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IL-6-induced human B cell differentiation

Natkunam, Yasodha, Ph.D.

City University of New York, 1993

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IL-6-INDUCED HUMAN B CELL DIFFERENTIATION

by

Yasodha Natkunam

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

1993

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

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ABSTRACT

IL-6-INDUCED HUMAN B CELL DIFFERENTIATION

by

Yasodha Natkunam

Advisor: Selina Chen-Kiang, Ph.D.

Terminal differentiation of B cells has been studied in an (Interleukin-6) IL-6-inducible system. We demonstrate that the hallmarks of terminal B cell differentiation are recapitulated in an Epstein Barr Virus-immortalized human lymphoblastoid cell line, CESS, induced by IL-6. IL-6 signaling leads to marked enhancement of synthesis of immunoglobulin (Ig) mRNAs encoding the secreted form-specific Ig heavy-chain. The enhanced mRNA synthesis leads to the increased synthesis and secretion of IgG. The regulation of Ig synthesis in CESS cells is subject to feedback control upon long term stimulation by IL-6. The IL-6-induced cells exhibit dramatic and stage-specific alterations in cell morphology which are characteristic of plasma cells *in vivo*. This implies that IL-6 not only regulates the molecular and biochemical markers of B cell differentiation, but also the architecture of the cell.

The mRNA encoding transcription factor, Oct-2, is temporally regulated by IL-6 in CESS cells, and is subject to feedback control upon long term IL-6 stimulation, suggesting a role for Oct-2 in mediating the IL-6-signals in the activation of Ig gene transcription. The steady state levels of mRNAs encoding the ligand binding (gp80)

and the signal transducing (gp130) subunits of the IL-6 receptor are also regulated and subject to feedback control upon long term IL-6 induction. This regulation suggests a possible mechanism for modulating IL-6 signaling in B cells during terminal differentiation. Induction of differentiation by IL-6 occurs in freshly isolated human tonsillar B cells after Epstein-Barr virus immortalization, and is not restricted to an Ig isotype. Thus, the IL-6-inducible CESS cell system provides a model to study the molecular mechanisms which underlie terminal B cell differentiation.

IL-6 regulates the expression of major histocompatibility complex (MHC) class II molecules in human B cells. IL-6-differentiated cells decrease the expression of MHC class II on the cell surface, which reflects another hallmark of plasma cells. This reduction in the surface expression of MHC class II is not restricted to a MHC class II locus. The reduction of surface MHC class II expression on differentiated cells is inversely correlated with the enhanced synthesis of IgG, as assessed by intracellular immunofluorescence. The mechanisms which regulate the reduction of MHC class II in differentiating B cells is primarily downstream of mRNA synthesis. The diminution of MHC class II on the cell surface corresponds to the enhanced synthesis of an endoplasmic reticulum-resident stress protein, GRP94, which is thought to bind MHC class II molecules in the endoplasmic reticulum. Our results suggest that the synthesis of GRP 94 may be preferentially induced by IL-6, and may be involved in the regulation of MHC class II expression during terminal B cell differentiation.

FORMAT OF THESIS

This thesis was prepared in accordance with guidelines of the City University of New York. Together with results obtained by Ziying Liu and Xiao Kui Zhang, the data contained in Chapter III has been submitted for publication. Chapters IV and V contain unpublished results. Each chapter contains an abstract, introduction, results and discussion, with a general introduction and conclusions at the beginning and end of the thesis. To minimize redundancy, the Materials and Methods, and Bibliography sections have been consolidated.

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I

INTRODUCTION

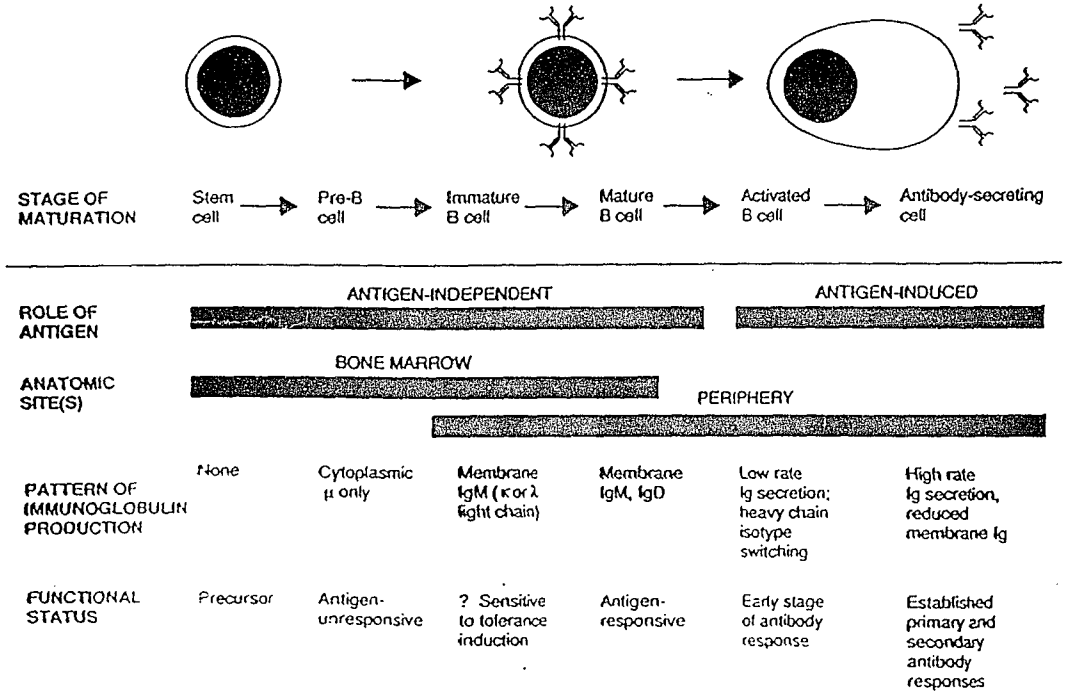
AN OVERVIEW OF B CELL DEVELOPMENT

The development of cells in the B lineage occurs in two phases. The first phase is antigen-independent, and in mammals, takes place in the fetal liver and in the adult bone marrow. This phase involves the development of pluripotent stem cells into B lymphocytes which are characterized by the expression of membrane-anchored immunoglobulin (mIg). In the transition from stem cells to B cells, DNA rearrangements lead to the assembly of functional transcription units encoding the heavy (H) and light (L) chains of Ig (Alt et al., 1987). These rearrangements occur in a very specific order: D_H to J_H, V_H to D_HJ_H, and V_L to J_L (where V, D, and J denote the variable, diversity, and joining regions of Ig), and generate productively rearranged transcription units from the germ-line configuration of the Ig heavy and light chain loci. As shown in Figure 1, the transcription of these rearranged loci lead to the expression of cytoplasmic Ig heavy chains (IgH), followed by cytoplasmic Ig light chains (IgL), and finally to the expression of mIg molecules in successive stages of B cell development (reviewed in Tonegawa et al., 1983; Honjo et al., 1983; Rolink and Melchers, 1991). Cells which generate non-productively rearranged alleles or which express mIgs of autoreactive or undesirable specificities are functionally silenced or deleted (Goodnow et al., 1988; Nemazee and Burki, 1989; Rolink et al., 1991).

Figure 1. Schematic representation of B cell development

[After Abbas, et al., 1991]

The ordered rearrangement of variable (V), diversity (D), and joining (J) regions during the maturation of B lymphocytes begin with D_H to J_H rearrangements at both heavy chain (H) loci. Then V_H to DJ_H rearrangements occur, and lead to cytoplasmic μ heavy chain expression in Pre-B cells. Subsequently, the light chain (L) loci rearrange (V_κ to J_κ and V_λ to J_λ) and the resulting Ig molecules are expressed on the surface of mature B cells. When mature B cells encounter the antigen to which their variable region is specific, they are activated to proliferate and differentiate into antibody-secreting plasma cells.



B cells which express mIg migrate to peripheral lymphoid organs such as the spleen, tonsils and lymph nodes. If further stimulation does not occur these cells remain as resting B cells. The second phase of B cell development is antigen-dependent and involves the specific binding of antigens to mIg molecules. This binding acts as a trigger to stimulate B cells to proliferate and terminally differentiate into antibody-secreting plasma cells. The secreted Ig (sIg) and mIg differ at the carboxyl termini of their heavy-chain polypeptides. These polypeptides are encoded by two alternatively spliced mRNAs which differ at their 3' ends (Alt et al., 1980).

B cell development progresses through a specific program of gene expression. This program involves changes in transcriptional activity and extracellular signals which modulate them. Transcriptional regulation appears to occur during all stages of B cell development: the step of commitment of pluripotent stem cells to the B cell lineage, the development of pre-B cells to mature B cells, and during the terminal differentiation of B cells to plasma cells. How the B lineage-specific and stage-appropriate developmental program unfolds has been the subject of intense investigation.

THE ROLE OF LYMPHOKINES IN B CELL DIFFERENTIATION

Lymphokines, also known as cytokines, are protein effector molecules produced during natural (innate) and specific (acquired) immunity, which regulate immune and inflammatory responses (as

defined by Abbas et al., 1991). Originally, cytokines were thought to be synthesized by leukocytes and mediate functions of leukocytes, which resulted in these molecules being called interleukins (ILs). Since cytokines were secreted by lymphoid cells during an immune response, they were also referred to as lymphokines. A clear understanding of lymphokine function began to emerge when several of these factors were cloned, and their pleiotropic roles investigated.

During B cell ontogeny, several lymphokines participate in regulating the different stages of B cell development. In the bone marrow hematopoietic stem cells and pre-B cells require lymphokines IL-3, IL-7 and IL-11 for growth and maturation (Namen et al., 1988; Itoh et al., 1990; Era et al., 1991; Rolink et al., 1991; Paul et al., 1990). The antigen-dependent phase of B cell differentiation also involves regulation by lymphokines. Initial observations suggested that the immune response to sheep red blood cells was T cell-dependent, and it was postulated that a factor produced by T cells (T cell replacing factor or TRF), could potentiate B cell proliferation and/or differentiation (Dutton et al., 1971; Schimpl and Wecker, 1972). Subsequently, Kishimoto et al. (1975), and Parker et al. (1979), showed that two signals, one from crosslinking mIgs, and the other from T cell-derived factors, cooperate to promote antibody secretion. Several cDNAs corresponding to these factors have been cloned and their participation in B cell regulation has been confirmed. IL-4, together with lipopolysaccharide (LPS), promotes class-switching by

inducing the rearrangement of γ1 and ε genes (Lutzker et al., 1988; Stavenezer et al., 1988). Recombinant IL-2 and IL-5 activate transcription of the gene encoding the joining component (J chain) of IgM and regulate the assembly and secretion of pentameric IgM (Tigges, et al., 1989; McFadden and Koshland, 1992). The role of IL-6 in terminal B cell differentiation has been confirmed by studies showing that IL-6 induces murine B cells to secrete a low level of Igs (Muraguchi et al.,1988).

Lymphokines act through specific receptors expressed on target cells. They exhibit agonistic or antagonistic effects on the functions of one another (McFadden and Koshland, 1992; Splawski et al., 1990; Kimata et al., 1992). Although many lymphokines are pleiotropic, their roles are specific with regard to cell type and to the stage of differentiation.

INTERLEUKIN 6

IL-6 was initially known as B cell differentiation Factor (BCDF, Hirano et al., 1985), B cell stimulatory factor (BSF-2, Hirano et al., 1986), interferon β_2 (IFN β_2, Zilberstein et al., 1986) and hepatocyte stimulatory factor (HSF, Andus et al., 1987), according to its biological functions. Molecular cloning (Hirano et al., 1986) indicated that all these factors were the same, and were designated IL-6. Human IL-6 consists of 212 amino acids encoded by 1100 nucleotides. Hydrophobic amino acids abundant in the first 28 amino acids specify a typical signal peptide required for the secretion of IL-6 (Hirano et al., 1986). The genomic genes of human and mouse

IL-6 share 60% sequence homology in the coding region, and 80% in the 300 nucleotides of the 5' flanking region and also in the 3' untranslated region. Both genes contain five exons and four introns, and exhibit a high degree of homology with the gene encoding the granulocyte colony stimulating factor (Yasukawa et al., 1987). A stretch of AT-rich nucleotides at the 3'- untranslated region of the IL-6 gene appears to regulate selective mRNA degradation. Figure 2 summarizes the 5'-flanking region of the human IL-6 gene. Several DNA motifs and stimuli responsible for the regulation of the IL-6 gene are indicated.

IL-6 is synthesized by many cell types. The molecular mass of human IL-6 varies in the range of 19-30 kDa, due to cell type-specific modifications of the protein by *O*-glycosylation, *N*-glycosylation, and serine-phosphorylation at several potential sites. IL-6 regulates the acute phase response, the immune response and hematopoiesis, and potentiates cell growth and differentiation in a cell type-specific manner. A variety of cells including T cells, B cells, hepatocytes, hematopoietic stem cells, nerve cells and mesangial cells respond to IL-6 stimulation. The multiple functions of IL-6 are summarized in Table 1.

The constitutive or unregulated production of IL-6 is implicated in the pathophysiology of several human diseases which include autoimmune diseases and malignancies (Table 2).

Figure 2. Schematic representation of the IL-6 promoter

[After Hirano et al., 1990.]

Potential transcriptional regulatory elements within the IL-6 promoter include glucocorticoid response elements (GRE), an Activator Protein-1 (AP-1) binding site, a *c-fos* serum response element (SRE) homology, a cyclic AMP response element (CRE), a NF-κB binding site regulated by IL-1 and tumor necrosis factor (TNF), a multi-response element (MRE) within the SRE regulated by IL-1, TNF, forskolin, serum, and phorbol ester (TPA), and an NF-IL-6 binding site regulated by IL-1, IL-6, TNF, and lipopolysaccharide (LPS).

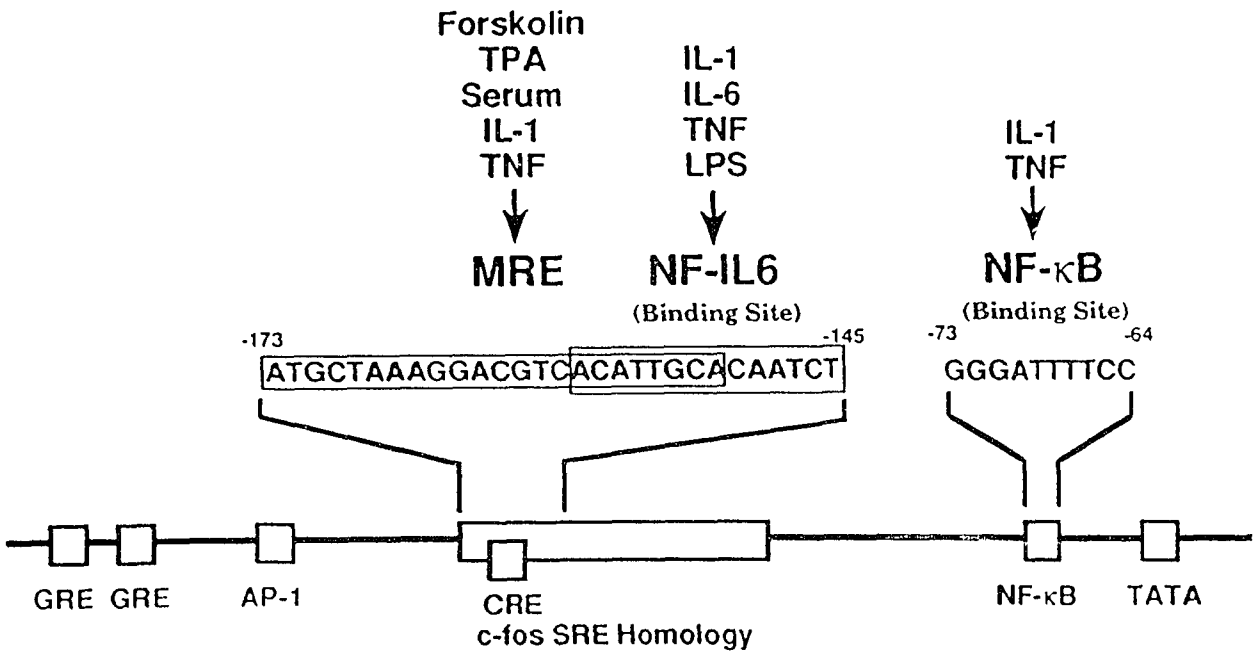


TABLE 1. THE FUNCTIONS OF IL-6

[After Hirano and Kishimoto, 1989; Hirano et al., 1990; Sehgal, 1990]

CELL PROLIFERATION AND DIFFERENTIATION

(a) Lymphoid

Induces B cell differentiation and Ig secretion.
Stimulates growth of plasmacytomas/myelomas/hybridomas and certain EBV-transformed B cell lines.
Stimulates proliferation and differentiation of cytotoxic T cells.

(b) Myeloid

Stimulates proliferation of hematopoietic stem cells.
Inhibits growth of certain myeloid leukemic cell lines.

(c) Other

Inhibits the growth of certain breast carcinoma cell lines.
Inhibits endothelial cell proliferation.
Induces macrophage differentiation.
Induces neuronal differentiation of PC12 cells.
Induces megakaryocyte maturation.
Enhances proliferation of keratinocytes and mesangial cells.

SYSTEMIC EFFECTS

Induces the mediators of the acute phase response in hepatocytes.
Elevates body temperature.
Stimulates the release of glucocorticoids and corticotropin-releasing factor.
Regulates metabolism.

TABLE 2. IL-6 AND HUMAN DISEASES

[After Hirano and Kishimoto, 1989; Hirano et al., 1990; Sehgal, 1990]

PROLIFERATIVE DISEASES

Psoriasis: keratinocytes secrete elevated levels of IL-6.
Mesangial proliferative glomerulonephritis: elevated systemic levels of IL-6 promote proliferation of mesangial cells.

AUTOIMMUNE DISEASES AND MALIGNANCIES

Cardiac myxoma
Castleman's disease
Rheumatoid arthritis
AIDS
Multiple myeloma
Lennert's T cell lymphoma
Hodgkin's lymphoma
Non-Hodgkin's lymphoma
Kaposi's sarcoma
Uterine cervical carcinoma

(The circulating levels of IL-6 are elevated in these diseases. The mechanism of action of IL-6 in causing these diseases is unknown.)

INFECTIONS

Meningitis: elevated levels of IL-6 in cerebrospinal fluid.
Intraamniotic infection: elevated levels of IL-6 in amniotic fluid.
Acute bacterial and viral infection: elevated levels of IL-6 in serum.

Of particular interest is the role of IL-6 in the generation of polyclonal and monoclonal plasma cell abnormalities. Plasmacytomas can be induced in BALB/c mice by the intra-peritoneal injection of mineral oil or pristane, both of which produce chronic inflammation and the secretion of IL-6. These plasmacytoma cells contain a reciprocal chromosomal translocation which brings the *c-myc* locus adjacent to the Ig gene locus (Nordan and Potter, 1986; Kawano et al., 1988). In C57BL/6 transgenic mice the overexpression of a human IL-6 cDNA under the control of the IgH enhancer results in plasmacytosis (Suematsu et al.,1989). In BALB/c transgenic mice, however, plasmacytomas with the chromosomal translocation t(12;15) were obtained (Suematsu et al., 1992). These transgenic studies suggest that the susceptibility to plasmacytomas may be genetically determined, and confirm that IL-6 plays a role in cell proliferation and oncogenesis.

THE IL-6 RECEPTOR SIGNAL TRANSDUCTION PATHWAY

IL-6 mediates its pleiotropic functions in different cell types through the heterodimeric IL-6 receptor (IL-6R). The gp80 and gp130 subunits of the IL-6R have been cloned and characterized (Yamasaki et al., 1988; Hibi et al., 1990). The gp80 subunit binds IL-6 and triggers the association of gp80 with the signal transducing subunit, gp130, which leads to the formation of a high affinity IL-6R (Taga et al., 1989; Hibi et al., 1990). This leads to a rapid and transient phosphorylation on tyrosine residues of gp130 (Nakajima and Wall, 1991; Lord et al., 1991; Ip et al., 1992). Recent

evidence suggests that the binding of IL-6 to gp80 may induce disulfide-linked homodimerization of gp130 which is associated with tyrosine phosphorylation of gp130 dimers (Murakami et al., 1993). The extracellular region of gp130 possesses structural features common to the receptors of prolactin, growth hormone, IL-2 (β chain), IL-3, IL-4, IL-7, GM-CSF, and G-CSF, and thus is classified as a member of the cytokine receptor superfamily (Hibi et al., 1990). Proline-X-Proline (where X represents any amino acid) is a conserved motif in the intracytoplasmic regions of several members of the cytokine receptor superfamily. Substitutions of the two proline residues by serine residues in this motif of gp130, abolished the signaling capacity of gp130 in prò-B cell transfectants. This motif appears to be important for triggering tyrosine kinase activation and signal transduction (Murakami et al., 1993). The gp130 subunit is shared by at least three other related cytokines, leukemia inhibitory factor (LIF), ciliary neurotropic factor (CNTF) and oncostatin M (Ip et al., 1992; Gearing et al., 1992; Taga et al., 1992). Unlike IL-6, signaling induced by LIF and CNTF lead to heterodimerization of gp130 and LIFRβ, a LIF-binding protein which resembles gp130 in structure, and the formation of high affinity receptors (Davis et al., 1993).

Very little is known about the cytoplasmic events which follow. Recent evidence suggests that in NIH 3T3 cells a *ras*-dependent, mitogen-activated protein (MAP) kinase may be involved in the phosphorylation of an IL-6-activated transcription factor, NF-IL6 (Nakajima et al., 1993).

Some of the nuclear targets of IL-6R signaling have been investigated. Immediate early response genes belonging to the AP-1 transcription factor family are induced in a cell type-dependent manner upon IL-6 stimulation. While *junB* is activated in B cell hybridomas (Nakajima and Wall, 1991), *junB*, *c-jun* and *junD* are regulated in M1 myeloid leukemia cells (Lord et al., 1991). Two members of the C/EBP family of transcription factors, NFIL-6 and NFIL-6β, are also induced by IL-6 (Akira et al., 1990; Konishita et al., 1992). NFIL-6 and NFIL-6β are encoded by intronless genes. They possess bipartite DNA-binding domains which contain a stretch of basic residues which allow interaction with DNA, adjacent to five heptad repeats of leucine residues at their carboxyl termini. These leucine zipper structures share a high degree of homology, and allow parallel coiled-coil interactions (O'Shea et al., 1989) among members of the C/EBP family (Landschulz et al., 1989). NFIL-6 and NFIL-6β bind to the regulatory regions of IL-6-inducible promoters as homo- or heterodimers and activate transcription in a synergistic manner (Konishita et al., 1992).

These studies show that some of the members of the AP-1 and C/EBP families which are induced by IL-6R signaling may regulate IL-6-inducible promoters. Since IL-6 activates Ig genes during B cell differentiation by transcriptional initiation (Raynal et al., 1989), transcription factors in the AP-1 and C/EBP families are likely intermediates in IL-6-induced activation of Ig genes. These transcription factors may interact with one another and with other transcription factors regulating Ig gene transcription. The p50

polypeptide of NF-κB, a member of the Rel family of transcription factors can associate with NF-IL-6 *in vitro* (LeClair et al., 1992). To understand how IL-6 regulates its target genes, further investigations of protein-protein interactions between IL-6-activated transcription factors, as well as their role in modulating the IL-6 signals, will be necessary.

THE B CELL ANTIGEN RECEPTOR COMPLEX

In murine and human B cells the IL-6R signal transduction pathway can provide one of the two signals required for terminal B cell differentiation (Kishimoto et al., 1975; Muraguchi et al., 1988; Raynal et al., 1989). The second signal required for B cell differentiation is derived from antigens that crosslink Ig molecules on the surface of B cells. This signal is specific to each B cell because the variable regions of Ig molecules are unique to a given antigen. The failure to crosslink mIgs appear to result in inefficient induction of Ig secretion (Parker et al., 1980). This observation suggests an essential role for mIg signaling in B cell differentiation.

Throughout B cell ontogeny, mechanisms exist to eliminate B cells which are non-functional or redundant. This process is regulated at each step of B cell development through interactions between B cell surface markers and signals from the extracellular matrix or stroma. At each stage, B cells bearing a specific surface marker are positively selected or programmed to die (Melchers et al., 1989; Rolink and Melchers, 1991). The most important surface marker involved in determining the maturation of B cells as they progress through successive stages of development is the mIg.

The mIgs expressed on B cells function as antigen-specific receptors. They transduce signals which initiate the processing of antigens which are presented to T cells, and induce resting B cells to proliferate and differentiate into plasma cells (Campbell et al., 1991). The mIgs can also inhibit the growth of immature B cells by functional silencing or by deletion of autoreactive B cell clones (Goodnow et al., 1988; Nemazee and Burki, 1989). The degree of mIg crosslinking facilitates qualitatively distinct signals which lead to clonal anergy or deletion (Hartley et al., 1991). The crosslinking of mIgs results in rapid plasma membrane depolarization, phosphatidylinositol turnover and [Ca^{2+}] mobilization (Monroe and Cambier, 1983; Fahey and DeFranco, 1987), whereas monovalent anti-Ig molecules fail to elicit these responses (Parker et al., 1980; Monroe and Cambier, 1983). Thus, the function of mIgs appear to depend on the stage of B cell development and on the efficiency of crosslinking.

The cytoplasmic domains of mIgs are completely conserved among different Ig isotypes, and between man and mouse. Mutational analyses indicate that intact transmembrane and cytoplasmic domains are essential for the functions of mIg, and that these functions can be uncoupled by specific mutations (Shaw et al., 1990). The three amino acid long cytoplasmic tail of the mIg has no direct signal transducing ability. An mIg-associated complex transmits signals elicited through the B cell antigen receptor. By subtractive hybridization and transfection studies, accessory molecules needed for the surface expression of IgM have been

identified. The IgM molecule is expressed on the surface of B cells in noncovalent association with IgM-α and Ig-β, which are encoded by the mb-1 and B29 genes (Hombach et al., 1988, 1990; Sakaguchi et al., 1988). All five Ig classes associate with IgMα, although their dependence on the α/β complex for membrane expression differs (Venkitaraman et al., 1991). B29 and mb-1 are expressed selectively in the B lineage. Whereas B29 is expressed throughout all stages of B cell development, mb-1 expression is restricted to pre-B and mature B cells. When B cells differentiate into plasma cells, mb-1 expression is thought to diminish (Sakaguchi et al., 1988).

The antigen receptor complex, together with signals elicited through lymphokine receptors, orchestrates B cell differentiation. These signals initiate the two major functions of B cells: antigen presentation and Ig synthesis. Although the antigen receptor complex is triggered upon crosslinking by antigens, for the presentation of antigenic peptides to T cells another molecule expressed on the surface of B cells, the MHC class II molecule, is required.

MHC CLASS II MOLECULES AND B CELL DIFFERENTIATION

MHC class I and class II molecules are cell surface glycoproteins which present antigenic peptides to T cells. Antigens generated within the target cell are processed and presented to $CD8^+$ T cells by MHC class I molecules which are expressed in almost all nucleated cells (Monaco, 1992). MHC class II molecules are

expressed in specialized cells known as antigen presenting cells which include B cells, macrophages, and dendritic cells. The MHC class II molecules associate with extracellularly derived antigens which are internalized and processed by proteolytic degradation, primarily by the lysosomal compartment (Neefjes and Ploegh, 1992). These peptides are presented to $CD4^+$ T cells. Transgenic mice lacking MHC class II molecules show an almost complete elimination of $CD4^+$ T cells which suggests that these molecules are essential for the positive selection of T cell subsets. Although the B cells of these mice are capable of differentiating into plasma cells, there is reduced production of serum IgG1 and lack of germinal centers in lymphoid organs (Cosgrove et al., 1991). This observation implies that in the absence of class II B cell differentiation is impaired.

MHC class II molecules are heterodimers composed of highly polymorphic α and β chains. In the B lineage, the expression of MHC class II is developmentally regulated. The surface expression of MHC class II occurs after mIgM in murine B cells, but precedes the expression of mIgM in the human. The MHC class II genes are a family of clustered genes whose expression is coordinately regulated at the transcriptional level (Kara and Glimcher, 1992). Transcriptional regulation of only a subset of the MHC class II promoters (Ono et al., 1991), and posttranscriptional mechanisms which may participate in the regulation of MHC class II (Schaiff et al., 1992), have also been reported. Several exogenous stimuli including lymphokines and glucocorticoids appear to modulate MHC class II expression (reviewed in Glimcher and Kara, 1992)

The promoters of all MHC class II genes contain two conserved

elements, the X and Y motifs, which are essential for constitutive and lymphokine-induced expression. Several other conserved elements common to all MHC class II promoters, and transcription factors which bind to them, have also been identified (Liou et al., 1990; Tsang et al., 1990; Voliva et al., 1992; Ray et al., 1992). The DRα gene promoter is the only MHC class II promoter which contains the octamer motif. When the octamer site is mutated, it severely reduces constitutive B cell-specific gene expression of DRα but does not affect expression in other cell types (Stimac et al., 1988; Sherman et al., 1989).

The αβ heterodimers associate with a third nonpolymorphic glycoprotein, the invariant chain (Ii), in the ER. The gene encoding the Ii chain, although not part of the MHC gene complex, shares promoter/enhancer sequences and is coordinately regulated with the MHC genes at the level of transcription. Shortly after its synthesis in the ER the αβ heterodimer of MHC class II combines with the Ii chain. This complex is then transported out of the ER, through the Golgi apparatus and to acidic endosomes (Lamb et al., 1991). Ii chains are then proteolytically degraded and the released αβ heterodimers bind antigenic peptides and are transported to the cell surface. The Ii chains appear to be necessary for the efficient assembly and trafficking of MHC class II molecules (Peterson and Miller, 1990). How and where the Ii chains associate with the αβ dimers, and how the assembly and the transport of the αβ heterodimers occur are not well understood. Ii chains may also play a role in segregating antigen presentation by the endogenous (MHC

class I-mediated) and exogenous (MHC class II-mediated) pathways by preventing the binding of MHC class II to peptides in the ER (Teyton et al., 1990; Roche and Creswell, 1990 and 1991).

In the absence of the Ii chain cell surface expression of MHC class II can occur, albeit with low efficiency (Sekaly et al., 1986). In Ii^- cells, DR molecules associate with specific stress proteins, GRP94 and ERp72, and are thought to be retained in the ER (Schaiff et al., 1992). Retention of DR molecules in the ER may occur because the αβ heterodimers fail to achieve the proper conformation in the absence of Ii. Alternatively, even in the presence of the Ii chain, stress proteins may transiently associate with properly folded proteins, but are displaced by the Ii chain in a competitive interaction with MHC class II molecules (Schaiff et al., 1992). In transgenic mice lacking Ii chains, MHC class II transport from the ER through the Golgi is aberrant, and the expression of surface MHC class II is reduced. As a result, antigen presentation to $CD4^+$ T cells is impaired, and $CD4^+$ T cells are negatively selected in the thymus (Viville et al., 1993).

Most studies pertaining to the expression of MHC class II in murine and human B cells have focussed on the early stages of the B cell lineage. Investigation of MHC class II expression during the terminal differentiation of B cells to plasma cells has been limited. By immunofluorescence microscopy of human plasma cells isolated from a variety of tissue sources and from plasma cell lines, Halper et al. 1978, have shown that terminal B cell differentiation is accompanied by a reduction of surface MHC class II. When B cells

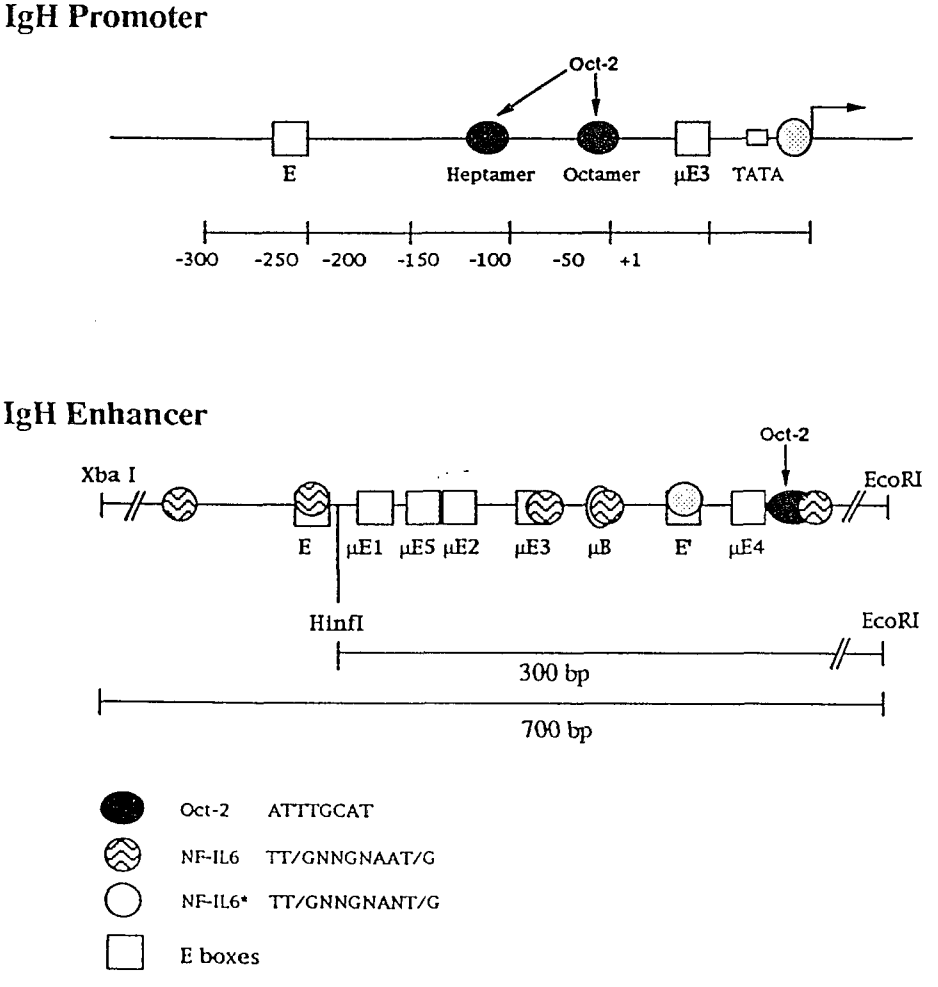
and plasmacytoma cells are fused, MHC class II$^-$ hybrids result, suggesting that a dominant suppressor factor in plasmacytomas is involved in the extinction of MHC class II gene expression in these hybrids (Venkitaraman et al., 1987; Latron et al., 1988). This factor has not been identified as yet. Although the regulation of MHC class II has been well studied and is thought to involve coordinate transcriptional activation of the genes encoding the α, β and Ii chains of MHC class II, little is known about how the diminution of surface MHC class II expression occurs. The mechanisms which govern this reduction during the terminal step of B cell differentiation needs further investigation.

THE TRANSCRIPTIONAL REGULATION OF Ig GENES

In contrast to the two surface molecules, mIg and MHC class II, differentiating B cells show a marked increase in intracellular Igs. This increase is potentiated by the activation of transcriptional initiation of the genes encoding the Ig heavy-and light chains, and the preferential accumulation of the secreted form-specific Ig heavy-chain mRNAs (Raynal et al., 1989). The transcriptional activation of Ig genes underlies the major function of plasma cells: the synthesis and secretion of antibodies. The regulation of the Ig genes occur during several stages of B cell ontogeny including the mature B cell and plasma cell stages. Promoter and enhancer elements (Figure 3A and 3B), which function preferentially in B cells regulate Ig gene transcription.

Figure 3. Schematic representation of a prototype IgH promoter and the IgH intronic enhancer.

Several sequence motifs are shared by the IgH promoter and enhancer regions: the octamer motif is common to all V_H promoters and is present in the IgH enhancer. A related heptamer site which binds Oct-2, and several E box elements (μE1-μE5, defined by methylation interference studies, Ephrussi et al., 1985), and potential binding sites (NF-IL6 and NF-IL6* sites) for NF-IL6, a bZip transcription factor induced by IL-6 (Akira et al., 1990), are also present. By gel mobility shift assays and transfection studies, many transcription factors which interact with these sites and their role in regulating transcription of Ig genes have been investigated.



Methylation interference studies have revealed the presence of at least six cis-elements in the murine IgH enhancer (Ephrussi et al., 1985). By gel-mobility shift and transfection assays, transcription factors which bind to some of these elements and activate Ig gene transcription such as TFE3, ITF-1 (Peterson and Calame, 1987; Beckman et al., 1990; Ruezinsky et al., 1991), and μB (Akira et al., 1985; Araki et al., 1988), have been identified. By transient transfection studies in a plasmacytoma cell line, a member of the C/EBP family, Ig/EBP, appears to regulate the transcription of Ig genes (Roman et al., 1990; Cooper et al. 1992). The expression of κ-light chains in pre-B cells requires the induction of the transcription factor NF-κB (Murre et al., 1989). These studies suggest that several transcription factors and their cognate binding sites in combination regulate the efficient transcription of Ig genes. Although cis- and trans-acting elements involved in Ig gene expression have been identified, the complex program of tissue-specific, and stage-specific regulation of Ig genes in B cells is still not understood.

Several copies of the octamer motif (consensus nucleotide sequence: ATGCAAAT) are found in the promoter and enhancer regions of Ig genes. The octamer binds Oct-1, a transcription factor expressed ubiquitously, and Oct-2, a factor expressed predominantly in B cells (Scheiderite et al., 1987; Staudt et al., 1988). Oct-2 is also expressed in neuronal cells, although alternative splicing leads to non-overlapping expression of Oct-2 transcripts and protein isoforms in B cells and in neuronal cells (Lillycrop and Latchman,

1992). Oct-1 and Oct-2 are members of the POU transcription factor family. They bind the octamer motif through the POU-specific and POU homeo domains, which are highly conserved among members of the POU family (Herr et al., 1988; Gerster et al., 1990).

Cell fusion studies show that the expression of Oct-2 is essential to stimulate lymphoid-specific promoters in non-lymphoid cells (Muller et al., 1988; Junker et al., 1990). The combination of multiple activation domains and specific protein phosphorylation is responsible for the differential transcriptional activation properties of Oct-1 and Oct-2 (Tanaka and Herr, 1990). These observations reveal that although the octamer motif is common to several transcription factors, lymphoid-specific transcriptional activation is facilitated by a number of mechanisms at the appropriate developmental stage.

Besides Oct-1 and Oct-2, the need for an additional B cell-specific component for the optimal transcription of Ig promoters has been recognized (Pierani et al., 1990). Luo et al. (1992), have shown that the promoter-specific Oct coactivator from B cells (OCA-B), in conjunction with Oct-1 or Oct-2, is a major determinant in potentiating transcription of the Ig heavy-chain promoter but not of the histone H2B promoter. The interaction of OCA-B with Oct-1 and Oct-2 confers promoter-specific transcriptional activation, and suggests that protein-protein interactions may modulate the functions of the Oct proteins.

Oct-2 is expressed throughout all stages of B cell development. This expression pattern has made any predictions

about the primary developmental stage-specific role of Oct-2 difficult. To address this question Corcoran et al. (1993), have created transgenic mice lacking Oct-2. In Oct-2$^-$ homozygotes all early stages of B cell development including D-J and V-DJ rearrangements and class-switching proceed normally. These mice, however, fail to secrete Igs upon LPS challenge. The evidence obtained from transgenic mice shows that Oct-2, although dispensable for earlier stages in B cell development, is critical for the terminal step of B cell differentiation and antibody secretion.

THE FATE OF THE DIFFERENTIATED B CELLS

To fully understand terminal differentiation of B cells the fate of the differentiated cells has to be taken into consideration. *In vivo* labeling studies show that plasma cells are short lived, and upon differentiation, the vast majority of these cells disappear after two weeks. A very small percent remain in circulation for almost two years (Miller, 1963). Antigen-driven B cell proliferation and the generation of plasma cells occur in germinal centers of lymphoid follicles in secondary lymphoid organs such as the spleen, tonsils, and lymph nodes. In lymphoid follicles a network of follicular dendritic cells take up antigens in the form of immune complexes and facilitate B cell proliferation and terminal differentiation. DNA-labeling studies show that a large number of cells in the germinal center die by apoptosis. Appropriate signals derived from crosslinking Igs or through the stimulation of surface receptors on B cells potentiate positive selection and germinal

center cell survival (Liu et al., 1989). Some of these cells remain in circulation for long periods of time as memory B cells (Liu et al., 1992). The expression of the bcl-2 encoded protein appears to mediate the survival and prolongs the lifespan of memory B cells (Nunez et al., 1991; Liu et al., 1991). Signals from the mIg complex and selection processes in the germinal centers regulate the fate of B cells during development.

A CLONAL MODEL SYSTEM FOR TERMINAL B CELL DIFFERENTIATION

During terminal B cell differentiation the expression of Ig molecules changes from the membrane anchored form to the secreted form. Studies aimed at identifying the mechanisms governing Ig gene regulation during terminal differentiation have produced conflicting results. Comparisons made between cell lines representative of different stages of B cell development have suggested that almost all intermediate steps in mRNA biosynthesis are important in Ig gene regulation (reviewed in Raynal et al., 1989).

To understand the regulatory steps involved in the terminal differentiation of B cells, a system in which this step of B cell development can be recapitulated *in vitro* is needed. When developing a system amenable for molecular analyses of B cell differentiation, the following criteria need to be considered:

(1) Clonality: Experiments using polyclonal cell populations may not lead to homogeneous responses. This heterogeneity of response may hamper detection and accurate interpretation of experiments. Thus, a clonal population in which distinct responses

can be assessed in detail is preferred when establishing a system to study differentiation. Once these responses have been characterized in a clonal system, the biological relevance and generality of the response can be tested in polyclonal cells or *in vivo*.

(2) Chromosomal translocations: Most B cell and all plasma cell lines are established from B cell or plasma cell neoplasms. Chromosomal translocations involving the Ig promoter/enhancer regions with proto-oncogenes such as *c-myc* have been well documented in these neoplasms. When trying to understand the normal regulation of Ig genes during B cell differentiation, B cell lines which do not contain translocations involving the Ig loci are preferable.

(3) Regulation: A system in which the program of differentiation and coordinate gene expression can be regulated is ideal to study specific regulatory events signaled by a distinct receptor.

Using a human lymphoblastoid cell line regulated by IL-6, a system has been developed in our laboratory in which the differentiation of B cells to antibody-secreting cells can be examined. CESS is an Epstein-Barr virus-immortalized human B cell line which expresses IgG1 (Bradley et al., 1982). A clone of CESS expressing a high density of sIg (92%) was selected by FACS-sorting based on the rationale that high surface expression of mIg is a characteristic of B cells early in differentiation (Raynal et al., 1989). This clone of CESS would enhance the change in Ig gene expression during differentiation and facilitate the detection of

molecular events which occur during this process. A 15-fold preferential accumulation of the secreted form-specific heavy-chain (γ1s) mRNA, but not of the alternatively processed membrane form-specific (γ1m) mRNA was seen when CESS cells were induced to differentiate using IL-6 (Raynal et al., 1989). A concurrent increase in accumulation of mRNAs encoding the non-productively rearranged μ heavy chain and λ light-chain was also seen. Nascent chain analysis suggested that the regulation of mRNA accumulation by IL-6 was due primarily to the activation of transcription (Raynal et al., 1989). An IgM-bearing cell line (SKW6.c14) at the pre-class-switched stage of B cell ontogeny also differentiates in response to IL-6-stimulation with increased μs mRNA accumulation (Liu and Chen-Kiang, unpublished). The results obtained in these two cell lines suggest that IL-6 is not specific to an Ig isotype or promoter, and can potentiate differentiation of cells at stages before and after class-switching.

The IL-6-inducible CESS cell system offers many advantages for the investigation of terminal B cell differentiation. Using this system, the regulated expression of genes can be analyzed by making comparisons before and after IL-6 induction in the same cells, than by making comparisons between cell lines or in cell hybrids. Thus, detailed molecular analyses of Ig synthesis and secretion, and the mechanisms which regulate this process during differentiation, can be addressed. The effects of IL-6 on CESS cells is not limited to the regulation of Ig genes. Therefore, the coordinate regulation of other genes involved in B cell

differentiation can be analyzed simultaneously. These genes include those encoding regulators of other B cell functions such as antigen presentation, and those which mediate the structural and biochemical modifications that B cells undergo during their transition to plasma cells. The IL-6R-mediated signals elicit specific and biologically meaningful responses. Because IL-6 induces B cell differentiation *in vivo,* it is a better candidate for the induction of B cell differentiation than a non-specific stimulant such as LPS. Although IL-6 induces proliferation of several cell types, and plays a role in the oncogenesis of plasma cells, IL-6 does not induce aberrant proliferation or transformation of CESS cells. Thus, the IL-6 signals in CESS cells specifically induce the differentiation phenotype, and allow this lymphokine to be a valuable tool for the investigation of molecular events during B cell differentiation. The CESS cell system is the only human system available to study terminal B cell differentiation and the regulation of IgG, the predominant class of Igs involved in the secondary immune response.

II

MATERIALS AND METHODS

Cell culture

The CESS lymphobastoid cell line was established from peripheral blood from a human patient with myelomonocytic leukemia, immortalized with EBV (Bradley et al., 1982), and expresses a low density of membrane IgG1 (Maraguchi et al., 1981; Kikutani et al., 1985). To enrich for cells expressing a high density of membrane IgG1, flow cytometry sorting was utilized as described (Raynal et al., 1989). CESS cells were cultured in RPMI 1640 medium (Gibco BRL, Gaithersburg, MR) supplemented with 10% fetal calf serum (Hyclone, Logan, UT) heat-inactivated at 56°C for 30 min. L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 mg/ml), and non-essential amino acids (2 mM) were also supplemented. The cells were maintained in suspension at a density of 2.5-5x10^5 cells/ml, with daily replacement of the medium. Similar growth conditions were used for the IgM expressing, EBV-immortalized lymphoblastoid cell line, SKW6.c14 (Saiki and Ralph, 1983), the human leukemia T cell line, Jurkat (Gillis and Watson), and the human histiocytic lymphoma cell line, U937 (Sundstrom and Nilsson, 1976).

IL-6 inductions

For IL-6 inductions, cells were grown in complete medium containing 40 units/ml of IL-6 (recombinant IL-6 expressed in

E. coli, kindly provided by Dr. T. Kishimoto). One unit (U) of IL-6 is defined as the activity that induces 50% of the maximum secretion of IgM in 10^4 SKW6.c14 cells in 4 days (Hirano et al., 1986). For the long term induction of CESS cells, IL-6 synthesized in a baculo virus expression system (kindly provided by Dr. Lester May) was used. 5 ng/ml of this IL-6 was established as equivalent to 40. U/ml of the IL-6 obtained from Dr. T. Kishimoto, and induced comparable numbers (15-20%) of CESS cells to differentiate as assessed by intracellular staining for IgG.

Isolation of tonsillar lymphocytes

To isolate tonsillar lymphocytes, human tonsils were minced, pressed through a sieve, and centrifuged (100 x*g*, 30 s) to remove debris and connective tissue. The leukocytes were collected by centrifugation (500 x*g*, 15 min, 4°C), resuspended in RPMI 1640 and layered on 1.077 gm/ml Ficoll (Sigma Chemical Company, St. Louis, MO). After centrifugation at 1000 x*g* for 30 min at 4°C in a swinging bucket rotor, the lymphocytes at the interface were collected, washed and resuspended in complete RPMI medium. Approximately 80-85% of these cells express membrane IgG and IgM, and the two subunits of the high affinity IL-6 receptor at levels comparable to that of CESS cells. In some experiments tonsillar lymphocytes were infected with the EBV as described (Hurley and Thorley-Lawson, 1988), and induced with IL-6 (40 U/ml) at day 3 after infection.

Electron microscopy

Cells were collected and fixed in 0.1M sodium cacodylate buffer containing 2.5% glutaraldehyde, 2.5% formaldehyde and 0.05% picric acid. Following fixation, cells were post-fixed in a solution of 1% osmium tetroxide and 1.5% potassium ferrocyanide, after which they were stained *en bloc* with 1% uranyl acetate in maleate buffer. After dehydration and embedding in EMbed 812 (Electron Microscopy Sciences, Fort Washington, PA), thin sections were obtained, stained with uranyl acetate and lead citrate, and examined on a Hitachi 7000 transmission electron microscope.

Intracellular immunofluorescence microscopy

Cells were collected by centrifugation and washed three times with PBS (137 mM NaCl, 2.7 mM KCl, 8 mM $Na_2HPO_4.7H_2O$, 1.4 mM KH_2PO_4, pH 7.4, 1 mM $MgCl_2$) containing 1% bovine serum albumin (BSA, Boehringer Mannheim Corporation, Indianapolis, IN). $1x10^5$ cells were spun onto each glass slide at 200 rpm for 4 min using a cytospin centrifuge (Shandon, Pittsburg, PA). The slides were incubated for 20 min at -20°C in a fixing solution containing 95% methanol and 5% glacial acetic acid, freshly prepared and chilled to -20°C, after which they were rinsed with distilled water.
The slides were incubated with the appropriate antibodies (as summarized in Table 3A). These incubations with antibodies were performed at 37°C in an opaque, humidified chamber for 45 min. The

cells were then rinsed two times with PBS/BSA (pH 7.4) and once with PBS (pH 7.8, without BSA or $MgCl_2$), for 15 min each time at room temperature. The slides were sealed in 2.5% DABCO (1,4-diazabicyclo [2.2.2] octane; Sigma) in glycerol. Visualization and photography were performed using a Zeiss Axiophot microscope. All photographs of stained cytospin preparations were taken under fixed exposure times.

Confocal microscopy

Cells were cytospun as described above and fixed in 4% formaldehyde (Fisher Scientific) in PBS for 30 min at room temperature (RT). After rinsing the slides twice in PBS for 1-2 min each time, the aldehyde fluorescence was quenched using 50 mM NH_4Cl in PBS for 20 min at RT. The slides were rinsed as before, and the cells were permeabilized with 0.1% Triton x-100 (t-Octylphenoxypolyethoxyethanol; Sigma) in PBS/BSA for 15 min on ice and rinsed once again. Incubations with the primary antibodies were performed for 45 min at RT (as summarized in Table 3B). The slides were rinsed twice in PBS/BSA for 15 min each time to remove excess primary antibody. Incubations with fluorochrome conjugated secondary antibodies were performed (as summarized in Table 3B), and the slides were rinsed twice in PBS/BSA and once with PBS for 15 min each time. The slides were sealed with 2.5% DABCO in glycerol, and analyzed using a BioRad MRC 600 confocal microscope.

Analysis of surface immunofluorescence and flow cytometry

Cells ($1x10^6$) were washed twice with PBS/BSA and incubated with appropriate antibodies diluted in PBS/BSA (as summarized in Table 4) for 30 min on ice. After incubation with the primary antibody, the cells were rinsed twice with PBS/BSA, and incubated with a fluorochrome-conjugated secondary antibody (as summarized in Table 4) for 30 min on ice. Visualization and photography were performed using a Zeiss Axiophot microscope. For flow cytometry, the cells were rinsed again, fixed in 3% formaldehyde, or analyzed without fixation. For isotype control for the goat anti-human antibodies used in the experiments, unconjugated goat anti-mouse IgG1 (isotypic antibody from an unrelated species) was used, and counterstained with the same secondary (fluorochrome-conjugated) antibodies which were used to detect the human antibodies (Table 4). The samples were either analyzed on an EPICS Profile II Analyzer (Coulter Corporation, Opa Locka, FL) with four decades of amplification, or sorted with an EPICS Elite Cell Sorter (Coulter) with four decades of amplification.

Table 3. Antibodies used for intracellular immunofluorescence and confocal microscopy

ANTIBODY	CONCENTRATION (diluted in PBS/BSA)	SOURCE
(A) INTRACELLULAR IMMUNOFLUORESCENCE OF IgG AND IgM		
(1) IgG		
Goat anti-human IgG-FITC	1:1000-1:2000	Cappel, Durham, NC.
Goat anti-human IgG-RITC	1:1000-1:2000	Cappel
(2) IgM		
Goat anti-human IgM-FITC	1:1000-1:2000	Cappel
Goat anti-human IgM-RITC	1:1000-1:2000	Cappel
(B) CONFOCAL MICROSCOPY		
(1) MHC class II		
* mAb CA 2.06 (DR, DP, DQ)	1:100	Dr. M. Peterlin
** Sheep anti-mouse Ig-FITC	1:500	Cappel
(2) GRP94		
* Rabbit anti-GRP94	1:100	Dr. P. Srivastava
** Goat anti-rabbit Ig-Texas red	1:500	Cappel.
(3) IgG AND IgM		
Goat anti-human IgG-FITC	1:500	Cappel
Goat anti-human IgG-RITC	1:500	Cappel
Goat anti-human IgM-FITC	1:500	Cappel
Goat anti-human IgM-RITC	1:500	Cappel

Abbreviations:

* Primary antibody (unconjugated)
** Secondary antibody (fluorochrome conjugated)

Table 4. Antibodies used for cell surface immunofluorescence and FACS analyses

ANTIBODY	CONCENTRATION (diluted in PBS/BSA)	SOURCE
(1) IgG		
Goat anti-human IgG-FITC	1:1000-1:2500	Cappel
(2) IgM		
Goat anti-human IgM-FITC	1:1000-1:2500	Cappel
(3) MHC class II		
*mAb CA 2.06 (DR, DP, DQ)	1:50	Dr. M. Peterlin
*mAb L243 (DR) supernatant	1:2	Dr. L. Mayer
*mAb B7/21 (DP) supernatant	1:2	Dr. L. Mayer
*mAb Genex 3.53 (DQ) supernatant	1:2	Dr. L. Mayer
*mAb W6/32 (class I) supernatant	1:2	Dr. L. Mayer
**Sheep anti-mouse-FITC	1:500	Cappel
(4) IL-6 Receptor		
*mAb MT18 (gp80)	1:100	Dr. T. Kishimoto
*mAb AM64 (gp130)	1:100	Dr. T. Kishimoto
**Sheep anti-mouse-FITC	1:500	Cappel.
(5) Isotype control		
Goat anti-mouse IgG1 (1mg/ml)	1:100 - 1:500	Dr. P. Boros
(6) Fcγ receptor		
Rabbit anti-human FcγRIIA	10 - 100 µg/ml	Dr. J. Odin Dr. P. Boros Dr. J. Unkeless

Abbreviations:

* Primary antibody (unconjugated)

** Secondary antibody (fluorochrome conjugated)

Northern blot analysis

Isolation of total RNA was performed using the guanidinium thiocyanate method (Chirwin et al., 1979). For isolating total RNAs from small numbers of cells (10^5-10^6 cells), the acid guanidinium thiocyanate-phenol-chloroform extraction method was utilized (Chomczynski and Sacchi, 1987). 10^5-10^6 cells were denatured in 1 ml of solution D (4M guanidinium thiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% sarcosyl, 0.1 M 2-mercaptoethanol), at room temperature. To the homogenate, 0.1 ml of 2 M sodium acetate, pH 4.0, 1 ml phenol (saturated with water), and 0.2 ml of chloroform-isoamyl alcohol (49:1), were added sequentially with thorough mixing by inversion of the tube after each of the reagents was added. The suspension was mixed vigorously for 10 seconds, and cooled on ice for 15 min. The homogenate was centrifuged at 10, 000 x*g* at 4°C for 20 min. After centrifugation, the aqueous phase containing RNA was transferred to a fresh tube, mixed with 2 volume equivalents of 100% ethanol, and the RNA was allowed to precipitate at -20°C for 1 hour. The samples were again spun at 10,000 *g* at 4°C for 20 min, and the resulting pellet was dissolved in 0.3 ml of solution D, and re-precipitated in ethanol at -20°C for 1 hour. After centrifugation for at 4°C for 10 min, the RNA pellet was dissolved in 50 µl 0.5% sodium dodecyl sulfate at 65°C for 10 min (Chomczynski and Sacchi, 1987).

The selection of polyadenylated RNA with poly(U)-agarose affinity chromatography was performed as described (Chen-Kiang and Lavery, 1989). RNAs were fractionated on 1.4% agarose-

formaldehyde denaturing gels in MOPS buffer (20 mM morpholinopropane sulfonic acid [Sigma], 5 mM sodium acetate, and 1 mM EDTA, pH 7.0), and analyzed by Northern blotting. Pre-hybridization and hybridization were performed in the presence of 50% formamide at 42°C overnight as described (Thomas et al., 1980). The only modification was the inclusion of 5x Denhardt's solution (50x Denhardt's solution contains 5 g polyvinylpyrrolidone, 5 g ficoll, and 5 g BSA in 500 mls H_20) in the hybridization mixture. The blots were washed four times with 2x SSC, 0.2% SDS for 10 min at 42°C, and twice with 0.1x SSC, 0.2% SDS for 20 min at 55°C (1x SSC contains 150 mM sodium chloride and 15 mM sodium citrate). All probes (summarized in Table 5) were labelled with [^{32}P] by the random priming reaction (Feinberg and Vogelstein, 1983).

Table 5. PROBES USED FOR NORTHERN BLOT ANALYSES

PROBE	INSERT	REFERENCE
human γ1	1.4 kb *Sal* I-*Sma* I fragment of the C1-C3 regions subcloned into pIBI30	Takahashi et al.1982
human μ	1.3 kb *Eco*RI fragment of the C1-C3 regions subcloned into pBR322	Ravetch et al.1981
GAPDH	1.25 kb *Pst* I fragment of the full length rat cDNA	Fort et al.1985
gp80 subunit of the IL-6 receptor	1.7 kb *Xho* I fragment of the 2.2 kb *Hind* III-*Xba* I cDNA insert (pBSF2R.236)	Yamasaki et al.1988
gp130 subunit of the IL-6 receptor	2.2 Kb *Pst*I fragmant of the human cDNA	Hibi et al.1990
Oct-2	whole plasmid	Staudt et al.1988
mb-1	whole plasmid	Rolink et al.1991
DRα	whole plasmid	Stetler et al.1982
DRβ	whole plasmid	Bell et al.1985
Ii chain	whole plasmid	Long et al.1983
GRP78	whole plasmid	Nakaki et al.1989
GRP94	whole plasmid	Maki, et al.1990
FcγRII	whole plasmid	Brooks et al.1989

Metabolic labeling and immunoprecipitation of IgG

[Performed by William McCully]

For metabolic labeling, $1x10^7$ cells were collected after induction with IL-6 and washed in 1xPBS. To deplete the endogenous methionine pool, the cells were resuspended at $5x10^6$ cells/ml in methionine-free RPMI with 10% dialyzed fetal calf serum and incubated for 1 hr at 37°C. The cells were labeled with [^{35}S]-methionine (250µC/ml, 1245 Ci/mMol; New England Nuclear) for 2 hrs at 37°C, after which they were pelleted, washed once with 1xPBS and frozen as cell pellets. Immunoprecipitation was performed according to Harlow and Lane (1985), using anti-human IgG (Cappel). Cell pellets were thawed in RIPA buffer (10 mM Tris, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 1% sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride), and centrifuged at 15,000 *xg* to remove debris. To reduce non-specific protein interactions, the lysates were preadsorbed with 10%(w/v) fixed *Staphylococcus aureus* Cowan strain bacteria (Boehringer Mannheim). The pre-cleared lysates were incubated with anti-human IgG (Cappel) at a dilution of 1: 100. The resulting antigen-antibody complexes were recovered by incubating with protein A-Sepharose (Pharmacia) and centrifugation. After washing three times with RIPA buffer, the samples were reduced and fractionated on a 10% SDS-polyacrylamide gel. The gel was treated for fluorography using En3Hance (New England Nuclear), and then dried and exposed to X-AR film.

III

Activation and Feedback Control of Oct-2 and Immunoglobulin Synthesis in IL-6-Induced Terminal Differentiation of human B Cells

ABSTRACT

We have studied terminal differentiation of human B cells in an IL-6-inducible system. IL-6 signaling leads to marked enhancement of the synthesis and secretion of Igs. The IL-6-induced cells exhibit cellular morphology characteristic of plasma cells *in vivo,* and lose the expression of major histocompatibility complex class II molecules on the cell surface. The regulation of Ig synthesis was assessed at the mRNA and protein levels, and is subject to feedback control upon long term IL-6 stimulation. The synthesis of the transcription factor Oct-2 is temporally regulated and is also subject to feedback control, which suggests a role for Oct-2 in mediating the IL-6-signals in the activation of Ig gene transcription. The two subunits of the IL-6 receptor are feedback regulated during the differentiation of CESS cells induced by IL-6, and indicate a potential mechanism by which IL-6 signaling in B cells may be modulated. Differentiation by IL-6 occurs in freshly isolated tonsillar B cells after Epstein Barr virus-immortalization, and is not restricted to an Ig isotype. Thus, the IL-6-inducible system provides a model to study the molecular mechanisms which underlie terminal B cell differentiation.

INTRODUCTION

Terminal differentiation of cells in the B lineage is characterized by a marked increase in the synthesis and secretion of immunoglobulins. A concomitant reduction of membrane-anchored Ig (mIg) is also seen (Rolink and Melchers, 1991). The terminally differentiated plasma cells exhibit a dramatically different cell morphology from that of mature B cells, and lose surface expression of MHC class II (Halper et al, 1978). Although the differences between activated B cells and plasma cells have been recognized through comparative studies, little is known about the mechanisms involved in this well orchestrated differentiation process.

Lymphokines are valuable tools for the investigation of the molecular mechanisms which govern B cell development, in particular, the expression of Ig. Several lymphokines participate in regulating B cells at different stages during their development. IL-2 and IL-5 facilitate the secretion of IgM in mouse B lymphoma cells by increasing the synthesis of the J chain and the assembly of pentameric IgM (Tiggs et al, 1989; McFadden and Koshland, 1991). IL-4 enhances the secretion of IgG1 and IgE in the presence of LPS by promoting the recombination of $\gamma 1$ and ε genes (Lutzker et al, 1988; Stavenezer et al, 1988; for a review, see Paul and Ohara, 1987). At the terminal stage of B cell development, IL-6 plays a role in inducing Ig secretion by activated peripheral mouse B cells (Miraguchi et al, 1988).

The secreted Ig (sIg) and mIg differ only at the carboxyl termini of their heavy chain polypeptides due to alternatively spliced mRNAs distinct at their 3' ends (Alt et al, 1980; Roger et al, 1980). Cells bearing a high density of mIg were selected from the EBV immortalized, IgG1-bearing, human lymphoblastoid cell line, CESS, and a population of these cells which secrete high levels of IgG in response to IL-6 was derived (abbreviated as CESS; Raynal et al, 1989). Using this cell population the enhanced Ig secretion was attributed to the preferential accumulation of the mRNA encoding the sIg, but not the mIg. The increase in steady state Ig mRNAs was controlled primarily at the level of transcriptional initiation. These results support a major role for transcriptional activation in the regulation of Ig genes during B cell differentiation (Raynal et al, 1989).

IL-6 is a pleiotropic cytokine that induces differentiation and regulates cell growth in a cell type-dependent manner (for a review, see Chen-Kiang et al, 1993; Kishimoto et al, 1992). The activation of transcription of Ig genes by IL-6 is not promoter- or isotype-specific (Raynal et al, 1989). This suggests that transcription factors which interact with sequences common to the Ig promoters and enhancers (for a review, see Staudt and Leonardo, 1991) may be involved in mediating the responses to IL-6.

Of particular interest is Oct-2, a POU domain-containing transcription factor expressed predominantly in the B lineage (Staudt et al, 1986, 1988; Landolfi et al; 1986; Scheidereit et al, 1988; Muller et al, 1988). Oct-2 activates transcription in

transfection and in cell-free systems (Scheidereit et al, 1988; Muller et al, 1988) by binding to an octamer motif, 5'-ATGCAAAAT-3', which is the most conserved sequence in the Ig promoters and enhancers (Falkner and Zachau, 1984; Parslow et al 1984; Singh et al, 1986, Currie and Roeder, 1989). Recent genetic evidence suggests that Oct-2 is critical for terminal differentiation of B cells. The disruption of both alleles of the *oct-2* gene in the mouse does not impair the development of Ig-bearing B cells, but prevents their differentiation into Ig-secreting plasma cells upon stimulation with LPS (Corcoran et al, 1993). The octamer-binding proteins are regulated by IL-6: Oct-1 and Oct-3 are regulated in human embryonal carcinoma cells, and Oct-1 in human T cells (Hsu and Chen-Kiang, 1993). Oct-2 may be regulated in response to IL-6 and may participate in the activation of transcription of Ig genes in B cells.

IL-6 transduces its signals through a specific receptor. The binding of IL-6 to its receptor, gp80, triggers the association of gp80 with a signaling subunit, gp130, and the formation of a high affinity receptor (Hibi et al, 1990). This rapidly leads to tyrosine phosphorylation of gp130 (Nakajima and Wall, 1991; Lord et al, 1991; Ip et al, 1992). Little is known about the subsequent events, although they may involve *ras*-dependent mitogen activated protein kinases (Nakajima et al, 1993). Transcription factors of the AP-1 and C/EBP families are likely to participate in nuclear signaling by IL-6 as they are rapidly and selectively regulated according to cell type upon IL-6 induction (Nakajima and Wall, 1991; Lord et al, 1991; Akira et al, 1990; Kinoshita et al, 1992). It is also unknown whether

the two subunits of the IL-6 receptor can be regulated.

Using an IL-6-inducible system, we have undertaken the investigation of the molecular mechanisms which underlie terminal differentiation of human B cells. We demonstrate that the syntheses of Oct-2 and Ig are regulated coordinately by IL-6 and that this regulation is subject to feedback control. The two subunits of the IL-6 receptor also appear to be regulated by this feedback mechanism. IL-6 signaling leads to marked enhancement in Ig secretion, reduction of surface MHC class II, and changes in cell morphology, which are hallmarks of terminal B cell differentiation.

RESULTS

A clonal model system for studying terminal B cell differentiation

Treatment of CESS cells with IL-6 resulted in a significant increase of IgG1 synthesis in approximately 20% of the cells on days 4 and 5 after induction, as evidenced by intracellular staining of IgG1 (Figure 4A). Induction by IL-6 is saturable, time dependent, transient and specific, and it can be inhibited by a monoclonal antibody against the IL-6 receptor (Raynal et al, 1989; Z. Liu and S. Chen-Kiang, unpublished). When analyzed further by transmission electron microscopy, the IL-6-induced cells displayed morphology characteristic of plasma cells: enlarged cytoplasm, well developed rough endoplasmic reticulum and an eccentric nucleus (Figure 4B). The resemblance between IL-6-induced cells and plasma cells suggests that IL-6 signaling may lead to changes in human B cells which are observed during terminal differentiation *in vivo.*

IL-6 induces the synthesis and secretion of Ig and reduces the expression of surface MHC class II.

The IL-6-induced enhancement of Ig synthesis correlated with a 25-fold increase of the secreted form-specific γ1 (γ1s) mRNA but not of the alternatively spliced membrane form-specific γ1 (γ1m) mRNA (Figure 5A and B), corroborating previous observations (Raynal et al, 1989). CESS cells, selected for expressing a high density of mIgG1, secrete Ig at negligible levels (Figure 5C).

Figure 4. Induction of differentiation of CESS cells by IL-6.

(A) CESS cells were incubated with 40 units/ml of IL-6 for days as indicated, permeablized and stained for IgG with a FITC-conjugated anti-human IgG antibody, and subjected to phase contrast (Phase), and immunofluoresence (IFM) microscopy.

(B) Electron microscopy was performed on CESS cells cultured in the absence (-IL-6) or presence of IL-6 for 4 days (+IL-6).

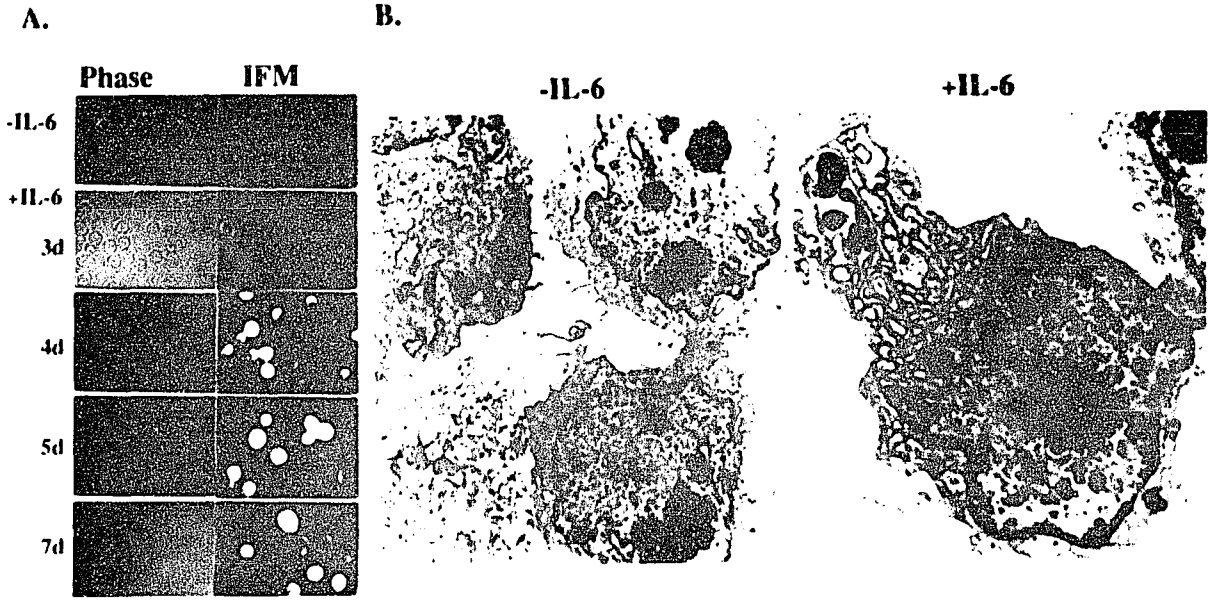


Figure 5. IL-6 enhances the synthesis and secretion of Ig and reduces surface MHC class II expression.

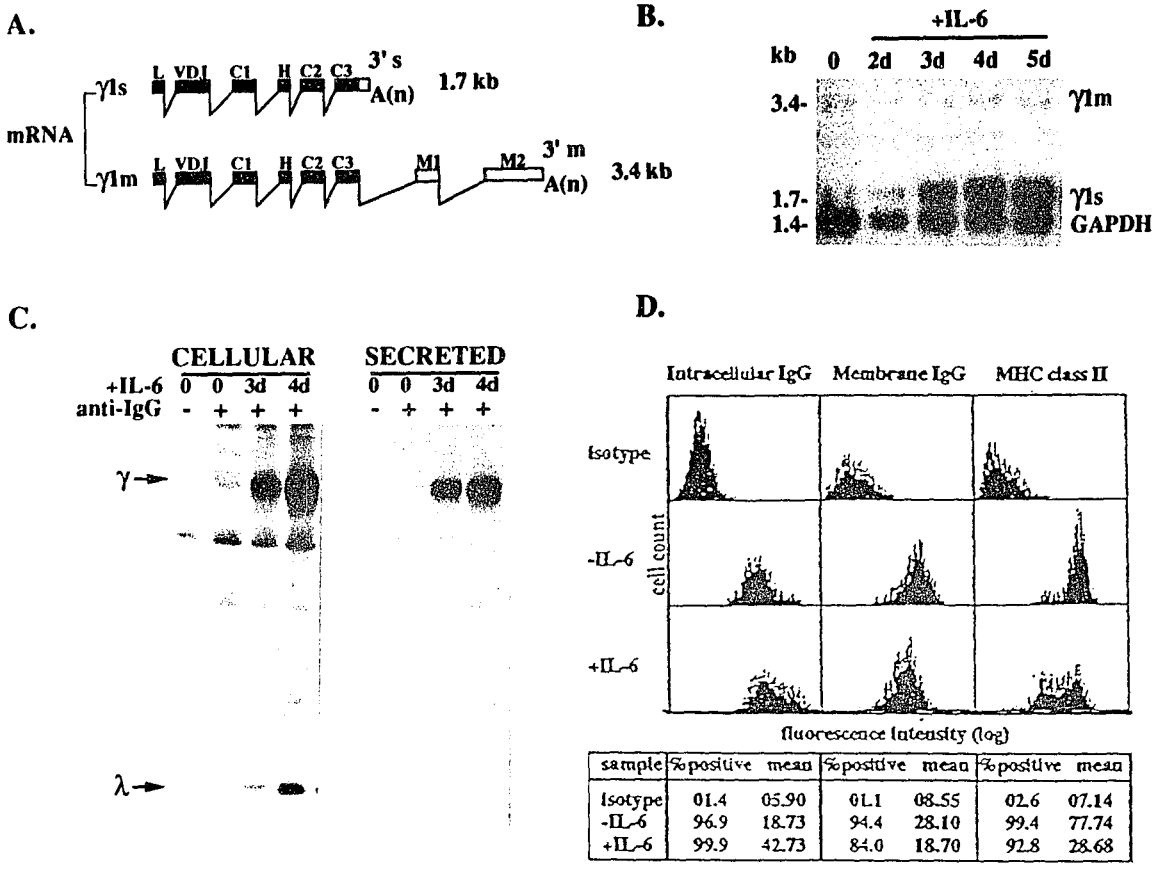
(A) A schematic representation of the alternatively spliced γ1s and γ1m mRNAs, with shared exons represented by solid boxes and specific exons represented by shaded boxes. The bent lines indicate introns.

(B) Northern blot analysis of γ1s and γ1m mRNAs on days after IL-6 induction as indicated.

(C) The synthesis (cellular) and secretion (secreted) of [^{35}S]-methionine labeled IgG in CESS cells were analyzed on days after IL-6 treatment as indicated by immunoprecipitation and SDS-PAGE. The migration of γ heavy chain and λ light chain are as marked. [The experiment presented in 5C was performed by William McCully, technician]

(D) The expression of intracellular IgG, membrane IgG and surface MHC class II in CESS cells cultured in the absence (-IL-6) or presence of IL-6 for 4 days (+IL-6) were analyzed by flow cytometry.

Figure 5.



sample	%positive	mean	%positive	mean	%positive	mean
Isotype	01.4	05.90	01.1	08.55	02.6	07.14
-IL-6	96.9	18.73	94.4	28.10	99.4	77.74
+IL-6	99.9	42.73	84.0	18.70	92.8	28.68

Analysis of newly synthesized [^{35}S]-methionine-labeled γ1 heavy- and λ light-chains in the cells by immunoprecipitation and SDS-PAGE, showed that the synthesis of IgG increased more than 50-fold at day 3, and was further augmented at day 4 of IL-6 induction (Figure 5C). The IgG polypeptides were efficiently secreted (the secreted λ light chain, although not visible in this autoradiograph, was present in autoradiographs of longer exposures). These biochemical analyses are consistent with results obtained by the reverse hemolytic plaque bioassay (Raynal el al, 1989), and provide quantitative evidence that the synthesis and secretion of IgG are significantly augmented by IL-6.

Whereas γ1s mRNA accumulates as a function of time of IL-6 induction, the levels of γ1m mRNA did not vary significantly (Figure 5A and 5B). The surface expression of the mIgG was diminished slightly by IL-6 induction, as assayed by flow cytometry analysis (Figure 5D). The expression of surface MHC class II molecules, however, showed a marked reduction on 50% of the cells at day 4 of IL-6 induction (Figure 5D). This reduction of MHC class II is inversely correlated with increases in sIg synthesis and secretion (Figure 5B, 5C, and 5D). Thus, IL-6 coordinately induces the reduction of surface MHC class II expression, and increases sIg synthesis and secretion, which are hallmarks of terminal B cell differentiation.

The regulation of Ig and Oct-2 synthesis by IL-6 is subject to feedback control

Despite the clonal origin of CESS cells, every cell induced with IL-6 does not reflect the differentiated phenotype. The regulation of Ig synthesis by IL-6 in CESS cells is, however, reproducible. To obtain CESS cell clones capable of uniformly responding to IL-6, CESS cells were cloned by limiting dilution. Thirty five single cell clones were analyzed, and responsiveness to IL-6 was assessed by intracellular staining for the enhanced synthesis of IgG at day 4 of IL-6 induction, as shown in Figure 4A. One clone exhibited 50% responsiveness, three clones exhibited 30% responsiveness and six others exhibited 15-20% responsiveness to IL-6. Clones in which 30-50% of cells were enhanced in Ig synthesis at day 4 of IL-6 induction, were analyzed further. After continuous growth in tissue culture, these clones did not maintain the high responsiveness to IL-6 initially observed, for more than 2 weeks. In successive inductions with IL-6 the numbers of differentiating cells declined to approximately 10-20%. Some clones which did not respond to IL-6 upon initial stimulation, began to respond to IL-6 at low levels (approximately 3-5%) in later inductions. These observations and the heterogeneous response of CESS cells induced with IL-6 raises the question as to the fate of IL-6-differentiated cells, and whether the differentiation of B cells by IL-6 is subject to feedback control.

Analyses of Ig mRNA and protein synthesis in the course of a long term induction (Figure 6A) showed that stimulation with IL-6 beyond day 5 leads to a gradual decline of γ1s mRNA. After day 10 in

the continuous presence of IL-6, the level of γ1s mRNA declined to that of uninduced cells (Figure 6B, lanes a-g; Figure 7). The synthesis of IgG, as assayed by intracellular immunofluorescence increased and decreased accordingly (Figure 6C, panels a-g). The entire population was subsequently refractory to IL-6 stimulation unless IL-6 was removed for at least 5 days before re-stimulation (10 days in the experiment presented in Figure 6; Figure 6B, lanes h-j and 6C, panels i-j). The synthesis of γ1s mRNA and IgG were regulated in the course of re-stimulation as in the first IL-6 treatment. The enhancement of Ig synthesis was maximal after 4 days in the presence of IL-6 and declined after 10 days in the continuous presence of IL-6 (Figure 6B, lanes i-k and 6C, panels i-l).

The basis for the increase of Ig mRNA by IL-6 is transcriptional activation (Raynal et al, 1989). Electrophoretic mobility-shift assays have shown that the binding of Oct-2 to the octamer site in the Ig heavy chain (IgH) enhancer is temporally regulated in cells induced by IL-6. This Oct-2 DNA-binding activity increased before the increase of γ1s mRNA synthesis (Z. Liu, X. Zhang and S. Chen-Kiang, unpublished). In the long term IL-6 induction the Oct-2 mRNA sysnthesis was regulated in parallel to the γ1s mRNA. The levels of Oct-2 mRNA rose to 9-fold above that of uninduced cells by day 5 and fell by day 7 in the continuous presence of IL-6 (Figure 6B, lanes a-g). Re-stimulation of CESS cells with IL-6 led to another cycle of regulation of Oct-2 mRNA, as in the case of γ1s mRNA (Figure 6B, lanes h-k; Figure 7). Regulation of Oct-2 mRNA synthesis by IL-6 is specific, because the mb-1 mRNA (Figure 6B),

which encodes an mIgM-associated protein (Sakaguchi et al., 1988) appears not to be regulated by IL-6.

Figure 6. Activation of Oct-2 and Ig synthesis by IL-6 is subject to feedback control.

(A) A schematic representation of an extended treatment of CESS cells with IL-6. Cells were incubated in the absence (-IL-6, open bars) or presence (+IL-6, solid bars) of IL-6 and samples were removed on the days indicated by arrows for Northern blot analysis or immunofluorescence, or both.

(B) Northern blot analysis of poly A^+ γ1s and γ1m, Oct-2 and mb-1 mRNAs isolated from $3x10^7$ cells on days as shown in panel A. The signals of GAPDH are used as references for RNA loading.

(C) Analysis of Ig synthesis by phase contrast (Phase) and immunofluorescence microscopy (IFM) in cells removed on the days indicated in panel A after permeabilization and staining with anti-IgG.

Figure 6.

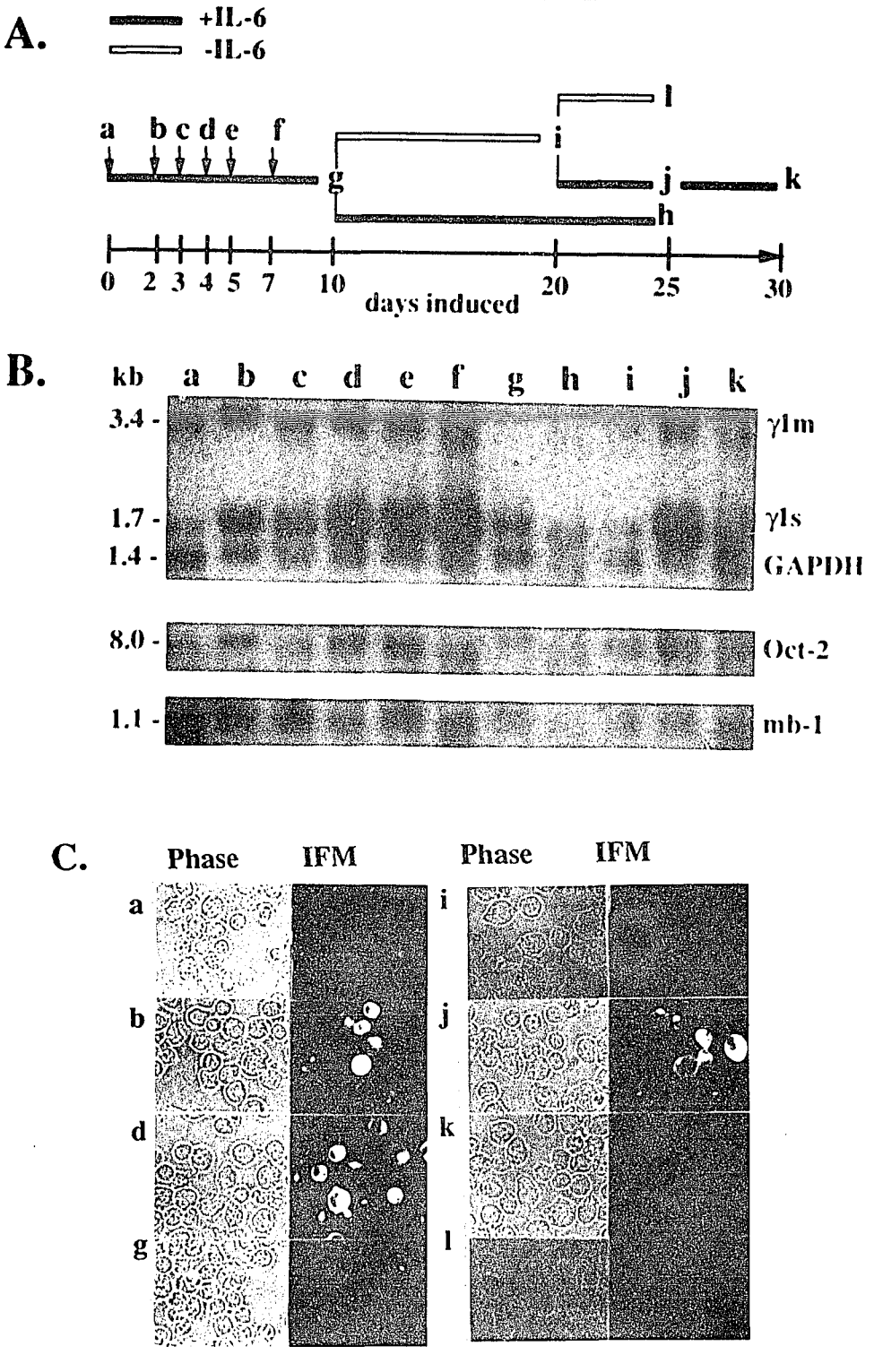
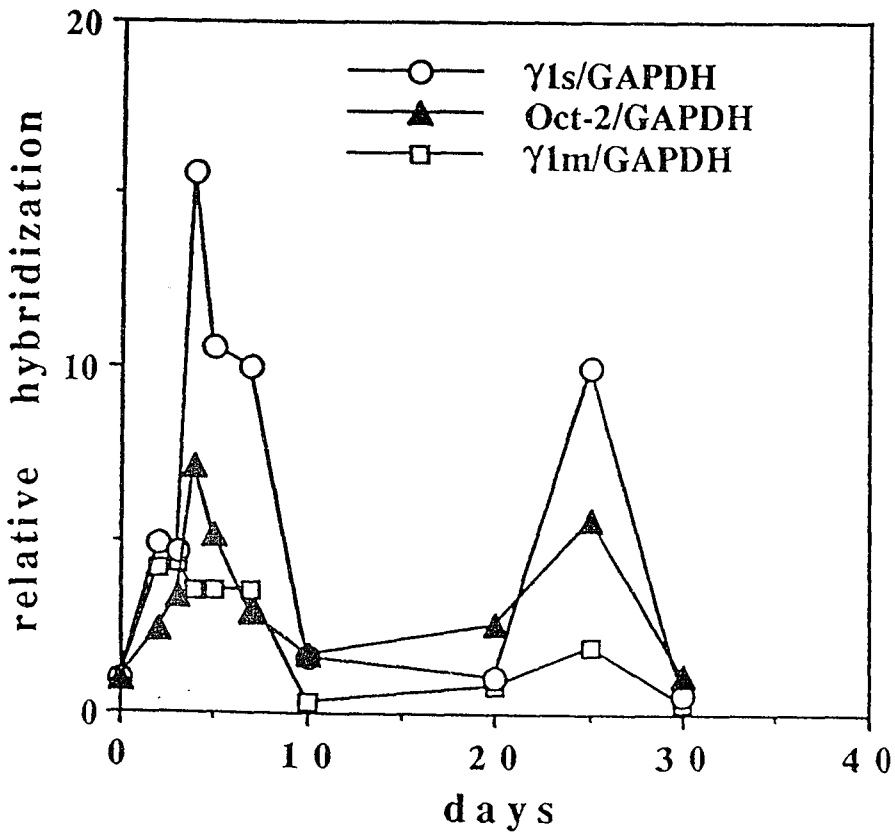

A.
+IL-6
-IL-6
a b c d e f g h i j k l
0 2 3 4 5 7 10 20 25 30
days induced
B.
kb a b c d e f g h i j k
3.4 -
1.7 -
1.4 -
γ1m
γ1s
GAPDH
8.0 -
Oct-2
1.1 -
mb-1
C.
Phase IFM Phase IFM
a b d g
i j k l

Figure 7. Temporal regulation of Oct-2, γ1s and γ1m mRNA synthesis by IL-6.

The hybridization signals shown in Figure 6B were quantified by densitometry scanning of autoradiographic signals within the linear range of an LKB scanner. Relative hybridization represents the ratios of γ1s to GAPDH, γ1m to GAPDH, and Oct-2 to GAPDH as a function of days of IL-6 treatment, with the ratios derived from the signals of day 0 arbitrarily set as 1.



These results suggest that the increases of Oct-2 DNA-binding activity (Z. Liu and S.Chen-Kiang, unpublished) is due in part to enhanced synthesis of Oct-2 mRNA. Since the changes in Oct-2 mRNA synthesis correlate with the changes in γ1s mRNA synthesis, Oct-2 may have a role in mediating the IL-6 signals to activate the transcription of the γ1 gene.

The loss of IL-6 responsiveness caused by prolonged IL-6 treatment may be attributed to the death of differentiated cells, feedback regulation, or both. Although an assessment of the fate of IL-6-induced cells will require the separation of the differentiated cells (~20%) from the undifferentiated cells, regaining IL-6-responsiveness after a period of IL-6 removal indicates that the regulation of Oct-2 and Ig synthesis by IL-6 is subject to feedback control.

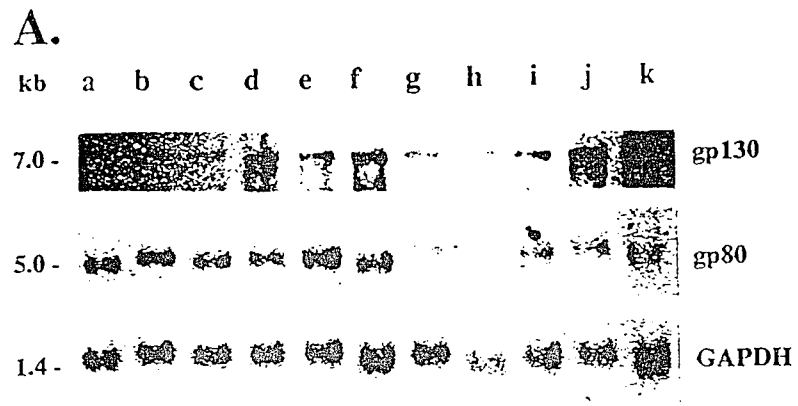
The two subunits of the IL-6 receptor are subject to feedback regulation by IL-6

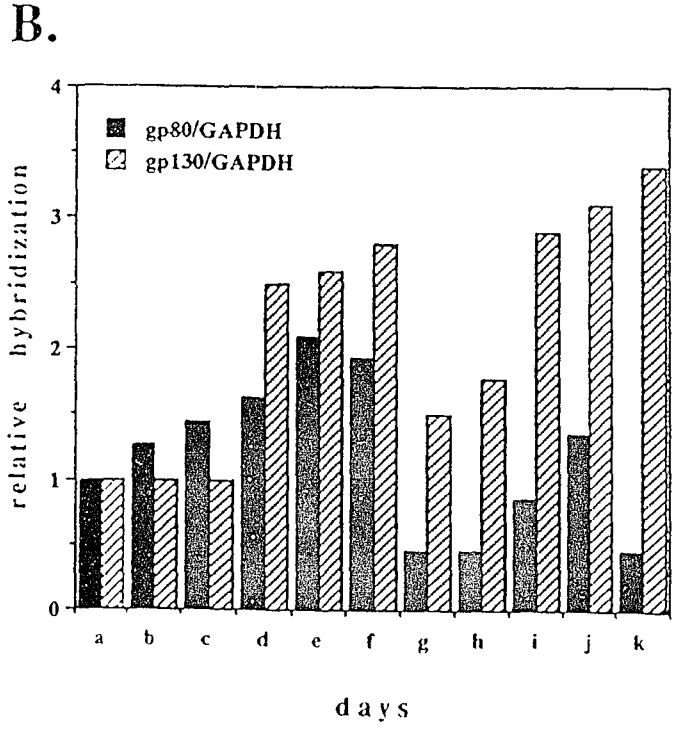
The feedback regulation of Ig and Oct-2 upon long term IL-6 stimulation may be reflected by regulation of molecules upstream of Ig and Oct-2 in the IL-6 receptor signaling pathway. To address this possibility, the two subunits of the IL-6 receptor, gp80 and gp130, were assessed in the course of the same long term IL-6 induction presented in Figures 6 and 7 (Figure 8). The mRNA encoding the IL-6-binding subunit, gp80, increased 2-fold by day 5 of IL-6 induction and declined to levels below that of uninduced cells by day 10 (Figure 8, lanes a-g).

Figure 8. The two subunits of the IL-6 receptor are regulated by IL-6.

(A) Northern blot analysis of poly A^+ gp80 and gp130 mRNAs isolated from $3x10^7$ CESS cells induced with IL-6 for the indicated number of days as shown in the schematic in Figure 6A.

(B) The hybridization signals shown in (A) were quantified by densitometry scanning of autoradiographic signals. Relative hybridization represents the ratios of gp80 to GAPDH, and gp130 to GAPDH as a function of days of IL-6 treatment, with the ratios derived from the signals of day 0 arbitrarily set as 1.





The mRNA encoding the signal transducing subunit, gp130, increased 3-fold by day 5 of IL-6 induction, and declined to 1.5-fold that of the level of uninduced cells by day 10 (Figure 8, lanes a-g). In the continued presence of IL-6 the mRNAs of both subunits remained at a reduced level (Figure 8, lane h). When IL-6 was removed for 10 days and the cells were re-induced with IL-6, mRNAs of both subunits increased in the course of re-stimulation with IL-6 (Figure 8, lanes h-k). Only the gp80 mRNA declined in the continued presence of IL-6 (Figure 8, lane K), by day 30 of IL-6 induction. A longer induction with IL-6 may be needed to better define whether the gp130 mRNA also declines after re-stimulation with IL-6. These data suggest that IL-6 signaling leads to the feedback regulation of the IL-6 receptor, and that this regulation may influence IL-6 receptor signals which mediate the feedback regulation of Ig and Oct-2 synthesis during B cell differentiation.

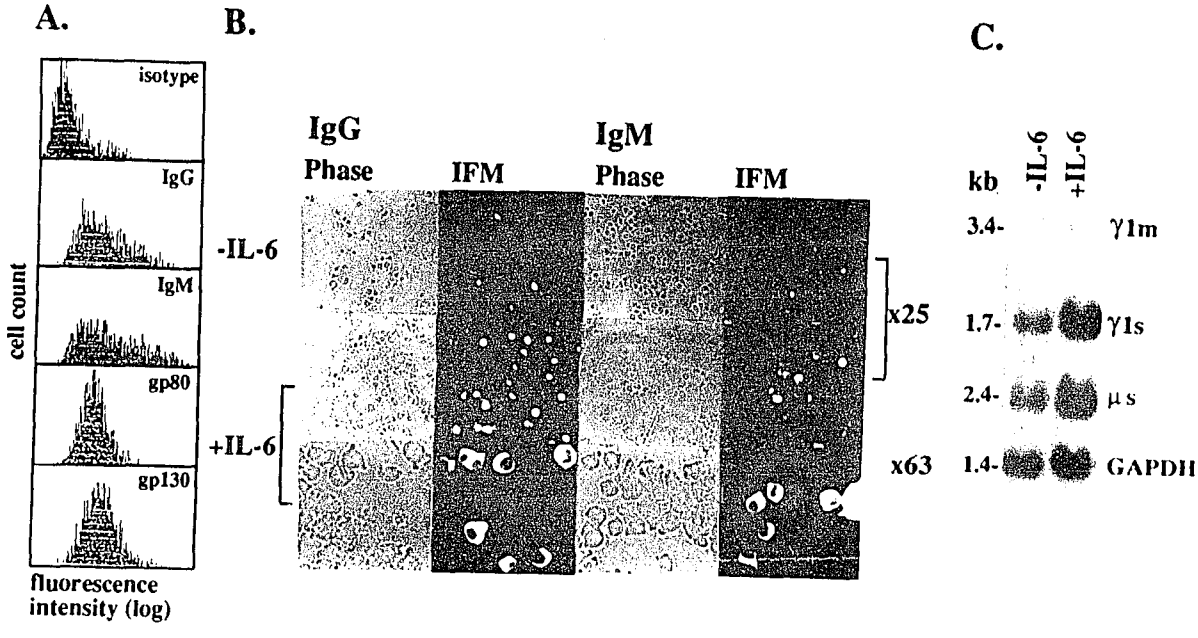
The differentiation of polyclonal human B cells by IL-6

Responsiveness to IL-6 is not restricted to B cells bearing IgG. An EBV-immortalized IgM-bearing human lymphoblastoid cell line SKW6-cl4, which represents B cells at the pre-class switched stage of B cell development, responds to IL-6 in a manner similar to CESS. The synthesis of μs mRNA and the secretion of IgM increased 20-25 fold upon IL-6 induction. Regulation of IgM synthesis is subject to feedback control and is preceded by the regulation of the Oct-2 DNA-binding activity (Z. Liu, R. Rowe and S. Chen-Kiang, unpublished).

Figure 9. IL-6 induces differentiation of polyclonal human tonsillar B cells.

(A) Flow cytometry analysis of surface IgG, IgM, and the two subunits of the high affinity IL-6 receptor, gp80 and gp130 present on tonsillar lymphocytes.

(B) After immortalization with EBV, tonsillar lymphocytes were incubated in the absence (-IL-6) or presence of IL-6 for 4 days (+IL-6), permeabilized, stained with anti-human IgM or IgG and subjected to phase contrast (Phase) and immunofluorescence (IFM) microscopy. X25 and X63 represent 250X and 630X magnifications, respectively. (C) Northern blot analysis of poly A+ γ1 and μ mRNA isolated from EBV-immortalized tonsillar B cells cultured in the absence (-IL-6) or presence of IL-6 for 4 days (+IL-6).



Lymphocytes freshly isolated from human tonsils were analyzed for the expression of IgM, IgG, and the two subunits of the IL-6 receptor (Figure 9A). In different preparations, approximately 20-50% of these cells express IgG, and 50-75% express IgM on the cell surface. About 72% of the cells were found to express the gp80 subunit, and 87% were found to express the gp130 subunit of the IL-6 receptor. The treatment of tonsillar B lymphocytes with IL-6 did not lead to enhancement of Ig synthesis, despite the expression of the two subunits of the IL-6 receptor (Figure 9A). These cells, however, were inducible by IL-6 after immortalization with EBV (Figure 9B). The expression of IgG, IgM, gp80 and gp130 was not tested after immortalization with IL-6. The synthesis and secretion of IgG and IgM were enhanced, and the cell morphology resembled that of plasma cells by day 4 after IL-6 induction (Figure 9B). The levels of γ1s and μs mRNAs were also elevated (Figure 9C). Since tonsillar B cells are polyclonal cells from a secondary lymphoid organ, the differentiation of these cells induced by IL-6 suggests a role for IL-6 in terminal B cell differentiation *in vivo*. These results also suggest that a subset of human B lymphocytes in secondary lymphoid organs are responsive to IL-6 signaling and are capable of differentiating into Ig-secreting cells, once they are activated to an IL-6-responsive state.

DISCUSSION

Terminal differentiation of human B cells by IL-6

We demonstrate that the hallmarks of terminal B cell differentiation are recapitulated in an IL-6-inducible CESS cell system. The expression of Ig, MHC class II, and the two subunits of the IL-6 receptor are coordinately regulated in these cells by IL-6 signaling. Dramatic and stage-specific alterations in cell morphology imply that IL-6 not only regulates the molecular and biochemical markers of B cell differentiation but also influences the architecture of the cell. IL-6 induces the differentiation of two human B cell lines which express IgG and IgM, and of polyclonal tonsillar B cells. This suggests that the responsiveness to IL-6 is not isotype-specific, and that the potential to differentiate is maintained in the presence of EBV-immortalization. The IL-6-inducible system serves as a useful model for investigating the cellular and mechanistic aspects of terminal B cells differentiation.

The syntheses of Oct-2 and Ig are subject to feedback control

The synthesis and secretion of Ig are regulated by a complex and precise program signaled by IL-6. In the CESS cell system, IL-6 regulates the preferential accumulation of the γ1s heavy chain mRNA by coordinate transcriptional initiation and elongation, as well as by RNA processing (Raynal et al, 1989). During terminal differentiation the secretory apparatus of B cells undergo expansion and re-

organization in order to accommodate the increase in Ig secretion (Weist et al., 1990). CESS cells differentiated by IL-6 appear to reflect this phenotype. Using the IL-6-inducible CESS cell system, the regulatory events which signal the morphological and molecular changes during IL-6-induced B cell differentiation were examined.

The cytoplasmic events of the IL-6 signal transduction pathway are not very well understood. Transcription factors of the AP-1 and C/EBP families are likely to participate in nuclear signaling by IL-6 as they are rapidly and selectively regulated upon IL-6 induction (Nakajima and Wall, 1991; Lord et al, 1991; Akira et al, 1990; Kinoshita et al, 1992). Our results show that transcription factor Oct-2 is regulated by IL-6 and is subject to feedback control upon long term treatment of CESS cells with IL-6. As Oct-1 can be regulated by NF-IL6 in human embryonal carcinoma and T cells (Hsu and Chen-Kiang, 1993), Oct-2 may also be regulated by NF-IL6 in the IL-6 signaling cascade in B cells. Although the structure of the *oct-2* promoter is unknown, investigating the events prior to Oct-2 in the regulatory cascade, and the restriction point for the feedback control, may give insight into the mechanism of IL-6 signaling in B cells.

The role of Oct-2 in terminal B cell differentiation

The physiological role of Oct-2 in the transcription of Ig genes has been challenged recently because, in the presence of a B cell derived co-activator, the nonlymphoid-specific Oct-1 protein can activate the transcription of Ig promoters in a cell-free system (Luo

et al, 1992). Although the DNA-binding activity of Oct 1 is activated by IL-6 in human embryonal carcinoma and T cells (Hsu and Chen-Kiang, 1993), it was not significantly regulated in human B cells. Oct-2 activates transcription directed by Ig promoters and enhancers (Staudt and Leonardo, 1991) and is critical for terminal differentiation of B cells (Corcoran et al, 1993). The temporal relationship between the regulation of Oct-2 and Ig expression, and the lack of significant regulation of Oct-1 (Liu & Chen-Kiang, unpublished), suggest that Oct-2 may mediate the IL-6 responses by activating the transcription of Ig genes.

Nuclear signaling by IL-6 may also be modulated by protein-protein interactions between transcription factors which are coordinately regulated. Oct-1 associates with proteins of viral and cellular origins (Gerster and Roeder, 1988; Stern et al, 1989; Ullman et al, 1991; Pomerantz et al, 1992), and activates transcription of Ig promoters in a cell-free system in the presence of a B cell-derived factor, OCA-B (Luo et al, 1992). The mechanism of Oct-2 action and its promoter-specificity have yet to be understood. Whether the activity of Oct-2 is modulated by protein-protein interactions is unknown. It would be of interest to investigate whether this B cell-derived factor can be regulated by IL-6 and to identify Oct-2-associated regulatory proteins in B cells. Apart from Ig genes, Oct-2 may be involved in the regulation of other promoters which contain the octamer motif in B cells. Whether IL-6 and Oct-2 are involved in the regulation of such promoters is likely to be informative with respect to the promoter specificity of IL-6

signaling, and the role of Oct-2 during terminal B cell differentiation.

The significance of feedback regulation of the two subunits of the IL-6 receptor by IL-6 signaling.

The heterodimeric IL-6 receptor transduces the IL-6 signals in target cells. Our results show that gp80 and gp130 are regulated during terminal B cell differentiation induced by IL-6. The steady state levels of mRNA encoding gp80 increases to 2-fold, and gp130, to 3-fold above that of uninduced cells at day 4 of IL-6 induction. This regulation is subject to feedback control upon long term IL-6 treatment of B cells. Given that only 20% of CESS cells differentiate upon IL-6 induction, the changes in the levels of mRNAs encoding gp80 and 130, although small, may be significant. Upon IL-6 stimulation, if each differentiating B cell is induced to express twice as many IL-6 receptor subunits on its surface, the effect on IL-6 receptor signal transduction in those cells may be significantly increased. In the continued presence of IL-6, the decreased expression of the IL-6 receptor subunits may also serve to regulate IL-6 signaling in each differentiating cell. Further investigations at the single cell level will be needed to assess the quantitative changes of the IL-6 receptor subunits on the surface of individual cells during terminal B cell differentiation. These results provide the first example of the regulation of the two subunits of the IL-6 receptor, and suggest a potential mechanism by which IL-6 may modulate its own signaling.

The biological significance of IL-6 signaling in cells of the B lineage

IL-6 has been implicated in the control of cell proliferation and survival. The most prominent example is the association between deregulated expression of IL-6 and multiple myeloma (Nordan and Potter, 1986). Studies in transgenic mice establish that the ectopic expression of IL-6 in the appropriate genetic background leads to chromosomal translocation and plasmacytoma, thereby confirming the role of IL-6 in oncogenesis (Suematsu et al, 1992). The mechanism by which IL-6 regulates cell growth, however, is not understood. An IL-6-inducible cellular activity can functionally substitute for the adenovirus E1A oncoproteins in transactivation and NF-IL6 reconstitutes this activity (Spergel and Chen-Kiang, 1991; Spergel et al, 1992). Implicit in these results is a link between components in the IL-6 signaling pathway and transforming proteins known to regulate the progression of the cell cycle.

A remaining question concerning terminal B cell differentiation is how the plasma cells undergo growth arrest *in vivo*. IL-6 differentiates human B cells with the cooperation of a second signal, whose function can be fulfilled by EBV-immortalization. The identification of this signal and the investigation of the fate of IL-6-differentiated cells may further our understanding of the balance between proliferation and differentiation during B cell development.

IV

The Expression of Surface MHC Class II is Reduced by IL-6 signaling.

ABSTRACT

We have found that IL-6 regulates the expression of major histocompatibility complex (MHC) class II in human B cells. The expression of MHC class II on the cell surface is dramatically reduced on differentiated cells upon IL-6 induction, and is not restricted to a particular locus of MHC class II. By investigating the expression of MHC class II at the mRNA and protein levels, we have observed that in B cells induced with IL-6, MHC class II is primarily regulated by mechanisms subsequent to mRNA synthesis. The reduction of MHC class II on differentiated cells is inversely correlated with the synthesis and secretion of the secreted form-specific Ig mRNA and protein. In cells exhibiting reduced expression of surface MHC class II, enhanced synthesis of an endoplasmic reticulum resident stress protein, GRP94, was seen. Our results suggest that the synthesis of GRP94 may be preferentially induced by IL-6, and may be involved in the regulation of MHC class II expression during terminal B cell differentiation.

INTRODUCTION

The MHC class II molecules are heterodimeric cell surface glycoproteins which present peptide antigens to class II-restricted T cells. They are composed of highly polymorphic α and β chains, and are expressed in specialized cells known as antigen presenting cells which include B lymphocytes. The genes encoding MHC class II molecules are developmentally regulated in the B lineage. Upon terminal differentiation, MHC class II expression is reduced on the surface of plasma cells, the stage at which the synthesis and secretion of sIgs are at their peak (Halper el al., 1978).

Activation of MHC class II expression in different cell lines can be mimicked by the use of a variety of agents including lymphokines such as IL-4 and IFNγ (Novelle et al., 1984; Noelle et al., 1986; Polla et al., 1986; Mond et al., 1986), and appears to be controlled primarily at the level of transcription (Tsang et al., 1988). Extensive dissection of the genes encoding the MHC class II complex has defined the sequence elements and the transcription factors which are important for MHC class II expression in transfection and in cell-free systems (for a review, see Kara and Glimcher, 1992).

The mechanism governing the reduction of MHC class II expression in the final step of B cell development is not yet understood. The reduction of MHC class II has been observed in mouse B lymphocytes treated with corticosteroids or LPS (Dennis and Mond, 1986; Gravallese et al., 1989), although LPS is also known

to increase class II expression (Monroe et al., 1983). Cell fusion studies suggest that the reduction of MHC class II upon terminal B cell differentiation may be due to a dominant repression mechanism operating at the level of transcription (Venkitaraman et al., 1987; Latron et al., 1988; Dellabona et al., 1989).

The expression of the αβ heterodimers of MHC class II is controlled at multiple levels of its biosynthesis. The assembly and transport of these dimers to the cell surface are facilitated by a third component, the invariant (Ii) chain (Jones et al., 1979; Roche and Cresswell, 1990; Layet and Germain, 1991; Roche and Cresswell, 1991; Anderson and Miller, 1992; Viville et al., 1993). In the absence of Ii, MHC class II molecules appear to be retained in the ER by stress proteins, in particular, GRP94 (Schaiff et al., 1992). These findings suggest that events downstream of mRNA synthesis may contribute to the regulation of surface MHC class II expression.

We have used the IL-6-inducible CESS cell system to address the mechanism of MHC class II regulation during terminal B cell differentiation. We show that IL-6 signaling in CESS cells leads to the reduction of surface MHC class II expression. Our results suggest that the mechanism of this regulation is not due to the reduced synthesis of mRNAs encoding the α, β and Ii chains of MHC class II, but may involve events subsequent to mRNA synthesis. The expression of GRP94 is induced by IL-6 and is co-localized to cells with enhanced Ig synthesis and reduced class II expression, thus suggesting a possible role for GRP94 in mediating the IL-6-induced reduction of surface MHC class II expression.

RESULTS

The expression of surface MHC class II is reduced by IL-6 signaling.

The expression of MHC class II and IgG was analyzed by immunofluorescence microscopy in CESS cells (Figure 10). CESS cells were induced to differentiate with IL-6 for 4 days, and surface MHC class II was detected with monoclonal antibody CA 2.06, specific for all MHC class II haplotypes, and counterstained with a fluorescein-conjugated secondary antibody (Figure 10A). The intracellular IgG1 was detected with a rhodamine-conjugated anti-human IgG (Figure 10B). While MHC class II was abundantly expressed on the surface of cells synthesizing a basal level of IgG, it was markedly reduced on the differentiated cells which are characterized by the increased synthesis of IgG (Figure 10C). These results demonstrate that Ig synthesis and surface MHC class II expression are inversely regulated by IL-6.

Analysis of the temporal regulation of MHC class II by flow cytometry showed that the expression of surface MHC class II diminishes as a function of time of IL-6 induction. Approximately 50% of the cells at days 4 and 5 after induction showed a reduced level of surface MHC class II expression (Figure 11A). IL-6-induced cells expressing high and low levels of surface MHC class II were separated by FACS-sorting and analyzed by immunofluorescence microscopy for the synthesis of IgG (Figure 11B).

Figure 10. Reduction of surface MHC class II expression in IL-6-differentiated cells.

CESS cells were stained for surface MHC class II (Panel A) and intracellular IgG (Panel B) simultaneously, after induction with IL-6 for 4 days. Panel C shows an overlapped exposure of panels A and B.

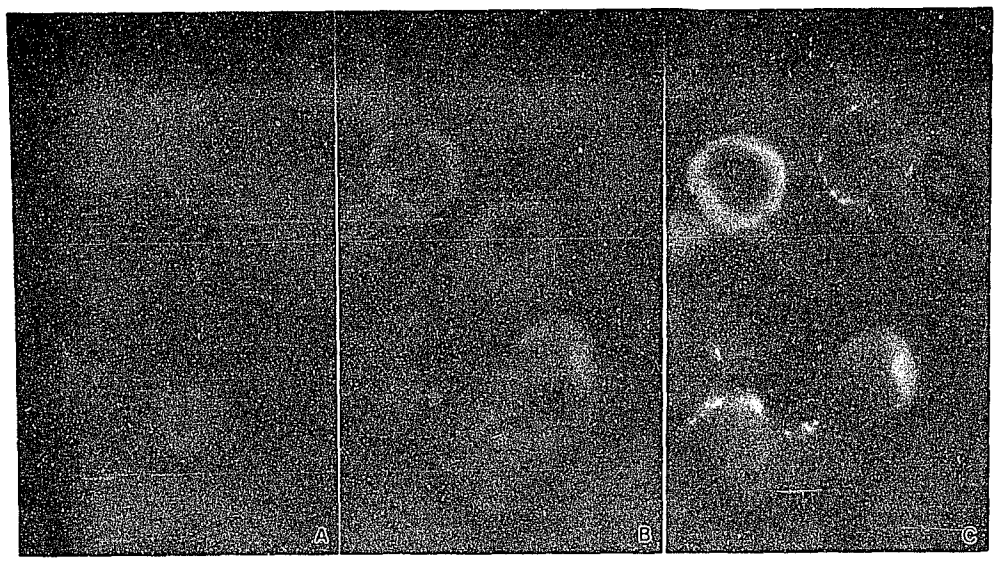
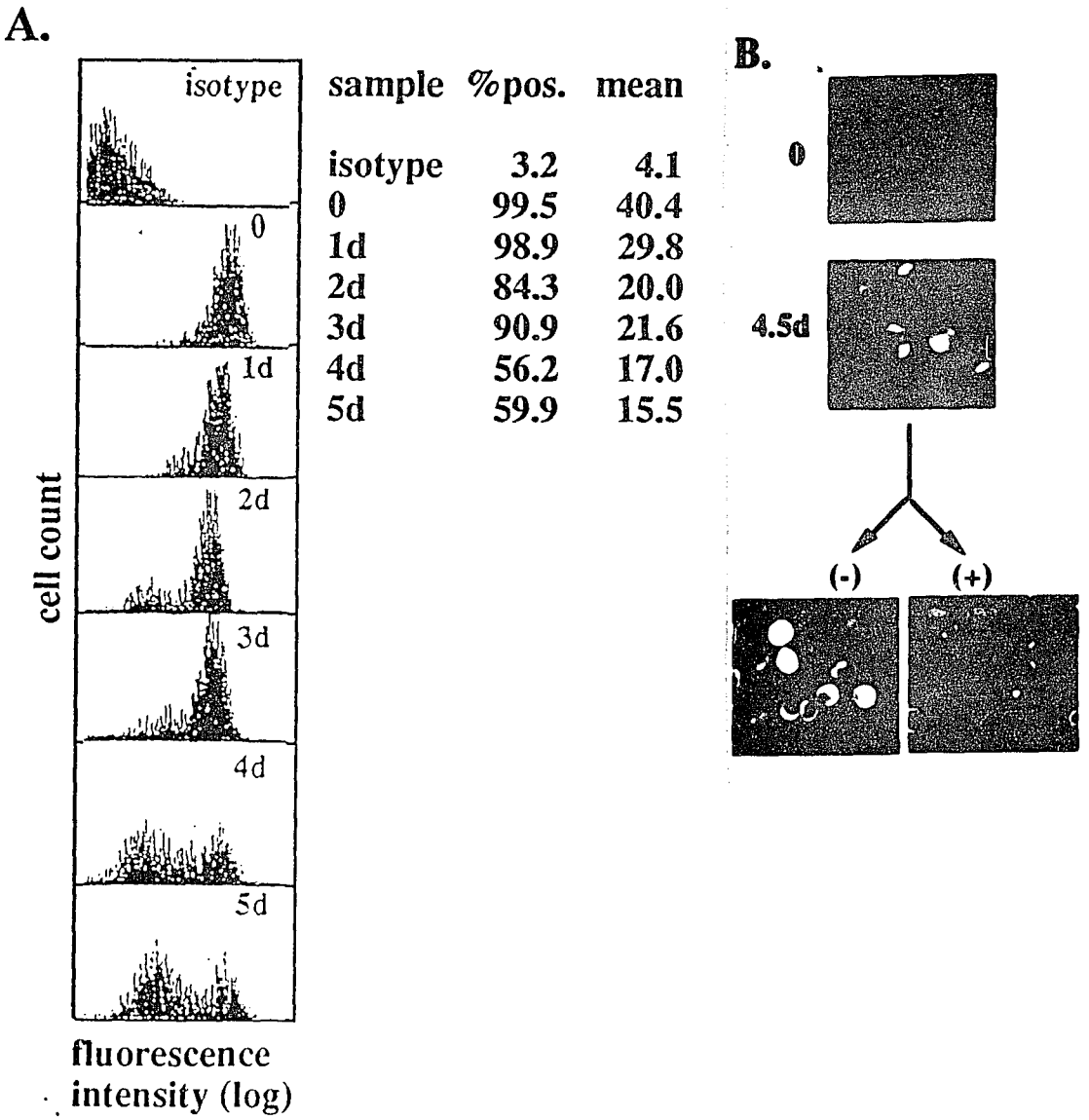


Figure 11. Surface MHC class II and intracellular Ig are inversely regulated by IL-6.

(A). The surface class II expression on CESS cells was assayed on the indicated days after IL-6 induction with mAb CA 2.06. The fluorescence intensity was measured by flow cytometry.

(B). CESS cells were induced with IL-6 for 4.5 days and sorted for the 25% of cells expressing the highest(+) or the lowest (-) surface class II by FACS. Uninduced cells (0), and induced cells before (4.5d), and after sorting (+,-) were stained for intracellular IgG.



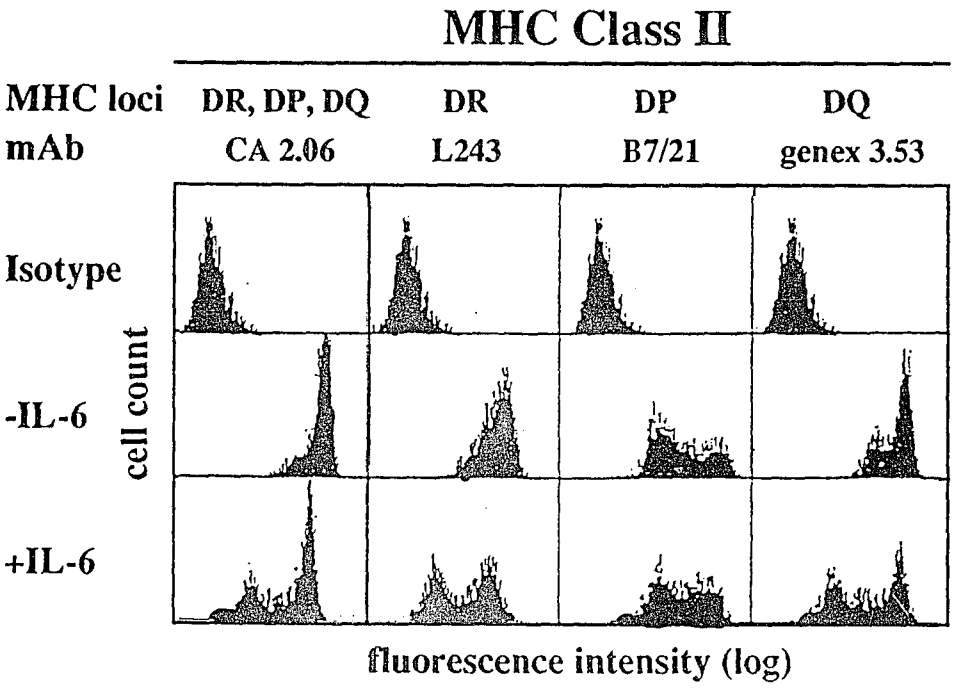
The cells which expressed low surface MHC class II showed a 3-fold enrichment for differentiated cells (approximately 45%, as assessed by intracellular staining for IgG synthesis) above that seen in the unsorted population induced with IL-6 (approximately 15%). Cells enhanced in Ig synthesis were rarely found among cells which maintained high levels of surface MHC class II expression at day 4.5 of IL-6 induction (Figure 11B). These results confirm the observations in Figure 10, and provide quantitative evidence that in cells enhanced in Ig synthesis, the expression of surface MHC class II is reduced.

The reduction of surface MHC class II expression is not restricted to a particular locus of MHC class II.

To examine which of the loci of MHC class II are expressed in CESS cells and to determine whether they are all subject to reduction upon differentiation, flow cytometry was performed with monoclonal antibodies specific to each of the major MHC class II loci, DR (mAb L243), DP (mAb B7/21), and DQ (mAb genex 3.53). As shown in figure 12, CESS cells expressed all three of these loci, although not uniformly. The surface expression of locus DR and DQ was dramatically reduced at 4.5 days of IL-6 induction. Although some CESS cells express the DP locus at comparable levels to loci DR and DQ, a second CESS cell population expressed low levels of this locus. Upon IL-6 induction the reduction of surface DP expression was not as significant as loci DR and DQ.

Figure 12. Reduction of surface MHC class II expression is not restricted to a locus of MHC class II.

CESS cells were induced with IL-6 and stained separately with monoclonal antibodies specific for the three MHC class II loci DR, DP and DQ. Monoclonal anti-mouse IgG was used as an isotype control. Fluorescence intensity was quantified by flow cytometry.



sample	%pos.	mean	%pos.	mean	%pos.	mean	%pos.	mean
isotype	3.0	4.2	3.0	4.2	3.0	4.2	3.0	4.2
-IL-6	99.9	99.5	99.7	46.3	98.0	30.9	99.7	72.4
+IL-6	94.0	28.5	76.3	18.4	96.6	32.7	89.0	27.1

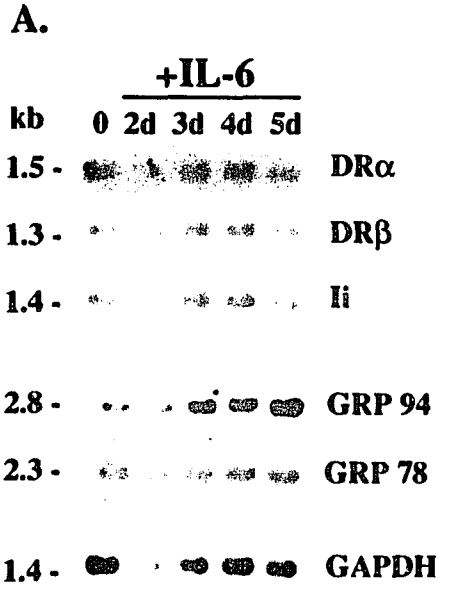
Preliminary results show that is no appreciable change in the expression of surface MHC class I (detected by mAb W6/32) after IL-6 induction (data not shown). These results indicate that the regulation of MHC class II by IL-6 is not restricted to a particular locus of MHC class II.

The reduction of surface MHC class II correlates with enhanced synthesis of GRP94

The IL-6-induced reduction of MHC class II expression could be due to transcriptional or postranscriptional controls. The regulation of MHC class II DR, the major species of the three polymorphic MHC class II molecules expressed in human B cells, was further analyzed. Northern blot analysis showed that there was no appreciable change in the levels of mRNA encoding DRα and DRβ, the two components of the heterodimeric DR complex, or the Ii chain, which is required for efficient assembly and transport of MHC class II molecules (Figure 13). These results suggest that the basis for the IL-6-induced reduction of surface MHC class II expression is unlikely to be the synthesis of mRNA encoding MHC class II molecules or the Ii chain but may lie further downstream in the MHC class II biosynthetic pathway. We investigated the synthesis of GRP94, a stress protein thought to retain MHC class II molecules in the ER in the absence of the Ii chain (Schaiff et al, 1992). The mRNA which encodes GRP94 was elevated to a level 5-fold above that in uninduced cells by day 5 of IL-6 induction (Figure 13). The mRNA which encodes GRP78/BiP, a related stress protein in the ER which binds to Ig heavy- and light-

Figure 13. IL-6 induces GRP94 mRNA.

(A) Northern blot analyses of DRα, DRβ, Ii, GRP94, GRP78 and GAPDH mRNAs in CESS cells induced with IL-6 for the indicated number of days. Each lane contains poly(A)+ RNA isolated from 10^7 CESS cells.



chains (Bole et al, 1986; Nakaki et al, 1989), did not increase significantly.

These results show that the regulation of MHC class II during terminal B cell differentiation is unlikely to be due to the diminished synthesis of the mRNAs encoding DRα, DRβ, or the Ii chain. The synthesis of GRP94 is increased by IL-6 signaling, and suggests that GRP 94 may play a role in the mechanism by which the reduction of surface MHC class II is regulated.

Increase in GRP94 protein correlates with reduction of class II expression

The co-localization of class II, Ig and GRP94 proteins was investigated using confocal microscopy. CESS cells were permeabilized and simultaneously stained for Ig and MHC class II, GRP94 and Ig, or GRP94 and MHC class II (Figure14A). In differentiated cells characterized by increased intracellular IgG, the level of intracellular MHC class II expression was reduced. This suggests that the reduction of surface MHC class II does not lead to increased accumulation of MHC class II molecules within the cell. Since the mRNAs encoding α, β, and the Ii chains were detected after IL-6 induction, the reduction in MHC class II may be due to the lack of synthesis or the enhanced degradation of the MHC class II proteins.

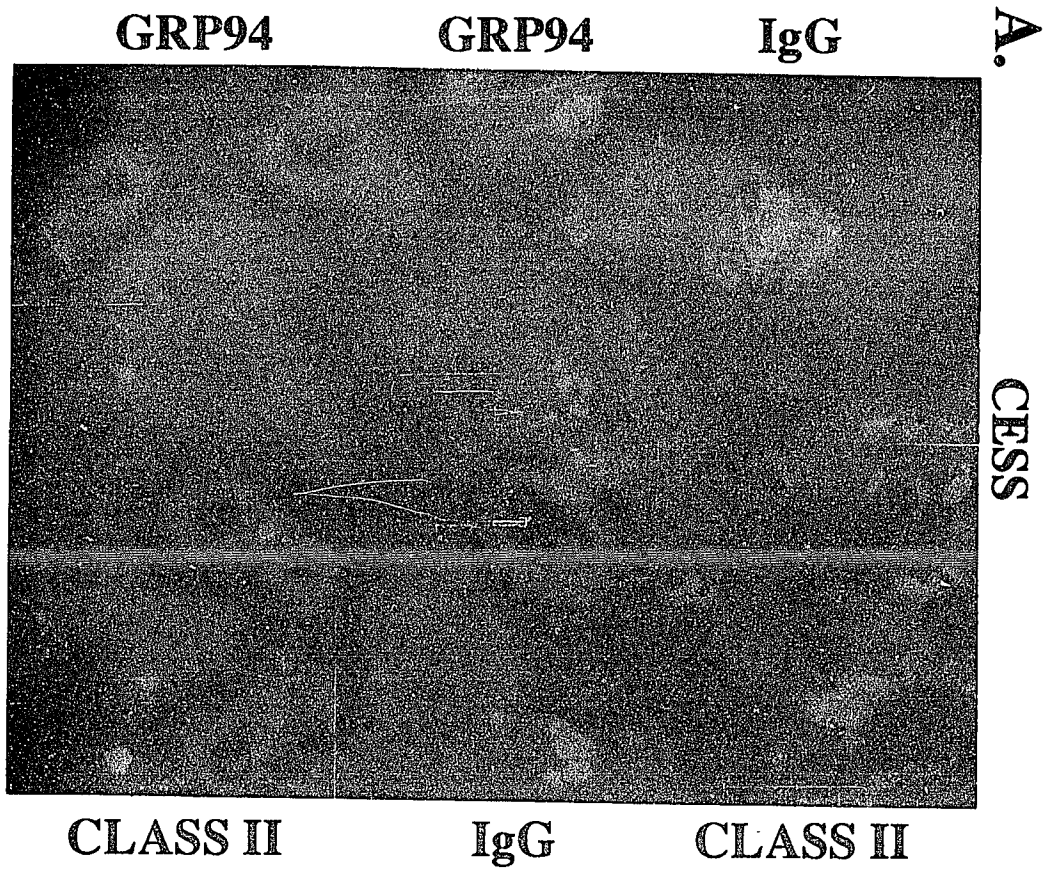
The GRP94 protein was elevated in differentiated cells characterized by increased intracellular IgG, and corresponded to the increase of GRP94 mRNA observed by Northern blot analysis in

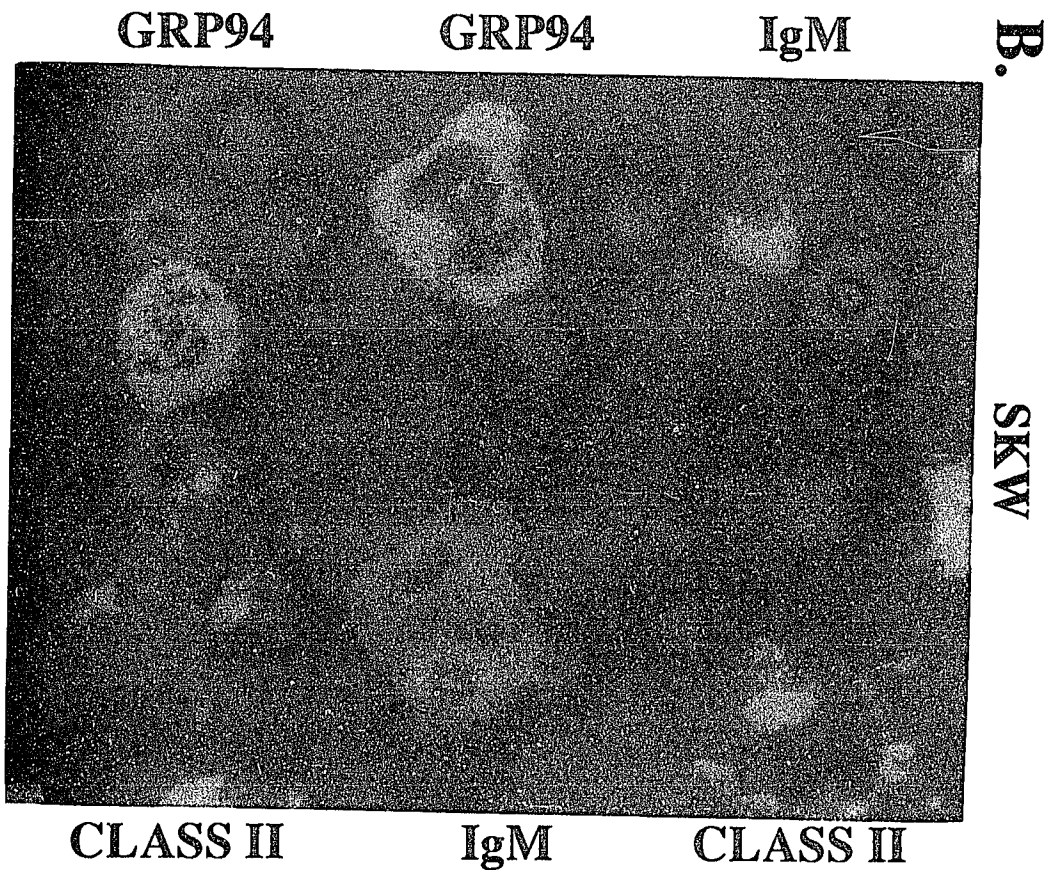
Figure 13. In cells with elevated levels of GRP94, MHC class II expression was reduced (Figure 14A), which corroborates the co-localization patterns of Ig and MHC class II in differentiated cells. Similar results were obtained in an IgM-bearing cell line SKW6.c14 (Figure 14B) in which the IgM, MHC class II, and GRP94 protein co-localization patterns corresponded with the observations in CESS cells. The results from these cell lines suggest that GRP94 co-localizes to cells which are reduced in MHC class II expression, and may function to decrease MHC class II expression during terminal B cell differentiation.

Figure 14. GRP94 protein is increased in cells with increased Ig synthesis and reduced MHC class II expression.

(A) Confocal microscopy analysis of CESS cells induced with IL-6 for 4.5 days, permeabilized, and stained for Ig and class II, GRP94 and Ig, or GRP94 and class II.

(B) Confocal microscopy analysis of SKW6.c14 cells prepared and stained as in (A).


GRP94
GRP94
IgG
A.
CESS
CLASS II
IgG
CLASS II


GRP94
GRP94
IgM
B.
SKW
CLASS II
IgM
CLASS II

DISCUSSION

During terminal B cell differentiation the Ig heavy- and light-chain genes are regulated by a complex and precise program signaled by IL-6. The activation of initiation of transcription provides the molecular basis for quantitative increases in the synthesis of Ig heavy- and light- chains (Raynal et al, 1989). The membrane form-specific Ig heavy chain mRNA is subject to additional control at the protein level, resulting in reduced expression of Ig on the cell surface. The secretion of Ig induced by IL-6 must be accompanied by the development of the secretory apparatus, as evident by the well expanded and organized ER displayed by IL-6-differentiated cells.

In contrast to the increased synthesis of sIg, IL-6 signals the reduction of surface MHC class II expression in B cells. Transcriptional regulation appears to be the primary control for the activation of MHC class II expression earlier in the B lineage (Noelle et al, 1986; Polla et al, 1986). The reduction of the constitutive expression of MHC class II in human B cells after fusion with mouse plasmacytoma cells was attributed to transcriptional repression (Dellabona et al, 1989). The identity of this putative repressor remains to be determined. We have shown that the genes encoding DRα, DRβ and the Ii chain continued to be transcribed after induction with IL-6, when the expression of surface MHC class II is reduced. Our results differ from the prevailing view that MHC class II expression is predominantly regulated at the level of transcription. They suggest that the regulation of MHC class II expression may

occur by translational or posttranslational mechanisms, or both. In this IL-6-induced terminal differentiation system, the Ig and MHC class II genes are appropriately regulated, and the cells undergo changes in morphology characteristic of plasma cells *in vivo*. Therefore, it is likely that the reduction of surface MHC class II expression by mechanisms downstream of mRNA synthesis is physiologically relevant to B cell differentiation *in vivo*.

A potential role for GRP94 in the reduction of MHC class II expression.

The enhanced synthesis of GRP94 and the reduction of surface MHC class II in IL-6-differentiated cells, suggests involvement of GRP94 in the regulation of MHC class II expression. GRP94 and GRP78/BiP are the two major ER proteins which are coordinately induced by glucose starvation and other stresses at the level of transcription (Liu and Lee, 1991). GRP94 is also regulated at the protein level in mouse splenic lymphocytes stimulated with LPS (Lewis et al, 1985). Our results have provided the first example that this stress protein can be regulated at the level of mRNA synthesis by a lymphokine, and that the regulation of GRP94 can be uncoupled from that of GRP78/BiP.

The function of GRP94 has not been well defined. Based on biochemical evidence obtained in transfection, Schaiff et al (1992) proposed that GRP94 binds to and retains MHC class II molecules in the ER in the absence of the Ii chain. Our results are consistent with this hypothesis but suggest that in B cells expressing the Ii chain,

an increase in GRP94 above the constitutive level may also lead to the retention of MHC class II molecules *en route* to the cell surface. The MHC class II molecules do not seem to accumulate intracellularly in cells which exhibit reduced expression of surface MHC class II. Therefore, the elevated levels of GRP94 may cause the MHC class II molecules to be inappropriately processed, targeted and degraded in the ER, a site which is thought to function as a clearinghouse for newly synthesized proteins (Klausner and Sitia, 1990). The modification, assembly and targeting of MHC class II molecules are known to be crucial for antigen presentation (Roche and Cresswell, 1990; Roche and Cresswell, 1991; Layet and Germain, 1991; Peters et al, 1991; Viville et al, 1993). Our results suggest that these events may also participate in the regulation of MHC class II during terminal B cell differentiation by mechanisms involving ER stress proteins.

The function of GRP94 in B cells may not be limited to interactions with MHC class II molecules. GRP94 associates with unassembled Ig heavy- and light-chains and forms ternary complexes which also contain GRP78/BiP. This suggests a functional interaction between the two stress proteins in the folding and assembly of Ig (Melnick et al, 1992). The coordinate regulation of GRP94 and sIg by IL-6 may have a role in the expression of mIg. The increase in sIg heavy chains could potentiate its assembly with the light chains, and result in the retention of unassembled mIg heavy chains by GRP94 and GRP78/BiP. This retention would reduce the expression of mIg during B cell differentiation.

In human B cells, IL-6 transduces its signals with a high degree of specificity. Whereas the Ig heavy- and light- chain genes are regulated at the level of transcription, the genes encoding the class II molecules, DRα, DRβ and the Ii chains, are not. While GRP94 is regulated by IL-6, GRP78/BiP appears not to be. During terminal B cell differentiation, to accommodate the high rate of secretion of Igs, the rough ER and other secretory organelles undergo expansion (Wiest et al., 1990). This expansion of the ER involves the increase in synthesis of several ER proteins in proportion to ER size. The expression of some selected proteins, however, are increased preferentially (Wiest et al., 1990). To ascertain that the synthesis of GRP94 is regulated by IL-6 preferentially, it is important to assess the regulation of other ER resident proteins which may be regulated by IL-6 during terminal B cell differentiation. Our results that GRP78/BiP is not significantly regulated by IL-6 supports the preferential regulation of GRP94 by IL-6. These results raise important questions as to how the specificity of IL-6-induced regulation is determined during terminal B cell differentiation.

Our results in the CESS cell system show that GRP94 may be involved in the IL-6-induced reduction of surface MHC class II expression. Transient transfection studies will be needed to directly test and confirm these results. By overexpressing GRP94 in CESS cells, the possible changes in surface MHC class II and the association of GRP94 with MHC class II, can be investigated. An efficient method to transfect CESS cells would need to be established for these investigations. The consequence of overexpressing a stress protein in B cells may not be trivial. Since

GRP94 and GRP78/BiP also associate with Ig, the overexpression of GRP94 may alter the surface expression and secretion of Ig. These questions, and the molecular role of GRP94 during terminal B cell differentiation, are amenable to investigation in the IL-6-inducible CESS cell system.

V

Crosslinking Human B Cell FcγII Receptors Leads to Cell Death, which is Delayed by IL-6

ABSTRACT

The function of the Fc receptor for IgG (FcγR) has been studied in human B lymphocytes. Crosslinking of FcγRII on the surface of EBV-immortalized human B lymphoblastoid cells by polyclonal anti-FcγRII IgG or $F(ab')_2$ leads to cell death that is dose- and time-dependent. The FcγR-mediated death can be delayed, but not suppressed, by IL-6. Similarly, crosslinking FcγRII on tonsillar B lymphocytes accelerates their death in culture, which can be delayed by IL-6. Although crosslinking of FcγRII does not interfere with induction of immunoglobulin synthesis by IL-6, its effect on cell survival is dominant. These results suggest that in contrast to the functions of FcγRs in other lymphoid and myeloid cells, human B cell FcγRs may function to regulate the number of mature B cells during terminal differentiation by active cell death.

INTRODUCTION

One of the hallmarks of terminal B cell differentiation is the growth arrest, or possibly death, of terminally differentiated plasma cells. Most plasma cells in the lymph nodes are short-lived with a half-life of less than three days, as shown by ^{3}H-thymidine labeling of rodents *in vivo* (Miller, 1963; Ho et al., 1986). The mIg and associated molecules, which function as antigen receptors (Venkitaraman et al., 1991), clearly have a central role in determining clonal specificity during B cell ontogeny. Crosslinking of mIg can lead to anergy or deletion of autoreactive B cell clones, apparently by inhibiting the growth of immature and mature B lymphocytes (Nemazee and Burki, 1989; Goodnow et al., 1989; Hartley et al., 1991), or to positive selection and expansion affinity maturation in the germinal centers (Liu et al., 1986). IL-6 plays an important role in terminal B cell differentiation, since it induces antibody synthesis and secretion by activated murine and human B cells (Kikutani et al., 1985; Raynal et al., 1989). However, the signals which regulate growth of B cells during the terminal stage of differentiation are not very well understood.

Receptors capable of binding the Fc portion of IgG (Fcγ receptors) are found on cells of the hematopoietic lineage. Fcγ receptors expressed on the surface of B cells may participate in regulating the proliferation of activated B cells and in modulating immune responses (Phillips and Parker, 1984). Activation of FcγRs requires the crosslinking of multiple FcγRs by immune complexes.

Upon crosslinking, FcγRs trigger a variety of responses in macrophages and neutrophils, including phagocytosis, antibody-dependent cellular cytotoxicity (ADCC) and the release of mediators of inflammation (Boros et al., 1991; Odin et al., 1990).

FcγRs form a multigene family and have structural features characteristic of members of the Ig super family. Human FcγRs are grouped into three subclasses, FcγRI (CD64), FcγRII (CD32), and FcγRIII (CD16), based primarily on antigenicity and the structure of the extracellular IgG binding domains (Odin et al., 1990; Ravetch and Kinet, 1991; Unkeless et al., 1992). Although the extracytoplasmic domains within any FcγR subclass are highly homologous, the intracytoplasmic domains are often highly divergent and may mediate different functions (Brooks et al., 1989).

FcγRs have no known intrinsic enzymatic or G-protein activities and thus, the signal transduction of FcγRs is not fully understood. Most studies of B cell FcγR function have been carried out in mice. Murine B cells express FcγRII-1, which differs from the FcγRII-2 splice-variant by a 47 amino acid cytoplasmic domain insertion that bears a serine phosphorylation site (Hunziker et al., 1990). The insertion in murine FcγRII-1 blocks FcγRII-mediated endocytosis and antigen presentation (Amigorena et al., 1992). The crosslinking of FcγRs on the surface of murine splenic B cells inhibits B cell proliferation and results in decreased Ig secretion (Phillips and Parker, 1985). The formation of a ternary complex of FcγR and mIg molecules results in inhibition of anti-Ig-induced proliferation and a blunting of the $[Ca^{2+}]$ influx associated with

anti-Ig stimulation (Phillips and Parker, 1984; Wilson et al., 1987). These effects of FcγR-crosslinking, however, can be overcome by IL-4 (Phillips et al., 1988). The human FcγRIIA is involved in phagocytosis and fluxes [Ca^{2+}] ions upon crosslinking when transfected into a murine macrophage cell line (Odin et al., 1991). Human B cells express predominantly FcγRIIB (Brooks et al., 1989). Although B cell Fcγ receptors appear to regulate B cell growth and differentiation, their specific function and mechanism of action are not very well understood.

We have observed that FcγRII-crosslinking induces cell death in an EBV-immortalized human lymphoblastoid cell line, and accelerates the death of tonsillar B lymphocytes. IL-6, a lymphokine which induces differentiation of human B cells into plasma cells, can delay, although not prevent, FcγRII-mediated cell death.

PRELIMINARY RESULTS

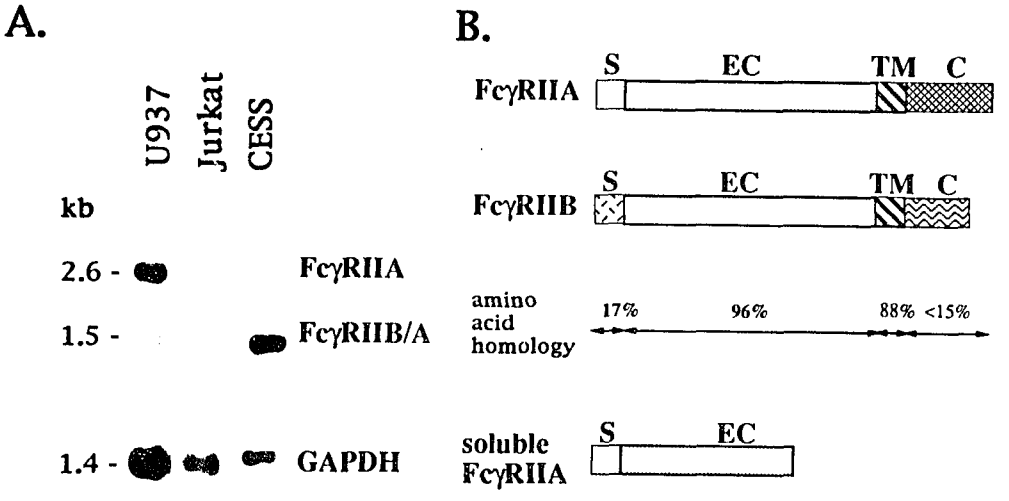
Anti-FcγRIIA crossreacts with FcγRIIB receptors on the surface of human B cells

We used the IL-6-responsive human lymphoblastoid cell line CESS to study growth control of B cells during terminal differentiation. To investigate the role of FcγR in the growth and differentiation of CESS, polyclonal rabbit antibodies against the extracellular domain of human FcγRIIA were generated (kindly provided by Drs. J. Odin, P. Boros and J. Unkeless). Briefly, a truncated human FcγRIIA cDNA clone (huFcγRIIA) which results in the secretion of human FcγRIIA protein was constructed, and the soluble protein was purified to homogeneity by affinity chromatography on a human IgG-Sepharose 4B column. Rabbits were immunized with truncated huFcγRIIA, and IgG was isolated by affinity chromatography on a protein A-Sepharose column. $F(ab')_2$ fragments were generated by digestion with pepsin, and Fc fragments and intact IgG removed by passage over a protein A-Sepharose column.

CESS, like most human and murine B cells, expresses only the 1.5 kb mRNA encoding FcγRIIB. Since a full length FcγRIIB probe was used, both FcγRIIA and FcγRIIB subclasses were detected in U937 cells and not in Jurkat T cells as predicted (Figure 15A). Since the extracellular domains of FcγRIIB and FcγRIIA are highly conserved (Figure 15B), we felt it likely that an antibody against huFcγRIIA would also react with the FcγRIIB expressed in CESS cells.

Figure 15. Northern Blot Analysis of Expression of Human FcγRII mRNA

(A) Total poly(A)+ RNA was isolated from 2 x 10^7 U937, Jurkat or CESS cells and analyzed as described in Materials and Methods. The full length huFcγRIIB probe detects huFcγRIIB mRNA (1.5 - 1.6 kb) and cross-hybridizes with the two alternatively spliced huFcγRIIA mRNAs (2.6 kb and 1.5 kb) (Brooks et al., 1989). As a control for RNA loading, the GAPDH probe was used to detect the 1.4 kb GAPDH mRNA. (B) Schematic representation of huFcγRIIA (325 amino acid residues) and huFcγRIIB proteins (310 and 291 amino acid residues by alternative splicing). The amino acid homology is based on the data presented in Brooks et al. (1989). S: signal peptide; EC: extracellular domain; TM: transmembrane domain; C: cytoplasmic domain.



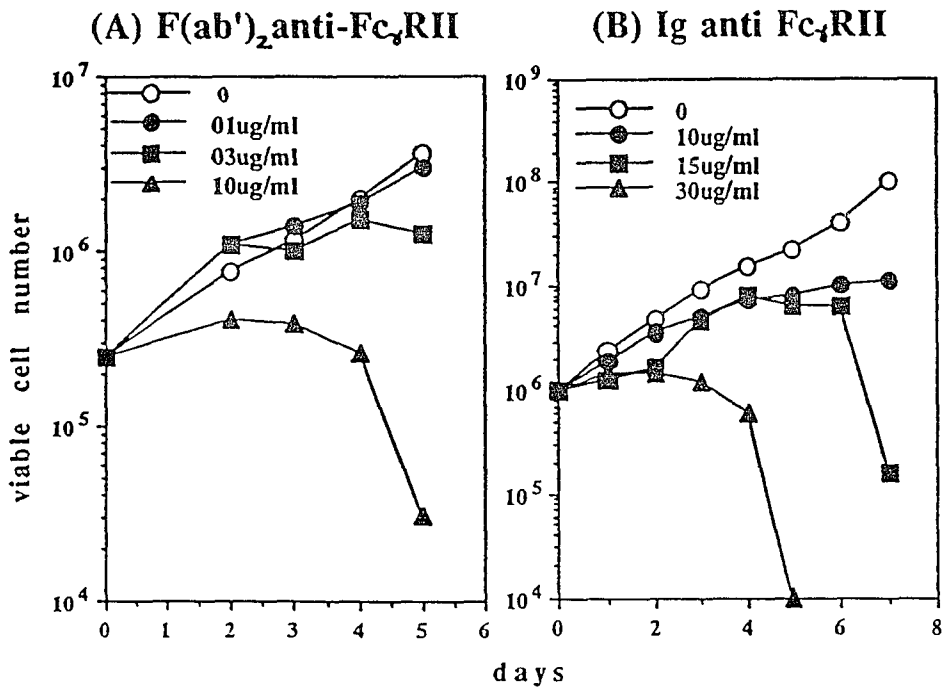
The use of a polyclonal serum was necessary because the anti-FcγRII monoclonal antibody IV.3 (mAb IV.3, Mederex) did not react with FcγRIIB expressed in CESS, suggesting that mAb IV.3 is directed against FcγRIIA epitopes not present in FcγRIIB. Affinity purified anti-FcγR IgG bound to CESS cells with high affinity, and the binding was inhibited by prior incubation of the anti-FcγRII IgG with increasing concentrations of recombinant FcγRIIA (Boros and Unkeless, data not shown). As expected, the anti-FcγRIIA bound to human neutrophils but not to human Jurkat T cells, which do not express FcγR (Figure 15A and data not shown). These results confirmed that FcγRIIB is expressed in CESS cells and that it can be recognized by polyclonal antibodies against huFcγRIIA.

Crosslinking of FcγRIIB induces death of human B cells

The function of Fc receptors in CESS cells was explored by crosslinking FcγRIIB with IgG or $F(ab')_2$ anti-FcγRII. To analyze cell death as opposed to growth arrest, we distinguished viable cells from dead cells by exclusion of trypan blue (0.08% in PBS). Three aliquots were counted from each sample (variation among the counts was < 5%) and the average plotted. Crosslinking FcγRIIB with the $F(ab')_2$ fragment of anti-FcγRII induced the death of CESS cells in a dose- and time-dependent manner (Figure 16A). The ratio of dead cells to viable cells increased as a function of time. Relative to a control culture, in the presence of 10 μg/ml of $F(ab')_2$ anti-FcγR the number of viable cells was reduced by day 2, and diminished to only

Figure 16. Crosslinking of FcγRIIB Induces Death of CESS Cells

CESS cells were incubated with (A) $F(ab')_2$ anti-FcγRII at 1μg/ml, 3μg/ml, and 10μg/ml, or with (B) intact IgG anti-FcγRII at 10μg/ml, 15μg/ml, and 30μg/ml, and viable cell counts were determined by trypan blue staining of cells at intervals as indicated. Uninduced controls are denoted as 0.



1% by day 5.

The kinetics of cell death was reproducible in more than 20 experiments in which IgG and $F(ab')_2$ anti-FcγRII preparations were used. The induction of cell death by $F(ab')_2$ anti-FcγR was more efficient than that by the intact IgG (Figure 16B), indicating that crosslinking of FcγRIIB alone is critical for triggering the death of CESS cells. Crosslinking of CESS mIg by either the $F(ab')_2$ fragment or the intact IgG of anti-human IgG did not influence survival or proliferation (Figure 17A and B). $F(ab')_2$ anti-FcγRII neither bound to Jurkat T cells nor affected their proliferation (Figure 17C). Taken together, these results suggest that induction of death of human B cells by anti-FcγRII is specifically mediated by the crosslinking of FcγRIIB.

The relationship between IL-6-induced differentiation and crosslinking of B cell FcγRIIB

Since the synthesis of IgG is induced by IL-6 in CESS cells, the interplay of IL-6-driven differentiation and FcγRIIB-mediated cell death was studied. IL-6 delayed the onset of cell death induced by crosslinking FcγRIIB by approximately 2 days (Figure 18). Cell death induced by crosslinking FcγRII could not be suppressed by IL-6, even when an 8-fold excess of the concentration of IL-6 needed for inducing differentiation was used (Figure 18).

Figure 17. Crosslinking of mIg Does Not Induce Cell Death

CESS cells were incubated with (A) F(ab')2 goat anti-human IgG1 at 10μg/ml or 20μg/ml, or with (B) intact goat anti-human IgG at 15μg/ml or 40μg/ml, or rabbit anti-human IgG at 15μg/ml or 40μg/ml. (C) Jurkat cells were incubated with 10μg/ml of $F(ab')_2$ anti-FcγRII. Uninduced controls are denoted as 0. Viable cell counts were determined as in Figure 16.

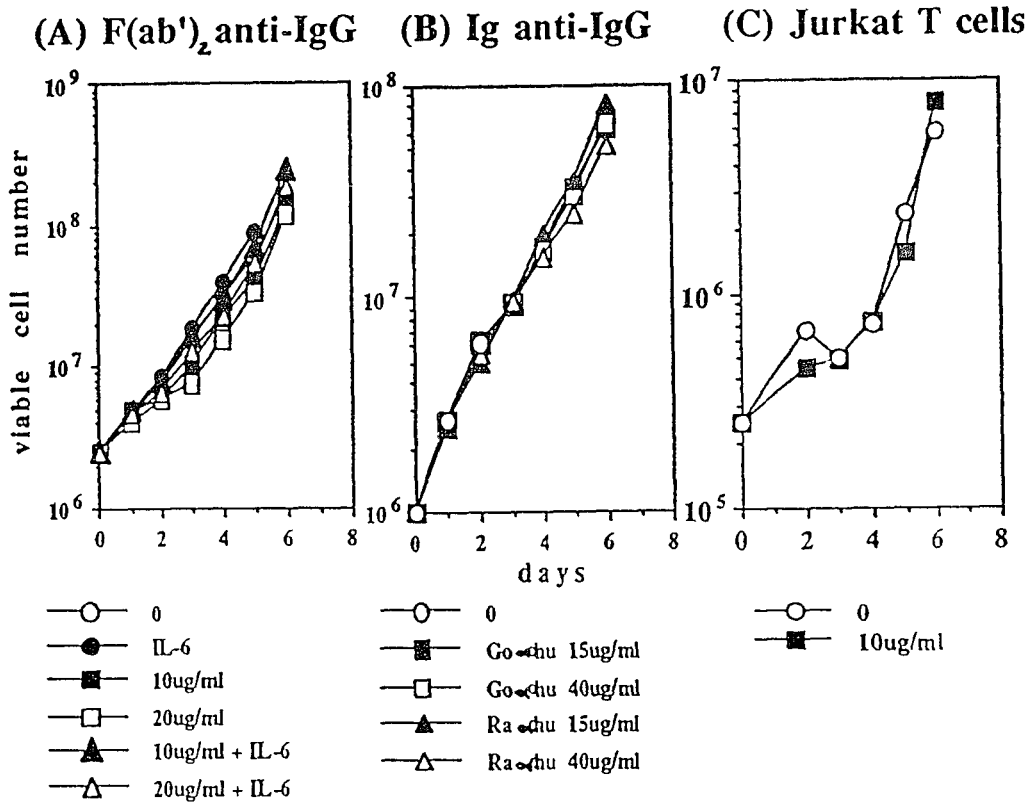
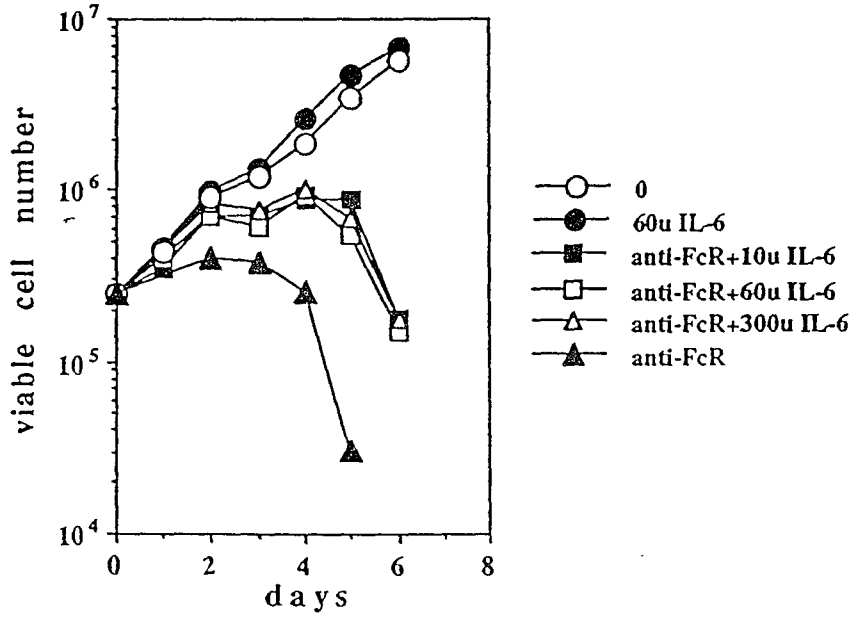


Figure 18. IL-6 Delays The Cell Death Induced by Crosslinking of FcγR

CESS cells were incubated without anti-FcγRII or IL-6, or with 60 u/ml of IL-6, 10μg/ml of $F(ab')_2$ anti-FcγR, 10μg/ml of $F(ab')_2$ anti-FcγRII and 10 u/ml of IL-6, 10μg/ml of $F(ab')_2$ anti-FcγRII and 60 u/ml of IL-6, or 10μg/ml of $F(ab')_2$ and anti-FcγRII and 300 u/ml of IL-6. Viable cell counts were determined as in Figure 16.



IL-6 alone did not influence the proliferation of CESS cells nor did it affect proliferation of CESS cells when mIgs were crosslinked by $F(ab')_2$ fragments of anti-human IgG (Figure 17A and 18). The effect of crosslinking FcγRIIB on the IL-6-induced differentiation was examined at the single cell level. As visualized by differential interference contrast microscopy, CESS cells that were treated with anti-FcγRII for 4 days were enlarged relative to control, or IL-6-induced cells, and showed prominent membrane blebbing, which was unaffected by the inclusion of IL-6 (Figure 19). IL-6 induces 15-20% of CESS cells to synthesize large amounts of intracellular IgG, as shown by immunofluorescence staining of permeabilized cells. Crosslinking of FcγRIIB did not seem to change the percentage of cells that differentiate in response to IL-6 (Figure 19). The IL-6-differentiated cells appear not to share the morphological alterations induced by crosslinking of FcγRIIB. The IgG-positive cells were not enlarged and did not bleb.

Crosslinking of FcγRII accelerates the death of tonsillar lymphocytes

To ensure that the events following FcγRIIB crosslinking in CESS cells were not a consequence of EBV-transformation, we examined lymphocytes from tonsils in a similar manner. Tonsillar lymphocytes consisted of 80-85% B cells, of which 30-50% were IgG- and 50-35% were IgM-positive, depending on the preparation. In culture the number of viable cells declined slowly, with loss of only 20-30% of the initial cell number after 8 days.

Figure 19. Crosslinking of FcγRIIB Does not Interfere With IL-6-induced B Cell Differentiation

CESS cells were incubated for 4d without anti-FcγRII or IL-6 (control), with 40 u/ml of IL-6 (IL-6), with 10 μg/ml of $F(ab')_2$ anti-FcγR and with both (anti-FcγR + IL-6) and examined by differential interference contrast microscopy (DIC), or immunofluorescence microscopy (IFM).

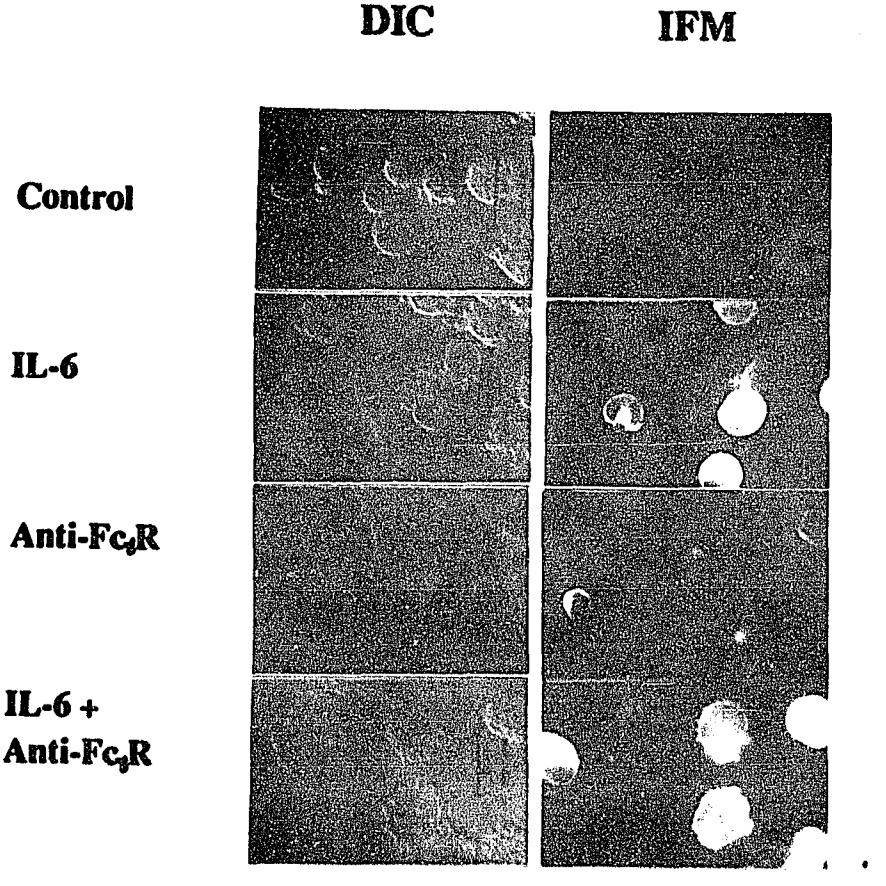
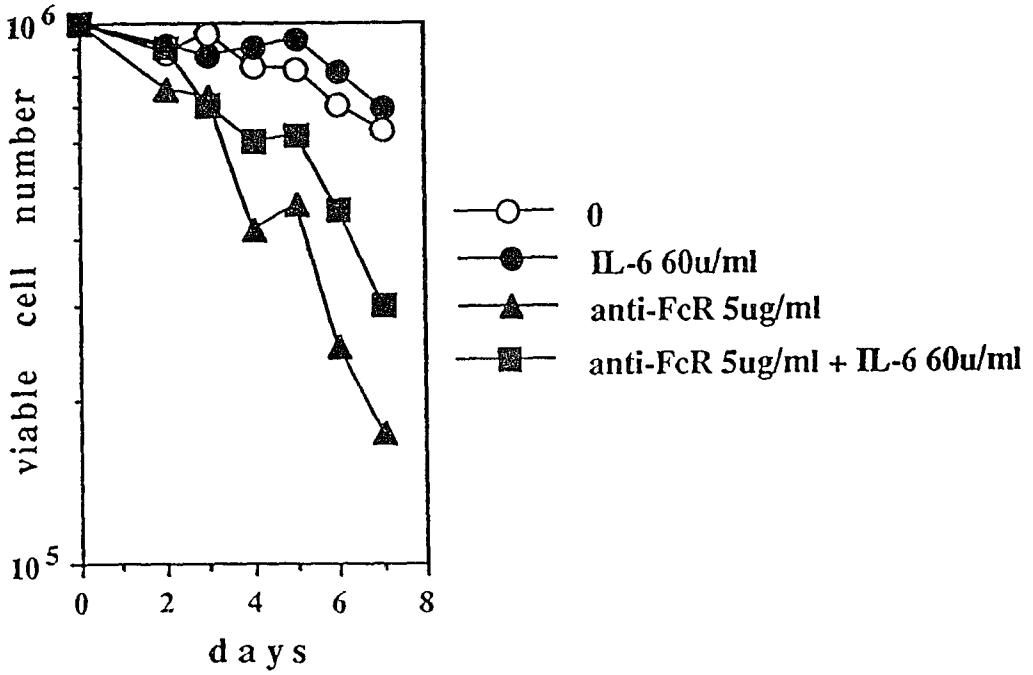


Figure 20. Crosslinking of FcγRIIB accelerated the death of tonsillar lymphocytes.

Lymphocytes were isolated from tonsils as described in Materials and Methods, and incubated without $F(ab')_2$ anti-FcγRII, with 60 U/ml of IL-6, with 10 µg/ml of $F(ab')_2$ anti-FcγRII, or with IL-6 and $F(ab')_2$ anti-FcγRII. Viable cell counts were determined as in Figure 16.



Crosslinking of FcγRIIB with $F(ab')_2$ anti-FcγRII accelerated the death of tonsillar cells, resulting in greater than 90% dead cells after 8 days (Figure 20). As seen with CESS cells, IL-6 did not influence the survival of the control cells, and IL-6 delayed, but did not overide, the cell death induced by crosslinking FcγRII.

Thus, induction of death of human B cells via crosslinking of FcγRII, first revealed in a clonal cell line CESS, also occurred in freshly isolated polyclonal B lymphocytes. Since very few, if any, of the tonsillar B cells are cycling, FcγRIIB-mediated cell death is the result of active interference of cell survival and is not due to the inhibition of cell proliferation.

DISCUSSION

We have presented evidence suggesting that FcγRIIB in human B cells may function to induce cell death. Programmed cell death, or apoptosis, in addition to control of cell proliferation serves to regulate appropriate cell mass during normal development in many lineages (see Williams, 1991; Duvall and Wyllie, 1986). For example, crosslinking of CD3/T cell receptor complex on the surface of immature thymocytes is thought to lead to their elimination by apoptosis (Smith et al., 1989). Likewise, the control of clonal diversity of B lymphocyte is at least in part due to deletion of self-reactive B cell clones by crosslinking antigen receptors expressed on immature and mature B lymphocytes (Nemazee and Burki, 1989; Hartley et al., 1991). Our results suggest a potential means by which mature B cells may be eliminated physiologically by FcγRII-crosslinking.

The significance of B cell death resulting from crosslinking of human B cell FcγRIIB needs to be interpreted in terms of the physiology of the immune response. The dose- and time-dependent cell death we have observed suggests that the consequence of FcγRII-mediated signaling depends on the degree of crosslinking. Also of interest is the interaction between crosslinking FcγR and mIg on the surface of B cells. The *in vitro* studies of murine B cell FcγR function have utilized the the anti-murine FcγRII/III mAb, 2.4G2 (Unkeless, 1979). Rabbit $F(ab')_2$ anti-μ triggers the proliferation and differentiation of murine B cells. The intact anti-γ, which can interact with both mIg and FcγRII, is inhibitory (Phillips and Parker,

1984). The inhibitory effect resulting from FcγR engagement is reversed by mAb 2.4G2 (Phillips and Parker, 1985). Although cell death was not invoked in these studies, it is likely that suppression of immune response and B cell proliferation by intact IgG may in part be due to the death of B cells that is induced by crosslinking of FcγR and mIg.

We observed that IL-6 can delay cell death induced by FcγRII-crosslinking, allowing B cells to differentiate before their eventual elimination. Phillips et al showed that IL-4 reverses the inhibitory effects of FcγRII-crosslinking (Phillips et al., 1988). Together, these results point to interactions between FcγR and cytokine receptor-mediated signaling pathways that need to be interpreted in the context of B cell functions. The biochemical events triggered by crosslinking FcγRII in human B cells are not well understood. The FcγRIIB differs from FcγRIIA and FcγRIIC primarily in the cytoplasmic domain, which has been shown to be critical for transducing the FcγRII-mediated signals in mouse B cells (Hunziker et al., 1991; Amigorena et al., 1992). IL-6 induced Ig synthesis by transcriptional regulation of Ig genes (Raynal et al., 1989), does not appear to be blunted by FcγR-crosslinking. Among its pleiotropic functions (Hirano and Kishimoto, 1989), IL-6 has also been significantly linked to B cell proliferation and neoplasia (Nordan and Potter, 1986; Suematsu et al., 1992). The IL-6 signaling pathway that leads to differentiation and growth control, however, has only begun to be elucidated (Taga et al., 1989; Akira et al., 1990; Nakajima et al., 1993; Murakami et al., 1993). To understand the

interplay of signals mediated by FcγRII and the IL-6 receptor, it will be important to first investigate the expression of these receptors during B cell development and differentiation, as well as their signaling pathways.

To confirm the observations that anti-FcγRIIA leads to cell death of CESS and tonsillar B cells, and to characterize whether this cell death is due to apoptosis, further investigations are needed. Since we and others have obtained evidence that not all anti-FcγRII antibodies result in producing cell death, a careful assessment of how this cell killing activity is triggered has to be undertaken. It is perhaps necessary to generate several polyclonal and monoclonal anti-FcγRII antibodies, and investigate their specificies in inducing cell death in several B cell lines, and in tonsillar or peripheral blood B cells. It would be of interest to assess whether these anti-FcγRII antibodies, alone or in combination with crosslinking Igs, regulate cell growth by progammed cell death during terminal differentiation of B cells *in vitro* and *in vivo*.

CONCLUSIONS

Differentiation is an actively and continuously regulated process of sequential gene expression, which requires an exquisite balance between positive and negative regulators to ensure the expression and the maintenance of phenotypic choices (Blau and Baltimore, 1991). Pluripotent hematopoietic stem cells in the bone marrow, which become committed to develop along the B lineage, undergo several stages regulated at multiple levels before differentiating into plasma cells. In this study, cellular and molecular aspects of the terminal step of B cell differentiation induced by IL-6 have been investigated in the EBV-immortalized cell line, CESS.

IL-6-induced CESS cell differentiation is characterized by the increased synthesis of the secreted form-specific Ig mRNA and the secretion of IgG1. Although the membrane form-specific Ig mRNA does not appear to be regulated by IL-6, the expression of membrane-bound Ig was seen to diminish slightly upon IL-6 induction. More striking is the reduction of surface MHC class II expression at day 4 of IL-6 induction. Cells induced with IL-6 also undergo stage-appropriate morphological alterations characteristic of plasma cells *in vivo*. These characteristics of IL-6-induced CESS cells establish this system as a model for further investigating B cell differentiation.

IL-6 is a pleiotropic lymphokine which exerts its effects in a cell type- and developmental stage-specific manner. The inverse

correlation between Ig synthesis and MHC class II expression during the differentiation of CESS cells attests to the specificity of IL-6 signaling in B cells. Although IL-6 signaling is specific, not all CESS cells respond to IL-6 in a similar manner, despite their clonal origin. While only 15-20% of these cells are enhanced in Ig synthesis at day 4 of IL-6 induction, as assessed by intracellular Ig staining, approximately 50% of CESS cells show a reduction in surface MHC class II expression. The marked differences in Ig and MHC class II expression suggest that IL-6 regulates these genes by distinct mechanisms.

The heterogeneity of response of CESS cells induced with IL-6 was further attested to by the difficulty in obtaining clones of CESS which exhibit a more uniform response to IL-6 induction. CESS cell-derived clones in which 50% of the cells initially showed an increased synthesis of Ig upon IL-6 induction, over time became reduced to only 15% of the cells. Long term induction studies with IL-6 showed that Ig synthesis is subject to feedback regulation at the mRNA level in the continued presence of IL-6 beyond 10 days. Unless IL-6 is removed for at least 5 days, these cells are refractory to further IL-6 stimulation, indicating that IL-6 signaling in B cells may be feedback-regulated.

To further understand how the feedback regulation of IL-6 signaling may operate, the mRNA levels of the two subunits of the IL-6 receptor were analyzed in the course of a long term IL-6 induction. mRNAs encoding both subunits were increased at day 4 and 5 of IL-6 induction, but were reduced to levels below that in

uninduced cells in the continued presence of IL-6 beyond day 5. The mRNAs of both receptor subunits were again elevated upon re-stimulation of IL-6 after a refractory period of 10 days. These results present the first evidence for the regulation of the IL-6 receptor subunits, and suggests a potential mechanism by which IL-6 may modulate its own signaling in B cells. Deciphering the intermediate steps that may be involved in this feedback mechanism will be of great interest for future investigations.

The Pou domain-containing transcription factor, Oct-2, which is essential for terminal B cell differentiation and Ig secretion (Corcoran et al., 1993), was also subject to feedback control in CESS cells. This regulation of Oct-2 mRNA synthesis correlates with Ig mRNA synthesis upon IL-6 stimulation, and suggests that Oct-2 is a key player in regulating Ig gene expression during terminal B cell differentiation. A possible candidate for regulating Oct-2 synthesis in B cells is the C/EBP transcription factor family member, NF-IL6, whose synthesis is induced by IL-6 (Akira et al., 1990). In human embryonal carcinoma and T cells, another Pou-domian containing transcription factor, Oct-1, is known to be regulated by NF-IL6 (Hsu and Chen-Kiang, 1993). By an analogous mechanism, NF-IL6 may potentiate the regulation of Oct-2 in B cells. Oct-1, in association with a B cell-derived co-activator, can function to activate Ig gene transcription in a cell-free system (Luo et al., 1992). This suggests that Oct-1 can substitute for Oct-2 in activating Ig genes. Since Oct-1 and Oct-2 share structural and functional similarities, a testable hypothesis is that these proteins may also be similarly

regulated by transcription factors such as NF-IL6, in their respective physiological settings.

The reduction of surface MHC class II expression by IL-6 is regulated by mechanisms distinct from that of Ig genes. The induction of surface MHC class II molecules in B cells earlier in development is thought to occur predominantly by coordinate transcriptional activation of genes encoding the α, β, and the invariant chains of MHC class II (Glimcher and Kara, 1992). Although the prevailing view is that upon terminal differentiation the reduced expression of surface MHC class II molecules may be due to transcriptional repression mechanisms, our results in this study suggest that this is unlikely. The mRNAs encoding the α, β, and the invarient chains of MHC class II are still synthesized in CESS cells with reduced surface MHC class II expression. In cells exhibiting reduced MHC class II, GRP94, a stress protein thought to sequester MHC class II molecules in the ER, is induced at the mRNA and protein levels. This synthesis of GRP94 mRNA is preferantially induced and temporally regulated by IL-6 in CESS cells. The mRNA encoding a related ER protein, GRP78/BiP does not seem to be induced by IL-6, and serves as a good control for the specifity of IL-6 signaling in the regulation of GRP94. In contrast to previous observations in invarient chain-negative cell lines (Schaiff et al., 1992), our results imply that even in the presence of Ii chain expression, elevated levels of GRP94 may be involved in MHC class II regulation. These results await further analyses by transfection studies, to confirm that GRP94 may regulate the reduction of surface MHC class II expression.

This study attests to the distinct and complex regulatory mechanisms which govern the terminal step of B cell development, and shows that IL-6-signaled human B cell differentiation is subject to active and continuous regulation as invoked by Blau and Baltimore, 1991.

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