine-induced signal transduction events in B cells are still not very clear. IL2 stimulated a murine B cell line, BCL1, to proliferate and express J chain, but neither Ca^{++} flux, IP_3, DAG, PKA nor cGMP-dependent protein kinase activities could be detected (Tigges et al., 1989). These data indicate that classical second messenger systems are not involved. In support of this concept, more recent data have shown that IL2 stimulates BCL1 cells to rapidly hydrolyze an inositol-containing glycolipid into two possible second messengers, a myristylated diacylglycerol (myr-DAG) and an inositol phosphate-glycan. (Eardley et al., 1991). These data implicate the glycosyl phosphatidylinositol system in an intracellular relay of the IL2 signal. However, these studies were performed in a malignant cell line and may not reflect normal physiology. Recent studies using murine pro-B cell lines showed that IL2 induced the activation of the src family protein tyrosine kinases p^{59fyn}, $p^{53/56lyn}$ and p^{56lck} (Kobayashi et al., 1993; Minami et al., 1993), suggesting that tyrosine kinases may participate in IL2 mediated signal transduction in murine B cells. The data described above have all been confined to studies in the murine system. Little if any information is available regarding similar studies in man. One recent study showed that the proliferative response of activated human B cells to IL2 was inhibited by increasing intracellular cAMP (Kolb et al., 1993). The differentiation signal(s) delivered by IL2 in human B cells is still not clear.

The signal transduction mediated by IL4 has been extensively studied. Binding of IL4 to its receptor transduces the signal that activates a tyrosine kinase phosphorylating cellular substrates, the receptor itself and a substrate called 4PS in many cell types, including B cell lines as well as primary LPS-stimulated B cells (Keegan et al., 1994). A phosphorylated 4PS interacts with the SH2 domain of the enzyme PI-3'-kinase and increases its enzymatic activity. IL4 induces CD23 expression in human tonsillar B cells via PIP_2 hydrolysis and delayed cAMP generation (Finney et al., 1990). However, conflicting results have also been reported where experimentally increased intracellular cAMP levels inhibited IL4-induced CD23 expression in Burkitt lymphoma cells (Galizzi et al., 1988). Whether the discrepancies of IL4 signaling described above are due to the apparent differences existing between two species and/or due to the behavior of normal and malignant human B cells remains to be determined.

Binding of IL6 to IL6 receptor (IL6R) triggers the association of IL6R and gp130. IL6R has only a short intracytoplasmic tail which can not transduce a signal (Yamasaki et al., 1988). However, the associated gp130 is known to be involved in the formation of high affinity IL6 binding sites (Kishimoto et al., 1992, Yawata et al., 1993). Binding of IL6 to its receptor induces disulfide-linked homodimerization of gp130 which is associated with tyrosine kinase activity. The dimerization of signal transducing molecules and activation of

associated tyrosine kinases are important for IL6 signal transduction and cellular responses to IL6 (Murakami et al., 1993).

The available data have shown that a single cytokine may deliver different signals by its receptor and different cytokines may use similar signal pathways to perform their function. The most recent data have suggested that tyrosine kinases and serine/threonine kinases may be involved in signal transduction pathways used by IL2, IL4 and IL6 (Goldstein et al., 1993). Human IL2, IL4 and IL6 stimulate IgM secretion by the EBV-transformed cell line SKW6.4. The stimulation of SKW6.4 cells by these cytokines induces the rapid serine/threonine phosphorylation of 47, 49, and 91 Kd proteins. The serine/threonine kinase inhibitor, H7, inhibited IgM secretion by IL2, IL4 or IL6 stimulated SKW6.4 cells. IgM secretion was also inhibited by the tyrosine kinase inhibitor, genistein. These data suggest that the activation of both serine/threonine and tyrosine kinases may be involved in the IL2, IL4 and IL6 stimulation of IgM secretion by SKW6.4 cells.

The involvement of the cAMP signal pathway in B cell function has been suggested. cAMP by itself has no effect on B cell proliferation, however, it inhibits IL2-driven human B cell proliferation and enhances IL4-mediated proliferation (Vazquez et al., 1991), suggesting that the combination of cAMP with different cytokine signals can play a differing role in human B cell proliferation. Effects of cAMP on human B cell differentiation have been described. Experimentally increasing intracellular cAMP levels with various

agents inhibited the formation of Ig-secreting cells and PMA-enhanced IgM production by the human B cell line, LA350 (Shearer et al., 1988; Patke et al., 1991), as well as IgM production by SKW cells (Goldstein et al., 1990) and IgG production by CESS cells (R. Huang and L. Mayer, unpublished data). This inhibitory effect was blocked by H8, a PKA inhibitor, suggesting that PKA might be a negative regulator in IgM production by SKW. Similar results were also obtained in a murine system. Crosslinking of class II MHC I-E molecules induced increases in cAMP levels in murine B cells, which in turn inhibited IgM production (Takahama et al., 1989). The increase in intracellular cAMP has also been shown to potentiate the immune complex induced ablation of IgM responses (Stein et al., 1991). All these data suggest that cAMP has a negative effect on B cell differentiation. However, opposite results were also observed in murine B cells (Gibert et al., 1985) where cAMP plus IL1 enhanced polyclonal Ig production and increased intracellular cAMP promoted an IgG1 class switch in murine B cells (Lycke, 1993). From these studies the effect of cAMP appears to be system depedent, inhibiting in most cases and stimulating in others.

The difficulty in understanding the signal transduction pathways of cytokines in B cells may reflect their complexity inside the cells. It is known that a single cytokine can act as both a positive and negative signal dependent on the type and developmental state of the target cells (Arai et al., 1990). On the other hand, different cytokines through different receptors can evoke apparently identical biological responses (Miyajima et al., 1992). These data suggest that cytokine

signaling pathways inside a cell are not linear and may form a network with multiple sites for cross-talk among cytokine pathways. Therefore a ligand binding to its receptor may not be the only determinant of the cellular response. A single receptor may couple to multiple signal transduction systems, Likewise, multiple receptors may couple to the same signal transduction pathway via common transducers or effectors.

MATERIALS AND METHODS

Cell Culture Medium

Cells were grown in RPMI 1640 (GIBCO, Grand Island, NY), 2 mM glutamine (GIBCO), 100 u/ml penicillin and streptomycin (GIBCO), and 10% heat inactivated fetal calf serum (FCS) (GIBCO), henceforth termed culture medium (CM). For the generation of activated T cell culture supernatants, phenol red free RPMI 1640 and 0.05% (w/v) bovine albumin (Sigma) were used to replace RPMI 1640 and FCS in CM.

Cell Separation and Culture

1. *Preparation of peripheral blood lymphocytes.* Peripheral blood mononuclear cells (PBMC) were isolated from leukocyte concentrate packs from normal blood donors. Cells were diluted 1/3 with PBS (5.4 mM KCl, 1.5 mM KH_2PO_4, 140 mM NaCl, 8 mM Na_2HPO_4, pH 7.4), and separated by Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, NJ) density gradient centrifugation (Sherries et al., 1989). The mononuclear cells were collected from the interface and washed three times with PBS. Cells were resuspended in RPMI 1640 and cell density was adjusted to $5x10^6$ cells/ml. T cells were then separated using a rosetting technique. Mononuclear cells were mixed with neuraminidase treated sheep red blood cells (SRBC) and kept at 4^0C overnight. The cell mixture was then applied to a Ficoll-Hypaque density gradient centrifugation for 30 minutes at 1800 rpm. Non-T cells from the interface were collected and washed three times with PBS and resuspended in CM. Adherent cells were depleted by

incubating non-T cells in tissue culture dishes (25 million cells per dish in 10 ml CM) for 1 hour at 37^0C in a humidified 5% CO_2 incubator. B cell enriched non-adherent cells were then aspirated and rosetting and depletion procedures were repeated. Resultant B cells were then analyzed by FACS (EPICS PROFILE II). In general, these B cell preparations contained <1% $CD3^+$ cells, <2% $CD14^+$ cells and >90% $CD20^+$ cells. Rosetted T cells were collected from the pellet and washed three times with PBS, and then treated with 0.75% ammonium chloride on ice for 10 minutes to lyse SRBC. Lysis was stopped by adding PBS. The T cell suspension was then washed three times with PBS and resuspended in CM. The contaminating adherent cells in the T cell suspension were then eliminated using the same depletion procedure described above. Resultant T cells were <1% $CD14^+$, <1% $CD20^+$ and >95% $CD3^+$.

2. *Preparation of tonsillar B cells.* Tonsil tissues were obtained from patients undergoing tonsillectomy. Tonsillar lymphocytes were prepared by washing tonsil tissues in ethanol and CM and teasing the tissue apart in fresh medium. The tissue debris and cells were transferred to conical tubes and the debris was allowed to settle. The cells were washed twice and resuspended in CM. Mononuclear cells were isolated by Ficoll-Hypaque density gradient centrifugation. B cells were isolated from mononuclear cells by adherence depletion of monocytes twice and rosetting twice with neuraminidase treated SRBC. The resulting B cell enriched population contains <1% $CD3^+$ cells, <1% $CD14^+$ cells and >95% $CD20^+$ cells, as measured by immunofluorescent staining on FACS.

Monoclonal Antibodies and Reagents

Monoclonal antibodies (mAb), 446 and 929, were generated in this laboratory. mAb446 specifically recognizes the γ-chain of the CD3 complex. mAb 929 was generated against 446-BCDF. This antibody does not block either Ig secretion by IL2 stimulated PB B cells and IL6 stimulated CESS cells, or IL2 dependent CTLL cell proliferation and IL6 dependent B9 cell proliferation. Anti-CD23 mAb was a kind gift of Dr. Peggy Crow (Hospital for special surgery, New York city). Anti-CD40 mAb was a generous gift of Dr. Shu Man Fu (Univ. of Virginia, VA). 5C8 is a blocking antibody against the CD40 ligand and was kindly provided by Dr. Lederman (Columbia Univ., NY). Rabbit anti-human IgM polyclonal antibody was purchased from Accurate Chemical & Scientific Corp., NY. Recombinant IL2 was purchased from Boehringer Manheim. rIL4 was purchased from Genzyme. rIL6. a kind gift of Dr. Edward Siden, was purified after expressing in a baculovirus system. Fixed Staphylococcal aureus Cowan I strain organisms (SAC) was purchased from Calbiochem, LaJolla, CA. Dibutyryl cyclic AMP (D-cAMP), forskolin, aminophylline, 3-isobutyl-1-methylxanthine (IBMX), pertussis toxin, cholera toxin, H7, staurosporin, calphostin C, and angiotensin were purchased from Sigma, St. Louis, MO. 1,2-bis(2-aminophynoxy) ethane N,N,N',N'-tetraacetic acid (BAPTA) and Indo-1 AM were purchased from Molecular probes, Junction City, OR. Protein kinase C (PKC) assay kit was purchased from GIBCO BRL, MD. The cAMP EIA assay kit was purchased from Advanced Magnetics Inc., MA.

Generation of 446-BCDF

T cells were cultured in phenol red free RPMI 1640, supplemented with 0.05% bovine albumin, and 0.25 ug/ml anti-CD3 mAb 446 (IgG1) (for control, 0.25 ug/ml isotype matched mouse IgG1 was utilized). After 48 hours of culture, cell free supernatants were obtained by centrifugation at 1800 rpm for 10 min and filtration (0.22u filter, Nalgene). Supernatants were concentrated 20 fold by positive pressure Amicon filtration (YM10 filter) and dialyzed against 10 mM NaCl pH 7.4 (final dialysis dilution 1:100,000). The concentrated T cell supernatant was then passed over a DE 52 cellulose column (Whatman Biosystem, England) and the fraction enriched in 446-BCDF activity was eluted at 50 mM NaCl. The contaminating IL6 activity in the 50 mM NaCl eluted fraction was then removed by passage over an anti-human IL6 affinity column (AMGEN Biologicals, CA). The effluent was tested in a B9 cell proliferation assay to ensure that contaminating IL6 was completely removed. The IL6-depleted effluent was then dialyzed against phenol red free RPMI 1640 and used in our assays. Characterization of 446-BCDF is described in Results section.

Cytokine Bioassays

1. *CTLL cell proliferation assay*

The IL2-dependent murine cytotoxic T lymphoid line, CTLL, was washed three times with PBS. $5x10^3$ CTLL cells were cultured in triplicate in round bottom 96-well plates. Recombinant IL2 and 446-BCDF were used to make a series of dilutions in corresponding wells. CTLL cells were cultured at 37^0C for 48 hours in the presence of 5%

CO_2. 1 uCi [^{3}H] TdR was added to each well during last 18 hours. Cells were then harvested onto glass filter mats (Skatron, Sterling, VA). CPM was determined by liquid scintillation counter (Model LS 3801, Beckman Instruments, Somerset, NJ).

2. *B9 cell proliferation assay*

The IL6-dependent murine B cell hybridoma, B9, was washed 3 times with CM. $5x10^3$ B9 cells were cultured in triplicate microwell cultures (Flow Laboratories Inc., McLean, VA) in the presence of differing amounts of 446-BCDF before or after passage over the anti-IL6 affinity column. Recombinant IL6 was used to serve as a positive control. Cells were cultured for 72 hours and 1 uCi [^{3}H]TdR/well (ICN Radiochemicals, Irvine, CA) was added during the last 18 hours of culture. Cells were harvested onto glass filter mats (Skatron, Sterling, VA) and CPM determined by a liquid scintillation counter.

3. *CD23 expression assay*

The Ramos cell line G4 was obtained from American Type Culture Collection. The Ramos cell line consists of human B lymphocytes derived from a Burkitt lymphoma which responds to IL4 with upregulation of the expression of the low affinity IgE receptor (CD23) (Rousset et al., 1988). Ramos cells were washed three times with PBS. $5x10^4$ Ramos cells were seeded in each well of 96-well flat bottom microtiter plates. Human recombinant IL4 was used as an IL4 standard. Different concentrations of 446-BCDF were added to the corresponding wells in the microtiter plate. After 48 hours, cells were washed three times, stained with 10 ug of anti-CD23

monoclonal antibody (BLAST-2) and then with 10 ug of FITC conjugated goat anti-mouse Ig (Cappel Laboratories). Mouse IgG1 was used as the isotype control for the anti-CD23 mAb. Constitutive expression of CD23 on Ramos cells was determined in cultured unstimulated cells. After washing, the fluorescence was analyzed by FACS. The mean channel reflects the CD23 expression on a logarithmic scale.

4. *B cell differentiation assay*

Isolated B cells were adjusted to $2X10^6$/ml in CM and 50 ul of cell suspension was added to triplicate microwell cultures (final volume 100 ul). SAC (Pansorbin) (Calbiochem, Burlingame, CA) was added to cultures at a final concentration of 0.001% (v/v). Unless otherwise indicated, the concentration of 446-BCDF was constant at 10% (v/v). In some experiments either forskolin (0.001-10 ug/ml), aminophylline (0.001-10 uM), dibutyryl cAMP (0.001-10 uM), H7 (0.032-20 uM), pertussis toxin (0.001-10 ng/ml), cholera toxin (0.001-10 ng/ml), angiotensin II (10^{-10}-10^{-6} M), staurosporin (0.39-100 nM), or Calphostin C (0.0625-1 uM) were added to SAC plus 446-BCDF stimulated B cell cultures at the onset of culture. In other experiments varying concentrations of BAPTA were used to pretreat B cells for 2 hours then washed 3 times or added at the indicated time points and then exposed to SAC and 446-BCDF. Cell free supernatants were obtained after 7 days of culture and assayed for Ig by ELISA. Briefly, supernatants were added to the ELISA plate (Nunc) precoated with 10 ug/ml polyspecific goat anti-human Ig antibodies (TAGO). After 1 hour incubation at 22^0C, the plate was

washed 5 times with ELISA buffer and then incubated with 10 ug/ml alkaline phosphatase conjugated goat anti-human κ and λ light chain antibodies (TAGO) for 1 hour at 22^0C. The plate was then washed and reacted with phosphatase substrate (Substrate 104, Sigma). Optical density values (absorbance at 405 nm) were measured in an automated ELISA reader (Genetic Systems). Purified human Ig (Sandoz) was used as a positive control to generate standard curves for Ig concentrations. The Ig concentration in the supernatant was then determined by regression analysis.

cAMP Assays

1. *RIA method.* $2x10^6$ B cells were cultured in 1 ml RPMI 1640 in the presence or absence of either 446-BCDF (10% v/v), IL-2 (20 u/ml) or IL-6 (400 u/ml). In several experiments IBMX (0.5 mM) was added and cells incubated for 30 minutes before addition of cytokine. At 5, 30, 60, 120, 240 min, and 24 hours of culture, cAMP was extracted by adding 2 ml of boiling ddH_20. The diluted cell suspension was boiled for 3 minutes (19), cooled on ice and centrifuged. The supernatant was lyophilized to dryness and then reconstituted to 1/8 its original volume. B cell cAMP concentrations were determined using a radioisotopic test kit (Amersham, Arlington Heights, IL). cAMP concentrations are expressed as pM/million cells.

2. *EIA method.* cAMP samples were prepared as described above except that B cells were pretreated 30 min with 0.5 mM IMBX to inhibit PDEs. B cells were then stimulated with either cholera toxin (1

ng/ml), pertussis toxin (1 ng/ml), or angiotensin (10^{10}-10^{6} M) with or without 446-BCDF (10% v/v) for 60 min. cAMP samples were assayed according to the manufacturer's protocol. 100 ul of polyclonal rabbit anti-cAMP antibody was added in duplicate into goat anti-rabbit antibody precoated wells of a 96-well microtiter plate except the substrate blank wells (for substrate control). 100 ul of washing buffer (0.05 M NaC_2HO_3, 0.1% bovine γ-globulin, 0.1% NaN_3, pH 6.2), cAMP standard, or sample were then added into the corresponding wells. The plate was gently agitated by tapping the edge of the holder for about 1 min. The wells were then covered with a plate sealer and incubated at 4°C. After 2 hours' incubation, 100 ul of cAMP alkaline phosphatase conjugate were added to each well except the substrate blank wells. The plate was tapped gently to mix. The wells were covered and incubated at 4°C. After one hour's incubation, solution from all wells was removed. The plate was washed 6 times with 400 ul washing buffer per well. 300 ul pNNP substrate solution were then added into all wells. The plate was covered and incubated for 3 hours at 37°C. The reaction was stopped by adding 50 ul of 0.2 N NaOH. The plate was gently tapped to mix. The absorbance of wells was recorded at 405 nm. The cAMP concentration of each sample was calculated according to the standard curve by regression analysis.

Inositol Phosphate Assay

B cells were prepared as described above. Cells were washed with inositol free MEM medium and resuspended in MEM at the concentration of $2x10^{6}$/ml. 3-5 uCi/ml of [^{3}H]-myo-inositol (Dupont)

were added to the cell suspension. Cells were incubated at 37°C in the presence of 5% CO_2. After 18 hours loading with [^{3}H]-myo-inositol, cells were washed two times with MEM and resuspended at final concentration desired (usually 10^7 cells/ml). 10 ul of cell suspension was dried onto filter paper and % loading determined by a liquid scintillation counter. Lithium chloride was then added to the cell suspension to prevent IP breakdown at a final concentration of 20 mM. After a 15 minute incubation at 37°C, 2-4x10^6 cells/sample were stimulated with specific agents for 1 min at 37°C. The reaction was stopped by centrifugation and removal of supernatants. 1 ml of ice-cold 10 mM formic acid was added to each sample to lyse cells by thorough pipetting. Samples were kept on ice for at least one hour. Anion exchange columns (Analytic Grade Anion Exchange Resin, BioRed) were washed with 2.5 ml of 3M ammonium formate containing 100 mM formic acid, and equilibrated with 10 ml of 10 mM formic acid containing 10 mM inositol. Samples were then applied onto the washed columns. 10 mM formic acid containing 10 mM inositol was used to wash the column. Eluates were collected and counted for CPM in NEN 963 scintillation fluid. The columns were washed until CPM counts were less than 500 per sample. To remove glycophosphate inositol, each column was washed with 10 ml of 60 mM sodium formate with 5 mM Borax. Washes were counted in NEN 963 scintillation fluid until CPM counts were less than 500 per sample. Columns were then washed with 5 ml 1 M ammonium formate with 0.1 M formic acid. Effluents were collected and 10 ml of NEN 963 scintillation fluid was added to each sample. The DPM/sample was then determined on a liquid scintillation counter.

Measurement of Intracellular Free Calcium Concentration

Indo-1 AM, a calcium binding fluorescent dye whose spectral properties change with binding of free Ca^{++}, was used to measure changes in Ca^{++} concentration. B cells were incubated for 15 min at 37^0C with 5 uM indo-1 AM (Molecular Probes, Junction City, OR). After loading, the cells were washed once with PBS and maintained at 22^0C in the dark. Five minutes prior to analysis the cells were warmed to 37^0C in phenol red free RPMI 1640 with Ca^{++} and adjusted to a concentration of $10x10^6$ cells/ml. $1x10^6$ cells were analyzed for a change in Ca^{++} level in each experiment by spectrofluorometer (SLM 8000). Constant stirring was maintained during each experiment. For conversion of the indo-1 signal to calcium concentration (nM), the four parameters (Rmin, Rmax, Sb2, and Sf2) were determined in a series of experiments using an SLM 8000 spectrofluorometer with excitation measured at 355 nm and emission measured simultaneously at 405 nm and 490 nm (SLM-Aminco, IL). The ratio of OD 490/405 nm can be converted to calcium concentration by comparison of the OD ratios of total intracellular Ca^{++} (Triton X 100 lysis) and extractable Ca^{++} (EGTA) and generation of a linear regression curve. Stimuli (mock TCS, anti-IgM mAb HB57, 446-BCDF, SAC) were added to stimulate B cells with or without either 1 mM EGTA or 50 uM BAPTA pretreatment through the injection port on the SLM.

Protein Kinase C Assay

PKC Assay System (GIBCO) was used to measure PKC activity according to the manufacturer's protocol. 1 ml of a B cell suspension

($10X10^6$/ml) was aliquoted to each eppendorf tube and stimulated with one of the following agents (0.001% SAC, 10% 446-BCDF, 100 ng/ml PMA or medium) for 10 min at 37^0C. Supernatants were removed by microfuging for 1 min. Cell pellets were washed once with ice cold PBS and resuspended with 0.5 ml of extraction buffer (20 mM Tris, pH 7.5, 0.5 mM EDTA, 0.5 mM EGTA, and 25 ug/ml each of aprotinin and leupeptin). Cell suspensions were transferred to 13x100 mm tubes and sonicated for 15 seconds on ice. Membrane and cytosol were then separated by microfuging for 2 hours at 4^0C. The cytosol fraction was transferred to eppendorf tubes and kept on ice. The membrane pellet was resuspended with 0.5 ml extraction buffer with 0.5 % Triton X 100 and incubated for 30 min at 22^0C. Cell debris was then removed by microfuging for 2 min at 4^0C. Supernatants were transferred to Eppendorf tubes and kept on ice. Cytosolic and membrane fractions were then loaded onto labeled DEAE cellulose columns (Whatman DE_{52}) (0.6 ml bed volume) pre-equilibrated with buffer B (20 mM Tris, pH 7.5, 0.5 mM EDTA, 0.5 mM EGTA). Columns were then washed with 3 bed volumes of buffer C (20 mM Tris, pH 7.5, 0.5 mM EDTA, 0.5 mM EGTA, 10 mM β-mercaptoethanol, 0.2M NaCl). Partially purified PKCs from both cytosolic and membrane fractions were then assayed. Two conditions were set up to measure PKC activity present in either cytosol or membrane fractions. Each condition was set up in triplicate. 15 ul of extract was added to each tube followed by 10 ul of Buffer C. 10 ul of inhibitor solution (100 uM PKC pseudosubstrate peptide PKC [19-36], 20 mM Tris, pH 7.5) was then added to control tube. 5 ul of lipids (100 uM PMA, 2.8 mg/ml phosphatidylserine, Triton X 100 mixed

micelles) was added to assay tube. The total volume of each tube was adjusted to 40 ul with ddH_2O. Tubes were then incubated at 22^0C for 20 min to allow the inhibitor to bind. After incubation, 10 ul of ^{32}P/substrate (250 uM PKC specific substrate peptide Ac-MBP[4-14], 100 uM ATP, 20 uci/ml [γ-^{32}P]ATP, 5 mM $CaCl_2$, 100 mM $MgCl_2$, 20 mM Tris pH 7.5) was added to each tube and tubes were incubated at 30^0C for 5 min. After incubation, 20 ul of the first sample was removed and spotted onto the corresponding phosphocellulose disc. The rest of the samples were repeated at 15 second intervals. When all the discs on one sheet of phosphocellulose were spotted, the sheet was washed 2 times with 500 ml of 1% phosphoric acid and 2 times with ddH_2O. Each wash lasted 5 min with rocking. After washing, each phosphocellulose disc was placed into labeled scintillation vials and 1 ml of scintillation fluid was added. Peptide-incorporated ^{32}P was counted by scintillation counter. Calculations of specific PKC activity in cytosol or membrane preparations were performed using the formulas provided by the manufacturer.

PTK assay

PB B cells ($5x10^6$) or tonsillar B cells ($5x10^6$) were stimulated with SAC (0.001% or 0.1% v/v), 446-BCDF (10% v/v), or Rabbit anti-human IgM polyclonal antibody (10 ug/ml) (Accurate Chemical & Scientific Corp, NY). Control cells were incubated with medium alone. Western blotting was performed essentially as described (Roifman et al., 1991). After stimulation with the appropriate agent at varying time, cells were washed with ice-cold 1 mM Na_3VO_4 and microfuged for 15

seconds at 4°C. The reaction was stopped by lysis of cells in SDS sample buffer, followed by immediate boiling for 5 min. Lysates were frozen and thawed four times and microfuged for 5 min. Supernatants were analyzed by 7.5% SDS-PAGE. A prestained SDS molecular weight standard mixture (Sigma) was used as molecular weight markers. The separated proteins were electrophoretically transferred to nitrocellulose in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol, 0.015% SDS, pH 8.3). The transfer was accomplished at 500 V for 2 hours. Immediately following transfer, the unreacted sites on the nitrocellulose sheet were blocked overnight with blocking solution (1% BSA in PBS with 0.05% Tween-20) in a rocking platform at room temperature (RT). After blocking, the blot was washed five times with washing buffer (PBS containing 0.05% Tween 20). The blot was then incubated with 50 ug of affinity purified anti-phosphotyrosine monoclonal antibody (4G10, IgG2bκ, UBI, NY) in 20 ml of blocking buffer with a rocking platform for 2 hours, followed by 5 washes with washing buffer. After washing, the blot was incubated with alkaline phosphatase conjugated goat anti-mouse IgG (Sigma) in 20 ml of blocking solution (1:1000) on a rocking platform. After one hour incubation at RT, the blot was washed five times with washing buffer and then incubated with the color developer (0.05 mg/ml Nitro blue tetrazolium, 0.05 M Tris, 4 mM MgCl2, 0.01 mg/ml 5-bromo-chloro-3-indolyl phosphate, pH 10). When protein bands were ideally visualized the reaction was terminated by rinsing briefly with water. The blot was then dried and photographed.

Characterization of a novel human B cell differentiation factor, 446-BCDF

1. Identification of 446-BCDF in anti-CD3-stimulated T cell supernatant. Initial studies in our laboratory have identified a novel factor, 446-BCDF, which is able to induce a significant increase in Ig secretion from human peripheral blood (PB) B cells (Sherris et al., 1989). The effects of 446-BCDF appear to be independent of IL2, IL4, and IL6 in that antibodies to any of these cytokines or their receptors fail to inhibit 446-BCDF activity. 446-BCDF is present in anti-CD3 monoclonal antibody (mAb 446, IgG1) stimulated PB T cell culture supernatant (TCS) but not in mouse IgG1 stimulated TCS. As shown in figure 1, mAb 446-stimulated TCS induced Ig secretion by human PB B cells in a dose dependent fashion, while isotype control mouse IgG1-stimulated TCS did not induce any significant Ig secretion at any concentration used in the assay. To partially purify 446-BCDF, crude TCS was concentrated 20 fold by Amicon filtration and loaded onto a DE52 anion exchange column. The fraction enriched in 446-BCDF activity was eluted at 50 mM NaCl henceforth termed "446-BCDF". Superose 12 permeation chromatography and chromatofocusing of this partially purified 446-BCDF reveal that this new cytokine has an apparent M_r of 32 Kd and pI of 6 (Sherris et al., 1989), which is distinct from the reported pI of IL6 (5-5.1) and the M_r of IL2, IL4, IL10, and IL13. 446-BCDF was capable of inducing SAC-activated PB B cells to secrete Ig in a dose dependent fashion. As shown in figure 2, enriched 446-BCDF induced a 8-fold increase in Ig secretion by SAC-activated B cells, but not resting B cells. These

findings suggest that 446-BCDF stimulated B cell differentiation is a two step event (SAC and 446-BCDF). This result is consistent with the accepted concept that initial activation of resting B cells is a prerequisite for subsequent lymphokine-induced proliferation and differentiation events. Given the lack of purity of this cytokine optimization of fractionated 446-BCDF was determinated in in vitro assays of Ig secretion. In most experiments 10% (v/v) 446-BCDF was sufficient to induce a maximal increase in Ig secretion by SAC activated B cells. This concentration of 446-BCDF was then used in subsequent 446-BCDF mediated B cell differentiation assays.

2. 446-BCDF does not contain IL2 activity. T cells have been known to secrete many cytokines which directly participate in and regulate immune reactivity. Among those T cell-derived cytokines which regulate human B cell function are IL2, IL4, and IL6. To test for the presence of these cytokines in partially purified 446-BCDF, several assays were performed. The murine cytotoxic T lymphoid line (CTLL) has been widely used as a sensitive bioassay for both human and murine IL2. The proliferative growth of this cell line is totally dependent on the presence of IL2 (Gillis et al., 1977). This cell line was used to detect IL2 activity in our partially purified 446-BCDF. As seen in figure 3, the fraction eluted with 50 mM NaCl did not contain IL2 activity. CTLL cells responded to a human recombinant IL2 in a dose dependent fashion, however, concentrations as high as 50% 446-BCDF did not induce any significant CTLL cell proliferation. CTLL cells can respond to IL4 as well (Lindqvist et al., 1993). Therefore, the lack of proliferation

would also suggest the absence of IL4. In order to directly address this issue an IL4 bioassay was performed.

3. 446-BCDF does not contain detectable IL4 activity. Ramos, a B lymphocyte line derived from a Burkitt lymphoma, has been established as a sensitive bioassay for human IL4 based on its ability to upregulate CD23 (low affinity Fcε receptor) expression in the presence of IL4 (Siegel et al., 1990). As seen in figure 4, Ramos cells constitutively express low levels of CD23 (mean channel 2.659). Human IL4 significantly upregulated the expression of CD23 in a dose dependent manner. This assay is quite sensitive since as little as 5 units/ml (3 pM) human IL4 was able to enhance CD23 expression in Ramos cells. Partially purified (50 mM) 446-BCDF did not contain detectable IL4 activity since concentrations as high as 25% 446-BCDF were unable to upregulate CD23 expression in these cells.

4. 446-BCDF activity is not contributed by IL6. Partially purified 446-BCDF contains IL6 which supports IL6-dependent proliferation by the murine plasmacytoma line B9 (Nordan et al., 1986). After passage over an anti-IL6 affinity column, the IL6 activity was completely removed from the 50 mM NaCl fraction as assessed by B9 cell proliferation assay (figure 5). The IL6 depleted fraction was then tested for BCDF activity. IL6-depleted and nondepleted fractions were used to stimulate SAC-activated PB B cells for seven days and secreted Ig was measured by ELISA. As seen in figure 6, the depletion of IL6 from the 50 mM NaCl fraction did

not reduce BCDF activity, suggesting that BCDF activity in 50 mM NaCl fraction was not contributed by IL6. This IL6-depleted fraction was designated 446-BCDF and used in subsequent studies at 10% vol/vol.

5. 446-BCDF activity is not soluble CD40 ligand. The regulation of B cell activation and differentiation by T cells requires two interactions: cognate and noncognate (cytokines). Although the cognate or cognate-dependent phenomenon has been well described by many groups, the molecular mechanism was not clear until recently when CD40 and its ligand gp39 were identified and cloned (Noelle et al., 1992). Now it is clear that the interaction of CD40 on the B cell surface with its ligand on activated T cells triggers B cell proliferation and Ig secretion (Armitage et al., 1993; Splawski et al., 1993). Although there is no evidence to date for the existence of a naturally occuring soluble form of CD40 ligand (CD40L) (Noelle et al., 1992), to rule out the possibility that soluble CD40L was present in 446-BCDF, an anti-CD40L mAb, 5C8 which inhibits CD40-CD40L interactions (Lederman et al., 1992), was used in our experiments. Differing concentrations of mAb 5C8 were added to the corresponding wells containing 10% 446-BCDF. The mixture was incubated at 37°C for 2 hours and then SAC-activated PB B cells were added. Ig secretion was then measured by ELISA after 7 days. As seen in figure 7, 446-BCDF induced a 7 fold increase in Ig secretion by B cells and mAb 5C8 did not significantly block 446-BCDF activity at any concentration used. In contrast, 5C8 completely inhibited anti-CD3 stimulated T cell induction of B cell differentiation in a

coculture system (Y. D. Kuang, personal communication). This suggests that soluble CD40L may not be present in 446-BCDF contributing to 446-BCDF mediated B cell differentiation.

6. The synergistic effect of 446-BCDF and anti-CD40 on Ig secretion by SAC-activated B cells. Recent data has demonstrated that anti-CD40 mAbs can mimic the effect of CD40L on B cell proliferation and differentiation (Splawski et al., 1993). This provided us another approach to address the possible relationship between 446-BCDF and CD40L in B cell differentiation. Data from our laboratory has shown that 10% BCDF and 10 ug/ml of anti-CD40 can stimulate maximal Ig secretion by SAC-activated PB B cells respectively. These two agents, 446-BCDF and anti-CD40, were then used in a B cell differentiation assay. As shown in figure 8, anti-CD40 alone did not induce resting B cells to secrete Ig, however, in the presence of SAC, anti-CD40 induced a 6-fold increase in Ig secretion by PB B cells. 446-BCDF induced a 8-fold increase in Ig secretion by SAC-activated B cells. These two factors displayed a synergistic effect on SAC-activated B cells as they induced a 18-fold increase in Ig secretion. However, in the absence of SAC, BCDF and anti-CD40 did not enhance Ig secretion by resting B cells. These data suggest that 446-BCDF and anti-CD40 are two distinct entities, and may function through different signaling pathways. The signal(s) delivered by SAC is essential for both 446-BCDF and anti-CD40 mediated B cell differentiation, and can not be replaced by either factor.

7. 446-BCDF activity can be specifically blocked by mAb 929. We have recently generated a monoclonal antibody against 446-BCDF, mAb 929. This mAb can specifically inhibit 446-BCDF induced Ig secretion by SAC-activated PB B cells. As seen in figure 9, mAb 929 blocked 446-BCDF induced Ig secretion in a dose dependent fashion, while an IgG2b isotype control did not. Additional data from our laboratory demonstrated that mAb 929 did not inhibit IL2-mediated Ig secretion by PB B cells, IL2-dependent CTLL cell proliferation, IL6-mediated IgG secretion by CESS cells, IL6-dependent B9 cell proliferation, and PWM induced B cell differentiation. These results further support the concept that 446-BCDF is a novel human BCDF distinct from IL2 and IL6.

8. 446-BCDF induces polyclonal Ig secretion by SAC-activated PB B cells. As shown above, 446-BCDF can significantly enhance Ig secretion by SAC-activated PB B cells. Although in vitro studies have shown that there is individual variability of the response of PB B cells to 446-BCDF stimulation, 446-BCDF can generally induce more than a 5-fold increase in Ig secretion. In 8 separate experiments from 8 different donors, 10% (v/v) of either control TCS or 446-BCDF were used to stimulate SAC-activated PB B cells for 7 days. Cell culture supernatants were then collected and assayed for Ig concentration by ELISA. As shown in Table I, in this series of studies, 446-BCDF induced a 7 to 123 fold increase in Ig secretion by SAC-activated PB B cells compared to control TCS. These results demonstrate that 446-BCDF is a potent human B cell differentiation factor. The culture supernatants from the same

sources were used to analyze Ig isotypes. Table I illustrates that 446-BCDF stimulates polyclonal Ig secretion by SAC-activated PB B cells. In 8 separate experiments, 446-BCDF induced a 6 to 141 fold increase in IgG secretion, a 22 to 362 fold increase in IgM secretion, and a 5 to 98 fold increase in IgA secretion. However, none of these experiments showed any significant IgE secretion. The IgE level of both control and 446-BCDF groups in all experiments were lower than 0.5 ng/ml, generally considered insignificant. While these findings may reflect the lack of an appropriate costimulatory signal (eg, CD40 for IL4), these results might support a role for 446-BCDF in normal immune responses.

9. Kinetics of Ig secretion by 446-BCDF stimulated SAC-activated PB B cells. The effect of 446-BCDF on the kinetics of Ig secretion is seen in figure 10. 446-BCDF stimulated PB B cells demonstrated significantly increased Ig secretion on day 6. Ig secretion continued to increase by day 12. On day 15, the Ig level was slightly lower than that of day 12, which might be due to the degradation of Ig. In contrast to 446-BCDF, control TCS did not induce any significant Ig secretion by day 12 although there was a slight increase in Ig secretion by day 15 which might be due to the accumulation of spontaneously secreted Ig.

10. Human tonsil B cells respond to 446-BCDF. Human tonsil B cells are generally considered to be preactivated. Therefore the ability of 446-BCDF to induce Ig secretion in stimulated and unstimulated human tonsil B cells was also examined. As

demonstrated in figure 11, human tonsil B cells responded to 446-BCDF both in the presence (figure 11A) and absence (figure 11B) of SAC. This finding may reflect their preactivated state. Although tonsil B cells did respond to 446-BCDF alone, the presence of SAC significantly enhanced Ig secretion (figure 11A). The Ig secretion by SAC-stimulated tonsil B cells was increased by more than 20-fold at any concentration of 446-BCDF compared to that by non-SAC stimulated tonsil B cells. The results further support the concept that the signal(s) delivered by SAC is important for human B cell differentiation.

In order to determine whether there was a qualitative difference in 446-BCDF induced Ig secretion by SAC-stimulated and non-SAC-stimulated tonsil B cells, Ig isotypes were analyzed (figure 12). The results demonstrated that Ig secretion by 446-BCDF stimulated tonsil B cells, like PB B cells, were also polyclonal in either the presence or absence of SAC. Both groups of tonsil B cells produced IgG, IgM, and IgA although quantities were dramatically different. These data demonstrate that, in contrast to peripheral blood B cells, tonsil B cells are present in a preactivated state and able to respond to 446-BCDF without a costimulatory factor. SAC is a potent costimulatory factor which is able to significantly enhance Ig secretion by 446-BCDF stimulated tonsil B cells.

In four separate experiments from four different donors, Ig secretion by 446-BCDF/SAC stimulated tonsil B cells was analyzed. As shown in Table II, tonsil B cells from four different donors all responded to

446-BCDF, and secreted IgG, IgM, and IgA. However there were individual differences in the level of Ig secreted. Compared to control, 446-BCDF induced a 8 to 44 fold increase in IgG secretion, a 3 to 67 fold increase in IgM secretion, and a 6 to 27 fold increase in IgA secretion.

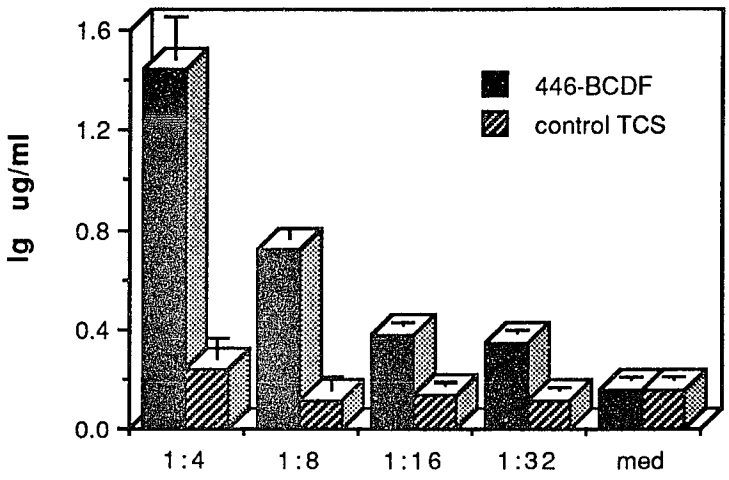

Figure 1. Identification of 446-BCDF in anti-CD3 stimulated T cell supernatant. Crude T cell supernatants from anti-CD3 mAb 446 (IgG1) or mouse IgG1 stimulated T cells were incubated at the indicated dilutions for 7 days with 10^5 cells/well PB B cells in the presence of SAC (0.001%). On day 7, the supernatants were assayed for the Ig secretion by ELISA.

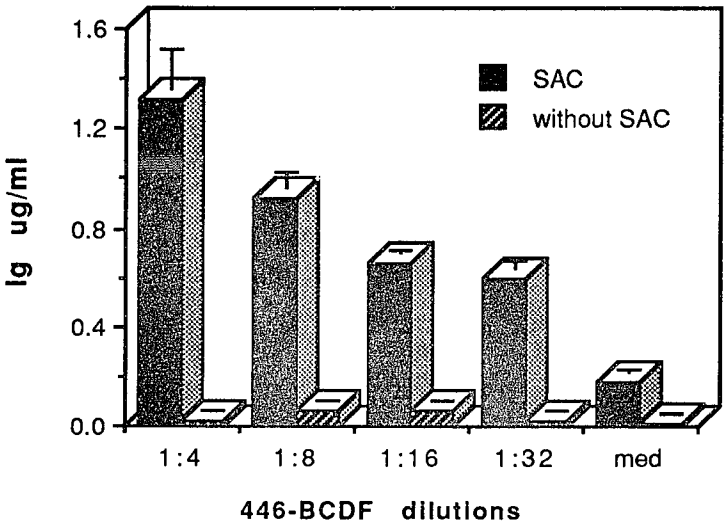

Figure 2. Induction of Ig secretion in SAC-activated B cell by 446-BCDF. Varying amounts of 446-BCDF were incubated for 7 days with 10^5 cells/well B cells in the presence or absence of SAC (0.001%). On day 7, the supernatants were assayed for the Ig secretion by ELISA.

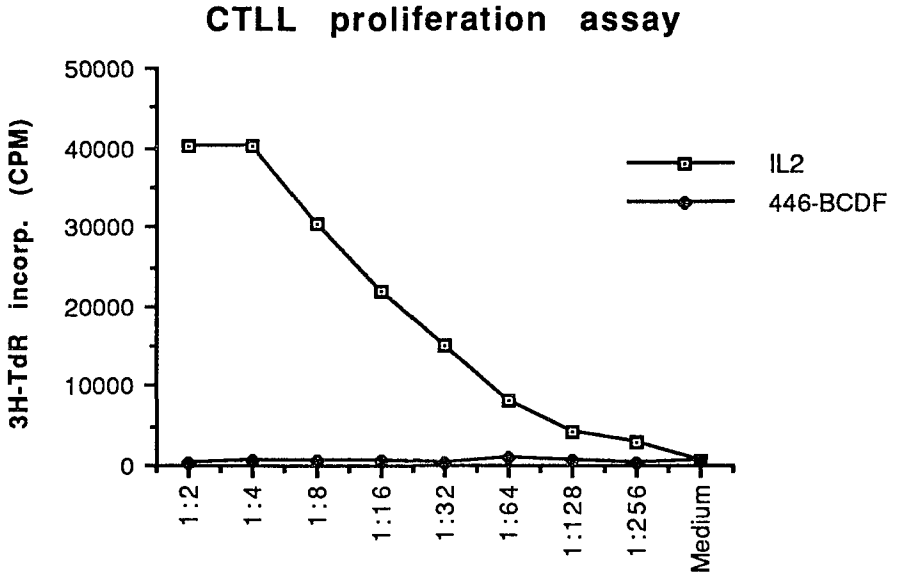

Figure 3. The proliferation assay of IL2-dependent cell line, CTLL. Recombinant human IL2 (20 units/ml) and 446-BCDF were used to make a series of dilutions. $5x10^3$ CTLL cells were seeded in each corresponding well in round bottom 96-well plates. Cells were cultured in triplicate at 37°C for 48 hours, pulsed with 1 μCi [^{3}H] TdR/well during last 18 hours, and harvested for scintillation counting.

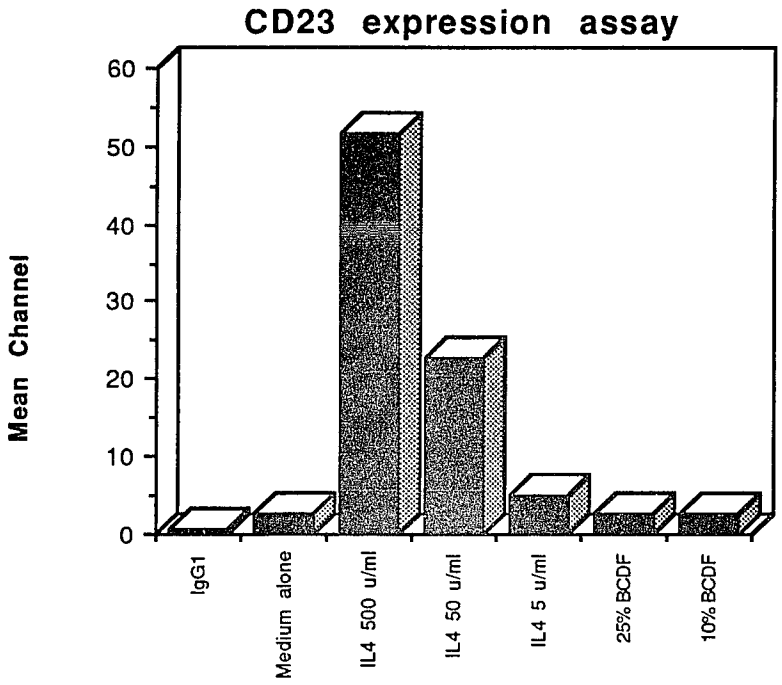

Figure 4. Flow cytometric analysis of the induction of CD23 expression. 10^6 Ramos cells were cultured in medium in the presence of the indicated agents for 48 hr at 37°C. IgG1 was used as the isotype control for the anti-CD23 monoclonal antibody. The medium alone sample reflected the constitutive expression of CD23 on Ramos cells. Cells were harvested and stained for the expression of CD23 by indirect immunofluorescence described in Materials and Methods. Mean channels reflect CD23 expression on a logarithmic scale.

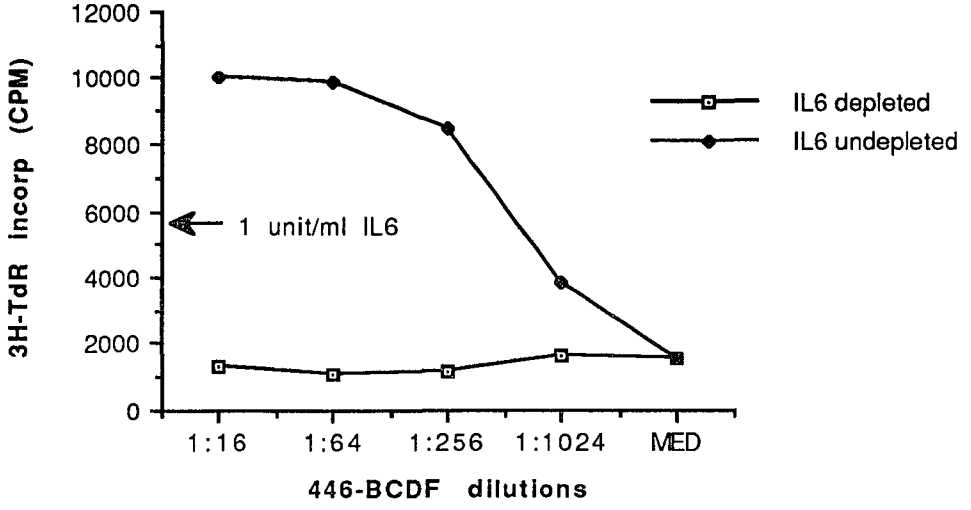

Figure 5. Removal of IL6 activity from a 446-BCDF preparation. mAb 446-stimulated T cell supernatant was fractionated on a DEAE column, the 50 mM NaCl fraction was collected and either left alone or passed over an anti-IL6 affinity column. $5X10^3$ B 9 cells were cultured in triplicate microwell cultures with differing dilutions of the indicated 446-BCDF preparations for 72 hours, pulsed with 1 μCi [^{3}H]TdR/well during the last 18 hours, and harvested for scintillation counting. IL6 activity was determined by ^{3}H-TdR incorporation in B9 cells. Recombinant IL6 served as the positive control. 1 unit IL6 reflects the half maximal ^{3}H-TdR incorporation in B9 cells.

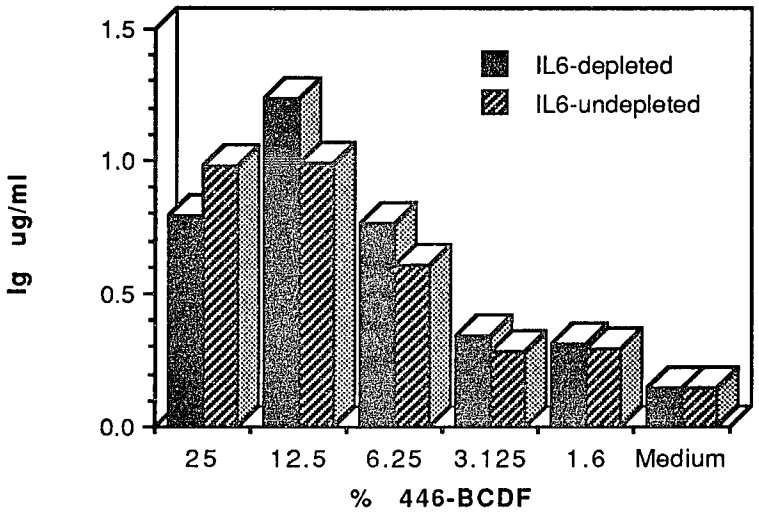

Figure 6. Induction of Ig secretion in SAC-activated PB B cells by 446-BCDF depleted of IL6. The 50 mM NaCl fraction of 446-BCDF untreated or passed over an anti-IL6 affinity column were used to stimulate SAC-activated PB B cells (10^5 cells/well) at the indicated concentrations. Cells were cultured in triplicate at 37°C for 7 days. Ig secretion was measured by ELISA.

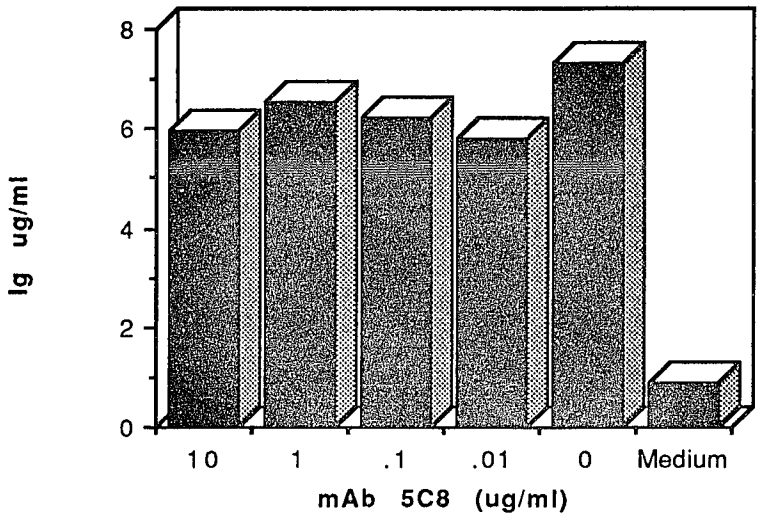

Figure 7. The effect of the anti-CD40 ligand mAb 5C8 on Ig secretion induced by 446-BCDF. 446-BCDF (10% v/v) was incubated with the blocking anti-CD40 ligand mAb, 5C8, at the indicated concentrations for 2 hr at 37^0C. 10^5 PB B cells were then added to the appropriate wells in the presence of SAC (0.001% v/v). Cells were cultured in triplicate for 7 days. Ig secretion was then measured by ELISA.

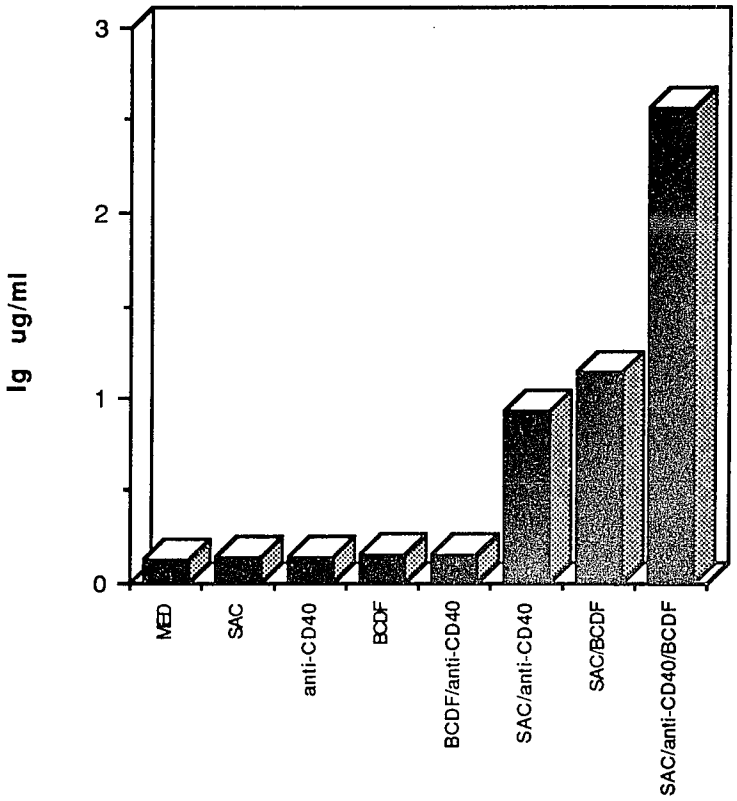

Figure 8. The synergistic effect of 446-BCDF and anti-CD40 on Ig secretion by SAC-activated B cells. 446-BCDF (20% v/v) and/or 10 ug/ml of the stimulatory anti-CD40 mAb (626.1) were incubated for 7 days with 10^5 cells/well PB B cells in the presence or absence of SAC (0.001% v/v). On day 7, supernatants were collected and assayed for Ig secretion by ELISA.

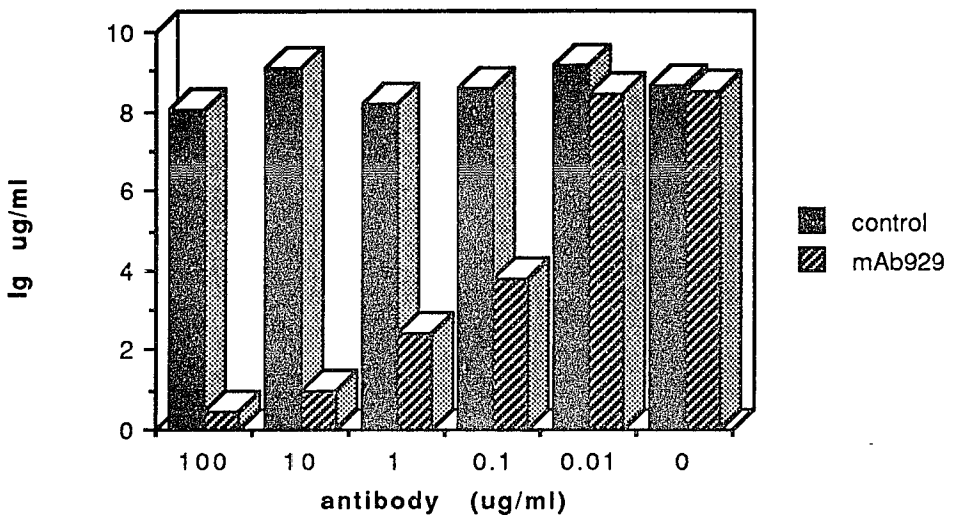

Figure 9. Specific inhibition of 446-BCDF activity by mAb 929. 446-BCDF (10% v/v) was incubated with either mAb 929 or a monoclonal IgG2b isotype control, MPC 11, at the indicated concentrations for 2 hr. SAC-activated PB B cells (10^5 cells/well) were then added to the corresponding wells, cultured in triplicate at 37^0C for 7 days. Ig secretion was measured by ELISA. Baseline Ig secretion by SAC activated B cells was 0.51 ug/ml.

Table I. Analysis of Ig isotypes secreted by 446-BCDF stimulated PB B cells

Expt.	Total Ig (μg/ml)		Stimulation index	IgG (μg/ml)		IgM (μg/ml)		IgA (μg/ml)		IgE (ng/ml)	
	Control	BCDF		Control	BCDF	Control	BCDF	Control	BCDF	Control	BCDF
1	0.21	8.39	39.95	0.13	4.96	0.02	1.43	0.06	2.02	<0.5	<0.5
2	0.95	6.84	7.2	0.89	5.88	0.04	0.86	0.02	0.11	<0.5	<0.5
3	0.05	2.72	54.4	0.01	1.41	0.02	0.45	0.02	0.86	<0.5	<0.5
4	0.11	9.12	82.9	0.07	1.49	0.02	7.24	0.02	0.39	<0.5	<0.5
5	0.06	5.96	99.33	0.02	1.43	0.02	3.08	0.02	1.45	<0.5	<0.5
6	0.08	9.87	123.37	0.04	5.6	0.02	4.17	0.02	0.11	<0.5	<0.5
7	1.26	22.89	18.17	0.97	5.01	0.25	17.49	0.04	3.93	<0.5	<0.5
8	0.35	6.28	17.94	0.28	4.71	0.04	1.15	0.03	0.42	<0.5	<0.5

10^5 cells/well PB B cells were incubated with either 446-BCDF (10% v/v) or control TCS (isotype control stimulated T cell supernatant) in the presence of SAC (0.001% v/v). Supernatants were harvested on day 7. Total Ig and Ig isotype secretion was measured by ELISA. Results are expressed as the mean of triplicate cultures. Stimulation index is expressed as the ratio of the amount of Ig induced by 446-BCDF over the amount of Ig induced by control TCS. Ig secretion induced by control TCS was not significantly higher than baseline Ig level in each experiment.

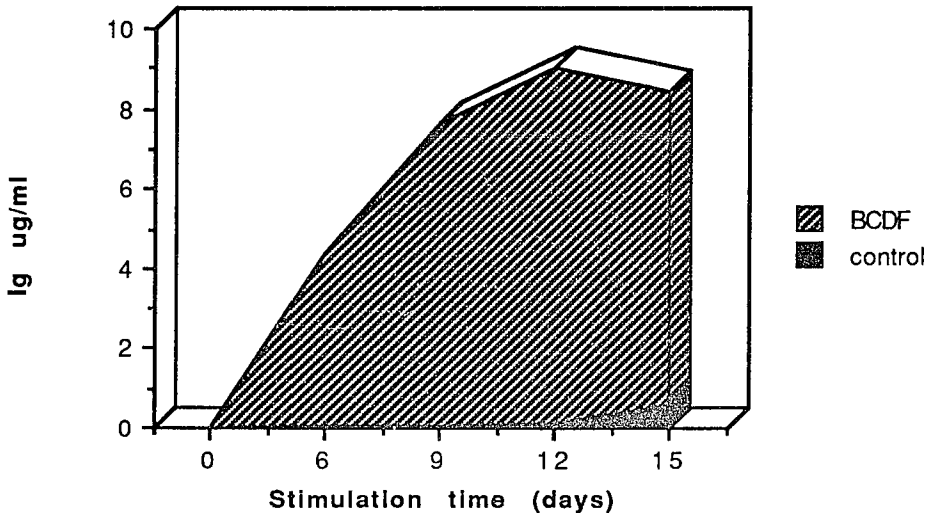

Figure 10. Kinetics of Ig secretion by 446-BCDF-stimulated B cells in the presence of SAC. Purified B cells (10^5 cells/well) were incubated with either 446-BCDF (20% v/v) or control TCS and SAC (0.001% v/v). Supernatants were harvested on the days indicated and analyzed by ELISA. In general, majority of B cells did not survive beyond day 12 in culture. These data are representative of 3 experiments.

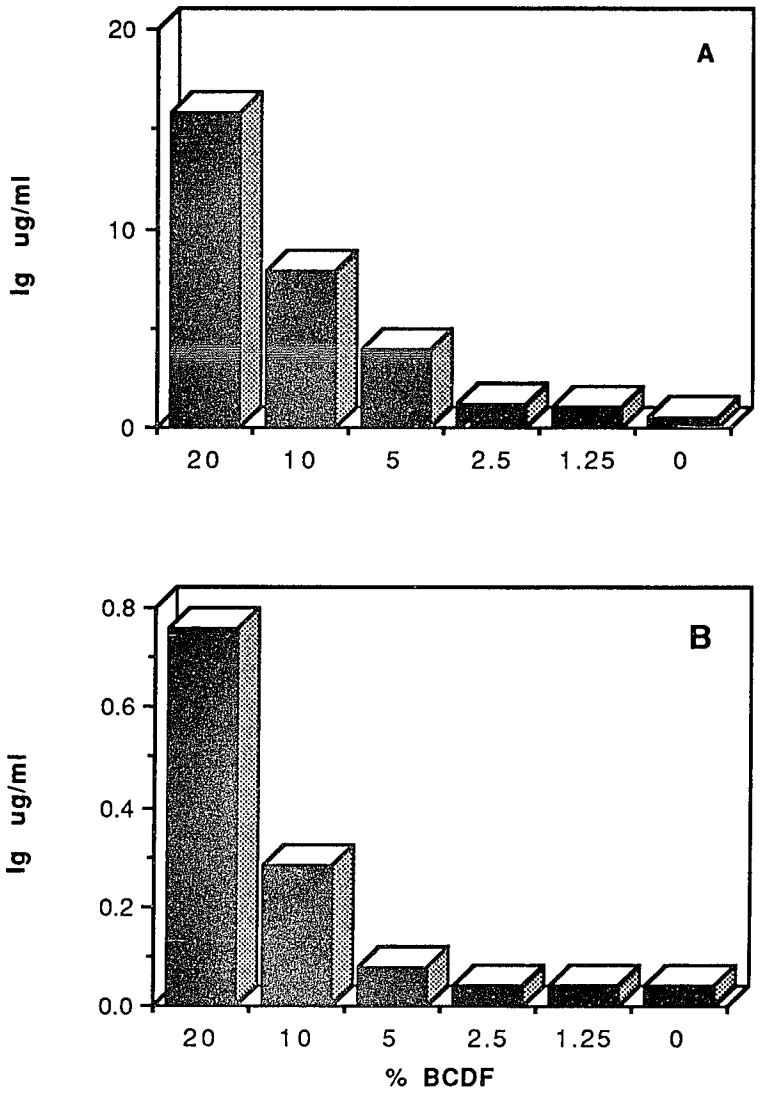

Figure 11. The response of tonsil B cells to 446-BCDF. Purified tonsil B cells (10^5 cells/well) were cultured in triplicate with 446-BCDF (10% v/v) in the presence (A) or absence (B) of SAC (0.001% v/v). Ig secretion was measured on day 7 by ELISA.

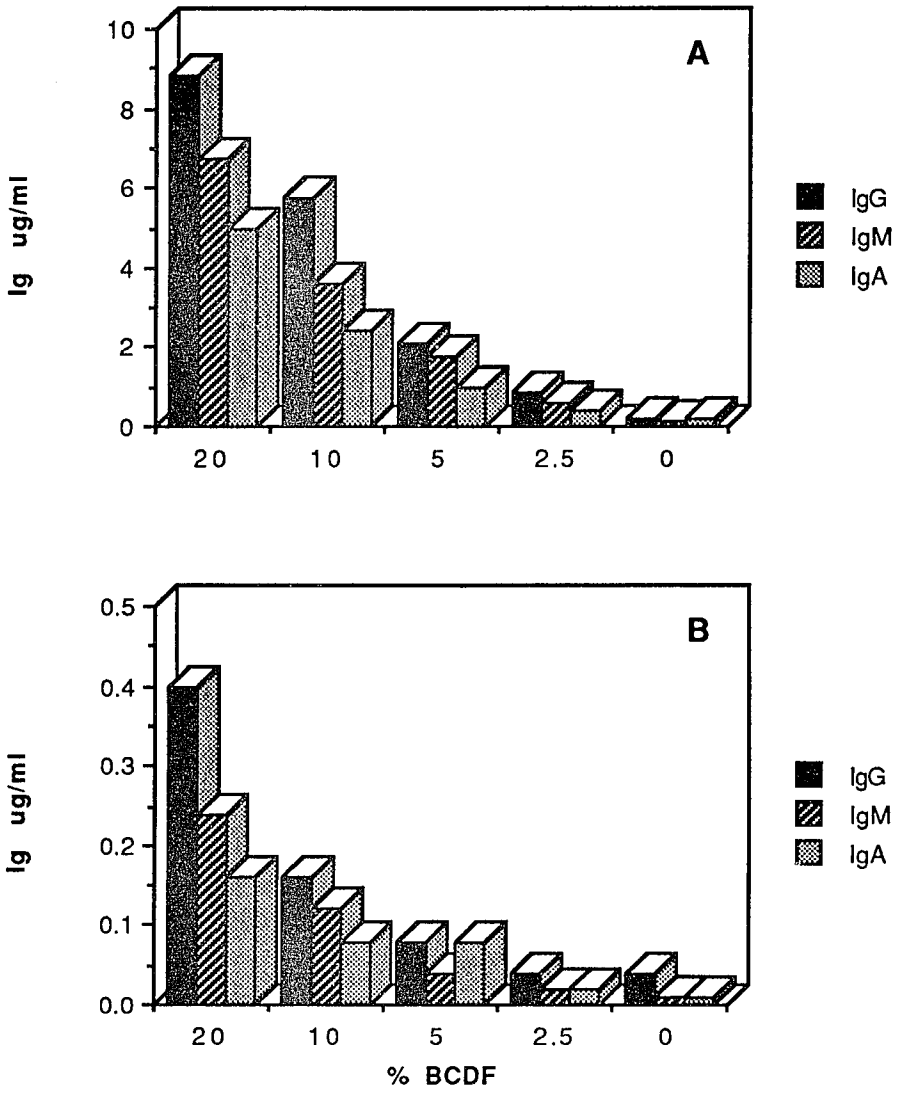

Figure 12. Polyclonal response of tonsil B cells to 446-BCDF in the presence or absence of SAC. Purified tonsil B cells (10^5 cells/well) were cultured in triplicate with 446-BCDF (10% v/v) in the presence (A) or absence (B) of SAC (0.001% v/v). On day 7, IgG, IgM, and IgA levels were determined by ELISA. Results are representative of two experiments from two different tonsils.

Table II. Analysis of Ig isotypes secreted by 446-BCDF stimulated tonsil B cells in the presence of SAC

Expt.	Ig Isotypes								
	IgG (μg/ml)			IgM (μg/ml)			IgA (μg/ml)		
	Control	BCDF	S.I.	Control	BCDF	S.I.	Control	BCDF	S.I.
1	0.04	0.32	8	0.04	0.12	3	0.08	0.6	7.5
2	0.08	1.76	22	0.04	0.84	21	0.48	3.04	6.3
3	0.21	8.84	44	0.1	6.77	67	0.18	4.94	27.4
4	0.61	4.86	8	0.04	0.16	4	0.2	1.88	9.4

Purified tonsil B cells (10^5 cells/well) were incubated with 446-BCDF (10% v/v) or control TCS in the presence of SAC (0.001% v/v). Cells were cultured in triplicate for 7 days. Supernatants were collected and assayed for IgG, IgM, and IgA by ELISA. The results reflect four experiments from four different donors. S.I. (stimulation index) is the ratio of the amount of Ig induced by 446-BCDF over the amount of Ig induced by control TCS. Ig secretion induced by control TCS was not significantly higher than baseline Ig levels in each experiment.

Running Title

Reciprocal relationship of cAMP and BCDF induced Ig secretion.

B cell differentiation factor induced B cell maturation. I. Regulation via reduction in cAMP.

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Footnotes

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Abbreviations used

BCDF - B cell differentiation factor
BCGF - B cell growth factor
CREB - cAMP responsive element
SAC - Staphylococcus aureus Cowan I
PKC - protein kinase C
PKA - protein kinase A
PTX- pertussis toxin
CTX- cholera toxin
PDE-phosphodiesterase

Abstract

We have previously described a novel human B cell differentiation factor, 446-BCDF, that is distinct biochemically and functionally from other cytokines. Since signal transduction pathways involved in human B cell differentiation have been incompletely studied and are poorly understood, we assessed the effects of 446-BCDF on various intracellular second messenger systems. After exposure to 446-BCDF, the intracellular cAMP concentration started to decrease at 5 minutes and was significantly lower at 30 min and reached the lowest level at 4h. In most cases, cAMP concentrations returned towards baseline by 24 hours. A cAMP analogue (dibutyryl cAMP), a stimulator of adenyl cyclase (forskolin), and phosphodiesterase inhibitors (aminophylline and IBMX) which inhibited the 446-BCDF induced decrease in intracellular cAMP, inhibited 446-BCDF induced B cell differentiation, suggesting that the fall in intracellular cAMP was a critical event in this process. To understand the mechanism involved in the reduction of cAMP, B cells were treated with pertussis toxin (10 ng/ml), a Gi protein inhibitor. Pertussis toxin blocked 446-BCDF induced B cell differentiation as well, suggesting that 446-BCDF may function by stimulation of a Gi-linked receptor resulting in the inhibition of adenylate cyclase with a consequent reduction in cAMP. Other cytokines known to promote Ig secretion (IL2 and IL6) also caused a reduction in cAMP, suggesting that this pathway may be generally important in B cell differentiation. Taken together, these data suggest that at least one pathway of terminal maturation in B cells may involve the reduction of intracellular cAMP.

INTRODUCTION

The pathway of normal human B cell maturation involves an initial activation signal, clonal proliferation and terminal maturation to Ig secretion. While several studies have addressed signal pathways involved in B cell activation and proliferation, no analysis of differentiation inductive signals is available (1-3). B cell proliferation after anti-IgM crosslinking is associated with turnover of inositol phospholipids, Ca^{++} mobilization and PKC translocation, in a manner similar to T cell proliferation (1). In studies reported to date, IL6 does not appear to activate any of the conventional signal pathways described via the 80kd chain of the IL6R. Although the receptor has been isolated and cloned, the 80kd chain has only a short intracytoplasmic tail which can not transduce a signal (4). An associated chain, gp130, does possess a long intracytoplasmic tail with GTP binding sites, however no specific signal pathways have been defined (5). More recent data suggest that gp130 is associated with tyrosine kinases which drive B cell responses to IL-6 (6), however, the exact mechanism by which tyrosine kinases regulate IL6-driven B cell response is not known. Ambrus et al reported that IL-6 induced IgM secretion in the B lymphoblastoid cell line SKW 6.4 was blocked by agents which cause a rise in intracellular cAMP (7). Experimentally increasing intracellular cAMP levels with various agents inhibited the formation of Ig-secreting cells and PMA-enhanced IgM production by the human B cell line, LA350 (8-9), as well IgG production by CESS cells (R. Huang and L. Mayer, unpublished data). Similar results were also obtained in a murine

system. Crosslinking of class II MHC I-E molecules induced increases in cAMP levels in murine B cells, which in turn inhibited IgM production (10). An increase in intracellular cAMP has also been shown to potentiate ablation of IgM responses induced by immune complexes (11). All these data suggest that cAMP has a negative effect on B cell differentiation. However, opposite results were also observed in murine B cells where intracellular cAMP increase after crosslinking class II molecules and enhanced LPS-driven B cell differentiation (12). Furthermore, cAMP plus IL1 was reported to enhance polyclonal Ig production in murine B cells (13). From these studies the effect of cAMP appears to be system dependent, inhibiting in most cases and stimulating in others.

We have recently described a human B cell differentiation factor, 446-BCDF, which is secreted by anti-CD3 stimulated T cells (14-15). This factor is biochemically distinct from the known cytokines acting on B cell differentiation (mw = 32 kDa, pI = 6.0) and cannot be neutralized by anti-IL2 and anti-IL6 Abs. It is a potent BCDF which acts on SAC activated human B cells inducing polyclonal Ig secretion. We have chosen to look at differentiation signal transduction pathways after receptor/ligand interaction, utilizing 446-BCDF. This cytokine stimulates a significant decrease in intracellular cAMP which is inhibitable by pertussis toxin, a known inhibitor of Gi and Go proteins. Terminal differentiation may, therefore, be associated with alterations in cAMP as an early event.

METHODS

Cell isolation and culture

Peripheral blood mononuclear cells (PBMNC) were isolated from leucocyte concentrate packs from normal blood donors by Ficoll-Hypaque (Pharmacia Chemicals, Piscataway, N.J.) density gradient centrifugation (14). T cells were depleted by rosetting with neuraminidase treated sRBC (14). The non T cell fraction was enriched for B cells with removal of monocytes by plastic adherence (16). The resulting B cell preparation was greater than >80% sIg^+ and >90% $CD20^+$, <1% $CD3^+$ and <2% $CD14^+$. Isolated B cells were cultured in RPMI 1640 (Gibco, Grand Island, NY), 10% fetal calf serum (Gibco), 1% penicillin/streptomycin (Gibco) and 2 mM glutamine (Gibco) henceforth termed culture medium (CM). Cells were cultured in a humidified 37°C incubator with 5% CO_2.

Generation of 446-BCDF

Monocyte depleted T cells were cultured in RPMI 1640, 0.1% BSA in the presence of anti-CD3 mAb 446 (10 ug/ml) bound to tissue culture dishes (60 x100 mm - Falcon, Oxnard, CA) as previously described (14). After 48 hours of culture at 37°C, cell free supernatants were obtained by filtration (0.22u filter, Nalgene), supernatants were concentrated twenty-fold by positive pressure Amicon filtration (YM10 filter) and dialysed against 10 mM NaCl pH 7.4 (final dialysis dilution 1:100,000). The concentrated T cell supernatant was then passed over a DEAE-sepharose column and the fraction enriched in 446-BCDF activity was eluted at 50 mM NaCl. We have previously determined that 446-BCDF, devoid of IL6 is

eluted at 50 mM NaCl while IL6 is eluted at 150 mM NaCl (14). The 50 mM fraction was dialysed against PBS and used at a final concentration of 10% vol/vol in our assays. In initial studies this concentration was determined to be maximal for stimulation of Ig secretion from SAC activated B cells. Higher concentrations of 446-BCDF yielded either no greater Ig secretion or an actual decrease (14). This partially purified BCDF contains no detectable IL-2, IL-6, IL-4, IL-10, or γ-IFN.

B cell differentiation assay

Isolated B cells were adjusted to $2x10^6$/ml in CM and 50 ul cell suspension was added to triplicate microwell cultures (final volume 100 ul). Fixed Staphylococcus aureus Cowan I organisms (SAC-Pansorbin, Calbiochem, Burlingame, CA) were added to cultures at a final concentration of 0.001% vol/vol. 446-BCDF concentration was constant at 10% vol/vol. In some experiments aminophylline (Sigma, St. Louis, MO, 0.01-10 uM), dibutyryl cAMP (Sigma, 0.01-10 uM), forskolin (Sigma, 0.01-10 ug/ml), IBMX (Sigma, 0.0625-1 mM), cholera toxin (Sigma, 0.001-10 ng/ml) or pertussis toxin (Sigma, 0.001-10 ng/ml) were added to SAC plus BCDF stimulated cultures at the onset of culture. At the end of the culture period cell viability (by trpan blue exclusion) and number were determined. Cell numbers and viability were not different in treated or untreated cultures. Cell free supernatants were obtained after 7 days and total Ig was measured by ELISA (17).

cAMP assay

1. RIA method. $2x10^6$ B cells were cultured in 1 ml RPMI 1640 at 37^0C in the presence or absence of either 446-BCDF (10% v/v), IL-2 (20 u/ml) or IL-6 (400 u/ml). In some experiments IBMX (0.5 mM) was added and cells incubated for 30 minutes before addition of cytokine. At 5, 30, 60, 120, 240 min, 24 and 48 hours of culture, cAMP was extracted by adding 2 ml of boiling ddH_20. The diluted cell suspension was boiled for 3 minutes (18), cooled on ice and centrifuged. The supernatant was lyophilized to dryness and then reconstituted to 1/8 its original volume. B cell cAMP concentrations were determined using a radioisotopic test kit (Amersham, Arlington Heights, IL). cAMP concentrations are expressed as pM/million cells.

2. EIA method. cAMP samples were prepared as described above except that B cells were pretreated 30 min with 0.5 mM IMBX to inhibit phosphodiesterases and prevent breakdown of cAMP. B cells were then stimulated with either cholera toxin (1 ng/ml), or pertussis toxin (1 ng/ml) with or without 446-BCDF (10% v/v) for 60 min. cAMP samples were assayed according to the manufacturer's protocol. 100 ul of polyclonal rabbit anti-cAMP antibody was added in duplicate into goat anti-rabbit antibody precoated wells of a 96-well microtiter plate except for the substrate control wells. 100 ul of washing buffer (0.05 M NaC_2HO_3, 0.1% bovine γ-globulin, 0.1% NaN_3, pH 6.2), cAMP standard, or sample were then added into the corresponding wells. The plate was gently agitated and incubated at 4^oC for 2 hours. 100 ul of cAMP alkaline phosphatase conjugate were then added and the plate was incubated at 4^oC. After one hour's incubation, solution from all wells was removed. The plate was

washed 6 times and 300 ul pNNP substrate solution were then added into all wells. The plate was covered and incubated for 3 hours at 37°C. The reaction was stopped by adding 50 ul of 0.2 N NaOH. The plate was gently tapped to mix. The absorbance of wells was recorded at 405 nm. The cAMP concentration of each sample was calculated according to the standard curve by regression analysis.

In order to determine the effect of various agents on 446-BCDF's ability to lower cAMP, either aminophylline, forskolin, pertussis toxin, or cholera toxin were added to cultures prior to co-culture with B cells. cAMP was then measured as described above. mAB 929 was recently produced as a specific anti-446-BCDF mAB. This mAb inhibits 446-BCDF induced Ig secretion but has no effect on IL2, IL6, or PWM induced Ig secretion by B cells or B cell lines. This mAb was used to determine the specificity of 446-BCDF activity.

Results

446-BCDF induces a decrease in intracellular cAMP

Since previous studies have shown that cAMP might play a role in promoting B cell differentiation (7-13), we elected to measure the effect of the differentiation signal delivered by 446-BCDF on intracellular cAMP. B cells were stimulated with SAC alone, SAC and 446-BCDF, or these two agents in the presence of aminophylline (10 uM). Control cultures of unstimulated B cells were included in all studies. After culture for 5, 15, 30, 60, 120, 240 minutes, and 24 hours, B cells from parallel cultures were boiled for 3 min stopping the response to the activation signals. As seen in Figure 1, in as early as five minutes, there was a decrease in intracellular cAMP which

achieved significance at 30'. The level of cAMP remained low for at least four hours and, in most cases, returned towards baseline by 24 hours. This pattern of response was consistent in all experiments performed (more than 40), although there was variability in endogenous cAMP levels between various individual B cell preparations. However, in experiments where multiple determinations were made using B cells from one individual, the endogenous level of cAMP and the response to 446-BCDF was consistent (data not shown). SAC, a costimulatory agent used in our B cell differentiation assays had no effect on cAMP levels (Figure 1). However, BCDF by itself was capable of inducing cAMP in the absence of SAC (see below).

Aminophylline effectively inhibited the reduction of cAMP (Figure 1- corresponding with the inhibition of Ig secretion - see below). Interestingly, aminophylline alone failed to induce an increase in cAMP in isolated B cells at any concentration used (data not shown).

Agents that effect an increase or prevent a decrease in intracellular cAMP inhibit cytokine mediated B cell differentiation

The reduction in intracellular cAMP induced by 446-BCDF raised the question as to whether this event was important for 446-BCDF induced B cell differentiation. To answer this question, SAC activated B cells were co-cultured with 446-BCDF in the presence or absence of varying concentrations of aminophylline (0.01-10 ug/ml), dibutyryl cAMP (0.01-10 ug.ml), or forskolin (0.01-10 ug/ml) at the onset of culture. These agents have been shown to affect

intracellular cAMP concentrations in other cell systems by three distinct mechanisms (inhibition of PDE activity, addition of cAMP analogue, or activation of adenyl cyclase respectively). In the absence of any of these agents 446-BCDF induced between a 10-100 fold increase in total Ig secretion as measured by ELISA on day 7 of culture. The addition of any of the three compounds completely inhibited this response in a dose dependent fashion (Figure 2). This effect did not reflect direct cell toxicity as both control and treated cultures exhibited similar viabilities and cell numbers at the end of the culture period (data not shown).

Interestingly, neither forskolin nor aminophylline had any effect on the intracellular cAMP levels of normal isolated B cells. Therefore, as noted above (figure 1), these agents may inhibit 446-BCDF induced Ig secretion by preventing BCDF's effects on reducing cAMP, suggesting that a threshold lower level of intracellular cAMP may be important for the initiation of differentiation. Increasing the concentration of 446-BCDF did not overcome the effects of these agents on either cAMP levels or Ig secretion (data not shown).

IBMX, a more potent phosphodiesterase inhibitor, was capable of stimulating an increase in intracellular cAMP in PB B cells. As seen in Figure 3, the addition of IBMX induced a 5 fold increase in cAMP. Addition of 446-BCDF (10% v/v-the optimal concentration for Ig secretion) lowered cAMP levels by about 35% in these cultures but this did not achieve baseline values. Addition of greater amounts of 446-BCDF to these cultures failed to completely reverse the increase in cAMP mediated by IBMX (data not shown), suggesting that the signals delivered by 446-BCDF may not be directed at

phosphodiesterase (PDE) but rather act higher up in the signal pathway. Prevention of the reduction in intracellular cAMP induced by 446-BCDF appeared to correlate with the ability of IBMX to inhibit Ig secretion by 446-BCDF stimulated B cells. As shown in figure 4, IBMX inhibited 446-BCDF induced Ig secretion in a dose dependent manner without affecting the cell viability and number.

Pertussis toxin and cholera toxin inhibit 446-BCDF activity

There are two potential pathways whereby intracellular cAMP concentration can be decreased; 1) by inhibition of adenylate cyclase mediated production (stimulation of a Gi linked receptor) or 2) by enhanced removal of cAMP (i.e. by consumption in an enzymatic pathway, breakdown by phosphodiesterase, or movement to another cellular compartment). As mentioned above, 446-BCDF failed to completely reverse the effect of IBMX on cAMP, suggesting that 446-BCDF may induce a reduction in cAMP at a higher level than PDE, possibly by stimulating a Gi-linked receptor. To test this hypothesis, PB B cells were pretreated with IBMX (0.5 mM) for 30 min to inhibit PDE activity and prevent the breakdown of cAMP, and then cultured for 60 min with either CTX (1 ng/ml) or PTX (1 ng/ml) alone or in the presence of 446-BCDF (10% v/v). Intracellular cAMP levels were measured and results shown in figure 5. The optimal concentration of 446-BCDF (10% v/v) induced a 60% reduction in intrcellular cAMP in IBMX pretreated PB B cells. Although neither CTX nor PTX at the concentrations used in our assays stimulated a significant rise in intracellular cAMP in PB B cells, they did prevent the decrease in intracellular cAMP induced by 446-BCDF. Shearea et al reported that

CTX induced an increase in intracellular cAMP in an IBMX pretreated human EBV-transformed cell line and inhibited IgM secretion (8). Failure to induce a significant increase in intracellular cAMP in human PB B cells with either CTX or PTX may reflect a different sensitivity of G proteins to these agents betwen normal PB B cells and cell lines. Since PTX is a potent Gi protein inhibitor, prevention of 446-BCDF induced reduction in cAMP by PTX suggests that 446-BCDF may function through a Gi-linked receptor. CTX, a Gs protein stimulator, had a similar effect on cAMP, probably via stimulation of Gs protein competing with 446-BCDF stimulated Gi protein for the common target, adenylate cyclase. Prevention of the reduction in intracellular cAMP also appeared to correlate with the ability of both PTX and CTX to inhibit 446-BCDF activity since both agents inhibited 446-BCDF induced Ig secretion by SAC activated B cells in a dose dependent manner (figure 6)

Is the reduction in cAMP 446-BCDF specific?

Since other factors might be contaminating the 50 mM fraction associated with 446-BCDF activity, we added mAb 929 (446-BCDF specific mAb- Kuang, Cidon, Mayer submitted) to preparations of 446-BCDF before addition to the B cell cultures. This mAb reversed the effects of 446-BCDF on intracellular cAMP levels seen (Figure 7). In contrast, an IgG2b isotype control did not have any significant effect on cAMP level, suggesting that the reduction in intracellular cAMP is specifically induced by 446-BCDF.

To further answer the specificity of these findings, IL-2 and IL-6 were added to isolated B cells since these cytokines have been

reported to induce human B cell differentiation under different conditions. As seen in Figure 8, both IL-2 (a) and IL-6 (b) effect a decrease in intracellular cAMP in PB B cells although less than that seen with 446-BCDF. However, only IL-2 can stimulate Ig secretion by SAC activated primary B cells. IL-6 has activity on cell lines (CESS and SKW) and only induces human B cell differentiation in the presence of IL-2. Therefore, the reduction in cAMP mediated by 446-BCDF may be necessary but not sufficient to drive terminal B cell differentiation and may be a common finding mediated by B cell differentiation factors.

Discussion

The pathways for T and B cell proliferation have been extensively explored over the past several years with most evidence favoring the activation of the PI pathway associated with Ca^{++} mobilization and PKC translocation. However, events relating to terminal differentiation have been incompletely studied. A number of reports have supported the concept that differentiation and proliferation pathways are distinct and nonoverlapping (19, 20). That is, that proliferation is an anti-differentiation event and vice versa. We have recently described a novel B cell differentiation factor, 446-BCDF, which is a potent inducer of terminal B cell differentiation to plasma cells. In the current study we assessed the pathway(s) involved in cytokine mediated terminal B cell differentiation. We have demonstrated that 446-BCDF mediates a time dependent decrease in intracellular cAMP which correlates with terminal maturation. Furthermore, this decrease in cAMP may be regulated by

a Gi linked receptor, since Ig secretion mediated by 446-BCDF is inhibitable by pertussis toxin. Several previous studies have documented that agents which cause a rise in intracellular cAMP are inhibitory for human B cell differentiation in B lymphoblastoid cell lines (7, 9) as well as murine B cells (10, 11, 19). However no measurement of intracellular cAMP was performed in these studies. Bishop has reported that cross-linking of surface Ia molecules on murine splenic B cells enhances Ag driven differentiation, but in contrast to our studies, intracellular cAMP was increased (12). The major differences between these two studies was the model system (human vs. mouse) and the stimulus for differentiation (cytokine vs. sRBC in an Ag specific B cell line).

A reduction in cAMP alone is essential but may not be sufficient to induce Ig secretion by human B cells. Both IL-2, which can independently induce human PB B cell maturation, and IL-6, which cannot, induce a reduction in cAMP. Previous studies have suggested that SAC/IL-2 induced B cell differentiation can be inhibited by agents which generally increase cAMP levels (23). However, direct measurement of cAMP was not determined in these studies. In our studies, IL2 induced a reduction in intracellular cAMP. Increasing intracellular cAMP by addition of dibutyl cAMP inhibited IL2 induced Ig secretion by SAC activated PB B cells (data not shown), suggesting that a reduction in intracellular cAMP may be important for IL2 mediated human B cell differentiation, similar to that seen with 446-BCDF. In most systems the presence of a preactivating agent such as SAC is required so that the combination of signals (BCDF and SAC) may activate distinct pathways necessary

for terminal differentiation. SAC has been reported to stimulate tyrosine kinase activity in B cells (22) although the consequences of such activation have not been addressed. In addition, while there is evidence that SAC alone can increase cAMP levels in B cells, this is at concentrations at least two orders of magnitude greater than the dose used in our studies (23). Furthermore, when directly measured, no change in intracellular cAMP was noted when cells were stimulated with SAC alone and SAC (0.001% v/v) did not inhibit the 446-BCDF mediated decrease in cAMP. It is postulated that SAC stimulation allows B cells to express appropriate receptors for growth and differentiation factors (24). Thus it is a critical component of the activation pathway.

There are few exogenous agents known to lower endogenous cAMP. Angiotensin II, whose receptor is Gi linked, was shown to reduce intracellular cAMP and synergized with 446-BCDF to trigger terminal B cell differentiation in SAC-activated B cells (data not shown). However as seen for IL6 a reduction in cAMP alone may not be sufficient to drive B cell maturation. Additional signals may be required.

The experiments where pertussis toxin inhibits 446-BCDF's differentiation activity point toward a Gi linked receptor pathway, however pertussis toxin may have other effects on B cells which may alter their ability to synthesize Ig (such as inhibition of the microtubular assembly-25). Still, failure of 446-BCDF to reverse cAMP levels in IBMX stimulated B cells suggests that the reduction in cAMP is coming from a point higher than phosphodiesterase.

One obvious question remains. What are the consequences of a reduction in intracellular cAMP in B cells? cAMP is a critical component of the activation of protein kinase A. In the absence of cAMP, altered kinase activities may exist, promoting differentiation over proliferation. cAMP regulation of cAMP responsive elements within the Ig gene is well recognized (26) and may be affected by PKA. Potentially a reduction in intracytoplasmic cAMP may alter CREB mediated proliferative signals, allowing for terminal differentiation. The answer to this question requires careful analysis of nuclear events following BCDF/receptor interactions.

References:

1. Cambier, J. C. and J. T. Ranson 1987. Molecular mechanism of transmembrane signaling in B lymphocytes. Ann. Rev. Immunol., 5:175.

2. Nichizura, Y. 1984. The role of protein kinase C in cell surface signal transduction and tumor promotion. Nature 308:693.

3. Gold M. R., D. A. Law, and A. L. DeFranco. 1990. Stimulation of protein tyrosine phosphorylation by the B-lymphocyte antigen receptor. Nature 345:810.

4. Yamasaki, K., T. Taga, Y. Hirata, H. Yawata, Y. Kawanishi, B. Seeds, T. Taniguchi, T. Hirano, and T. Kishimoto. 1988. Cloning and expression of the human interleukin-6 (BSF-2/IFN$_\beta$2) receptor. Science 241:825

5. Hibi, M., M. Murakami, M. Saito, T. Hirano, T. Taga, and T. Kishimoto. 1990. Molecular cloning and expression of an IL-6 signal transducer, gp130. Science 247:1149

6 Murakami, M., M. Hibi, N. Nakagawa, T. Nakagawa, K. Yasukawa, K. Yamanishi, T. Taga and T. Kishimoto. 1993. IL6-induced homodimerization of gp130 and associated activation of a tyrosine kinase. Science, 260:1808.

7. Goldstein, H., D. Koerholz, L. Chesky, X. Fan, and J. ambrus. 1990. Divergent activities of protein kinase in IL-6 induced differentiation of a human B cell line. J. Immunol., 145:952.

8 Shearer, W. T., C. L. Patke, E. B. Gilliam, H. M. Rosenblatt, K. S. Barron and F. M. Orson. 1988. Modulation of a human lymphoblastoid B cell line by cyclic AMP. Ig secretion and phophotidylcholine Metabolism. J. Immunol., 141:1678.

9. Patke, C. L., F. M. Orson, and W. T. Shearer. 1991. Cyclic AMP-mediated modulation of immunoglobulin production in B cells by prostaglandin E1. Cell Immunol., 137:36.

10 Takahama, Y., S. Ono, K. Ishihara, M. Muramatsu and T. Hamaoka. 1989. Disparate functions of I-A and I-E molecules on B cells as

evidenced by the inhibition with anti-I-A and anti-I-E antibodies of polyclonal B cell activation. Eur. J. Immunol., 19:2227.

11. Stein, S. H., and R. P. Phipps. 1990. Macrophage-secreted prostaglandin E2 potentiates immune complex induced B cell unresponsiveness. Eur. J. Immunol., 20:403.

12. Bishop, G. A. 1991. Requirements of class II-mediated B cell differentiation for class II crosslinking and cyclic AMP. J. Immuno., 147:1107.

13 Gibert, K. M. and K. M. Hoffmann. 1985. cAMP is an essential signal in the induction of antibody production by B cells but inhibits helper function of T cells. J. Immunol., 135: 2084.

14. Sherris, D., W. Stohl, and L. Mayer. 1989. Characterization of lymphokines mediating B cell growth and differentiation from monoclonal anti-CD3 antibody-stimulated T cells. J. Immunol., 142:2343.

15. Mayer, L., D. Sherris, R. Huang, and M. Cidon. 1991. Cytokines regulating human B cell growth and differentiation. Clin. Immunol. and Immunopathol., 61:S28.

16. Mayer, L., and R. Shlien. 1987. Evidence for function of Ia molecules on gut epithelial cells in man. J. Exp. Med. 166:1471.

17. Mayer, L., S. P. Kwan, C. Thompson, H. S. Ko, N. Chiorazzi, T. Waldmann, and F. Rosen. 1986. Evidence for a defect in "Switch" T cells in patients with immunodeficiency and hyperimmuno-globulinemia M. N. Engl. J. Med. 314:409.

18. Ramussen, H., W. Lake, and J. E. Allen. 1975. The effect of catecholamines and prostaglandins upon human and rat erythrocytes. Biochim. Biophys. Acta 411:63.

19. Mayer, L. 1986. Terminal maturation of resting B cells by proliferation independent B cell differentiation factors. J. Exp. Med., 164:383.

20. Golding, B., S. R. Pillemer, P. Roussou, E. A. Peters, G. C. Tsokos, J. E. Ballow, and T. Hoffman. 1988. Inverse relationship between proliferation and differentiation in a human TNP-specific B cell

line. Cell cycle dependence of antibody secretion. J. Immunol., 141:2564.

21. Ishihara, K., S. Ono, Y. Takahama, F. Hirayama, H. Hirano, K. Itoh, K. Dobashi, S. Murakami, Y. Katoh, M. Yamaguchi, and T. Hamaoka. 1989. Different effect of prostaglandin E2 on B-cell activation by two distinct B-cell differentiation factors, B151-TRF1/IL-5 and B151-TRF2: selective inhibition of B151-TRF2-induced antibody response through increases in intracellular cyclic AMP levels. Immunology 68:154.

22 Roifman, C. M., K. Chin, A. Gazit, G. B. Mills, C. Gilon, and A. Levitiki. 1991. Tyrosine phosphorylation is an essential event in the stimulation of B lymphocytes by Staphylococcus aureus Cowan I. J. Immunol., 146:2965.

23. Gaciong, Z., J. M. Alexiewicz, M. Linker-Israeli, I. A. Shulman, T. O. Pitts, and S. G. Massry. 1991. Inhibition of immunoglobulin production by parathyroid hormone. Implications in chronic renal failure. Kidney Int. 40:96.

24. Ruskanen, O., W. B. Pittaard, K. Miller, G. Pierce, R. U. Sorensen, and S. H. Polman. 1980. Staphylococcus aureus Cowan I-induced immunoglobulin reproduction in human cord blood lymphocytes. J. Immunol. 125:820.

25. Melamed, I., G. P. downey, K. Aktories, and C. M. Roifman. 1991. Microfilament assembly is required for antigen-receptor mediated activation of human B lymphocytes. J. Immunol., 147:1139.

26. Dash, P. K., K. A. Karl, M. A. Colios, R. Prywes, and Kandel. 1991. cAMP response element-binding protein is activated by ca^{2+}/calmodulin as well as cAMP-dependent protein kinase. Proc. Natl. Acad. Sci. USA. 88:5061.

Figure legends

Figure 1. 446-BCDF stimulates a time dependent decrease in intracellular cAMP. 4 x 10^6 B cells were stimulated with SAC (0.001% v/v) alone, SAC plus 446-BCDF(10% v/v), or SAC plus 446-BCDF and aminophylline (10 ug/ml) in RPMI 1640. At varying time points (depicted in the graph) the cells were pelleted and immediately boiled in ddH_2O. Cell Lysates were lyophilized and resuspended in assay buffer. cAMP was measured by the RIA method as described in Materials and Methods. This finding was seen in over 30 experiments.

Figure 2. Effects of cAMP-modulating agents on Ig secretion induced by 446-BCDF. Isolated peripheral blood B cells (10^5/well) were cultured in triplicate microwell cultures with SAC (0.001% v/v) and 446-BCDF (10% v/v) in the presence or absence of varying concentrations of either dibutyryl cAMP (DcAMP), forskolin (FSK), or aminophylline (AP) for 7 days. Cell free supernatants were collected and tested for secreted Ig by ELISA. All three agents (dibutyryl cAMP, forskolin, aminophylline) inhibited B cell differentiation without affecting cell viability. Baseline secretion of Ig without BCDF was 0.47 ug/ml.

Figure 3. The cAMP increase induced by IBMX is not completely reversed by 446-BCDF. PB B cells were treated with either medium, 10% 446-BCDF, 0.5 mM IBMX, or 446-BCDF plus IBMX for 60 min. Intracellular cAMP was then isolated and assayed by the RIA

method as described in Materials and Methods. Results are representative of over twenty experiments.

Figure 4. IBMX inhibits Ig secretion induced by 446-BCDF. SAC activated PB B cells (10^5 cells/well) were incubated with the indicated concentrations of IBMX in the presence of 10% 446-BCDF. After 7 days, Ig secretion was assayed by ELISA. Medium indicates Ig secretion by SAC activated B cells alone. These experiments were performed 4 times with similar results.

Figure 5. Both CTX and PTX prevent a reduction of cAMP induced by 446-BCDF. PB B cells were pretreated with 0.5 mM IBMX for 30 min at 37^0C and then cultured with either 446-BCDF (10% v/v), CTX (1 ng/ml), or PTX (1 ng/ml) with or without 10% 446-BCDF for 60 min. cAMP was isolated and assayed as described in Figure 2.

Figure 6. Both pertussis toxin and cholera toxin inhibit Ig secretion induced by 446-BCDF. Isolated B cells (10^5/well) were cultured in triplicate microwell cultures in the presence of SAC (0.001%) and 446-BCDF (10% v/v) with or without varying concentrations of PTX or CTX. After 7 days, cell free supernatants were harvested and assayed for IgG secretion by ELISA. This graph represents the results of 5 experiments. Both PTX and CTX were markedly inhibitory for 446-BCDF induced Ig secretion. No difference in viability of B cells was noted between control (no toxin) and toxin treated cells. Baseline secretion of IgG without BCDF was 0.1 ug/ml.

Figure 7. mAb 929 specifically blocks the cAMP reduction induced by 446-BCDF. Either mAb 929 (20 ug/ml) or isotype control IgG2b (20 ug/ml) was added to 446-BCDF (10% v/v) and incubated at 37^0C for 30 min. PB B cells (10^5/well triplicate cultures) was then added to cultures. cAMP was isolated and assayed as described in Figure 1. Results are mean $\pm$ SD of triplicate cultures.

Figure 8. Both IL2 and IL6 induce a reduction in intracellular cAMP. PB B cells were stimulated with either recombinant IL2 (20 units/ml) (Fig. 8A) or rIL6 (100 units/ml) (Fig. 8B) at 37^0C for the indicated times. cAMP was isolated and assayed as described in Figure 1.

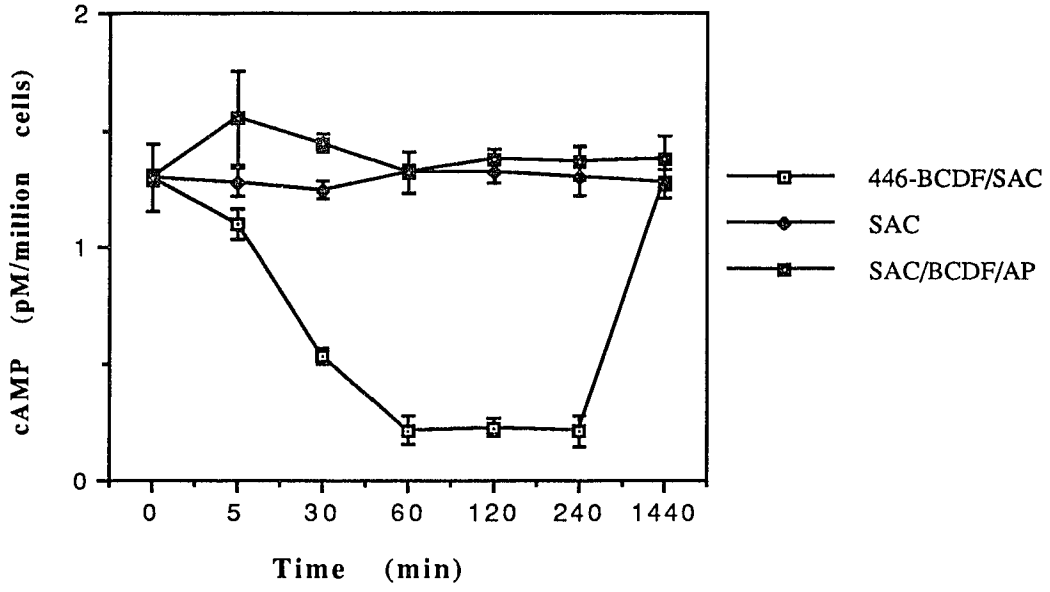
2
1
0
cAMP (pM/million cells)
0
5
30
60
120
240
1440
Time (min)
446-BCDF/SAC
SAC
SAC/BCDF/AP

Figure 1

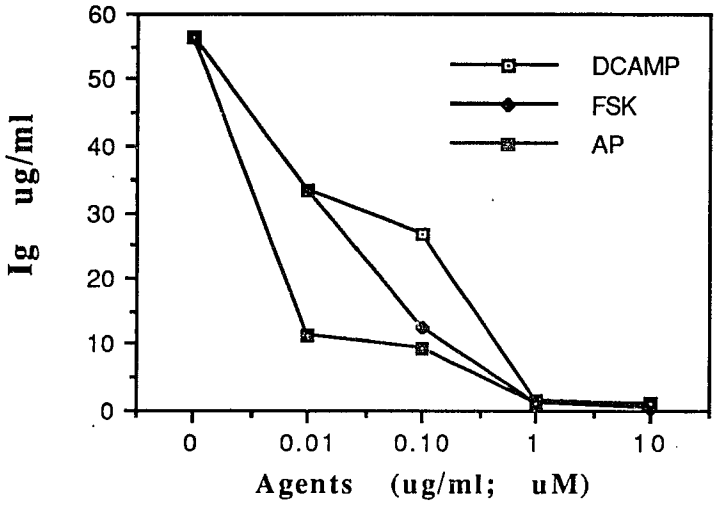
60
50
40
30
20
10
0
Ig ug/ml
DCAMP
FSK
AP
0
0.01
0.10
1
10
Agents (ug/ml; uM)

Figure 2

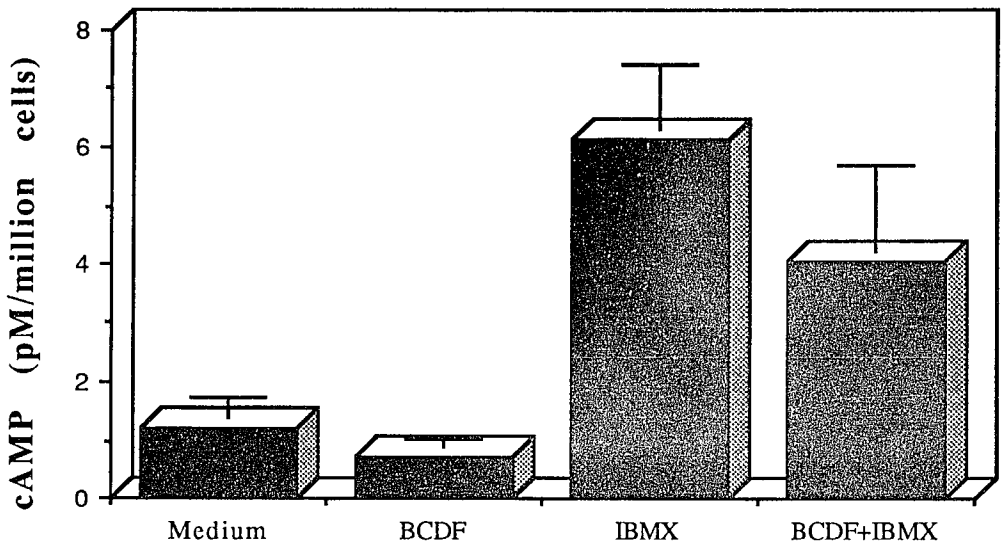

Figure 3

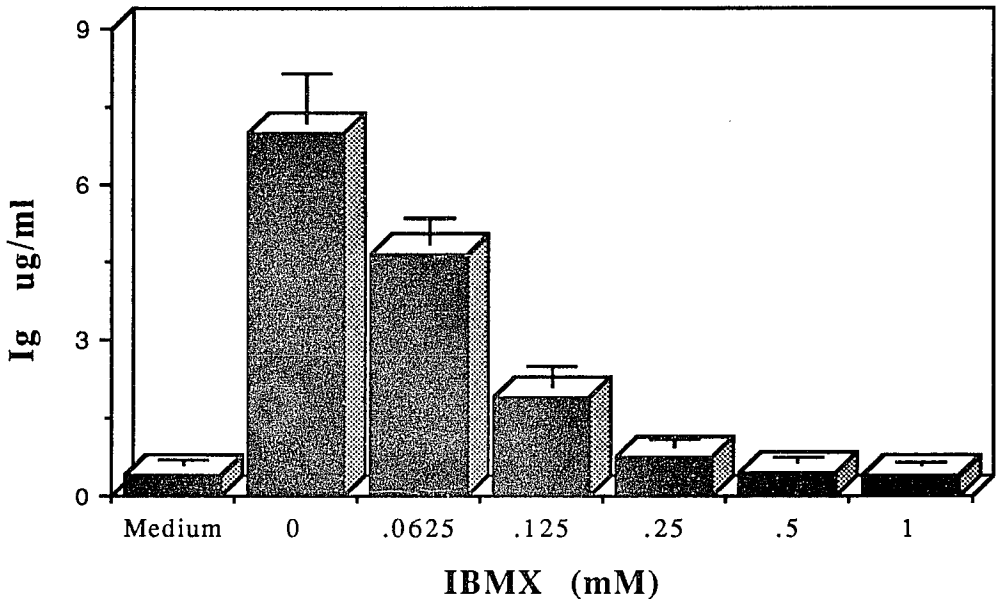

Figure 4

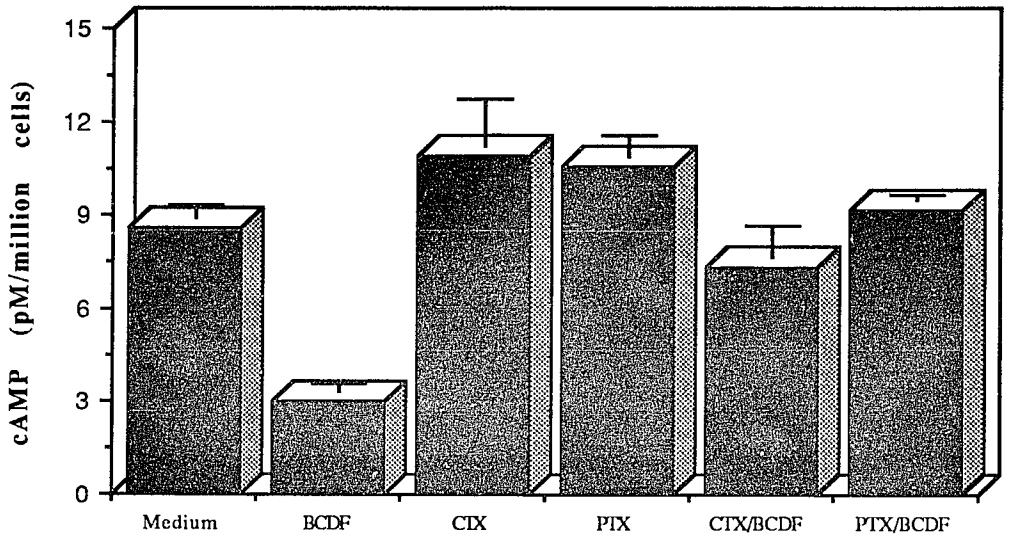
cAMP (pM/million cells)
15
12
9
6
3
0
Medium
BCDF
CTX
PTX
CTX/BCDF
PTX/BCDF

Figure 5

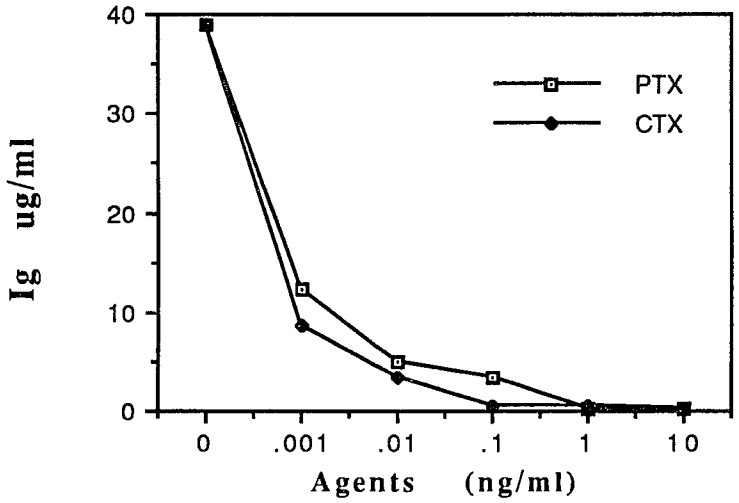

Figure 6

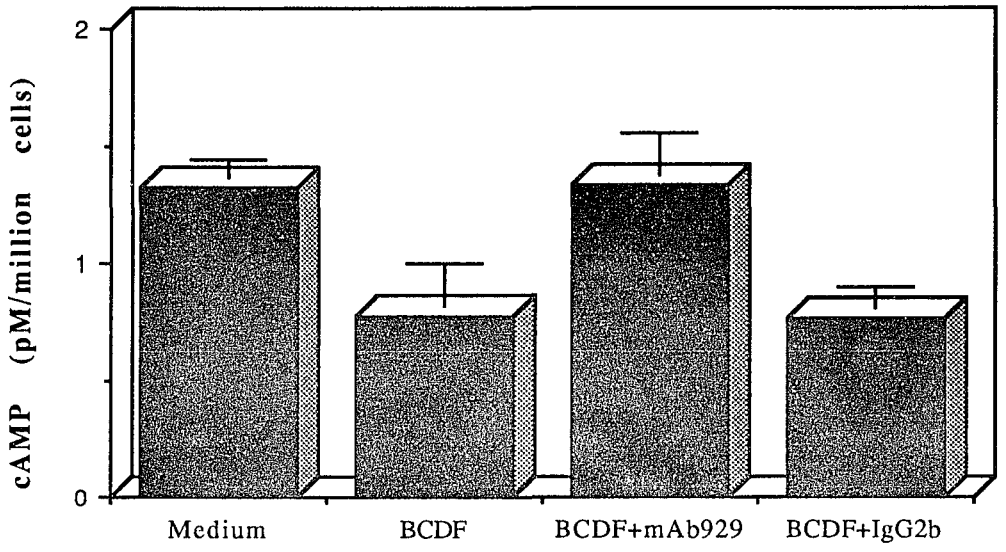
cAMP (pM/million cells)
2
1
0
Medium
BCDF
BCDF+mAb929
BCDF+IgG2b

Figure 7

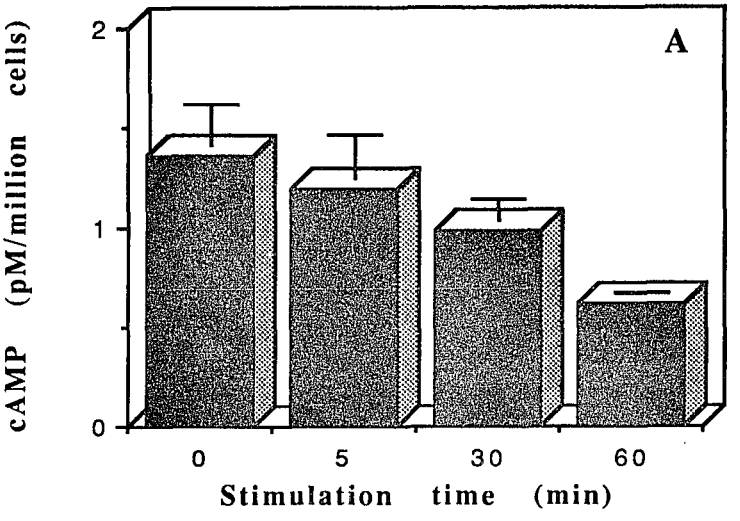

Figure 8a

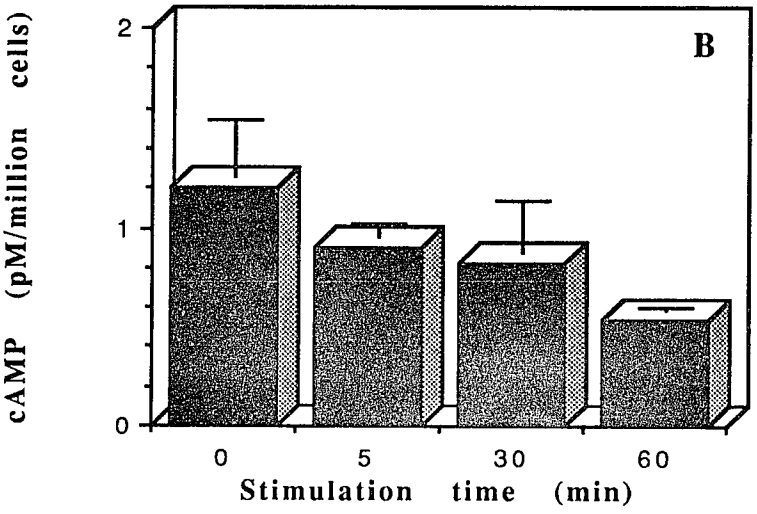

Figure 8b

COMPLEMENTARY RESULTS I

1. Effects of angiotensin II on intracellular cAMP and Ig secretion by B cells. In the adjoining paper we have shown that PTX inhibits 446-BCDF activity and 446-BCDF may be acting through a Gi protein linked receptor. The angiotensin II (AT II) receptor has been shown to couple to Gi proteins. Binding of AT II to its receptor inhibits adenylate cyclase activity in a variety of cell types, leading to a decrease in intracellular cAMP levels (Pobiner et al., 1991; Sims et al., 1992; Klett et al., 1993; Edwards et al., 1993). It was therefore interesting to use this potential cAMP-reducing agent to determine whether Ig secretion and intracellular cAMP levels have a reciprocal relationship. Since the effect of AT II on the intracellular cAMP levels in human B cells has not been elucidated, we first used this agent to act on IBMX pretreated PB B cells and measure the effect on intracellular cAMP levels. Results are shown in Figure 1a. AT II induced about a 20% to 30% reduction in intracellular cAMP at the concentrations used in our studies. Interestingly, this reduction appeared to correlate with the ability of AT II to enhance the Ig secretion (Figure 1b). AT II stimulated Ig secretion by SAC activated B cells with the maximal effect at 10 nM, a concentration known to be in the physiologic range, inducing a 12 fold increase (from 0.14 to 1.64 ug/ml) in Ig secretion. The optimal concentration of 446-BCDF (10% v/v) induced a 40 fold increase (from 0.14 to 5.66 ug/ml) in Ig secretion by SAC activated B cells, which was significantly higher than that induced by any concentration of AT II used in our studies. The effect of AT II on Ig secretion by SAC activated B cells was

further enhanced by 10% 446-BCDF. Maximal Ig secretion was observed in the presence of 10% 446-BCDF and 10 nM AT II. These data further support our concept that the reduction in intracellular cAMP may be an important signal to trigger human B cell differentiation, and suggest that in addition to inducing a decrease in intracellular cAMP 446-BCDF may also deliver other signals to induce human B cell Ig secretion.

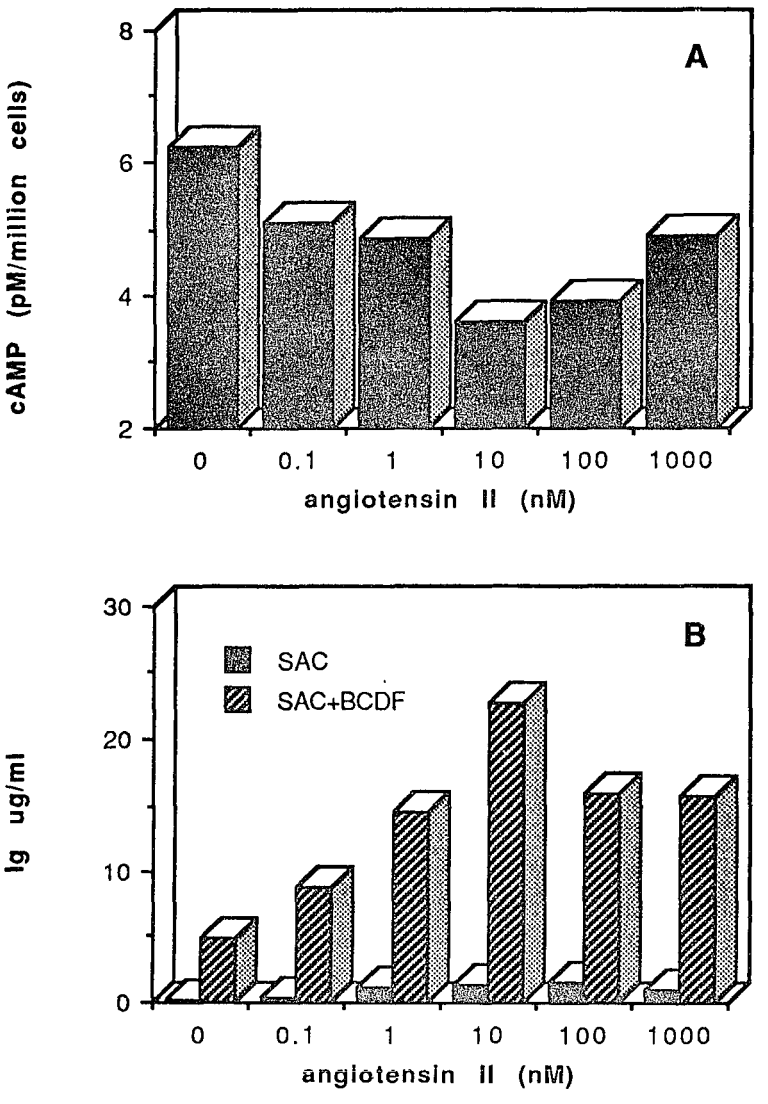

Figure 1. The effect of angiotensin II on intracellular cAMP and Ig secretion. (A) PB B cells were pretreated with 0.5 mM IBMX for 30 min at RT and then cultured with the indicated concentrations of angiotensin II for 60 min. cAMP was isolated and assayed by the EIA method. (B) SAC activated PB B cells (10^5 cells/well) were incubated at 37^0C with the indicated concentrations of angiotensin II in the presence or absence of 10% 446-BCDF. After 7 days, Ig secretion was measured by ELISA.

Running Title

BCDF induced calcium flux

B cell differentiation factor induced B cell maturation. II. Stimulation of intracellular calcium release.

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Footnotes

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Abbreviations used

BCDF - B cell differentiation factor
BCGF - B cell growth factor
DAG - diacylglycerol
HMW - high molecular weight
IP3 - inositol triphosphate
LMW - low molecular weight
MBP - myelin basic protein
SAC - Staphylococcus aureus Cowan I
PKC - protein kinase C
PKA - protein kinase A
PIPLC - phosphoinositol phospholipase C
PTX- pertussis toxin

Abstract

We have recently identified a non-IL-6 human B cell differentiation factor, 446-BCDF, derived from anti-CD3 stimulated peripheral blood (PB) T cells. This novel cytokine, which may act through a pertussis toxin sensitive Gi linked receptor, induces a 5-100 fold increase in immunoglobulin (Ig) secretion by SAC (0.001% v/v) activated PB B cells. Co-culture of B cells with 446-BCDF induces a decrease in intracellular cAMP which is necessary but not sufficient to drive terminal B cell differentiation. A second signal appears to be required. We therefore measured Ca^{++} flux in indo-1 AM loaded PB B cells. Stimulation with 446-BCDF resulted in an immediate rise in intracellular Ca^{++} comparable to that seen with the anti-IgM mAb HB57. Ca^{++} appeared to be mobilized from internal stores as pretreatment with BAPTA but not EGTA inhibited the response. Ca^{++} mobilization was critical for the induction of differentiation as BAPTA pretreatment of B cells completely inhibited Ig secretion without affecting viability. In contrast, neither SAC (0.001%), rIL-6 (50 to 500 U/ml), IL-2 (10 to 100 U/ml), γ-IFN (100-500 u/ml), nor IL-4 (10-1000 U/ml) could mobilize Ca^{++}. To determine whether the Ca^{++} flux was generated in the course of inositol phosphate turnover, we measured IP3 turnover and the translocation of PKC from cytosol to membrane. An increase in IP3 comparable to that seen with a monoclonal anti-human IgM antibody was noted and was specifically inhibited by the 446-BCDF specific mAb 929. Interestingly, no membrane PKC was demonstrable in either SAC or BCDF stimulated B cells although PMA (50 ng/ml) could directly activate PKC. To confirm these findings functionally, B cells were stimulated with SAC

and 446-BCDF in the presence of two known PKC inhibitors, staurosporin and calphostin. No inhibition of Ig secretion was detected at any concentration tested (0.39-100 nM staurosporin and 0.0625-1 uM calphostin C). These data suggest that induction of B cell differentiation is a Ca^{++} dependent event whose regulation may be under the control of novel signal pathways.

Introduction

Activation of B cells in vivo involves a series of cognate and noncognate interactions (1-3). Several recent studies have demonstrated that the crosslinking of surface immunoglobulin results in both the activation of PIPLC within the membrane as well as the participation of at least 2 CD3 like molecules MB-1 and B29 (4-6). Crosslinking of sIg is a prerequisite for clonal proliferation and if crosslinking is extensive, no additional signals are required for human B cell growth (7). In contrast, terminal differentiation of human B cells requires additional signals. These have been less well defined but include IL2 (8), IL4 (9), IL6 (10-11), IL10 (12), IL13 (13-14) and a potent B cell differentiation factor described in our laboratory, 446-BCDF (15). Clearly the signal pathways regulating growth should be distinct from those regulating differentiation. This scenario does not preclude the fact that clonal proliferation may render the B cell responsive to differentiation stimuli, inducing cell surface receptors for differentiation factors, altering intracellular substrates, or altering the stage of maturation. Neither IL6 nor 446-BCDF induce B cell proliferation suggesting that one signal alone may not be sufficient.

Although extensively studied most lymphokine-induced signal transduction events in B cells are still not clear. IL2 stimulates a murine B cell line, BCL1, to activate a nonclassical glycosyl phosphatidylinositol system (16), however, the importance of possible second messengers generated in this system is unknown. Tyrosine kinases and serine/threonine kinases were reported to be involved in IgM secretion by a EBV-transformed cell line SKW 6.4

(17). However, since these data were obtained in an EBV-transformed cell line and may not reflect normal physiology. For 446-BCDF we have previously shown that co-culture of primary B cells with this cytokine results in a rapid decrease in intracellular cAMP (R. Huang et al., submitted). Preliminary data have suggested that this phenomenon may be the result of 446-BCDF binding to a Gi linked receptor since Ig secretion stimulated by 446-BCDF can be inhibited by pre-culture of B cells with the Gi protein inhibitor pertussis toxin. We have been able to establish that reduction in cAMP alone is necessary but not sufficient to trigger terminal differentiation. Therefore, other signals may be required. Since G proteins are known to regulate Ca^{++} channels (18-19), we measured Ca^{++} flux in primary B cells after co-culture with 446-BCDF. In this paper we document that 446-BCDF, but not IL6, IL2, IL4 or γ-IFN, stimulates a rapid rise in intracellular Ca^{++} emanating from intracellular stores. This Ca^{++} flux is a critical event in 446-BCDF mediated terminal differentiation since inhibition of this flux inhibits Ig secretion.

Methods

Isolation of PBMNC

PBMNC were isolated from leukocyte concentrate packs provided by the Mount Sinai Blood Bank as previously described (15) using Ficoll-Hypaque (Pharmacia, Piscataway, NJ) density gradient centrifugation, T cells were isolated by rosetting with neuraminidase treated sheep RBC and the resultant non T cell population in the interface was enriched for B cells by removal of

monocytes by plastic adherence (20). The resultant B cell preparation was >90% $CD20^+$, <1% $CD3^+$, <2% $CD14^+$.

Generation of 446-BCDF and other cytokines

446-BCDF was isolated from anti-CD3 (mAb 446-IgG_1) stimulated purified monocyte depleted PB T cells by either co-culture of T cells on anti-CD3 coated tissue culture dishes, soluble anti-CD3 (10 ug/ml-non monocyte depleted T cells), or fixed hybridoma (446) cells (1:10 ratio-hybrid to T cell) as previously described (15). Contaminating IL6 is removed by passage over an anti-IL6 affinity column (21) and the effluent from the column fails to stimulate the IL6 dependent B9 cell line (22) while retaining its differentiation factor activity. We have previously shown that mAb 446 stimulated crude TCS contains no IL2 (as assessed by CTLL assay (23)) but does possess IL4 and γ-IFN. These latter two cytokines can be removed by passage of IL6 depleted 446-BCDF over a DEAE column. 446-BCDF eluted at 50 mM NaCl is devoid of IL4 and γ-IFN but still possesses potent BCDF activity. This semi-purified BCDF was then used in our assays at a concentration of 10% vol/vol shown to trigger maximal Ig secretion in SAC activated PB B cells.

rIL2, purchased from Boehringer Manheim, was used at a concentration range of 10-100 u/ml. rIL4, purchased from Genzyme, was utilized at a concentration range of 10-1000 u/ml. rIL6, a kind gift of Dr. Ed Siden, was purified from a Baculovirus system and used at a final concentration of 50-500 u/ml. γ-IFN was the kind gift of Genentech and was used at a final concentration of 100-500 u/ml.

B cell differentiation assay

Isolated B cells were cultured in RPMI 1640 (Gibco, Grand Island, NY), 10% fetal calf serum (Flow Labs, Maclean, VA), 1% penicillin/streptomycin (Gibco) and 2 mM glutamine, henceforth termed CM, in the presence or absence of fixed Staphylococcus aureus Cowan I strain organisms (SAC-0.001% v/v - Calbiochem, LaJolla, Ca), 446-BCDF (10% v/v) in triplicate 100 ul microwell cultures (10^5 cells/well) (Falcon, Oxnard, CA). Cultures were maintained in a humidified 5% CO_2 95% air 37°C incubator for 7 days. After this culture period, cell free supernatants were harvested and total secreted Ig was measured by ELISA (15) as previously described.

In some studies pertussis toxin (Sigma, St. Louis, MO) or BAPTA (Molecular Probes, Junction City, MO), an intracellular Ca^{++} chelator (24), was added to B cells two hours before culture, at the onset of culture, or at varying time periods after the onset of culture to determine the effect of these agents on 446-BCDF induced B cell differentiation. Pretreatment of B cells with BAPTA or PT for 2h was followed by removal of these agents by washing cells three times with PBS prior to co-culture with SAC and 446-BCDF.

In other experiments staurosporin (0.39-100 nM) or calphostin C (0.0625-1 uM), known specific PKC inhibitors, were added at the onset of culture. Neither agent at the concentrations used in our studies affected B cell viability or cell number.

Ca^{++} flux studies

Indo-1, a fluorescent indicator with spectral properties that change with the binding of free Ca^{++}, was used to measure changes in intracellular calcium concentrations. B cells were incubated with 5 uM indo-1 acetomethoxy ester (Molecular Probes, Junction City, MO). After loading, the cells were washed once with PBS and maintained at 25°C in the dark. Prior to analysis, the cells were warmed to 37°C for 5 min in PBS plus Ca^{++} and Mg^{++} and adjusted to a concentration of 10^7 cells/ml. Indo-1AM fluorescence analysis was performed on an SLM 8000C Spectrofluorometer (SLM, Aminco, Urbana, IL). A xenon short arc lamp in conjunction with a single gating monochromator provided excitation at 355 nm, while two photomultiplier tubes measured emission at 405 and 490 nm simultaneously. Ratio values of 405 nm emission intensity over 490 nm emission intensity, and absolute values of 490 nm emission intensity were acquired in 1 sec increments over a period of 360 sec for each test cuvette. A corresponding stimulus was injected into each cuvette at 60 sec with interruption of acquisition. A magnetic stirbar provided constant stirring throughout each experiment. At 260 sec a 10% Triton-X 100 solution was added to lyse the cell membranes and permit calcium saturation of all indo-1, providing a maximum calcium concentration value. Subsequently, at 300 sec, EDTA (1 mM) (Mallinckrodt Chemical Works, New York, NY) was added to chelate all calcium, thereby providing a minimum calcium concentration value. Indo-1 fluorescence emission ratios were converted to Ca^{++} concentration as described by Grynkiewicz (25).

Inositol Phosphate Assay

B cells were prepared as described above. Cells were washed with inositol free MEM medium and resuspended in MEM at the concentration of $2x10^6$/ml. 3-5 uCi/ml of [^{3}H]-myo-inositol (Dupont) were added to the cell suspension. Cells were incubated at 37°C in the presence of 5% CO_2. After 18 hours loading of [^{3}H]-myo-inositol, cells were washed two times with MEM and resuspended at final concentration desired (usually 10^7 cells/ml). 10 ul of cell suspension was dried onto filter paper and % loading determined by a liquid scintillation counter. Lithium chloride was then added to the cell suspension at a final concentration of 20 mM. After a 15 minute incubation at 37°C, $2\text{-}4x10^6$ cells/sample were stimulated with specific agents for 1 min at 37°C. The reaction was stopped by centrifugation and removal of supernatants. 1 ml of ice-cold 10 mM formic acid was added to each sample to lyse cells by thorough pipetting. Samples were kept on ice for at least one hour. Anion exchange columns (Analytic Grade Anion Exchange Resin, BioRad) were washed with 2,5 ml of 3M ammonium formate containing 100 mM formic acid, and equilibrated with 10 ml of 10 mM formic acid containing 10 mM inositol. Samples were then applied onto the washed columns. 10 mM formic acid containing 10 mM inositol was used to wash the column. Eluates were collected and counted for CPM in NEN 963 scintillation fluid. The columns were washed until CPM counts were less than 500 per sample. To remove glycophosphate inositol, each column was washed with 10 ml of 60 mM sodium formate with 5 mM Borax. Washes were counted in NEN 963 scintillation fluid until CPM counts were less than 500 cpm per

sample. Columns were then washed with 5 ml of 1 M ammonium formate with 0.1 M formic acid. The eluate was collected and 10 ml of NEN 963 scintillation fluid was added to each sample. The CPM/sample was then determined on a liquid scintillation counter. In order to determine the specificity of the cytokine effect, mAb 929 was added to 446-BCDF prior to its addition to B cells. mAb 929 has been shown to specifically inhibit 446-BCDF activity and not IL-2, IL-6 or pokeweed mitogen stimulated B cell differentiation (Kuang, Cidon, and Mayer submitted). Furthermore, cytokine purified from a mAb 929 affinity column possesses BCDF activity.

Assay for PKC

The PKC Assay System (GIBCO) was used to measure PKC activity according to the manufacturer's protocol. 1 ml of a B cell suspension ($10X10^6$/ml) was aliquoted into each Eppendorf tube and stimulated with one of the following agents (0.001% SAC, 10% v/v 446-BCDF, 50 ng/ml PMA, anti-IgM mAb HB57, or medium) for 10 min at 37°C. Supernatants were removed by microfuging for 1 min. Cell pellets were washed once with ice cold PBS and resuspended with 0.5 ml of extraction buffer (20 mM Tris, pH 7.5, 0.5 mM EDTA, 0.5 mM EGTA, and 25 ug/ml each of aprotinin and leupeptin). Cell suspensions were sonicated for 15 seconds on ice. Membrane and cytosol were then separated by microfuging for 2 hours at 4°C. The cytosol fraction was transferred to Eppendorf tubes and kept on ice. The membrane pellet was resuspended with 0.5 ml extraction buffer with 0.5 % Triton X 100 and incubated for 30 min at 22°C. Cell debris was then removed by microfuging for 2 min at 4°C. Supernatants

were transferred to Eppendorf tubes and kept on ice. Cytosolic and membrane fractions were then loaded onto labeled DEAE cellulose columns (Whatman DE_52) (0.6 ml bed volume) pre-equilibrated with buffer B (20 mM Tris, pH 7.5, 0.5 mM EDTA, 0.5 mM EGTA). Columns were then washed with 3 bed volumes of buffer C (20 mM Tris, pH 7.5, 0.5 mM EDTA, 0.5 mM EGTA, 10 mM β-mercaptoethanol, 0.2M NaCl). Partially purified PKCs from both cytosolic and membrane fractions were then assayed. Two conditions were set up to measure PKC activity present in either cytosol or membrane fractions. Each condition was set up in triplicate. 15 ul of extract was added to each tube followed by 10 ul of Buffer C, 10 ug of inhibitor solution (100 uM PKC pseudosubstrate peptide, PKC[19-36], 20 mM Tris, pH 7.5) was then added to control tube. 5 ul of lipids (100 uM PMA, 2.8 mg/ml phosphatidylserine, Triton X-100 mixed micelles) was added to assay tube. The total volume of each tube was adjusted to 40 ul with ddH_2O. Tubes were then incubated at 22^0C for 20 min to allow the inhibitor to bind. After incubation, 10 ul of ^{32}P/substrate (250 uM PKC specific substrate peptide Ac-MBP[4-14], 100 uM ATP, 20 uCi/ml [γ-^{32}P]ATP, 5 mM $CaCl_2$, 100 mM $MgCl_2$, 20 mM Tris pH 7.5) was added to each tube and tubes were incubated at 30^0C for 5 min. After incubation, samples were spotted onto phosphocellulose discs and washed 3 times with 1% phosphoric acid and 2 times ddH_2O. After washing, peptide-incorporated ^{32}P was counted by a scintillation counter. Calculations of specific PKC activity in cytosol or membrane preparations were performed using the formulas provided by the manufacturer.

Results

446-BCDF stimulates a rapid flux in intracellular Ca^{++}

Ca^{++} flux is an important contributory event to B cell growth stimulated by engaging the antigen receptor. Its role in differentiation events has not been addressed. As seen in Figure 1, 446-BCDF induced a brisk Ca^{++} flux that was comparable to that seen by crosslinking sIgM with mAb HB57 as previously described (Fig. 1c and 1b respectively). The Ca^{++} flux appeared to derive from intracellular pools since BAPTA but not EDTA pretreatment of B cells inhibited Ca^{++} flux (Figure 1e and 1f). SAC alone, an important co-stimulatory factor for 446-BCDF induced differentiation, used at various concentrations (0.001-0.01% v/v) did not stimulate Ca^{++} flux in these cells (Figure 1d). Similarly, a mock T cell culture supernatant (stimulated with an irrelevant IgG1 mAb) failed to induce a significant Ca^{++} flux (Figure 1a).

In contrast to our findings with 446-BCDF, no other cytokine could stimulate resting peripheral blood B cells to flux Ca^{++} (Figure 2). Neither IL2 (10-100 u/ml), IL4 (10-1000 u/ml), IL6 (5-500 u/ml), nor γ-IFN (50-500 u/ml) were capable of inducing Ca^{++} mobilization from either intracellular or extracellular stores.

Ca^{++} flux is necessary for 446-BCDF induced B cell differentiation

In order to determine whether Ca^{++} flux was a critical signal in cytokine mediated terminal B cell differentiation, we pretreated B cells with BAPTA (1 uM) for 2h prior to stimulation with 446-BCDF. Pretreatment with BAPTA was not only capable of inhibiting Ca^{++}

flux (Figure 1d), but also inhibited Ig secretion (Figure 3a). Possible cytotoxicity was ruled out both by a trypan blue exclusion assay and the activity of these B cells to proliferate when stimulated by low molecular weight BCGF (data not shown). These data suggest that the early Ca^{++} flux induced by 446-BCDF is critical for Ig secretion. To corroborate these findings, BAPTA was added to 446-BCDF stimulated B cell cultures at varying time points after the onset of culture (figure 3b). BAPTA added after 24 and 48 hours of culture was not inhibitory for Ig secretion, again suggesting that the effects seen were not related to nonspecific toxicity of this agent and that the requirement for Ca^{++} flux was an early event in culture.

Pertussis toxin inhibits 446-BCDF mediated Ca^{++} flux

It has been well documented that some Ca^{++} channels are regulated by G proteins and internal Ca^{++} release from G protein regulated Ca^{++} channels can be inhibited by pertussis toxin (18-19). To determine whether PTX can affect internal Ca^{++} release induced by 446-BCDF, Indo-1AM loaded B cells were pretreated with pertussis toxin for 2h, a time period previously defined as critical for ADP ribosylation and inhibition of Gi proteins. After PTX was removed by washing, cells were stimulated with 446-BCDF and Ca^{++} flux was measured. Pretreatment with PTX completely inhibited Ca^{++} flux (Figure 4a and b) as well as Ig secretion (Figure 5), suggesting that the intracellular Ca^{++} flux may be regulated by a PTX sensitive G protein.

446-BCDF stimulates inositol phosphate turnover but does not mediate PKC translocation or require PKC translocation for activity

In conventional stimulation systems, Ca^{++} flux can be triggered by the activation of G protein regulated PiPLC resulting in the generation of IP_3 and DAG. IP_3 mediates Ca^{++} flux while DAG stimulates PKC translocation to the membrane for activation. Addition of 446-BCDF to PB B cells resulted in a 60% to 3 fold increase in IP3 in four separate experiments, comparable to the response seen with anti-human IgM mAb HB57 (positive control) (Figure 6). This increase was specifically inhibited by the BCDF specific mAb 929, suggesting that IP3 was induced by 446-BCDF. However, when we used a PKC specific assay system to measure PKC activity after stimulation with 446-BCDF the majority of PKC remained in the cytoplasm (figure 7). Following stimulation with PMA (50 ng/ml), a direct activator of PKC, the enzyme translocates to the membrane. However, neither SAC nor BCDF could cause PKC translocation. In some experiments, however, there was a minor increase in membrane PKC following stimulation with SAC (0.001% v/v) alone. The sensitivity of this assay was brought into question as anti-human IgM mAb HB57 also failed to induce PKC translocation (Fig 7). To ensure then that PKC truly had no role in 446-BCDF induced Ig secretion, we utilized the PKC inhibitors staurosporin and calphostin. Both of these agents failed to inhibit SAC/446-BCDF stimulated Ig secretion (Figures 8a and b). These data document that PKC is not a critical factor in 446-BCDF induced differentiation and provide further evidence that SAC/446-BCDF signaling is distinct from other differentiation stimuli.

Discussion

Signal transduction pathways in differentiation events have been poorly defined. One of the major obstacles to such studies is that most cytokines acting on B cell differentiation are only costimulatory factors which may function through a complex network of signals. Some cytokines, such as IL2, may function through a nonclassical signal pathway (16). We have previously described a potent human BCDF, 446 BCDF, distinct from IL6 which is capable of inducing high rate Ig secretion in primary B cells. Interaction of this factor with its receptor results in a decrease in intracellular cAMP within 5' of culture. This finding may relate to the binding of 446-BCDF to a Gi linked receptor which would inhibit adenyl cyclase reducing steady state cAMP. Evidence for this was provided by the fact that Ig secretion induced by 446-BCDF could be inhibited by the Gi protein inhibitor pertussis toxin.

In this study we document that in addition to its effects on cAMP, 446-BCDF mediates a rapid Ca^{++} flux. This flux appears to be a critical early event in that it occurs immediately upon stimulation of B cells with 446-BCDF and inhibition of Ca^{++} flux by pretreatment of B cells with BAPTA abrogates Ig secretion stimulated by 446-BCDF.

In contrast to Ca^{++} mobilization described for proliferative signals in B cells, 446-BCDF does not appear to activate this pathway conventionally. Despite the presence of IP3, no translocation of PKC is detected and inhibition of PKC fails to inhibit Ig secretion induced by this cytokine. Lastly, Ca^{++} flux is inhibited by pretreatment with

pertussis toxin. Therefore, 446-BCDF appears to mobilize Ca^{++} via a G protein linked Ca^{++} channel independent of processes which have been described for B cell proliferation. It is interesting to speculate on the sequelae of the Ca^{++} flux induced by 446-BCDF. We have previously described a decrease in intracellular cAMP after stimulation with 446-BCDF. The question remains as to whether mobilized Ca^{++} may be an important co-factor in this process either activating a Ca^{++} dependent protein kinase or a Ca^{++} dependent phosphodiesterase which could account for the prolonged decrease in cAMP seen. Preliminary studies suggest that these two events are independent as inhibition of Ca^{++} flux by BAPTA fails to alter the reduction in cAMP mediated by 446-BCDF. Clearly additional signals are needed. Terminal B cell differentiation only occurs after co-stimulation with SAC in vitro or after in vivo activation of B cells (tonsil B cells are preactivated in vivo and generally do not require SAC to differentiate in the presence of 446-BCDF). SAC itself has no effect on cAMP levels and is able to induce a Ca^{++} flux only at concentrations higher than those used in this study. Previous reports have suggested that SAC stimulates tyrosine kinase activity (26) although we have only been able to demonstrate this at the higher concentrations as well.

Despite the requirement for SAC in vitro or pre-activation in vivo for Ig secretion it is clear from these experiments that SAC is not required for Ca^{++} flux since 446-BCDF alone is capable of stimulating a brisk response. These data also provide strong evidence for the fact that resting B cells constitutively express

receptors for 446-BCDF. Formal proof of this will require the purification of the cytokine and receptor binding assays.

References:

1. Sanders, V. M., J. M. Snyder, J. W. Uhr, and E. S. Vitetta. 1986. Characterization of the physical interaction between antigen-specific B and T cells. J. Immunol., 137: 2395.

2. Schimpl, A., and E. Wecker. 1972. Replacement of T-cell function by a T-cell product. Nature, 237: 5.

3. Kishimoto, T. 1985. Factors affecting B cell growth and differentiation. Annu. Rev. Immunol., 3: 133.

4. Cambier, J. C. and J. T. Ranson. 1987. Molecular mechanisms of transmembrane signaling in B lymphocytes. Annu. Rev. Immunol., 5: 175.

5. Reth, M., J. Hombach, J. Wienands, K. S. Campbell, N. Chien, L. B. Justement, and J. C. Cambier. 1991. The B-cell antigen receptor complex. Immunol. Today, 12: 196.

6. Hombach, J., T. Tsubata, L. Leclercq, H. Stappert, and M. Reth. 1990. Molecular components of the B-cell antigen receptor complex of the IgM class. Nature, 343: 760.

7. Rudith, S. M., K. H. Roux, R. J. Winchester, and P. K. Mongini. 1988. Anti-IgM-mediated B cell signaling. Molecular analysis of ligand binding requisites for human B cell clonal expansion and tolerate. J. Exp. Med., 168:247.

8. Nakaganda, N., T. Nakagawa, D. J. Volkman, J. L. Ambrus, and A. S. Fanci. 1987. The role of interleukin 2 in inducing Ig production in a pokeweed mitogen-stimulated mononuclear cell system. J. Immunol., 138: 795.

9. Defrance, T., B. Vanbervliet, J. Pene and J. Banchereau. 1988. Human recombinant IL4 induces activated B lymphocytes to produce IgG and IgM. J. Immunol., 141:2000.

10. Gauldie, J., C. Richards, D. Harnish, P. Lansdorp, and H. Baumann. 1987. Interferon beta 2/ B-cell stimulatory factor type 2 shares identity with monocyte-derived hepatocyte-stimulating factor and regulates the major acute phase protein response in liver cells. Proc. Natl. Acad. Sci. USA, 84: 7251.

11. Muraguchi, A., T. Hirano, B. Tang, T. Matsuda, Y. Horii, K. Nakajima, and T. Kishimoto. 1988. The essential role of B cell stimulatory factor 2 (BSF-2/ IL-6) for the terminal differentiation of B cells. J. Exp. Med., 167: 332.

12. Rousset, F, E., Garcia, T. Defrance, C. Peronne, N. Vezzio, D. Hsu, R. Kastelein, K. W. Moore, and J. Benchereau. 1992. Interleukine 10 is a potent growth and differentiation factor for activated human B lymphocytes. Proc. Natl. Acad. Sci. USA, 89:1890-1893.

13. Mckenzie, A. N. J., J. A. Culpepper, R. W. Malefyt, F. Briere, J. Punnonen, G. Aversa, A. Sota, W. Dang, B. G. Cocks, S. Menon, J. E. de Vries, J. Banchereau, and G. Zurawski. 1993 Interleukin 13, a T cell-derived cytokine that regulates human monocyte and B cell function. Proc. Natl. Acad. Sci. USA, 90:3735.

14. Cocks, B. G., R. W. Malefyt, J. P. Galizzi, J. E. de Vries, and G. Aversa. 1993. IL13 induces proliferation and differentiation of human B cells activated by the CD40 ligand. Int. Immunol., 6:657.

15. Sherris, D., W. Stohl, and L. Mayer. 1989. Characterization of lymphokines mediating B cell growth and differentiation from monoclonal anti-CD3 antibody-mediated T cells. J. Immunol., 142: 2343.

16. Eardley, D. D. and M. E. Koshland. 1991. Glycosyl phosphatidylinositol: a candidate system for interleukin 2 signal transduction. Science, 251:78.

17. Goldstein, H. and A. Kim. 1993. Immunoglobulin secretion and phosphorylation of common proteins are induced by IL2, IL4, and IL6 in the factor responsive human B cell line, SKW6.4. J. Immunol., 151:6701.

18. Rosales, C. and E. J. Brown. 1992. Signal transduction by neutrophil immunoglobulin G Fc receptors. Dissociation of intraplasmic calcium concentration rise from inositol. J. Biol. Chem., 267:5265.

19. Dahinden, C. A. 1993. RANTES and related chemokines activate human basophil granulocytes through different G protein-coupled receptors. Eur. J. Immuno., 23:761.

20. Mayer, L., S. M. Fu, and H. G. Kunkel. 1982. Human T cell hybridomas secreting factors for IgA-specific help, polyclonal B cell activation and B cell proliferation. J. Exp. Med., 156: 1860.

21. Mayer, L., D. Sherris, R. Huang, and M. Cidon. 1991. Cytokines regulating human B cell growth and differentiation. Clin. Immunol. and Immunopathol. 61:S28.

22. Helle, M., L. Boeije, and L. A. Aarden. 1988. Functional discrimination between interleukin 6 and interleukin 1 . Eur. J. Immunol., 18: 1535.

23. Gillis, S., M. M. Feron, W. Ou, and K. A. Smith. 1978. T cell growth factor: parameters of production and a quantitative microassay for activity. J. Immunol., 120: 2027.

24. MacDougall, S. L., S. Grinstein, and E. W. Gelfand. 1988. Detection of ligand-activated conductive Ca^{2+} channels in human B lymphocytes. Cell, 54: 229.

25. Grynkiewicz, G., M. Poenie, and R. Y. Tsien. 1985. A new generation of Ca^{2+} indicators with improved fluorescence properties. J. Biol. Chem., 260: 3440.

26. Roifman, C. M., K. Chin, A. Gazit, G. B. Mills, C. Gilon and A. Levitzki. 1991. Tyrosine phosphorylation is an essential event in the stimulation of B lymphocytes by Staphylococcus aureus Cowan 1. J. Immunol., 146:2965.

Figure legends

Figure 1. 446-BCDF induces internal calcium release. Peripheral blood B cells were loaded with indo-1AM. Intracellular calcium was determined by the conversion of the indo-1AM signal to $[Ca^{++}]i$ (nM). After recording the baseline $[Ca^{++}]i$, Specific agents were added to $1x10^6$ cells where indicated (arrow). a) Mock TCS (IgG1 isotype stimulated T cell supernatant), b) anti-IgM mAb HB57 (10 ug/ml), c) 446-BCDF (10% v/v), or d) SAC (0.001% v/v) was added to stimulate B cells in phenol red free RPMI 1640 with $[Ca^{++}]i$. B cells were incubated with EGTA (1mM) for 5 minutes (e) or BAPTA (50 uM) (f) for 2 hours at 37°C after which 446-BCDF (10% v/v) was added. This is representative of five experiments.

Figure 2. Ca^{++} flux is specific for 446-BCDF. Indo-1AM loaded B cells were stimulated with a) 446-BCDF (10% v/v), b) IL-2 (50 u/ml depicted here), c) IL-4 (10 u/ml depicted here), d) IL-6 (250 u/ml depicted here, or e) γ-IFN (500 u/ml depicted here). Calcium flux is measured as described in figure 1. This representative of 3 experiments.

Figure 3. Ca^{++} flux is critical for 446-BCDF mediated B cell differentiation. a). B cells ($10x10^6$) were pretreated with varying concentrations of BAPTA (0.8-2 uM) for 2 hours and washed with culture medium prior to stimulation with SAC (0.001% v/v) and 446-BCDF (10% v/v) ($1x10^5$/microwell-triplicate cultures). Ig secretion was measured on day 7 by ELISA. Medium alone sample represents

baseline Ig secretion induced by SAC activated B cells without 446-BCDF.

b). B cells (10^5/well) were cultured in triplicate with 446-BCDF (10%, v/v) and SAC (0.001%, v/v). BAPTA (1uM) was added at the onset of culture or at the indicated times. DMSO (0.1%, v/v), the diluent for BAPTA, was added to the corresponding control wells at the indicated times. Ig secretion was measured by ELISA after 7 days of culture. Baseline Ig secretion (Medium) was 0.04 ug/ml, representing SAC stimulated B cells without 446-BCDF.

Figure 4. PTX blocks Ca^{++} flux induced by 446-BCDF. Indo-1AM loaded B cells were pretreated with either culture medium (a) or pertussis toxin (1 ng/ml) (b) for 2 hours at 37°C. After washing, B cells were stimulated with 446-BCDF (10% v/v). [Ca2+]i concentration was determined as described for figure 1.

Fifure 5. PTX inhibits Ig secretion induced by 446-BCDF. Peripheral blood B cells (10^5/microwell-triplicate cultures) were cultured with SAC (0.001% v/v) and 446-BCDF (10% v/v) in the presence of varying concentrations of PTX for 7 days. Cell free supernatants were assayed for total Ig secretion by ELISA. Baseline equals Ig secretion by SAC activated B cells in the absence of 446-BCDF. Results are representative of 4 experiments.

Figure 6. 446-BCDF stimulates IP production. B cells ($4x10^6$ cells/ml) were incubated overnight with [^{3}H]-myo-inositol (3-5 uCi/ml) and were then treated with anti-human IgM mAb HB57 (20 ug/ml), or

BCDF (10% v/v) in the presence or absence of mAb 929 (20 ug/ml). Cells were then lysed and inositol phosphates separated by anionic exchange chromatography and assayed as described in Materials and Metheds. Medium alone sample reflected the baseline level of IP. Results are reprsentative of five experiments with similar results.

Figure 7. Neither 446-BCDF nor SAC stimulates PKC translocation. $10x10^6$ peripheral blood B cells were stimulated with either anti-human IgM mAb HB57, SAC (0.001% v/v), 446-BCDF (10% v/v), or PMA (50 ng/ml) for 10 minutes at 37°C. PKC in the cytosolic and membrane fractions were partially purified on DEAE columns. The activity of PKC was then determined as described in Materials and Methods. PMA served as a positive control and culture medium as a negative control. These results are representative of four experiments with similar results.

Figure 8. PKC inhibitors do not inhibit 446-BCDF activity. SAC activated PB B cells (10^5 cells/well) were stimulated with 10% 446-BCDF in the presence of either (A) staurosporin (0.39-100 nM) or (B) calphostin C (0.0625-1 uM) at the onset of culture. Medium alone sample were used to reflect the Ig secretion by SAC-activated B cells. Cells were cultured at 37°C for 7 days. Ig secretion was measured by ELISA. Baseline Ig level was 0.02 and 0.04 ug/ml in assay A and assay B, respectively.

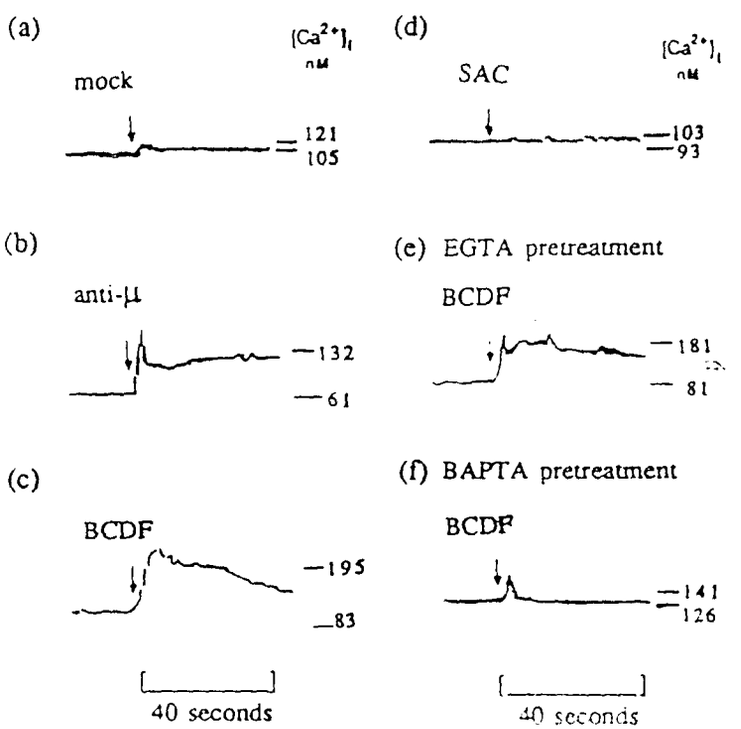
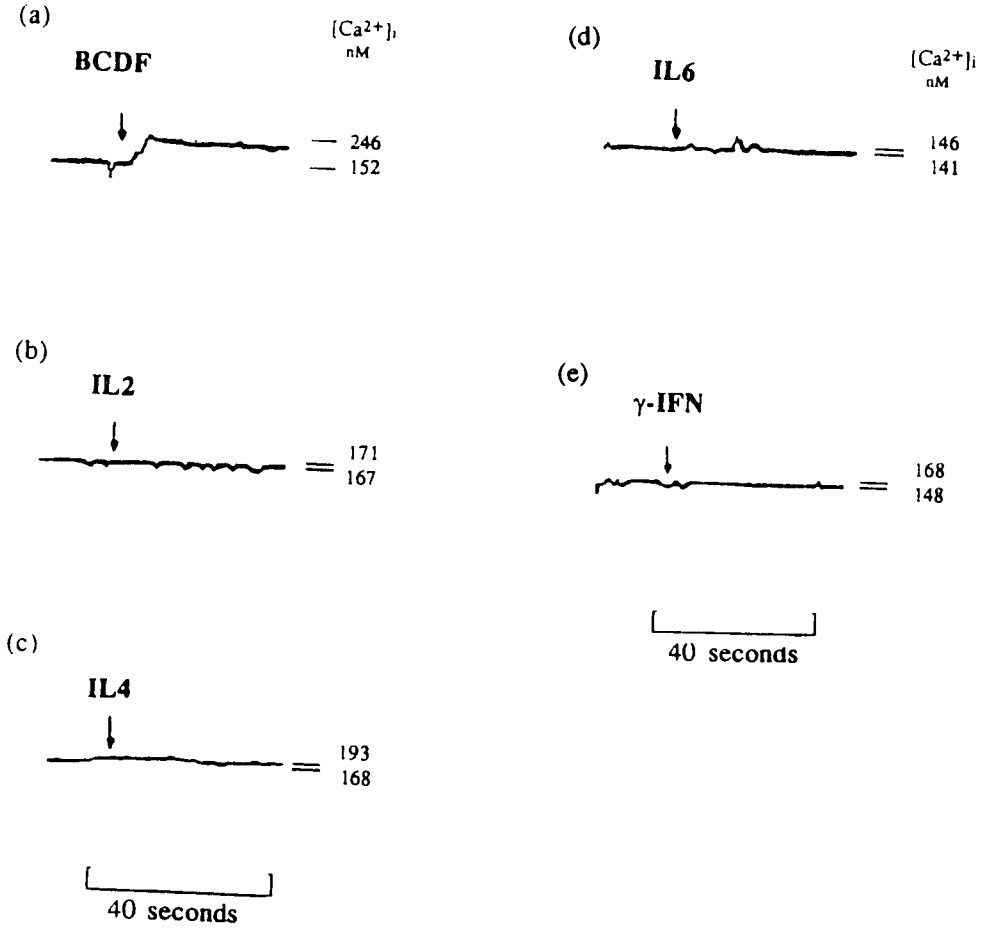

(a)
$[Ca^{2+}]_i$
nM
mock
121
105
(b)
anti-μ
132
61
(c)
BCDF
195
83
40 seconds
(d)
$[Ca^{2+}]_i$
nM
SAC
103
93
(e) EGTA pretreatment
BCDF
181
81
(f) BAPTA pretreatment
BCDF
141
126
40 seconds

Figure 1


(a)
BCDF
[Ca2+]i
nM
246
152
(b)
IL2
171
167
(c)
IL4
193
168
40 seconds
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IL6
[Ca2+]i
nM
146
141
(e)
γ-IFN
168
148
40 seconds

Figure 2

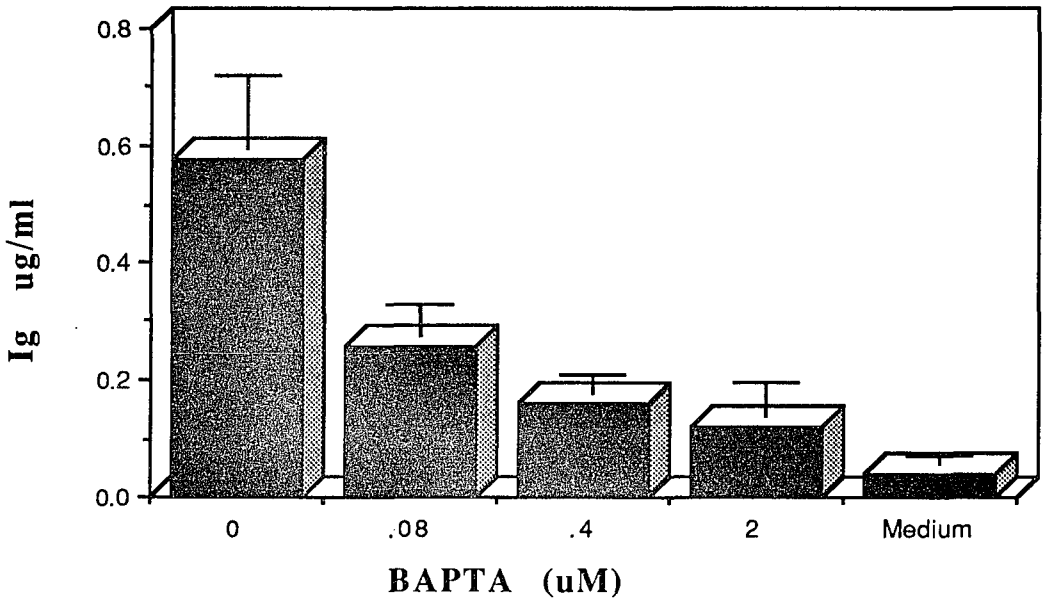

Figure 3a

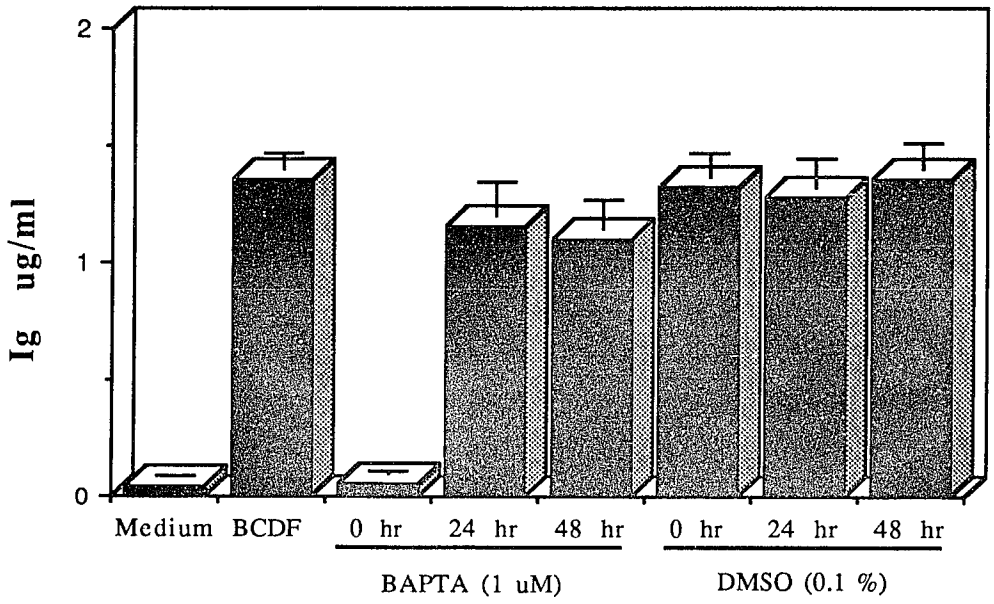
Ig ug/ml
2
1
0
Medium
BCDF
0 hr
24 hr
48 hr
0 hr
24 hr
48 hr
BAPTA (1 uM)
DMSO (0.1 %)

Figure 3b

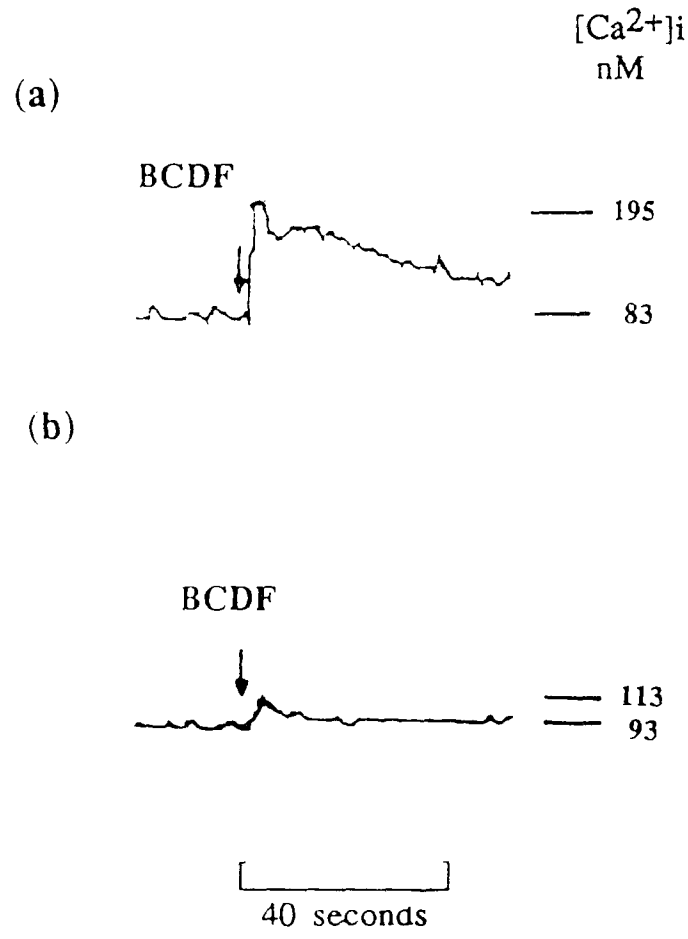

Figure 4

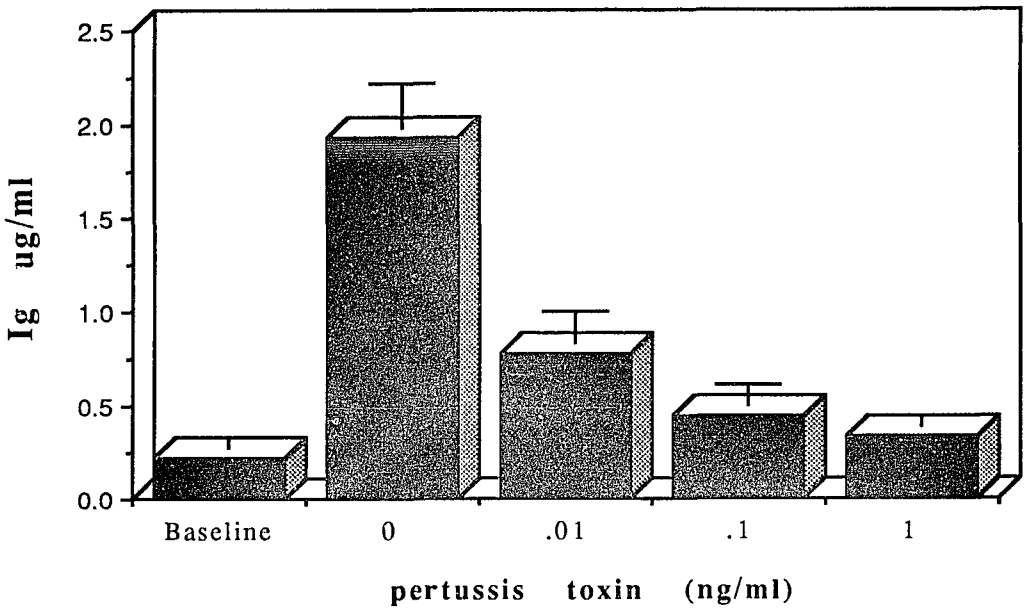

Figure 5

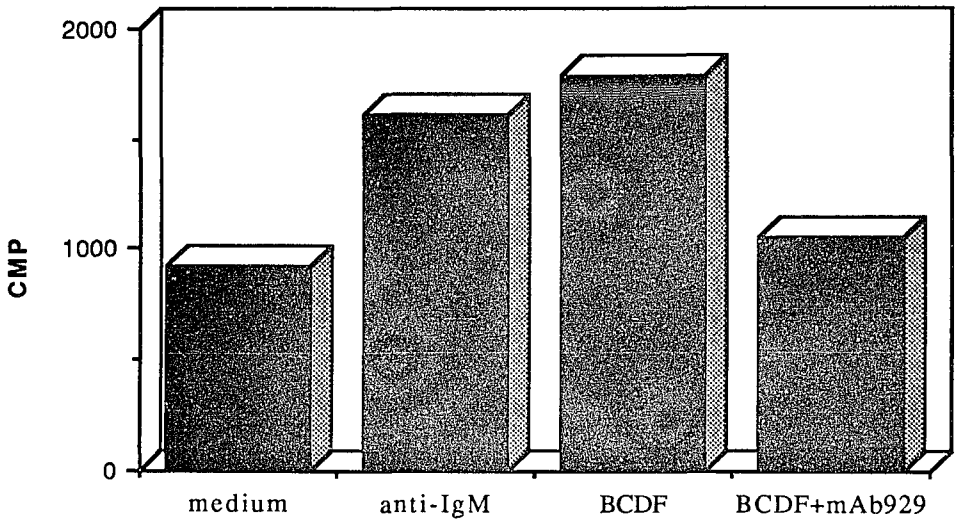

Figure 6

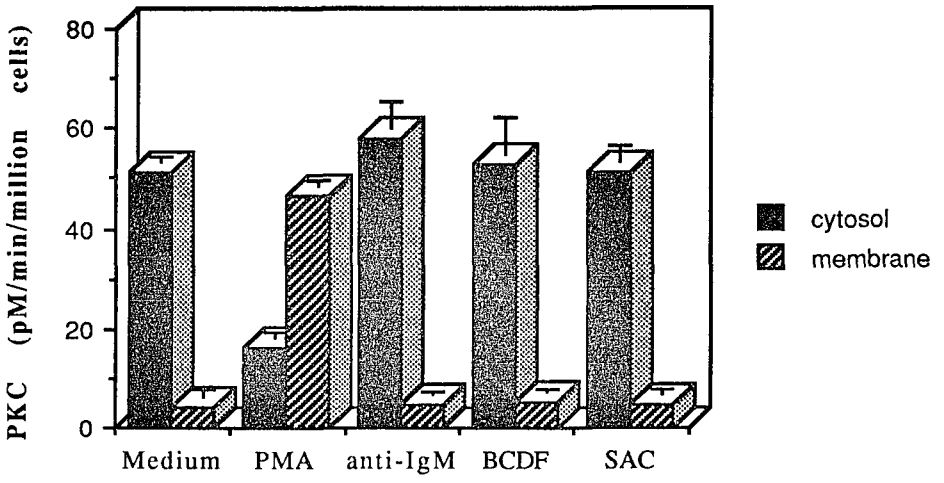
PKC (pM/min/million cells)
80
60
40
20
0
Medium
PMA
anti-IgM
BCDF
SAC
cytosol
membrane

Figure 7

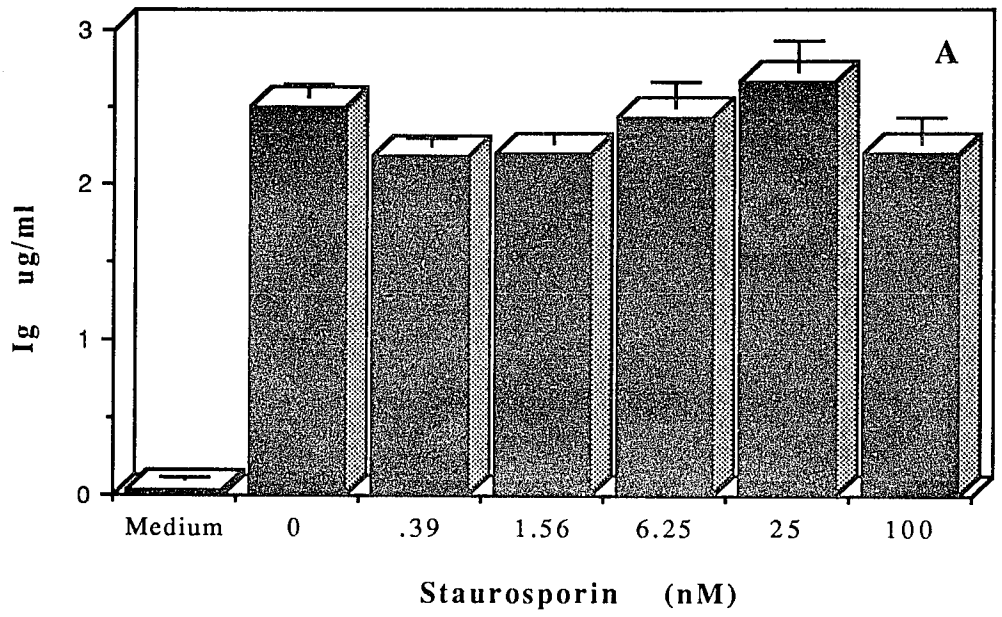

Figure 8a

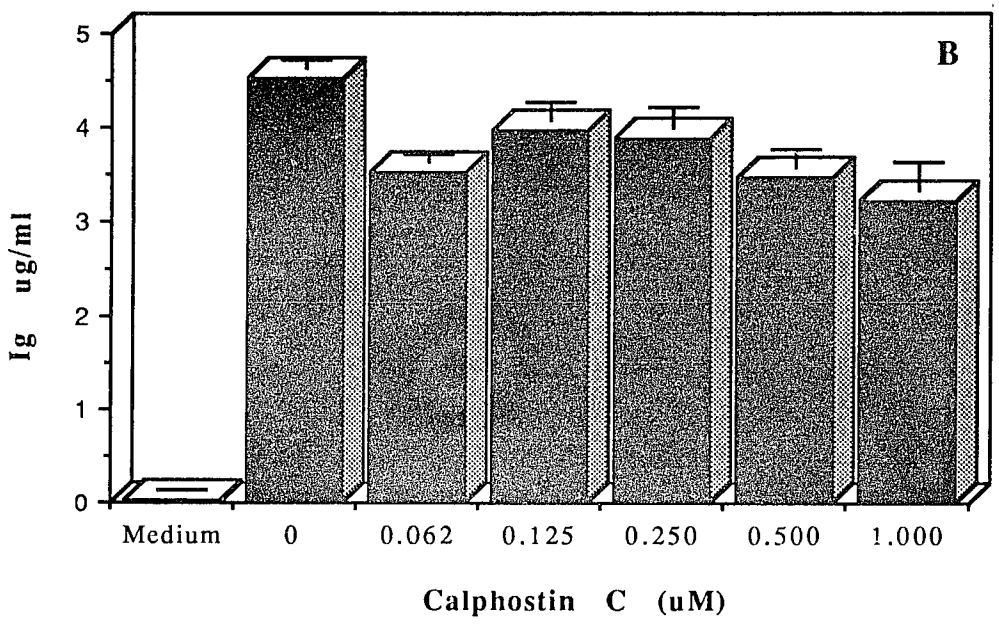
B
5
4
3
2
1
0
Ig ug/ml
Medium
0
0.062
0.125
0.250
0.500
1.000
Calphostin C (uM)

Figure 8b

COMPLEMENTARY RESULTS II

1. **Western blot analysis of tyrosine kinase activity in 446-BCDF stimulated B cells.** The involvement of tyrosine phosphorylation in B cell proliferation and differentiation has been well documented. Crosslinking the Ag receptor on B cells results in a rapid increase in PTK activity. Two important substrates of B cell Ag receptor complex associated PTK are PLCγ1 and PLCγ2 (Roifman et al., 1992a) which hydrolyze PIP2 to produce IP3 and DAG, causing intracellular Ca^{++} accumulation and PKC translocation. Inhibition of PTK activity has been shown to block anti-IgM induced tyrosine phosphorylation, B cell proliferation (Padeh et al., 1991), IP production, Ca^{++} flux, IL2 and transferrin receptor expression, c-fos mRNA expression (Roifman et al., 1992b), and IgM secretion by SKW6.4 cells (Goldstein et al., 1993), suggesting the importance of PTK in B cell function. 446-BCDF stimulates B cells to produce IP3 and causes an increase in intracellular Ca^{++} which is important second messenger for triggering 446-BCDF mediated B cell differentiation. It was interesting to determine whether PTK was involved in 446-BCDF driven B cell differentiation. PB or tonsillar B cells were stimulated with either SAC, or anti-IgM for 3 min, or with 446-BCDF for the indicated times with the anti-pty mAb 4G10. Western blotting was used to analyze tyrosine phosphorylation in our samples. As seen in Figure 1A, rabbit anti-human IgM polyclonal antibody induced rapid tyrosine phosphorylation of more than 10 proteins in PB B cells. In contrast, neither 0.001% SAC nor 10% 446-BCDF induced any detectable tyrosine phosphorylation. In tonsillar B

cells (Figure 1B), 0.1% SAC induced rapid tyrosine phosphorylation which has been reported (Roifman et al., 1991). However, as seen with PB B cells, neither 0.001% SAC nor 10% 446-BCDF induced any detectable tyrosine phosphorylation. The failure to detect tyrosine kinase activity in both 0.001% SAC and 446-BCDF stimulated B cells suggests that PTK may not be involved in 446-BCDF mediated signal transduction. Alternatively, PTK may be involved but the activity was not detectable by our assays. To address this issue, a PTK inhibitor genistein was used in a 446-BCDF mediated B cell differentiation assay.

2. **446-BCDF activity can be blocked by a protein tyrosine kinase inhibitor.** Genistein is a potent PTK inhibitor (Kuruvilla et al., 1993). It has been widely used to inhibit PTK activity in many cell systems. Since we can not rule out the possibility that undetectable PTK may contribute to 446-BCDF mediated B cell differentiation, genistein was used to address this issue. Varying concentrations of genistein were added to SAC activated B cell cultures with 10% 446-BCDF at the onset of culture. After 7 days of culture, supernatants were analyzed for Ig secretion by ELISA. As shown in figure 2, 446-BCDF induced a 7 fold increase in Ig secretion by SAC activated B cells. genistein inhibited 446-BCDF mediated Ig secretion in a dose dependent manner. In some experiments, 10 uM genistein was able to completely inhibit 446-BCDF activity. At higher concentrations, genistein (40 uM) was toxic to B cells during the 7 days of culture and accordingly was not used in our assays. These data indicated that although PTK activity could not be detected by

Western blotting, PTKs may play an important role in 446-BCDF mediated B cell differentiation.

3. **Effects of H7 on Ig secretion by 446-BCDF stimulated B cells and IL6 stimulated CESS cells.** The involvement of serine/threonine kinases in B cell differentiation has been suggested in IL2, IL4, or IL6 stimulated SKW cells (Goldstein et al., 1993). IgM secretion by SKW cells was blocked by the serine/threonine kinase inhibitor, H7. It was therefore of interest to investigate the role of serine/threonine kinases in 446-BCDF mediated B cell differentiation. SAC activated B cells were incubated with varying concentrations of H7 in the presence of 10% 446-BCDF. After 7 days of culture, supernatants were analyzed for Ig secretion. Figure 3(A) shows that H7 did not significantly inhibit Ig secretion by 446-BCDF stimulated B cells at any concentration used in the assay. In contrast, H7 significantly inhibited IgG secretion by IL6 stimulation of the EBV-transformed B cell line CESS (figure 3B). H7 (20 uM) completely blocked IgG secretion by CESS while this concentration of H7 was not able to significantly block Ig secretion by 446-BCDF stimulated PB B cells. These data suggest that serine/threonine kinases may play an important role in cytokine induced Ig secretion in EBV-transformed B cell but not in 446-BCDF mediated B cell Ig secretion.

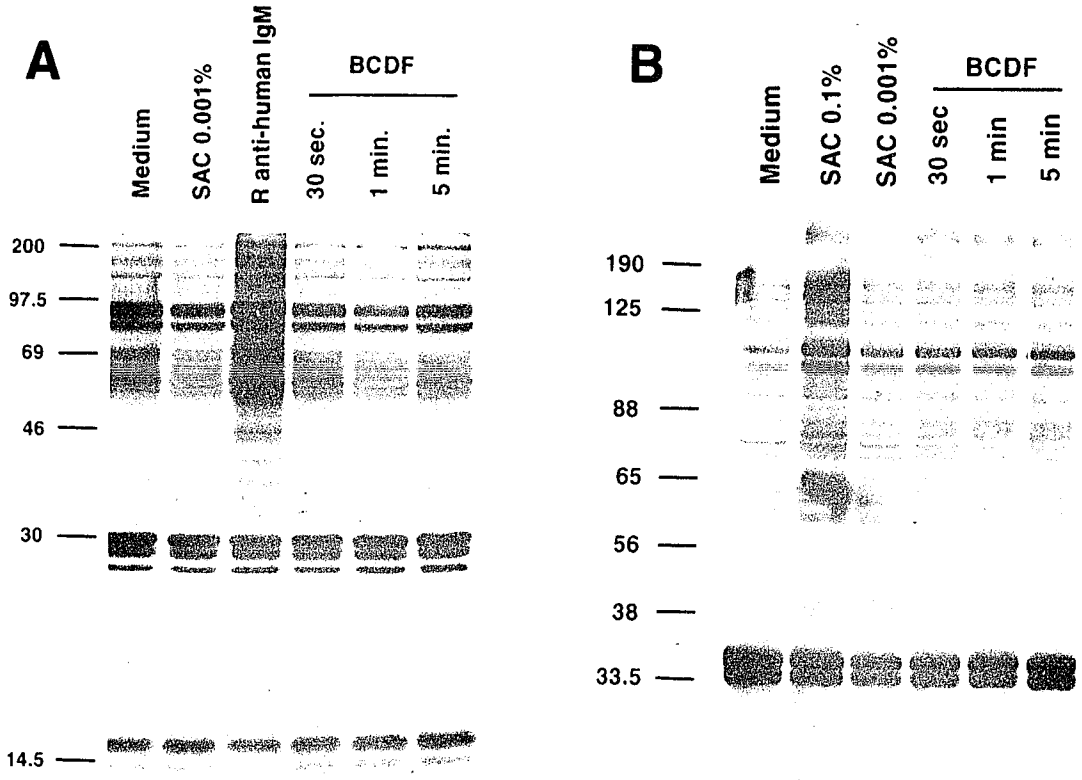

Figure 1. Effects of anti-IgM, SAC and 446-BCDF on protein tyrosine phosphorylation. $4x10^6$ PB B cells (A) or tonsillar B cells (B) were incubated with either 10 ug of rabbit anti-human IgM (positive control in PB B cell assay), 0.1% SAC (positive control in tonsillar B cell assay), 0.001% SAC for 3 min or 10% 446-BCDF for the indicated time. B cells cultured in medium was used as a negative control in both assays. Samples were then processed for Western blotting as described in Materials and Methods.

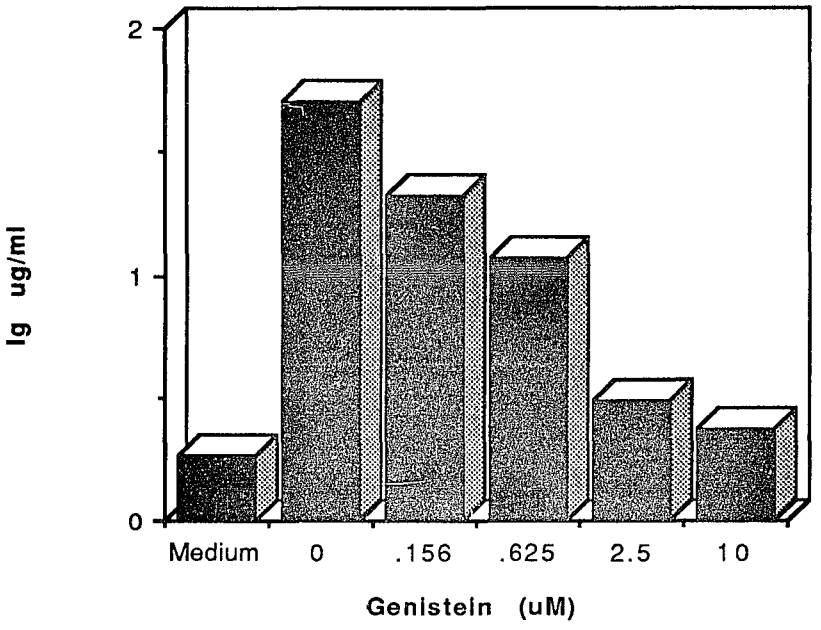

Figure 2. Effect of PTK inhibitor on 446-BCDF activity. PB B cells (10^5 cells/well) were incubated with 0.001% SAC in the presence of 10% 446-BCDF. At the onset of culture, varying concentrations of genistein were added to the corresponding wells of a 96-well microtiter plate. Cells were cultured in triplicate at 37^0C for 7 days. Ig secretion was analyzed by ELISA.

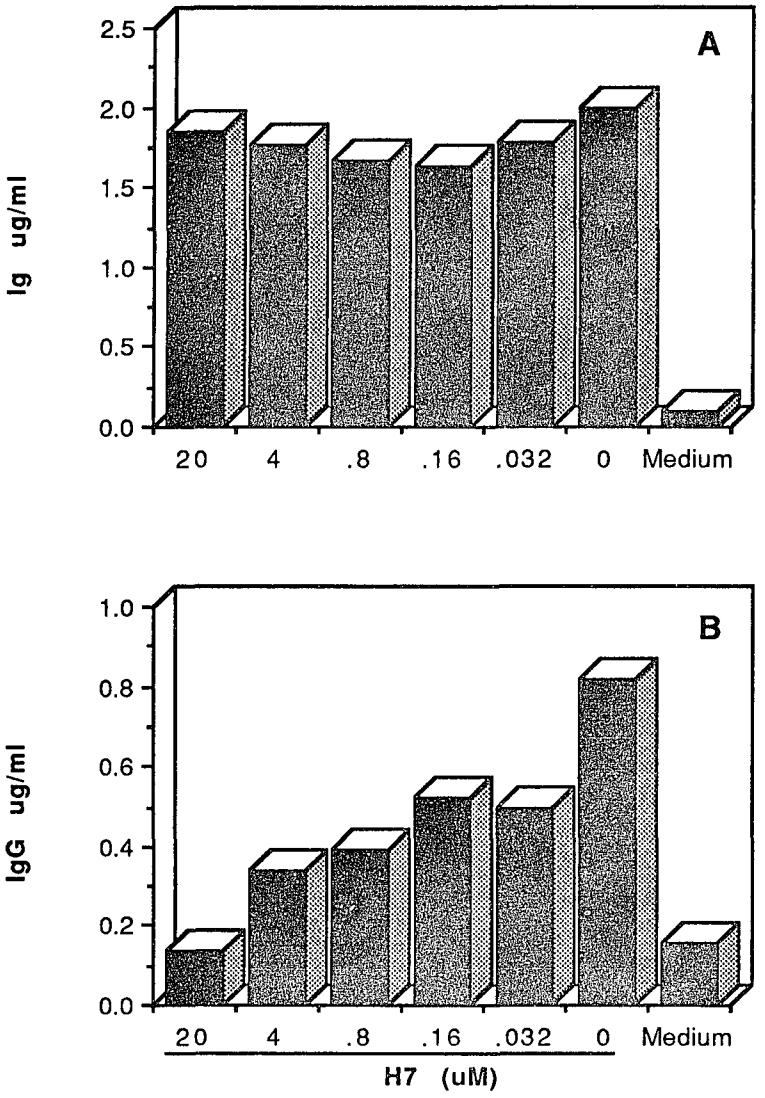

Figure 3. Effects of H7 on Ig secretion induced by 446-BCDF. (A), SAC activated PB B cells (10^5 cells/well) were incubated with 10% 446-BCDF. (B), CESS cells (10^4 cells/well) were incubated with 100 units/ml IL6. At the onset of culture, the indicated concentrations of H7 were added to the corresponding wells of 96-well microtiter plates. PB B cells were cultured in triplicate at 37^0C for 7 days and polyclonal Ig secretion was measured by ELISA. CESS cells were cultured for 5 days and IgG secretion was measured by ELISA.

General Discussion

The pathway of normal B cell maturation involves the sequential steps of initial activation, proliferation, and differentiation. Cytokines regulating this pathway can stimulate B cells to either proceed to the next stage or progress through several stages. We have recently identified a novel factor, 446-BCDF, which is able to stimulate a significant increase in Ig secretion by human B cells (Sherris et al., 1989). Superose 12 permeation chromatography and chromatofocusing of the biochemically purified 446-BCDF reveal that this potent B cell differentiation factor has an apparent M_r of 32 Kd and a pI of 6, distinct from the M_r of IL2, IL4, IL10, and IL13, and the reported pI of IL6. In this study, we further characterize this partially purified cytokine. 446-BCDF induces more than a 5 fold increase in polyclonal Ig secretion, including IgG, IgM, IgA, but no IgE. This cytokine did not stimulate proliferative responses of IL2 and IL6 dependent cell lines, and did not induce CD23 expression on B cells, an important function of IL4. Coupled with the previous findings that antibodies against IL2, IL4, IL6 or their receptors failed to inhibit 446-BCDF activity, the effect of 446-BCDF appears to be independent of these cytokines. IL10 and IL13 are two recently discovered cytokines acting on B cells and have a M_r of 18.5 and 10 Kd, respectively. IL10 stimulates B cell growth and, in the presence of IL4, induces IgE secretion. In contrast, 446-BCDF did not stimulate B cell growth (Sherris et al., 1989) and IgE secretion, and IL4 inhibits the effect of 446-BCDF on PB B cell Ig secretion (data not shown). IL13 is a costimulatory factor for B cell differentiation. IL13 alone

does not induce Ig secretion by SAC activated B cells (Mckenzie et al., 1993). In the presence of anti-IgM or CD40L, it induces IgE secretion. In addition, IL13 induces CD23 expression on B cells. All these suggest that IL13 is a IL4 like cytokine and distinct from 446-BCDF. Recent studies have demonstrated that the interaction between CD40 and CD40L can trigger B cell proliferation and differentiation. A panel of anti-CD40 mAbs has been generated to mimic the effect of CD40L on B cells. In this study, an anti-CD40 mAb stimulated SAC activated B cells to secrete Ig and had a synergistic effect with 446-BCDF on Ig secretion. Furthermore, the anti-CD40L mAb 5C8 failed to inhibit 446-BCDF activity. These data suggest that CD40L and 446-BCDF are two distinct entities and may function via different signal pathways. A specific monoclonal antibody 929 completely blocked 446-BCDF activity, however, mAb 929 did not inhibit either Ig secretion induced by IL2 and IL6, or IL2 and IL6 dependent cell line proliferation (Y. Kuang, personal communication). Taken together, these data indicate that 446-BCDF is a novel cytokine distinct from the known cytokines acting on B cell differentiation and 446-BCDF and CD40L are two distinct entities.

Although extensively studied, the precise mechanisms by which cytokines regulate lymphocyte function, especially B cell differentiation, have not been elucidated. One of the major obstacles to such studies is that most of cytokines acting on B cell differentiation are only costimulatory factors which may function through a complex network of signals and some cytokines, such as IL2, may function through a nonclassical signal pathway (Eardley et

al., 1991). 446-BCDF is a potent BCDF which directly acts on SAC activated B cells. With this newly characterized cytokine, we have been able to investigate the signal transduction pathways involved in human B cell differentiation. We have demonstrated that 446-BCDF mediates a time dependent and a dose dependent decrease in intracellular cAMP which appears to correlate with terminal B cell differentiation. Experimentally increased intracellular cAMP inhibited Ig secretion induced by 446-BCDF, supporting the concept that the reduction of intracellular cAMP may be critical for human B cell differentiation. This decrease in cAMP appeared to be regulated by the binding to a Gi linked receptor, since a potent Gi inhibitor, pertussis toxin, prevented the reduction of intracellular cAMP and inhibited Ig secretion induced by 446-BCDF. Cholera toxin, a Gs stimulator, had a similar effect on cAMP and Ig secretion, probably via stimulation of Gs completing with Gi for the common target, adenylate cyclase, preventing the decrease in cAMP. A potent PDE inhibitor, IBMX, stimulated a 5 fold increase in intracellular cAMP by prevention of cAMP hydrolysis. 10% 446-BCDF, an optimal concentration for B cell Ig secretion, or higher concentrations failed to return the cAMP level to the baseline, suggesting that signals delivered by 446-BCDF may not be directed at PDEs. These data further support the concept that 446-BCDF may function through a Gi linked receptor to mediate the reduction of intracellular cAMP.

The involvement of cAMP in human B cell differentiation has been well documented (Goldstein et al., 1990; Shearer et al., 1988; Patke et al., 1991). Experimentally increasing intracellular cAMP inhibited the

formation of Ig-secreting cells and IgM, IgG production by human B cell lines. Similar results were also obtained in murine systems (Takahama et al., 1989; Stein et al., 1991) although opposite results were reported in one murine system (Gilbert et al., 1985). These data suggest a negative effect for cAMP on B cell differentiation. An interesting question to be answered is whether a reduction of intracellular cAMP is an important signal to trigger B cell differentiation. Our data have shown that similar to 446-BCDF, both IL2 and IL6 induce a decrease in intracellular cAMP. Studies from our laboratory have also shown that Db-cAMP inhibits IL2 and IL6 induced Ig secretion by PB B cells and CESS cells, respectively. Furthermore, AT II also induced a reduction in cAMP and stimulated SAC activated B cells to secrete Ig. These data all point towards the importance of a reduction in cAMP in B cell differentiation. Interestingly, AT II significantly enhanced the effect of optimal concentrations of 446-BCDF on Ig secretion, suggesting that, in addition to inducing a decrease in cAMP, 446-BCDF may also deliver other signals allowing for maximal Ig secretion.

With regard to this latter point, our data demonstrate that 446-BCDF mediates a rapid, transient Ca^{++} flux in addition to its effect on cAMP. This Ca^{++} flux appears to be a critical early event in that it occurs immediately upon stimulation of B cells with 446-BCDF and inhibition of Ca^{++} flux by pretreatment of B cells with BAPTA blocks Ig secretion induced by 446-BCDF. A Ca^{++} flux was not observed in IL2, IL4, IL6 and IFN-γ stimulated B cells, suggesting that these cytokines may act on B cells via distinct signal transduction

pathways. The Ca^{++} flux induced by 446-BCDF appears to be mediated by inositol phosphate turnover since IP3 was increased after 446-BCDF stimulation. Interestingly, the Ca^{++} flux was inhibited by pretreatment with PTX. It has been well documented that some Ca^{++} channels are regulated by pertussis toxin sensitive G proteins (Kleuss et al., 1991; Rosales et al., 1992; Dahinden et al., 1993) and IP3 mediated internal Ca^{++} release from G protein regulated Ca^{++} channels can be inhibited by pertussis toxin (Rosales et al., 1992). There is therefore a precedent for a pertussis toxin sensitive IP3 mediated Ca^{++} flux. The inhibition of Ca^{++} flux by PTX correlates its ability to inhibit Ig secretion induced by 446-BCDF, further supporting the importance of an early Ca^{++} flux in Ig secretion. Although IP3 was increased with 446-BCDF stimulation and the accumulation of IP3 was prevented by mAb 929, it is not clear whether 446-BCDF stimulates PIP_2 turnover since no PKC activity was detectable using a specific PKC assay system. Furthermore, PKC does not appear to play a role in 446-BCDF mediated B cell differentiation in that specific PKC inhibitors failed to block 446-BCDF activity.

Several kinases have been shown to be involved in mediating Ca^{++} flux. PTK has been known to be involved in B cell function and maturation. Inhibition of PTK activity blocks human B cell proliferation, IP3 production, Ca^{++} flux, IL2 and transferrin receptor expression stimulated by anti-IgM, and IgM secretion induced by IL2, IL4, and IL6 in a human B cell line (Padeh et al., 1991; Roifman et al., 1992b; Goldstein et al., 1993). In our studies, PTK activity was

not detectable in 10% 446-BCDF and/or 0.001% SAC stimulated B cells using an anti-phosphotyrosine western blot technique. However, a PTK specific inhibitor, genistein, blocked 446-BCDF activity, suggesting that PTKs may play a role in 446-BCDF mediated B cell differentiation. It has been reported that SAC stimulates tyrosine phosphorylation in human B cells, however, PTK activity can be detected by western blot only when SAC is used at 0.01% or higher concentration (Roifman et al., 1991). Therefore, it is not surprising that PTK activity is undetectable in 0.001% SAC stimulated B cells. In addition to SAC, it is possible that 446-BCDF may also deliver a signal to activate PTK. Although serine/threonine kinases have been reported to play a role in IL2, IL4, and IL6 mediated IgM secretion by SKW cells, these kinases appears not to contribute to 446-BCDF mediated B cell differentiation since the serine/threonine kinase inhibitor H7 failed to inhibit 446-BCDF activity.

We have demonstrated that the newly characterized 446-BCDF is a potent BCDF distinct from the known cytokines acting on B cells. 446-BCDF stimulates a decrease in intracellular cAMP and a rapid, transient Ca^{++} flux. These two events are critical in 446-BCDF mediated Ig secretion. It is interesting that 446-BCDF functions through at least two signaling pathways. This is reminiscent of IL4's effects on human tonsil B cells where IL4 activates B cells via an immediate and transient elevation of IP3 and Ca^{++}, followed several minutes later by a sustained rise in intracellular cAMP (Finney et al., 1990; Rigley et al., 1991). These phenomena are very similar to those observed in 446-BCDF stimulated PB B cells although in the latter

case the cAMP level is reduced instead of increased. This difference may explain why IL4 and 446-BCDF have different effects on B cell differentiation. An increase in intracellular cAMP induced by IL4 upregulates CD23 expression (Finney et al., 1990) and inhibits Ig secretion. The question then is, how can one cytokine mediate two intracellular signals? It has been suggested that IL4 functions through two receptors (Rigley et al., 1991). Either distinct receptors or a high and low affinity receptor mediating different signals. There is growing awareness of multichain cytokine (IL2, IL6) receptors. These generally alter the affinity of the receptor rather than mediate different signals. Still, a multichain receptor may be another mechanism to stimulate more than one signal. It is possible that 446-BCDF may also function via two receptors: one activates the associated Gi protein to inhibit adenylate cyclase, leading to the reduction of cAMP; the other activates a PTX sensitive G protein to regulate IP3 mediated Ca^{++} flux. The reduction of intracellular cAMP may prevent a negative effect on Ig production and Ca^{++} may activate downstream targets to regulate Ig production. To develop a model where these two signaling events are linked is not self evident. PTKs can activate G proteins to induce inositol phosphate turnover and Ca^{++} flux. Interestingly, Ca^{++} may enhance the activity of a number of kinases or even Ca^{++} dependent PDEs. However, our data do not support the activation of PDE as a mechanism for reduction of intracellular cAMP. Furthermore, inhibition of the Ca^{++} flux with BAPTA fails to have an effect on the reduction of intracellular cAMP mediated by 446-BCDF. We are therefore left with a multisignal event that may result from binding one or more

receptors. Further characterization awaits the identification of 446-BCDF's receptor.

Finally, there have been recent findings that help creat an intriguing model. It is well recognized that B cells can act as antigen presenting cells taking up Ag through sIg. The interaction between an Ag specific B cell and an Ag specific T cells is the ultimate cognate interaction. In order to render B cells more efficient at Ag presentation they must express the CD28 ligand, B7-1, or B7-2. While these co-stimulatory molecules are not expressed on resting B cells, they are inducible upon T:B co-culture where the class II molecule on the B cells are crosslinked. Crosslinking class II molecules on B cells results in an increase in intracellular cAMP, which is directly responsible for B7-1/B7-2 upregulation. Therefore, given these data as well as ours, one can postulate that during T:B interaction Ag presentation and clonal proliferation is important. Once B cells dissociate they then can become susceptible to differentiation signals. Reducing cAMP may be a mechanism to prevent the B cell from proliferation or performing as an antigen presenting cell. This might explain the common findings with all differentiation factors used and develops a model where B cells can be driven down different pathways.

REFERENCES:

Ambrus, J. L, J. Pippin, A. Joseph, C. Xu, D. Blumenthal, A. Tamayo, K. Claypool, D. McCourt, A. Srikiatchatochorn, and R. J. Ford. 1993. Identification of a cDNA for a human high molecular weight B cell growth factor. Proc. Natl. Acad. Sci. U. S. A., 90:6330.

Arai, K., F. Lee, A. Miyajima, S. Mijatake, N. Arai, and T. Yokota. 1990. Cytokines: coordinators of immune and inflammatory responses. Annu. Rev. Biochem., 59:783.

Armitage, R. J. B. A. Macduff, M. K. Spriggs, and W. C. Fanslow, 1993. Human B cell proliferation and Ig secretion induced by recombinant CD40 ligand are modulated by soluble cytokines. J. Immunol., 150:3671.

Armitage, R. T., W. C. Fanslow, T. A. Sato, K. N. Clifford, B. M. Macduff, D. M. Anderson, S. D. Gimple, T. Davis-Smith, C. R. Maliszewski, E. A. Clark, C. A. Smith, K. H. Grabstein, D. Cosman, and M. K. Spriggs. 1992. Molecular and biological characterization of a murine ligand for CD40. Nature, 357:80.

Banchereau, J., P. dePaoli, A. Valle, E. Garcia, and F. Rousset. 1991. Long term human B cell lines dependent on interleukin 4 and antibody to CD40. Science, 251:70.

Beavo, J. A., R. S. Hansen, S. A. Hurwitz, T. J. Martins, and M. C. Mumby. 1982. Identification and properties of cyclic nucleotide phosphodiesterase. Mol. cell. Endocrinol., 28: 387.

Beckwith, M., W. J. Urba, D. K. Ferris, C. E. Freter, D. B. Kuhns, C. M. Morotz, and D. L. Longo. 1991. Anti-IgM mediated growth inhibition of a human B lymphoma cell line is independent of phosphatidylinositol turnover and protein kinase C activation and involves tyrosine phosphorylation. J. Immunol., 147:2411.

Bende, B., G. J. Jochems, T. H. Frame, M. R. Klein, R. V. van-Eijk, R. A. van-Lier, and W. P. Zeijlemaker. 1992. Effects of IL4, IL5, and IL6 on growth and immunoglobulin production of Epstein-Barr virus infected human B cells. Cell. Immunol., 143:310.

Benjamin, D., T. J. Knobloch, and M. A. Dayton. 1992. Human B cell interleukin 10: B cell lines derived from patients with acquired

immunodeficiency syndrome and Burkitt's lymphoma constitutively secrete large quantities of interleukin-10. Blood, 80:1289.

Berridge, M. T. 1985. The molecular basis of communication within the cell. Scientific America, 253: 142.

Bertolini, J. N., and E. M. Benson. 1990. The role of human interleukin 6 in B cell isotype regulation and differentiation. Cellular Immunol., 125:197.

Bertolini, J. N., C. J. Sanderson, and E. M. Benson. 1993. Human interleukin 5 induces staphylococcal A Cowan 1 strain-activated human B cells to secrete IgM. Eur. J. Immunol., 23:398.

Besterman, J. M. 1986. Rapid formation of diacylglycerol from phosphatidylcholine: a pathway for generation of a second messenger. Proc. Natl. Acad. Sci. USA, 83:6785.

Brouet, J. C., W. Vainchenker, D. Blanchard, U. Tseta, and J. Cartron. 1983. The origin of human B and T cells from multipotent stem cells: a study of the Tn syndrome. Eur. J. Immunol., 13: 350.

Buchler, W., U. Walter, B. Jastorff, and S. M. Lohmann. 1988. Catalytic subunit of cAMP-dependent protein kinase is essential for cAMP-mediated mammalian gene expression. FEB., 228:27.

Burch, R, M., A. Luini, and J. Axelrod. 1986. phospholipase A_2 and phospholipase C are activated by distinct GTP-binding proteins in response to α1-adrenergic stimulation in FRTLs thyroid cells. Proc. Natl. Acad. Sci, USA, 83:7201.

Cambier, J. C., and J. T. Ranson. 1987. Molecular mechanism of transmembrane signaling in B lymphocytes. Annu. Rev. Immunol., 5:175.

Cantley, L. C., K. R. Auger, C. Carpenter, B. Duckworth, A. Graziani, R. Kapeller, and S. Solfoff. 1991. Oncogenes and signal transduction. Cell, 64:281.

Clark, E. A,. and J. A. Ledbetter. 1994. How B and T cells talk to each other. Nature, 367:425.

Clark, M. R., R. J. Friedrich, K. S. Campbell, and J. C. Cambier. 1992. Human pre-B and B cell membrane mu-chains are noncovalently associated with a disulfide-linked complex containing a product of the B29 gene. J. Immunol., 1149:2857.

Clutterbuck, E., J. G. Shields, J. Gordon, S. H. Smith, A. Boyd, R. E. Callard, H. D. Campbell, I. G. Young, and C. J. Sanderson. 1987. Recombinant human interleukin 5 is an eosinophil differentiation factor but has no activity in standard human B cell growth factor assays. Eur. J. Immunol., 17:1743.

Cocks, B. G., R. W. Malefyt, J. P. Galizzi, J. E. de Vries, and G. Aversa. 1993. IL13 induces proliferation and differentiation of human B cells activated by the CD40 ligand. Int. Immunol., 6:657.

Coffman, R. L., A. O'Garra, and D. J. Volkman. 1990. Interleukins and Interferons acting on B lymphocytes. In Immunophysiology, ed by J. J. Oppenheim.

Cooper, M. D., 1987. Current concepts, B lymphocytes: Normal and abnormal development. J. Clin. Immunol., 1:81.

Daeipour, M. G. Kumar, M. C. Amaral, and A. E. Nel. 1993. Recombinant IL6 activates p42 and p44 mitogen-activated protein kinases in the IL6 responsive B cell line, AF-10. J. Immunol., 150:4743.

Dahinden, C. A. 1993. RANTES and related chemokines activate human basophil granulocytes through different G protein-coupled receptors. Eur. J. Immuno., 23:761.

Dash, P. K., K. A. Karl, M. A. Colicos, R. Prywes, and E. R. Kandel. 1991. cAMP response element-binding protein is activated by Ca^{++}/calmodulin as well as cAMP-dependent protein kinase. Proc. Natl. Acad. Sci. USA, 88:5061.

Defrance, T., B. Vanbervliet, J. Pene, and J. Banchereau. 1988. Human recombinant IL4 induces activated B lymphocytes to produce IgG and IgM. J. Immunol., 141:2000.

Defrance, T., P. Carayon, G. Billian, J. C. Guillemot, A. Minty, D. Caput, and P. Ferrara. 1994. Interleukin 13 is a B cell stimulating factor. J. Exp. Med., 179:135.

Eardley, D. D,. and M. E. Koshland. 1991. Glycosylphosphatidylinositol: a candidate system for interleukin 2 signal transduction. Science, 251:78.

Edwards, R. M., and E. J. Stack. 1993. Angiotensin II inhibits glomerular adenylate cyclase via the angiotensin II receptor 1 (AT1). J. Pharmacol. Exp. Ther., 266:506.

Farrar, W. L., and W. B. Anderson. 1985a. Interleukin 2 stimulates association of protein kinase C with plasma membrane. Nature, 315:233.

Farrar, W. L., T. P. Thomas, and W. B. Anderson. 1985b. Altered cytosol/membrane enzyme redistribution on interleukin 3 activation of protein kinase C. Nature, 315:235.

Finco, T. S., and A. S. Baldwin. 1993. Kappa B site-dependent induction of gene expression by diverse inducers of nuclear factor kappa B requires Raf-1. J. Biol. Chem., 268:17676.

Finney, M., G. R. Guy, R. H. Michell, J. Gordon, B. Dugas, K. P. Rigley, and R. E. Callard. 1990. Interleukin 4 activates human B lymphocytes via transient inositol lipid hydrolysis and delayed cyclic adenosine monophosphate generation. Eur. J. Immunol., 20:151.

Gibert, K. M., and K. M. Hoffmann. 1985. cAMP is an essential signal in the induction of antibody production by B cells but inhibits helper function of T cells. J. Immunol., 135: 2084.

Gillis, S., and K. A. Smith. 1977. Long term culture of tumor- specific cytotoxic T cells. Nature, 268:154.

Go, N. F., B. E. Castle, R. Barrett, R. Kastelein, W. Dang, T. R. Mosmann, K. W. Moore, and M. Howard. 1990. interleukin 10, a novel B cell stimulatory factor: unresponsiveness of X chromosome-linked immunodeficiency B cells. J. Exp. Med., 172:1625.

Gold M. R., D. A. Law, and A. L. Defranco. 1990. Stimulation of protein tyrosine phosphorylation by the B lymphocyte antigen receptor. Nature, 308:693.

Goldstein, H., and A. Kim. 1993. Immunoglobulin secretion and phosphorylation of common proteins are induced by IL2, IL4, and IL6 in the factor responsive human B cell line, SKW6.4. J. Immunol., 151:6701.

Goldstein, H., D. Koerholz, L. Chesky, X. Fan, and J. Ambrus. 1990. Divergent activities of protein kinase in IL6 induced differentiation of a human B cell line. J. Immuno., 145:952.

Grabstein, K. H., C. R. Maliszzewski, K. Shanbeck, T. A. Sato, M. K. Spriggs, W. C. Fanslow, and R. J. Armitage. 1993. The regulation of T cell dependent antibody formation in vitro by CD40 ligand and IL2. J. Immunol., 150:3141.

Harriman, G. R. D. Y. Kunimoto, J. F. Elliott, V. Paetkau, and W. Strober. 1988. The role of IL5 in IgA B cell differentiation. J. Immunol., 140:3033.

Harris, C. E., D. Staley, and P. M. Conn. 1985. Diacylglycerols and protein kinase C: potential amplifying mechanism for Ca++-independent Gonadotropin-releasing hormone-stimulated luteinizing hormone release. Mol. pharmocol., 27: 532

Hatakeyama, M., T. Kona, N. Kobayashi, A. Kawahara, S. D. Levin, R. M. Perlmutter, and T. Tanigichi. 1991. Interaction of the IL2 receptor with the src-family kinase $p56^{lck}$: identification of novel intermolecular association. Science, 252:1523.

Hirano, T., K. Yasukawa, H. Harada, T. Taga, Y. Watanabe, T. Matsuda, S. Kashiwamura, K. Nakajima, K. Koyama, A. Iwamatsu, S. Tsunasawa, F. Sakiyama, H. Matsui, Y. Takahara, T. Taniguchi, and T. Kishimoto. 1986. Complementary DNA for a novel human interleukin (BSF-2) that induces B lymphocytes to produce immunoglobulin. Nature, 324: 73.

Keegan, A. D., and J. H. Pierce. 1994. The interleukin 4 receptor: signal transduction by a hematopoietin receptor. J. Leuk. Biol., 55:272.

Kim, K. M., G. Alber, P. Weiser, and M. Reth. Differential signaling through the Ig-alpha and Ig-beta components of the B cell antigen receptor. 1993. Eur. J. Immunol., 23:911.

Kim, U-H., J. W. Kim, and S. G. Rhee. 1989. Phosphorylation of phospholipase C-γ by cAMP-dependent protein kinase. J. Biol. Chem., 264:2017.

Kishimoto, T., S. Akira, and T. Taga. 1992. IL6 receptor and mechanism of signal transduction. Int. J. Immunopharmacol., 14:431.

Klausner, R. D., J. J. O'shea, H. Yuong, P. Rose, J. A. Bluestone, and L. E. Samelson. 1987. T cell receptor tyrosine phosphorylation. Variable coupling for different ligands. J. Biol. Chem., 262:12654.

Klett, C., R. Nobiling, P. Gierschik, and E. Hackenthal. 1993. Angioyensin II stimulates the synthesis of angiotensinogen in hepatocytes by inhibiting adenylyl cyclase activity and stabilizing angiotensinogen mRNA. J. Biol. Chem., 268:25095.

Kleuss, C., J. Hescheler, C. Ewel, W. Rosenthal, G. Schultz, and B. Wittig. 1991. Assignment of G protein subtypes to specific receptors inducing inhibition of calcium currents. Nature. 353:43.

Kobayashi, N., T. Kono, M. Hatakeyama, Y. Minami, T. Miyazaki, R. M. Perlmutter, and T. Taniguchi. 1993. Function coupling of the sre-family protein tyrosine kinase p^{59fyn} and $p^{53/56lyn}$ with the interleukin 2 receptor: implication for redundancy and pleiotropism in cytokine signal transduction. Proc. Natl. Acad. Sci. USA., 90:4201.

Kolb, J. P., A. Abadie, N. Paul-Eugene, and B. Dugas. 1993. Intracellular signaling events associated with the induction of DNA synthesis in human B lymphocytes. II. Different pathways triggered by IL2 and IL4. Cell. Immunol., 146:131.

Kontny, E., A. Kvanta, and B. B. Fredholm. 1992. Activation of protein kinase C and elevation of cAMP interact synergistically to raise c-fos and AP-1 activity in Jurkat cells. Eur. J. Pharmocol., 227:333.

Kuruvilla, A., G. Putcha, E. Poulos, and W. E. Shearer. 1993. Tyrosine phosphorylation of phospholipase C concomitant with its activation by platelet-activating factor in a human B cell line. J. Immunol., 151:637.

Lanzavacchia, A. 1990. Receptor-mediated antigen uptake and its effect on antigen presentation to class II restricted T lymphocytes. Annu. Rev. Immunol., 8:773.

Lederman, S., M. J. Yellin, A. Krichevsky, J. Belko, J. J. Lee, and L. Chess. 1992. Identification of a novel surface protein on activated $CD4^+$ T cells that induces contact-dependent B cell differentiation. J. Exp. Med., 175:1091.

Lees-Miller, S. P., and C. W. Anderson. 1991. The DNA-activated protein kinase, DNA-PK: a potential coordinator of nuclear events. Cancer Res., 3:341.

Levitt, D., and M. D. Cooper. 1987. B cells In Basic and Clinical Immunology. Sixth edition., ed by D. P. stites et al. Appleton and lange, Norwalk, U.S.A.

Lindqvist, C., H. Lundstrom, C. Oker-Blom, and K. E. Akerman. 1993. Enhanced IL-4-mediated D10.G4.1 proliferation with suboptimal concentrations of anti-IL4 receptor monoclonal antobodies. J. Immunol., 150:394.

Londos, C., D. M. F. Cooper, and J. Wolff. 1980. Subclasses of external adenosine receptors. Proc. Natl. Acad. Sci. USA, 77:2551-2554.

Lycke, N. Y. 1993. Cholera toxin promotes B cell isotype switching by two different mechanisms. cAMP induction augments germ-line Ig H-chain RNA transcripts whereas membrane ganglioside GM1-receptor binding enhances later events in differentiation. J. Immunol., 150:4810.

Malefyt, R. W., J. Abrams, B Bennett, C. G. Figdor, and J. E. de Vries. 1991. Interleukin 10 (IL-10) inhibits cytokine synthesis by human monocytes: An autoregulatory role of IL-10 produced by monocytes. J. Exp. Med., 174:1209.

Marone, M., R. Petracca, and S. Viggorita. 1985. Adenosine receptors on human inflammatory cells. Int. Archs Allergy Appl. Immun. 77:259-263.

Maslinski, W., B. Remillard, M. Tsudo, and T. B. Strom. 1993. Interleukin 2 receptor signal transduction: translocation of active serine/threonine kinase Raf-1 from IL2 receptor into cytosol

depends on IL2 induced tyrosine kinase activation. Transplant. Proc., 25:109.

Massague, J., J. Andres, L. Attisano, S. Cheifetz, F. Lopez-Casillas, M. Ohtsuki, and J. L. Wrana. 1992. TGF beta receptor. Mol. Reprod Dev., 32:99.

May, L. T., U. Santhanam, S. B. Tayyer, J. M. Bhardeva, J. Ghrayeb, and P. B. Sehgal. 1988. phosphorylation of secreted forms of human beta 2 interferon/hepatocyte stimulatory factor/interleukin 6. Biochem. Biophysics. Res. Commun., 152: 1144.

Mckenzie, A. N. J., J. A. Culpepper, R. W. Malefyt, F. Briere, J. Punnonen, G. Aversa, A. Sota, W. Dang, B. G. Cocks, S. Menon, J. E. de Vries, J. Banchereau, and G. Zurawski. 1993 Interleukin 13, a T cell-derived cytokine that regulates human monocyte and B cell function. Proc. Natl. Acad. Sci. USA, 90:3735.

Minami, Y., T. Kono, K. Yamada, N. Kobayashi, A. Kawahara, R. M. Perlmutter, and T. Taniguchi. 1993. Association of p^{56lck} with IL2 receptor beta chain is critical for the IL2 induced activation of p^{56lck}. EMBO J., 12:759.

Miyajima, A., T. Kitamura, N Harada, T. Yokota, and K. Arai. 1992. Cytokine receptors and signal transduction. Annu. Rev. Immunol., 10:295.

Moller, G. 1992. Germinal centers in the immune response. Immunol. Rev., 126:5.

Monroe, J. G., V. L. Seyfert, C. S. Owen, and N. Sykes. 1989. Isolation and characterization of a B lymphocyte mutant with altered signal transduction through its antigen receptor. J. Exp. Med., 169: 1059.

Morla, A. O., J. Schreurs, A. Miyajima, and L. Y. J. Wang. 1988. Hematopoietic growth factors activate the tyrosine phosphorylation of distinct sets of proteins in interleukin 3 dependent murine cell lines. Mol. Cell. Biol., 8:22214.

Murakami, M., M. Hibi, N. Nakagawa, T. Nakagawa, K. Yasukawa, K. Yamanishi, T. Taga, and T. Kishimoto. 1993. IL6-induced

homodimerization of gp130 and associated activation of a tyrosine kinase. Science, 260:1808.

Nakagawa, N., T. Nakagawa, D. J. Volkman, J. L. Ambrus, and A. S. Fauci. 1987. The role of interleukin 2 in inducing Ig production in a pokeweed mitogen-stimulated mononuclear cell system. J. Immunol., 138:795.

Nakagawa, T., N. Nakagawa, D. J. Volkman, and A. S. Fauci. 1986. Sequential synergistic effect of interleukin 2 and interferon-γ on the differentiation of a Tac-antigen-positive B cell line. J. Immunol., 136,164.

Nichizuka, Y. 1986. The role of protein kinase C in cell surface signal transduction and tumor promotion. Nature, 308: 693.

Noelle, R. J., J. A. Ledbetter, and A. Aruffo. 1992. CD40 and its ligand, an essential ligand-receptor pair for thymus-dependent B cell activation. Immunol. Today, 13:431.

Nordan, R. P., and M. Potter. 1986. A macrophage-derived factor require by plasmacytomas for survival and proliferation in vitro. Science, 233:566.

Nordstedt, C., A. Kvanta, I. Van-der-Ploeg, and B. B. Fredholm. 1989. Dural effects of protein kinase C on receptor stimulated cAMP accumulation in a human T cell leukemia line. Eur. J. Pharmocol., 172:51.

Owaki, H., R. Varma, B. Gillis, J. T. Bruder, U. R. Rapp, L. S. Davis, and T. D. Geppert. 1993. Raf-1 is required for T cell IL2 production. EMBO J, 12:4367.

Padeh, S., A. Levitzki, A. Gazit, G. B. Mills, and C. M. Roifman. 1991. Activation of phospholipase C in human B cells is dependent on tyrosine phosphorylation. J. Clin. Invest., 87:1114.

Park, D. J., H. K. Min, and S. G. Rhee. 1992. Inhibition of CD3 linked phospholipase C by phorbol ester and by cAMP is associated with decreased phosphotyrosine and increased phosphoserine contents of PLC-gammar 1. J. Biol. Chem., 267:1496.

Patke, C. L., F. M. Orson, and W. Shearer. 1991. Cyclic AMP-mediated modulation of immunoglobulin production in B cells prostaglandin E. Cell. Immunol., 137:36.

Perlmutter, R. M., S. D. Levin, M. W. Appleby, S. J. Anderson, and J Alberola-Ila. 1993. Regulation of lymphocyte function by protein phosphorylation. Annu. Rev. Immunol., 11:451.

Plagemann, P.G.W., R.M. Wohlhueter, and J. Erbe. 1981. Facilitated transport of inosine and uridine in cultured mammalian cells is independent of nucleoside phosphorylases. Biochim. Biophys. Acta, 640:448-462.

Pobiner, B. F., J. K. Northup, P. H. Bauer, E. D. Fraser, and J. C. Garrison. 1991. Inhibitory GTP-binding regulatory protein Gi3 can couple angiotensin II receptors to inhibition of adenylyl cyclase in hepatocytes. Mol. Pharmacol., 40:156.

Prigent, A. F., P. Fonlupt, M. Dubois, G. Nemoz, L. Timouyasse, H. Pacheco, Y. Pacheco, N. Biot, and M. Fayolle. 1990. cyclic nucleotide phosphodiesterase and methyltransferases in purified lymphocytes, monocytes and polymorphonuclear leukocytes from healthy donors and asthmatic patients. Eur. J. of clin. Invest., 20: 323.

Rapp, U. R. 1991. Role of Raf-1 serine/threonine kinase in growth factor signal transduction. Oncogene, 6:495.

Ravichandran, K. S., and S. J. Burakoff. 1994. Evidence for differential intracellular signaling via CD4 and CD8 molecules. J. Exp. Med., 179:727.

Rawlings, D. J., D. C. Saffran, S. Tsukada, D. A. Largaespada, J. C. Grimaldi, L. Cohen, R. N. Mohr, J. F. Bazan, M. Howard, N. G. Copeland, N. A. Jenkins, and O. N. Witte. 1993. Mutation of unique region of Bruton's tyrosine kinase in immunodeficient XID mice. Science, 261:358.

Rigley, K. P., S. M. Thurstan, and R. E. Callard. 1991. Independent regulation of interleukin 4 (IL4)-induced expression of human B cell surface CD23 and IgM: functional evidence for two IL4 receptors. Int. Immunil., 3:197

Roifman, C. M., and G. Wang. 1992a. Phospholipase C-gamma1 and phospholipase C-gamma 2 are substrates of the B cell antigen receptor associated protein tyrosine kinase. Biochem. Biophys. Res. Commun., 183:411.

Roifman, C. M., and G. Wang. 1992b. Receptor-mediated activation of human B lymphocytes in a nonphosphotyrosine-dependent manner. J. Immunol., 149:1179.

Roifman, C. M., K. Chin, A. Gazit, G. B. Mills, C. Gilon, and A. Levitzki. 1991. Tyrosine phosphorylation is an essential event in the stimulation of B lymphocytes by Staphylococcus aureus Cowan 1. J. Immunol., 146:2965.

Rosales, C., and E. J. Brown. 1992. Signal transduction by neutrophil immunoglobulin G Fc receptors. Dissociation of intraplasmic calcium concentration rise from inositol. J. Biol. Chem., 267:5265.

Rousset, F., D. W. Malefijt, R. Slierendregt, B. Aubry, J-P. Bonnefoy, J. Y. Defrance, T. Banchereay, and D. Vaies, J. E. 1988. Regulation of Fc receptor for IgE (CD23) and class II MHC antigen expression on Burkitt's lymphoma cell lines by human IL4 and IFN-γ. J. Immuno., 140:2625.

Rousset, F, E., Garcia, T. Defrance, C. Peronne, N. Vezzio, D. Hsu, R. Kastelein, K. W. Moore, and J. Benchereau. 1992. Interleukine 10 is a potent growth and differentiation factor for activated human B lymphocytes. Proc. Natl. Acad. Sci. USA, 89:1890-1893.

Rudd, C. E., P. Anderson, C. Morimoto, M. Streuli, and S. E. Schlossman. 1989. Molecular interactions, T cell subsets and a role of the CD4/CD8: $p56^{lck}$ complex in human T cell activation. Immunol. Rev., 111:2965.

Saiki, O., and P. Ralph. 1981. Induction of human immunoglobulin secretion . I. Synergistic effect of B cell mitogen Cowan I plus T cell mitogens or factors. J. Immuno., 127:1044.

Saklatvala, J., and F. Guesdon. 1992. Interleukin 1 and tumor necrosis factor signal transduction mechanisms: potential targets for pharmacological control of inflammation. J. Rheumatol. Suppl. 32:65.

Schultz, L. A., G. M. Kammer, and S. A. Rudolph. 1988. Characterization of the human T lymphocyte adenosine receptor: comparison of normal and systemic lupus erythematous cells. FASEB J., 2:224-250.

Sharmas, S., S. Mehta, J. Morgan, and A. Maizel. 1987. Molecular cloning and expression of a human B cell growth factor gene in Escherichia coli. Science. 235:1489.

Shearer, W. T., C. L. Patke, E. B. Gilliam, H. M. Rosenblatt, K. S. Barron, and F. M. Orson. 1988. Modulation of a human lymphoblastoid B cell line by cyclic AMP. Ig secretion and phosphotidylcholine Metabolism. J. Immunol., 141:1678.

Sherris, D., W. Stohl, and L. Mayer. 1989. Characterization of lymphokines mediating B cell growth and differentiation from monoclonal anti-CD3 antibody-stimulated T cells J. Immunol., 142:2343.

Siegel, J. P., and H. S. Mostowski. 1990. A bioassay for the measurement of human interleukin 4. J. Immunol. Methods, 132:287.

Simonsson, A. C., J. W. Larrick, and C. A. Borrebaeck. 1991. In vitro immunization of human B lymphocytes. Mapping of lymphokine specific mRNA and the effect of recombinant factors. Hum. Antibodies Hybridomas, 2:148.

Sims, C., K. Ashby, and J. G. Douglas. 1992. Angiotensin II-induced changes in guanine nucleotide binding and regulatory proteins. Hypertension. 19:146.

Smith, C. J., B. Baskin, P. Humire-Greiff, J. N. Zhou, P. G. Olsson, H. S. Maniar, P. Kjellen, J. D. Lambris, B. Christensson, L. Hammarstrom, et al. 1994. Expression of Bruton's agammaglobulinemia tyrosine kinase gene, BTK, is selectively down-regulated in T lymphocytes and plasma cells. J. Immunol., 152:557.

Sozeri, O., K. Vollmer, M. Liyanage, D. Frith, G. Kour, G. E. Mark, and S. Stabel. 1992. Activation of the c-Raf protein kinase by protein kinase C phosphorylation. Oncogene, 7:2259.

Splawski, J. B., S. M. Fu, and P. E. Lipsky. 1993. Immunoregulatory role of CD40 in human B cell differentiation. J. Immunol., 150:1276.

Stein, S. H., and R. P. Phipps. 1990. Elevated levels of intracellular cAMP sensitize resting B lymphocytes to immune complex-induced unresponsiveness. Eur. J. Immunol., 21:313.

Stilest, G.L. 1992. Adenosine receptors. J. Biol. Chem., 267:6451-6454.

Swain, S. L., and R. W. Dutton. 1982. Production of a B cell growth-promoting activity, (DL)BCGF, from a cloned T cell line and its assay on the BCL1 B cell tumor. J. Exp. Med., 156:1821.

Takahama, Y., S. Ono, K. Ishihara, M. Muramatsu, and T. Hamaoka. 1989. Disparate functions of I-A and I-E molecules on B cells as evidenced by the inhibition with anti-I-A and anti-I-E antibodies of polyclonal B cell activation. Eur. J. Immunol., 19:2227.

Tamaki, T., Y. Kanakura, A. Kuriu, H. Ikeda, H. Mitsui, H. Yagura, I. Matsumura, B. Druker, J. D. Griffin, and Y. Kaanaayama. 1992. Surface immunoglobulin-mediated signal transduction involves rapid phosphorylation and activation of prooncogene product Raf-1 in human B cells. Cancer Res., 52:566.

Taylor, S. S., J. A. Buechler, and W. Yonemoto. 1990. cAMP-dependent protein kinase: framework for a diverse family or regulatory enzymes. Annu. Rev. Biochem, 59:971.

Thompson, P. A., J. A. Ledbetter, U. R. Rapp, and J. B. Bolen. 1991. The Raf-1 serine/threonine kinase is a substrate for the p^{56lck} protein tyrosine kinase in human T cells. Cell Growth Differ., 2:609.

Tigges, M. A.L. S. Casey, and M. E. Koshland. 1989. Mechanism of interleukin 2 signaling: mediation of different outcomes by a single receptor and transduction pathway. Science, 243:781.

Tohma, S., and P. E. Lipsky, 1991. Analysis of the mechanisms of T cell-dependent polyclonal activation of human B cells. J. Immunol., 146:2544.

Ullich, A., and J. Schlessinger. 1990. Signal transduction by receptors with tyrosine kinase activity. Cell, 61:203.

Vazquez, A., M. T. Autlredow, P. Galanaud, and G. Leca. 1991. Modulation of IL2 and IL4-dependent human B cell proliferation by cyclic AMP. J. Immunol., 146:4222.

Vercelli, D., H. H. Jabara, B. Lee, N. Woodland, R. S. Geha, and D. Y. M. Leung. 1988. Human recombinant interleukin 4 induces FcεR2/CD23 on normal human monocytes. J. Exp. Med., 167:1406.

Weiss, E. R., D. J. Kelleher, C. W. Woon, S. Soparkar, S. Osawa, L. E. Heasley, and G. L. Johnson. 1988. Receptor activation of G proteins. FASEB J, 2:2841.

Whetton, A. D., P. N. Monk, S. D. Consalvey, S. J. Huang, T. M. dexter, and C. P. Downes. 1988. Interleukin 3 stimulates proliferation via protein kinase C without increasing inositol lipid turnover. Proc. Natl. Acad. Sci. USA, 85:3284.

Yamasaki, K., T. Taga, Y. Hirata, H. Yawata, Y. Kawanishi, B. Seeds, T. Taniguchi, T. Hirano, and T. Kishimoto. 1988. Cloning and expression of the human interleukin 6 (BSF-2/INFb2) receptor. Science, 241:825.

Yawata, H., K. Yasukawa, S. Natsuka, M. Murakami, K. Yamasaki, M. Hibi, T. Taga, and T. Kishimoto. 1993. Structure function analysis of human IL6 binding and for IL6 signal transduction trough gp130. EMBO J., 12:1705.

Zhang, Ke., E. A. Clark, and A. Saxon. 1991. CD40 stimulation provides an IFN-γ independent and IL4-dependent differentiation signal directly to human B cells for IgE production. J. Immunol., 146:1836-1842.

Zurawski, G., and J. E. de Vries. 1994. Interleukin 13, an interleukin 4-like cytokine that acts on monocytes and B cells, but not on T cells. Immunol. Today, 15:19.