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CCAAT/ENHANCER BINDING PROTEIN-RELATED TRANSCRIPTION FACTORS: REGULATORS OF CYTOKINE EXPRESSION AND MYELOMONOCYTIC DIFFERENTIATION

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JAMES DANIEL BRETZ

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Richard C. Schwartz
Major professor

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CCAAT/ENHANCER BINDING PROTEIN-RELATED TRANSCRIPTION FACTORS: REGULATORS OF CYTOKINE EXPRESSION AND MYELOMONOCYTIC DIFFERENTIATION

By

James Daniel Bretz

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ABSTRACT

CCAAT/ENHANCER BINDING PROTEIN-RELATED TRANSCRIPTION FACTORS: REGULATORS OF CYTOKINE EXPRESSION AND MYELOMONOCYTIC DIFFERENTIATION

By

James Daniel Bretz

The regulation of hematopoiesis is important for the understanding of immune system function and the mechanisms of leukemia. The myelomonocytic lineages, all derivatives of a single stem cell type, have important functions in antigen clearance, antigen presentation, and intercellular signaling of immune responses. Inhibition of differentiation of myelomonocytic cells can lead to uncontrolled growth, impaired immune function and leukemia.

Transcription factors are integral to the regulation of cellular function. Transcription factors CRP2 and CRP3 are members of the C/EBP-related protein (CRP) family. CRPs have been implicated in the regulation of myelomonocytic differentiation.

In this thesis I will demonstrate that lineage switch macrophages derived from lymphoid pre-B cells are capable of normal functions such as the capacity to release cytokines in response to LPS and antigen presentation. These lineage switch macrophages also gain the capacity to express CRP2 and CRP3. I will also demonstrate that ectopic expression of CRP2 and CRP3 in murine P388 B lymphoblasts confers the capacity for lipopolysaccharide (LPS)-inducible transcription of

cytokine genes *in vivo*, and acts as an inducer of certain aspects of myelomonocytic differentiation. I will also propose the utility of this experimental system for further discrimination of the genetic mechanisms of myelomonocytic differentiation.

DEDICATION

To

Phyllis Jo Hallet Bretz
and
Janet Lynn Salzwedel

for their unwavering support,
which made this possible.

ACKNOWLEDGMENTS

I would like to thank my mentor, Richard C. Schwartz, whom I consider a friend as well as mentor. Peter F. Johnson and his lab at the National Cancer Institute for giving my research a much needed boost. Jerry Dodgson and his lab for their helpful discussions, and reagents and equipment provided. My fellow graduate students in Rich's lab, Tim Weichert and Shu Chih Chen, for providing an educational, interesting and pleasant work environment (and challenging euchre games). And especially, to all the people who gave me a second chance and allowed me to prove I was worthy of their confidence in me. Their are so many of you (I hope you know who you are, and realize what a big difference you have made in my life).

PREFACE

In this thesis, two chapters are based on collaborative efforts. Chapter 2 involves the derivation and characterization of lineage switch macrophages. The isolation of these cells and preliminary characterization was accomplished by Shu-Chih Chen. RT-PCR was performed by Hsun-Lang Chang. The cytokine assays were performed by Alfred Ayala.

Chapter 3 involves manipulation and characterization of CRP transfected cell lines. Western analyses were performed and anti sera and oligonucleotides supplied by Peter F. Johnson, Mark Baer and Simon Williams at the National Cancer Institute. Hsien-Meng Hu constructed the pBABE-CRP2 expression vector.

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Hematopoiesis: an introduction

Hematopoiesis is the process of producing blood cells and related cellular components of the immune system. The process consists of differentiation of a single totipotent stem cell into a variety of cellular lineages (Figure 1) (Kuby 1992, Roitt et al 1989). There are two major lineages: lymphoid and myeloid. Both derive from the totipotent stem cell. Pluripotent lymphoid stem cells give rise to the T and B lymphocyte populations. Pluripotent myeloid stem cells (CFU-GEMM) give rise to monocyte/macrophage, neutrophil, megakaryocyte, erythrocyte, basophil/mast cell, and eosinophil cell lineages, each with distinct and complex functions. These lineages give rise to extremely specialized cells involved in immune system function and oxygen transport.

The microenvironment, including cell-cell contact, of hematopoietic cells is important for differentiation (Mayani et al 1992, Kincade 1992). Hematopoeisis occurs in a number of specialized sites. The bone marrow is the source of hematopoeitic cells of the early lymphoid lineage as well as myeloid, and erythroid lineages in adult mammals (Metcalf 1988). Stromal cells of the bone marrow provide factors for proliferation and differentiation (Mayani et al 1992). Fetal liver is also a source of stem cells (Metcalf 1988). Resting

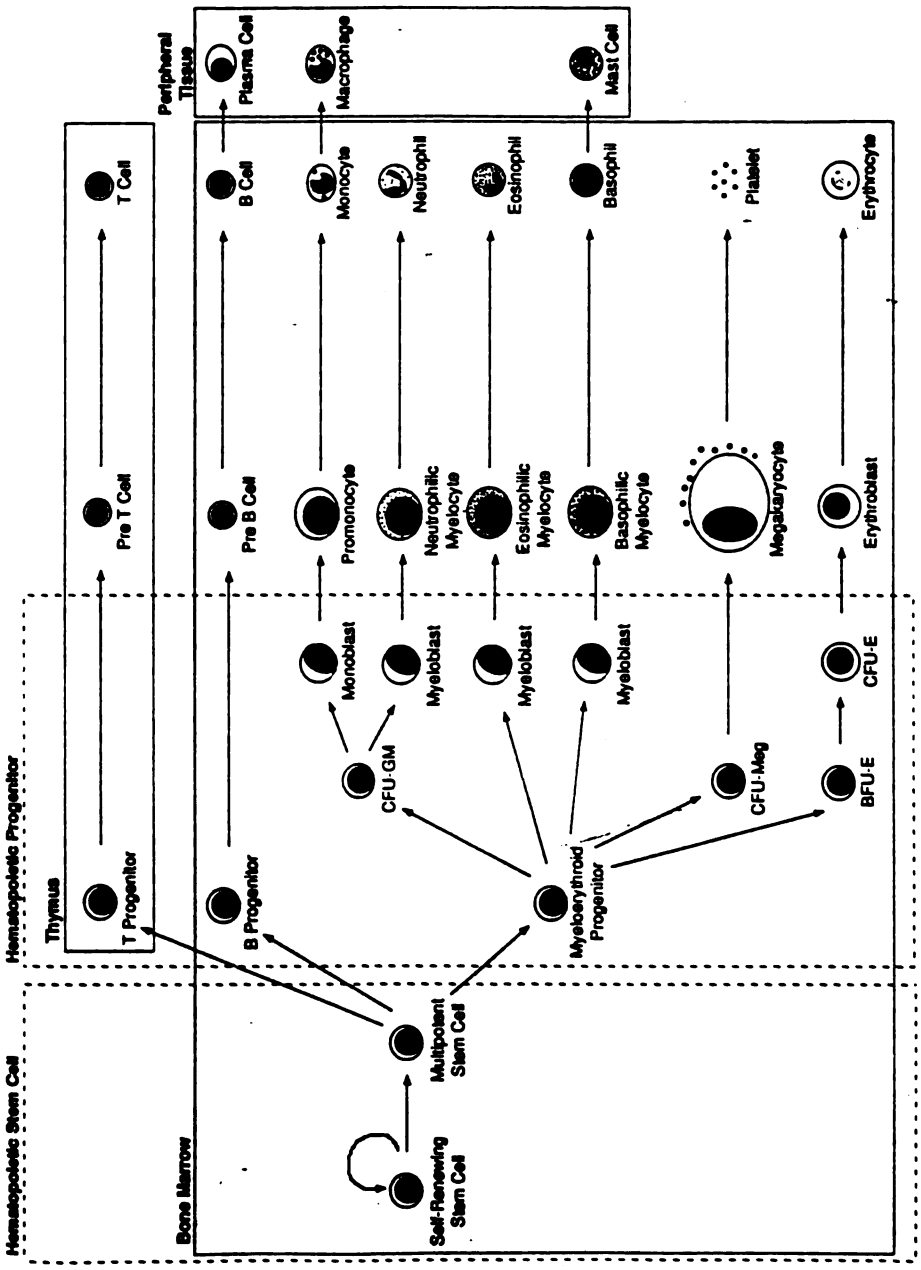


Figure 1: Hematopoieses: All lineages derive from a totipotent stem cell (from Nicola 1989).

B lymphoid cells transport to the adult spleen or lymph nodes. T progenitor cells transport to the thymus where they complete their differentiation (Metcalf 1988). A more detailed discussion of hematopoiesis relevant to this thesis will follow.

Normally, early commitment to an individual lineage produces progenitor cells restricted to that lineage through terminal differentiation. Terminal differentiation occurs in the circulating blood, spleen, lymph nodes and, as is the case for macrophages, the resident peripheral tissue. Erythropoiesis continues in the spleen in adults (Metcalf 1988).

Differentiation is generally considered antithetical to proliferation. As cells differentiate they become less proliferative. Consistent with this, inhibition of hematopoietic differentiation can lead to leukemogenic proliferation. Alternatively, induction of hematopoiesis can inhibit leukemogenic proliferation.

Myelomonocytic differentiation

A bipotential stem cell (CFU-GM) gives rise to both the neutrophilic granulocyte and monocyte/macrophage lineages (Figure 2) (Roitt et al 1989). A number of cell surface markers are specific for these lineages (CD 13, CD11b (Mac I), CD11c, CDw12, CD17, CD31, and CDw32) (Kuby 1992). CD34 is specific for the myeloid progenitor stage (Roitt et al 1989).

The currently defined stages of monocyte/macrophage

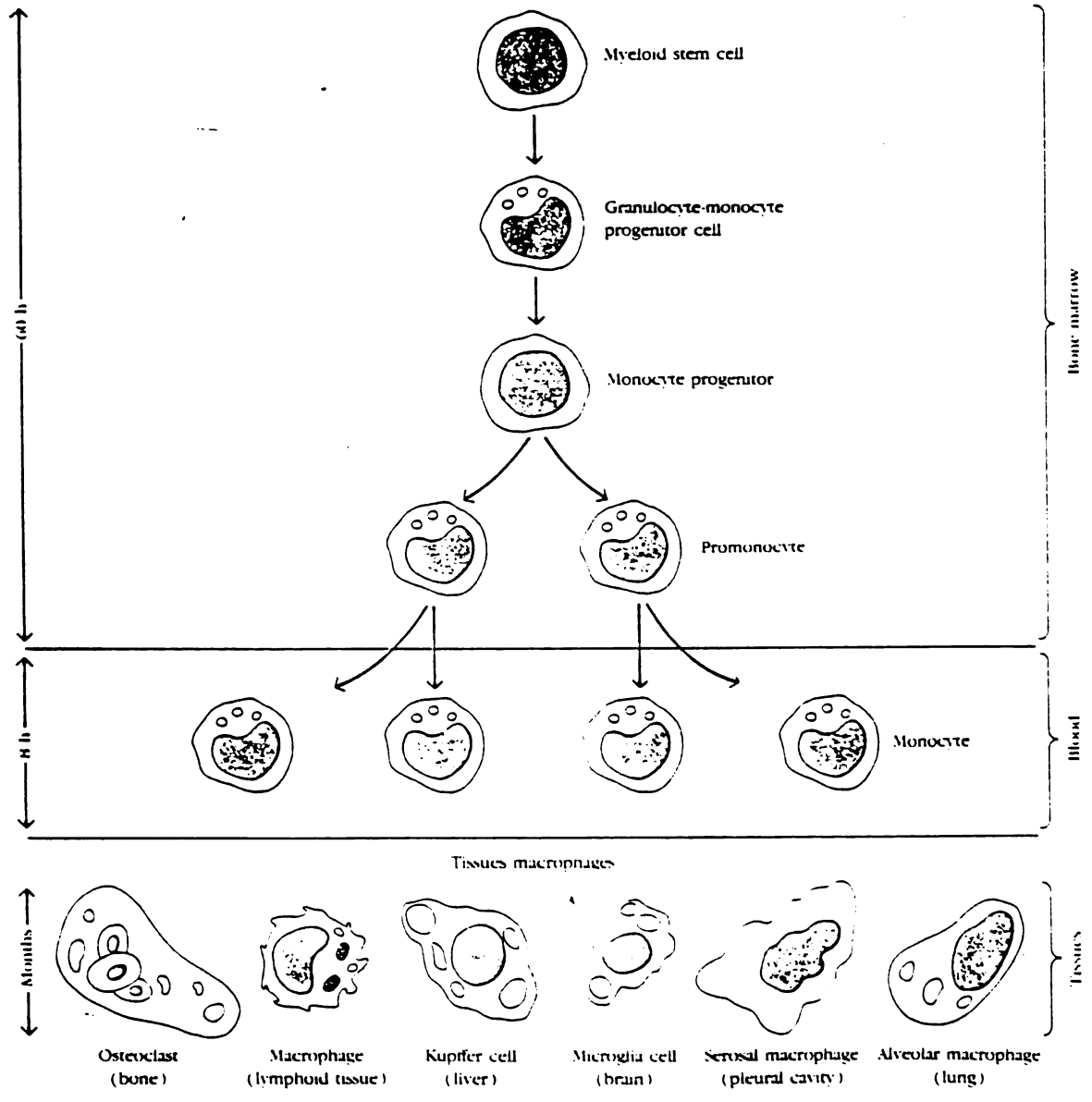


Figure 2: Monocyte/macrophage differentiation (from Kuby 1992).

differentiation from stem cell to terminal stage are: CFU-GEMM (bone marrow derived, GM-CSF responsive myeloid stem cell), CFU-GM (bone marrow derived, M-CSF responsive granulocyte/monocyte progenitor), monoblast (renewable, bone marrow derived, monocyte committed precursor), promonocyte (no longer under goes cell division), monocyte (translocates to peripheral blood, $CD14^{+}$), macrophage (after migration to peripheral tissue, $CD23^{+}$) and activated macrophage ($CD26^{+}$) (Roitt et al 1989, Kurland 1984, Metcalf 1988). CD14, CD23 and CD26 cell surface marker expression is maintained through terminal expression. The expression of proto-oncogene and transcription factor c-fos is upregulated with differentiation along this pathway (Gonda and Metcalf 1984, Lord et al 1993). Cells of this lineage can also be identified by gross morphology (comparitively large cells, large cytoplasm-to-nucleus ratio), cytochemical stains [non-specific esterase (Yam et al 1970)], and functional assays. The important functions of this lineage are not acquired until after activation which occurs in the peripheral tissues in response to signals such as endotoxin (LPS) or cytokines (Turpin and Lopez-Berestein 1993). Such functions include antigen processing and presentation, phagocytosis of particulate matter including cells, mediation of the inflammatory response, secretion of factors controling hematopoiesis, keratinization, brain wound repair, iron storage and calcium metabolism (Turpin and Lopez-Berestein 1993, Adams 1982 and 1989). Depending on the extravascular tissue into which they

have infiltrated, macrophages may be called Kupfer cells (liver), Langerhans cells (skin), microglia (brain), osteoclasts (bone) or tissue macrophages of the spleen, lymph node, etc. (Turpin and Lopez-Berestein 1993).

Neutrophilic granulocyte differentiation can be characterized by function, gross morphology, cytochemical staining (chloroacetate esterase, leukocyte alkaline phosphatase [LAP] , myeloperoxidase [MPO]) (Yam et al 1970, Rambaldi et al 1990) , lactoferrin (LF) expression, and cell surface markers. The stages of this lineage are defined as the myeloblast ($CD33^+$) (Roitt et al 1989), promyelocyte (MPO^+, chloroacetate $esterase^+$, $CD33^+$), myelocyte (MPO^+, chloroacetate $esterase^+$, $CD33^+$) metamyelocyte (MPO^+, LF^+, chloroacetate $esterase^+$, $CD33^+$) and neutrophil (MPO^+, LF^+, chloroacetate $esterase^+$, polymorphonuclear, $CD33^-$) (Valteiri et al 1987). Differentiation occurs entirely in the bone marrow (Metcalf 1988). The neutrophils will then translocate to the peripheral blood and eventually to the extravascular tissue. The lifespan of the neutrophil in peripheral tissue is short (~3 days) compared to macrophages which may live for years.The functions of neutrophilic granulocytes include phagocytosis and destruction of antigen (Roitt et al 1989, Kuby 1992).

Colony stimulating factors

Four colony stimulating factors, first identified by their ability to stimulate colony formation of bone marrow cultures, have been shown to promote survival, proliferation,

differentiation, and maturation of myeloid cells. IL-3 (multi-CSF), granulocyte-macrophage colony stimulating factor (GMCSF), macrophage colony stimulating factor (MCSF), and granulocyte colony stimulating factor (GCSF) are a family of glycoproteins that act through receptors on their target cells (Kuby 1992). Expression of CSFs is usually not constituitive but requires an inductive stimulus on the synthesizing cell.

IL-3 is a 20-28kd glycoprotein synthesized by T lymphocytes (Metcalf 1988). IL-3 influences the differentiation of all myeloid progenitors and may even act on the totipotent stem cell. More specifically, IL-3 promotes survival and proliferation of these stem cells. Other functions include proliferation and maturation of mast cells and proliferation of erythroid cells (Hapel et al 1985).

GMCSF is a 21-23 kd glycoprotein produced in vivo by a wide variety of cell types (T lymphocytes, macrophages, fibroblasts, bone marrow stromal cells, endothelial cells) (Metcalf 1988, Golde et al 1990). Similar to IL-3, it acts on all myeloid progenitors in survival and proliferation (Metcalf 1988). It induces differentiation and proliferation of immature granulocytes and monocytes (Golde et al 1990). It also has a number of effects on the function of mature myeloid cell types. GMCSF enhances phagocytosis, intracellular killing, cytotoxicity, and antigen processing of macrophages, (Golde et al 1990). Neutrophils have enhanced phagocytosis, intracellular killing, chemotaxis, antibody dependent cytotoxicity (ADCC), protein synthsis, and vascular adhesion,

cytotoxicity (ADCC), protein synthsis, and vascular adhesion, due to GMCSF (Golde et al 1990). In addition GMCSF primes neutrophils for degranulation, release of arachidonic acid and leukotriene B_4, and oxidative metabolism. (Golde et al 1990). Mature eosinophils also respond with enhanced functions (Golde et al 1990).

MCSF is a glycoprotein homodimer comprised of 43kd subunits that can be synthesized by fibroblasts and bone marrow stromal cells in mouse and also macrophages and endothelial cells in humans (Metcalf 1988). MCSF induces differentiation and proliferation of progenitors of the monocyte lineage (Kuby 1992). It can also induce cytokine expression (GMCSF, GCSF, megakaryocyte potentiator [an enhancer of platelet production]), ADCC, tumor killing, and osteoclast production and activation (Metcalf 1988). The MCSF receptor gene also known as c-fms, was originally discovered as the tranforming gene in an acute oncogenic retrovirus (Hampe et al 1984, Sherr et al 1985). C-fms has been found expressed in some tumors, and with MCSF, induces growth through a paracrine or autocrine mechanism (Roussel and Sherr 1989).

GCSF is a ~25kd glycoprotein synthesized by macrophages, fibroblasts, bone marrow stromal cells and, in humans, endothelial cells (Fukanaga et al 1990, Metcalf 1988). GCSF promotes differentiation and proliferation of neutrophil progenitors. For mature neutrophils, it enhances phagocytosis and ADCC (Kuby 1992).

B lymphocyte differentiation

Stages of B lymphocyte differentiation are generally defined by the state of immunoglobulin gene expression (see Figure 3). Cell surface markers are also used experimentally to determine the stage of development. Both T and B lymphocytes are derived from the same lymphoid stem cell characterized by its expression of the enzyme terminal deoxynucleotidyl transferase (TdT), but lacking immunoglobulin (Ig) or T cell receptor gene rearrangements (Gregoire et al 1979). TdT inserts nucleotides in the Ig heavy chain gene providing increased variability (Desiderio et al 1984). The progenitor of the B lineage expresses the cell surface marker B220 (CD45) which is diagnostic for the entire lineage (Kuby 1992). Commitment to the B lineage is first observable with μ heavy chain Ig gene rearrangement (Roitt et al 1989). Cytoplasmic expression of the μ heavy chain Ig gene is diagnostic of a pre-B lymphocyte. CD20 is a cell surface marker, possibly part of a calcium channel (Tedder et al 1990), whose expression starts at this point and continues through terminal differentiation (Clarke and Lane 1991). This is followed by light chain gene (either kappa or lambda) rearrangement and expression (Tsubata and Nishikawa 1991). This allows for the heavy chain and light chain to combine and be expressed as IgM on the cytoplasmic membrane which is diagnostic of an immature or resting B lymphocyte. The immature B lymphocyte will then relocate to the spleen or lymph nodes (Ikuta et al 1992). A mature B lymphocyte is

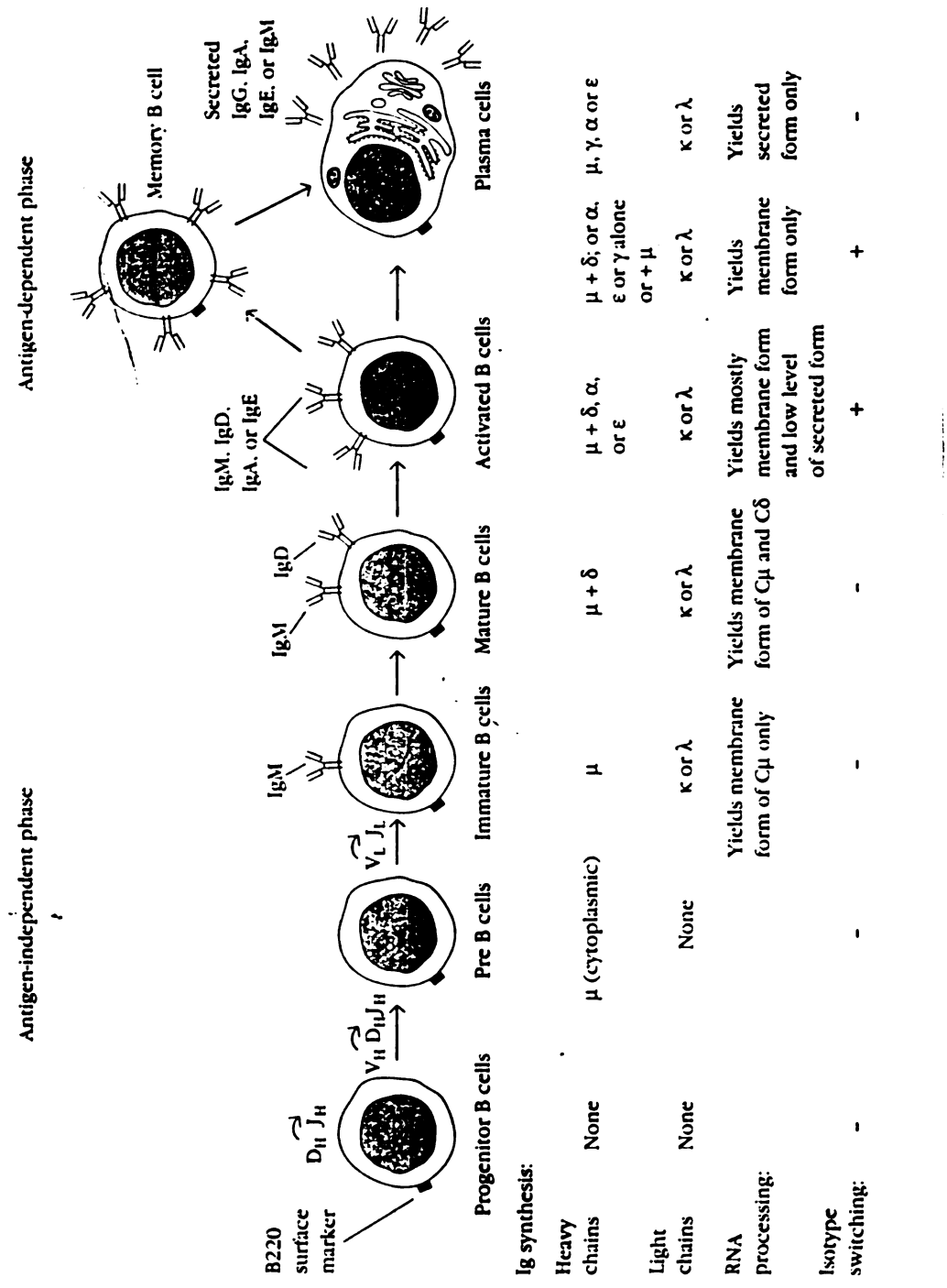


Figure 3: B lymphocyte differentiation (from Kuby 1992).

generated after IgD is coexpressed with IgM on the cell surface. After initial antigen stimulation, the cell becomes an activated B lymphocyte secreting low levels of Ig after isotype switching but maintaining surface Ig. The expression of CD39, possibly involved in B lymphocyte-dependent T lymphocyte activation (Harriman et al 1993), is specific for mature B lymphocytes (Clark and Lane 1991). Isotype switching may continue after further Ag stimulation and a changeover to exclusively the secreted form of Ig. This is the terminal plasma cell stage of differentiation which is marked by the expression of a number of specific surface markers including CD27, CD38, and plasma cell associated-1 (PCA-1) (Clark and Lane 1991, Kuby 1992).

The B lymphocytes used in this thesis work were characterized by southern analysis of Ig gene rearrangements and immunocytostaining for B220 expression.

Long-term B lymphocyte cultures

In the studies reported in this thesis, the v-Ha-ras transformed pre-B lymphocytes that underwent B lymphocyte to macrophage conversion were derived from the Witte-Whitlock long-term culture system (see chapter 2) (Bretz et al 1992). This system allows for continuous culture of primary cells of the B lineage for extended periods (>1 year) (Whitlock and Witte 1982 and 1987). Committed B lymphocyte precursors can be followed through differentiation to mature B lymphocytes.

The culture is derived from bone marrow cells obtained

from the femurs of three to four week old mice. These cells are grown in culture medium supplemented with 5% fetal calf serum (lot tested; serum lot varies and is critical for successful cultures), and 10^{-5}M 2-mercaptoethanol. A high density layer of adherent cells (feeder layer) derived from the bone marrow provides factors required to maintain and grow the non- adherent B lymphocytes. The bone marrow also provides B lymphocyte progenitors. Typically the predominant cell type early after establishment in culture (3-8 weeks) is pre-B. This is observed by the appearance of foci of small round cells growing on the feeder layer. After 3-6 months, mature B lymphocytes become predominant. Cells can be maintained for extended periods after transfer to low density feeder layers (Whitlock and Witte 1987). High density feeder layers have a tendency to overgrow and after some time they will die.

The cells maintained in this culture system can be grown in numbers suitable for biochemical analysis and can be cloned, infected with retroviral vectors (Whitlock et al 1983), analyzed by FACS, or used in reconstitution experiments of immune-deficient mice.

Inducible differentiation of cell lines

There are problems with using primary cell cultures for the study of hematopoiesis. The inability to maintain a homogenous population of cells for extended periods of time in culture can be overcome by the use of spontaneous or induced

(chemically or by viral infection) leukemic cell lines. But these cell lines are usually locked into a stage of differentiation. The isolation of cell lines capable of differentiating in response to chemical or biological agents has overcome this problem. These cells can be induced and studied along their differentiative path to determine the mechanisms involved. Three myeloid cell lines have relevance to my research.

M1 is a murine monoblastic leukemia. Various subclones have been isolated that can differentiate in response to one or more of the following factors: IL-6, leukemia inhibitory factor (LIF), and IL-1 (Lotem and Sachs 1992). Induction may be indirect by some of these factors; one factor inducing expression of another factor(s). LPS causes an abortive differentiative effect where some immediate response genes are expressed but the cells fail to reach the terminal stage of differentiation. (Lord et al 1990). Cytokine induction leads to terminal differentiation into a functional macrophage. The end stage is non-tumorigenic. This system was used to determine the immediate response genes for myeloid differentiation (MyD^+) (Lord et al 1990a and 1990b). MyD genes include the transcription factors c-jun, jun B, jun D, but curiously not c-fos which is upregulated during normal and leukemic differentiation (Lord et al 1993, Gonda and Metcalf 1984). Expression of transcription factors c-myb (Selvkumaran et al 1992) and c-myc (Hoffman-Leibermann et al 1991) block induced differentiation. Expression of these genes is

normally suppressed in the course of differentiation.

32DC13 is an IL-3 dependent myeloblastic cell line derived from a murine long term bone marrow culture (Greenberger et al 1993). If IL-3 is removed and GCSF is added to their growth medium, 32DC13 cells differentiate into mature neutrophilic granulocytes (Valtieri et al 1987). This method has led to the discovery that expression of evi-1 (a zinc finger transcription factor gene) (Moroshita et al 1992) or id (an inhibitor of certain bHLH transcription factors) (Benezra et al 1990, Kreider et al 1992) can inhibit GCSF induced differentiation. As with M1, a number of transcription factors are immediate early response genes to GCSF induction (c-fos, c-jun, jun B, egr-1), but c-myc expression remains unaltered (Krieder and Rovera 1992). In Chapter 4 I will show that transcription factors CRP2 and CRP3 may be involved in the differentiation of myeloid cells.

The human cell line HL-60 was isolated from a patient with acute promyelocytic leukemia (Rovera et al 1979). This line has been very useful due to its ability to differentiate along either the neutrophilic granulocyte or the monocyte/macrophage pathway. Dimethyl sulfoxide and retinoic acid are the most commonly used compounds for induction of granulocyte differentiation (Breitman et al 1980, Collins et al 1978). 1,25 dihydroxyvitamin D_3 and phorbol-12-myristate-13-acetate are used for induction of macrophage differentiation (Murao et al 1983). The induced cells become growth arrested and functional at the terminal stage.

Expression of the zinc finger transcription factor egr-1 is essential for and restricts differentiation to the macrophage lineage in this system (Nguyen et al 1993).

Lineage switching

According to historical hematopoietic dogma, commitment to one lineage starts an irreversible path of differentiation along that lineage. This dogma has recently been challenged by the discovery of lineage switched cells. A lineage switch is defined as the changing of a cell line (or leukemia) of one lineage to another lineage.

The first examples were discovered by clinical observation in the course of human leukemia. In the early 1980s it was shown that some patients with a leukemia of one lineage (usually lymphoid) would relapse after remission with a leukemia of a different lineage (usually myeloid) (Stass et al 1984, Stass et al 1986). It was not determined if these cases were a true lineage switch (perhaps caused by the chemical agents of therapy) or merely one leukemia being eradicated by chemotherapy allowing expansion of a secondary leukemia of a different phenotype. In the latter case both leukemias would have been derived from the same pluripotent stem cell. This has yet to be completely resolved.

Occurences of a lineage switch in culture have also been observed. More recently, lineage switch has been observed through the expression of certain oncogenes. Davidson et al. (1988) used LPS to stimulate v-Ha-ras-transformed lymphoid

cell lines to differentiate into macrophage-like cells after long term culture selection. Klinken et al. (1988) induced B lymphoid cells derived from a transgenic mouse expressing c-myc from the immunoglobulin mu enhancer to convert to macrophages by expressing the v-raf oncogene. Borzillo et al (1990) have expressed human c-fms (MCSF receptor) in murine pre-B lymphocytes in culture and induced lineage switch by stimulating with human MCSF. The above lineage switch systems evaluated the macrophage nature of the resultant cells by morphology, adherence, surface markers (including CD11b [Mac I] expression), phagocytosis, esterase activity, and lysozyme activity. All of these studies were lacking in assays to determine complete macrophage functionality of the macrophage-like cells derived from pre-B lymphocytes. In chapter two, we address this question. Our studies show that ras-transformed pre-B lymphocytes can undergo lineage switch on passage in animals and differentiate into functional macrophages with Ag presentation and cytokine production capabilities (Bretz et al 1992). This validates the lineage switch model as a system in which to study the regulation of myeloid differentiation (Chapters 3 and 4).

Inflammatory cytokines

Inflammation is the body's response to injury or infection. It allows the body to direct the immune system to the site of injury. Several cytokines are involved in signaling the inflammatory response. Inflammatory cytokines

such as IL-1, IL-6, TNF, MCP-1, IFNγ, MIP-1, MIP-2, IL-8, and others are released by cells at the site of injury. Inflammatory response activities such as chemotaxis of immune cells to the site of tissue damage, adhesion of these cells to vascular endothelial cells and permeabilization of vascular tissue to allow the migration of immune system cells to the site of injury, as well as activation of antigen clearance mechanisms (e.g. phagocytosis, degranulation) are all signaled by these cytokines (Kuby 1992, Roitt et al 1989). Inflammatory cytokines are also involved in stimulating growth and differentiation of immune cells.

The inflammatory cytokines interleukin 6 (IL-6), interleukin 1 (IL-1), tumor necrosis factor (TNF), and monocyte chemoattractant protein (MCP-1) are germaine to my research. These cytokines have a wide variety of activities in inflammation as well as parallel activities that enhance the immune response such as the fever and acute-phase responses. In the following summary, I will limit descriptions to these cytokines' major activities in the inflammatory response.

Interleukin 6

IL-6 is a 20-30kd glycoprotein that has a wide variety of activities that play an important role in the immune response. These activities include terminal differentiation and proliferation of B lymphocytes, stem cell survival, elicitation of acute-phase response, differentiation and

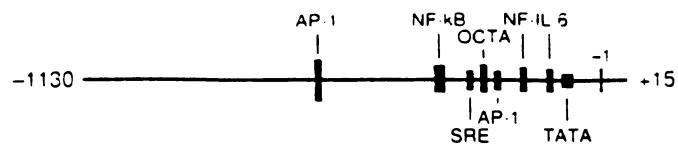


Figure 4: Cis-acting elements of IL-6 (upper) and IL-1β (lower) regulation (from Akira and Kishimoto 1992) and (Zhang and Rom).

of T lymphocytes and macrophages (Akira and Kishimoto 1992).

When stimulated appropriately, monocyte/macrophages, fibroblasts, keratinocytes, endothelial cells, mesangial cells, glial cells, chondrocytes, and T and B lymphocytes can produce IL-6. Depending on the producing cell, IL-6 can be induced by LPS, IL-1, TNF, IFNβ and PDGF. Elevated levels of IL-6 have been found in several pathological conditions including certain lymphoid malignancies, rheumatoid arthritis,and HIV infection (Akira and Kishimoto 1992).

Cis-acting elements of IL-6 gene transcription regulation are summarized in Figure 4. The NF-IL6 element confers LPS and IL-1 inducibility in a human glioblastoma cell line as detected by a chloramphenicol acetyl transferase (CAT) assay (Isshiki et al 1990). In chapter three, I will show that CRP2 (NF-IL6) and CRP3 can confer LPS inducibility of IL-6 when ectopically expressed in a lymphoblastic cell line.

Interleukin 1

Interleukin 1 activity describes the effects of two structurally related 17kD proteins that activate the same receptor (Scales 1992). Macrophages are the major in vivo source of both IL-1α and IL-1β although many other cell types also express them including lymphocytes and neutrophils. Macrophages can be induced to produce IL-1 by endotoxin (LPS), as well as by cytokines IL-2, GMCSF, TGFβ, TNFα, and all three interferons. Activities of IL-1 include T lymphocyte proliferation, B lymphocyte activation in response to antigen,

induction of acute phase proteins by the liver, induction of expression of adhesion molecules by vascular endothelial cells, and oxidative metabolism by neutrophils.

Cis-acting elements involved in IL-1β gene regulation and the factors that bind them are summarized in Figure 4 (Zhang and Rom 1993). The NF-IL6 enhancer elements of the IL-1 β promoter confer LPS inducibility in CAT assays using THP-1 cells (a human myelomonocytic leukemia line). In Chapter 3, I will show that anti-sense CRP2 and CRP3 gene transcripts can block LPS inducibility of IL-1β when expressed ectopically in a lineage switched macrophage cell line.

Tumor necrosis factor

Similar to IL-1 in that two distantly related 17kD proteins bind to the same receptor, TNF(α and β) also has an overlap of activities with IL-1 (Tsuji and Torti 1992). TNFα is produced by macrophages in response to LPS, gamma interferon, or IL-6. TNFβ is produced by T lymphocytes. TNF induces lymphocyte proliferation, activation of neutrophils, induction of expression of adhesion molecules by vascular endothelial cells, tumor killing by macrophages and acute phase protein expression in liver, probably through IL-6 up regulation (Tsuji and Torti 1992, Dawson 1991). It is a mediator of septic shock (Buetler et al 1988). TNF inhibits adipocyte specific gene expression and may play a role in cachexia (wasting, wieght loss and muscle weakness) in immune disorders such as AIDS (Dawson 1991).

Regulation of TNFα gene transcription has not been well characterized. The TNFα core promoter can confer TPA inducibility when joined to a number of unrelated enhancer sequences (Leitman et al 1992). A TNF-responsive element contains AP-1 and ATF/CREB binding sequences but these factors are not involved in its regulation (Leitman et al 1991). Four LPS-responsive sites bind NFκB in primary macrophages (Drouet et al 1991).

Monocyte chemoattractant protein-1

Monocyte chemoattractant protein-1 (MCP-1) was first discovered in murine fibroblasts as a platelet-derived growth factor (PDGF)-induced immediate early response gene and designated JE (Cochran et al 1983). It was later found by homology to be related to a number of cytokines and identical to the human monocyte chemoattractant protein-1 (MCP-1) (Rollins et al 1989). This 16-18kD glycoprotein is specific for monocyte/macrophage chemotaxis with no activity on neutrophils or lymphocytes (Rollins et al 1989, Leonard and Yoshimura 1990). A wide variety of cells can be induced to express MCP-1. The literature is often contradictory as to that cell types respond to what stimuli. Peripheral blood monocytes (PBMC) may (Yoshimura et al 1989) or may not (Brach et al 1992) upregulate MCP-1 in response to LPS. This may be due to the need for given factors to act in synergy with other factors that may or may not be present from one assay system to the next or, in the case of PBMCs, possible contaminating

MCP-1 expressing cells. LPS, IL-1, IL-4, TNF, PDGF, and GMCSF have all been shown to induce MCP-1 in at least one cell type [peritoneal macrophages (Introna et al 1987), pulmonary fibroblasts (Rolfe et al 1992), kidney tissue (Xia et al), endothelial cells (Shyy et al 1993), alveolar epithelial cells (Paine et al 1993), or chondrocytes (Villiger et al 1992)]. In addition to chemotaxis, MCP-1 also is involved in activation of macrophages (Rollins et al 1991).

Cis-acting elements involved in MCP-1 gene regulation have not been mapped in detail nor have any specific transcription factors been implicated. A TPA-inducible element has been mapped to a region 88 to 141 base pairs upstream of the transcription start site without specific knowledge of the transcription factors involved (Timmers et al 1990). But a possible AP-1 binding site (normally conferring TPA inducibility) in the -70 to -38 region does not confer TPA inducibility to a reporter gene construct (Timmers et al 1990). A novel 7 base pair element 3′ of the MCP-1 stop codon apparently is necessary for serum inducibility in conjunction with a 5′ element that is also required (Freter et al 1992). No LPS inducible elements have as yet been identified. In chapter 3 I will show that LPS inducibility can be confered by expression of CRP2 or CRP3. I will also identify a putative promoter element.

This is a dizzying array of cytokines and functions that make up the signaling required to maintain homeostasis. Hopefully the relevant facts have been communicated in a

Hopefully the relevant facts have been communicated in a meaningful way for proper understanding of the thesis to follow.

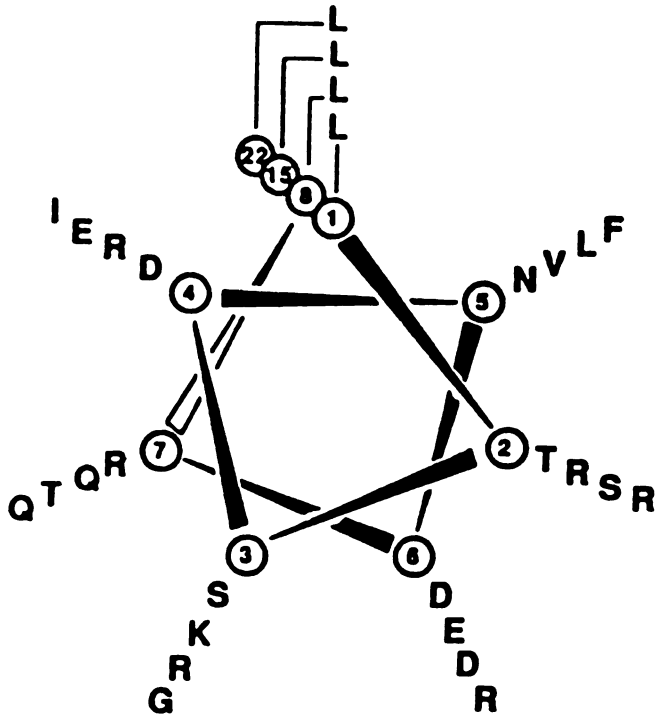
C/EBP-related transcription factors

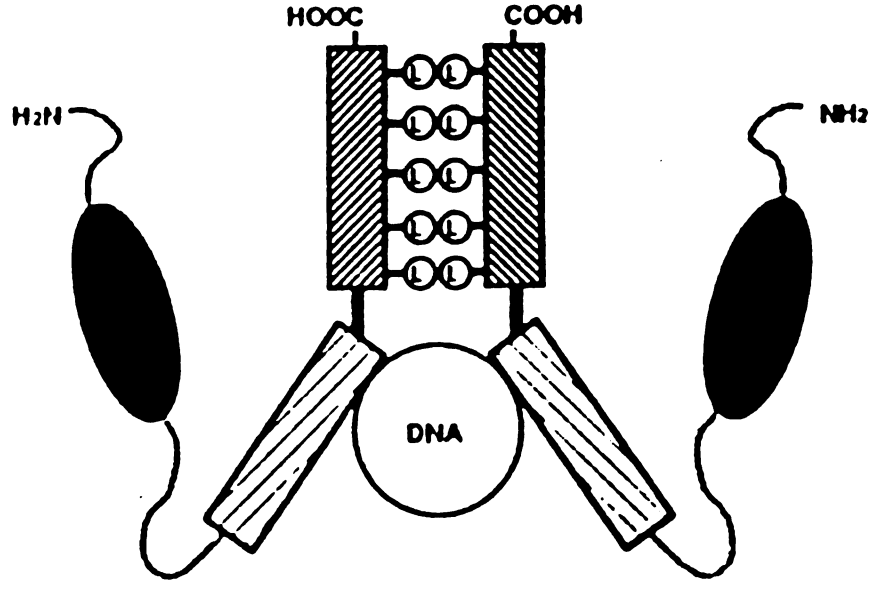
C/EBP related proteins (CRPs) are a family of enhancer binding transcription factors (see Figure 5) (Williams et al 1991, Cao et al 1991). They have been grouped together based on amino acid homology and their ability to dimerize. CRPs have extensive homology between their carboxy terminal regions (~100 a.a.). This region is the basic region-leucine zipper (bZIP) element which constitutes the DNA binding and dimerization domains.

The leucine zipper domain is an alpha helix that has a leucine residue every seven amino acids (Landschulz et al 1988a). Computer modeling has predicted that this sequence places the hydrophobic leucines on the same side of the alpha helix in a linear fashion. X-ray crystallography has shown the alpha helices of leucine zippers are in a coiled-coil configuration (Ferre-D' Amere et al 1993, Ellenberger et al 1992). This allows the hydrophobic leucines of one alpha helix to interact with those of a dimerization partner to form a dimer (see Figure 6). The dimerization allows juxtaposition of the basic regions which contact the DNA. The CRPs can both homodimerize as well as heterodimerize within the family (Williams et al 1991, Cao et al 1991). Heterodimerization outside of the family generally does not occur but an

NAME	SYNONYMS	DERIVATIVE PROTEINS	TISSUE EXPRESSION
C/EBP	C/EBPα, C/EBP-42	C/EBP-30	LIVER, MYELOID CELLS, ADIPOSE TISSUE
CRP1	----	----	UNKNOWN
CRP2	NF-IL6, C/EBPβ, LAP, IL-6DBP, AGP/EBP,	LIP, LOP	LIVER, MYELOID CELLS, ADIPOSE TISSUE
CRP3	C/EBPδ, NF-IL6β	----	LIVER, MYELOID CELLS, ADIPOSE TISSUE
IgEBP-1	C/EBPγ	----	UBIQUITOUS
CHOP	GADD153	----	UBIQUITOUS

Figure 5: C/EBP-related protein family nomenclature





C/EBP

Figure 6: Leucine zipper schematic. An axial view (upper figure) of the α-helix of a leucine zipper (from Vinson et al 1989). The leucines are all on the same side. Leucine zipper (lower figure) allows for dimerization and DNA binding (from Akira et al 1993).

exception has recently been reported with IgEBP dimerizing with ATF4 (Vinson et al 1993). Heterodimerization with multiple partners may allow for exquisite fine tuning of DNA binding and transcriptional control. Interactions with other enhancer binding factors such as Sp1, NFκB and the glucocorticoid receptor, which occur through non-bZIP dimerization, have also been investigated (P. Johnson personal communication, LeClair et al 1992, Stein et al 1993, Nishio et al 1993).

The basic region is coterminal to the leucine zipper and, as the name suggests, consists predominantly of basic amino acid residues. The basic amino acids interact with DNA bases and phosphate oxygens along the major groove of double stranded DNA (Vinson et al 1989). This allows the protein to bind in a sequence specific manner. Models of this structure have been described as a "scissors grip" or "forceps grip" (Vinson et al 1989, Ellenberger et al 1992).

The CRPs bind DNA in a sequence specific manner. The consensus sequence T T/G N N G N A A T/G has been determined by DNA footprinting, electrophoretic mobility shift, and interference assays on a number of gene promoter sequences (Akira et al 1990). This sequence is found in the promoter of a number of genes (Figure 7). These include genes that fall into five general categories: cytokines, liver specific genes (including many acute-phase proteins), immunoglobulin genes, adipocyte specific genes and viral enhancers (Akira et al 1992, Shirakawa et al 1993, Brooks et al 1992, Christy et al

Cytokine genes	
AGATTGTGCAATGT	IL-6 (human)
GGATTTGGAAAGTT	TNF-α (human)
AACTTTCGCAAACA	G-CSF (mouse)
ATCAGTTGCAAATC	IL-8
ACGTTGCACAACCT	IL-1β
Liver-specific genes	
TGATTTTGTAATGG	albumin distal element 1 (DE1)
AGATTGAGCAATCT	albumin -3.5kb HS site
GTCTTAAGCAAAGC	α_1-antitrypsin site C
GTATTAGGACATGT	transthyretin site 2
ATGTTGAGTAAGAT	transthyretin site 3
ACCTTTTGCAATCC	apolipoprotein B element IV
ACAAGTTGCAACAT	carbamyl phosphate synthetase 1
(acute-phase proteins)	
GTGTGAAGCAAGAG	haptoglobin site A
GAATTACGAAATGG	haptoglobin site C
AAGTTGTGCAATGG	α_1-acid glycoprotein
TAGTGGCGCAAACT	C-reactive protein
CAGTGATGTAATCA	hemopexin site A
Immunoglobulin genes	
ATCTTAAGCAACTG	Igκ enhancer
GAATTGAGCAATAA	IgH enhancer
GCATTTTGTAATAA	VH V1 promoter
TCATGAGGCAAGGC	VH 17.2.25 promoter
Adipocyte-specific genes	
AAGTTGAGAAATTT	422(aP2)
GGCTGAGGAAATAC	stearolyl-CoA desaturase
Virus enhancers	
GGGTGTGGAAAGTC	simian virus 40 enhancer
TGGTTTTGCAAGAG	polyoma enhancer
ATCTGTGGTAAGCA	murine sarcoma virus enhancer
Consensus	
TTNNGNAAT	
(G) (G)	

Figure 7: C/EBP-related protein recognition sequences (adapted from Akira et al 1990)

1989, Kardassis et al 1992, Johnson et al 1987). The cytokine genes for IL-6, TNFα, GCSF, and IL-1β are of importance in our current research. I will propose the addition of the MCP-1 to this list (see Chapter 3).

The amino terminal section of CRPs contains the transcription activation domain. Mutations in this region inhibit the ability to trans-activate expression of target genes in cotransfection assays (Freidman et al 1990, Pei and Shih 1991) without disrupting dimerization or DNA binding. Three CRP family members (CHOP, LIP and LOP) do not have an activation domain (Ron and Habener 1992, Descombes et al 1991, P. Johnson personal communication). Due to their ability to heterodimerize and bind DNA with other family members they can act as repressors of transcription. This adds to the capacity for fine tuning of transcriptional control.

IgEBP-1 and CHOP are ubiquitously expressed proteins (Roman et al 1990, Ron and Habener 1992). However, all other CRPs exhibit some tissue specificity (see Figure 7). This implies a possible involvement in control of tissue specific expression and differentiation.

CRP2 and CRP3 are highly expressed in mature macrophages and neutrophils but have not been detected in abundance in other hematopoietic lineages (Natsuka et al 1992, Scott et al 1992, this thesis). They have also been shown to be upregulated in induced differentiation of M1 (Natsuka et al 1992) and 32Dc13 (Scott et al 1992) myeloid cell lines. For M1, upregulation is observed as soon as three hours post

induction.

CRP2 and CRP3 are initially upregulated in hormone induced differentiation of 3T3-L1 mouse fibroblasts to adipocytes (Cao et al 1991). CRP2 and CRP3 expression then declines as C/EBP expression increases. Anti-sense C/EBP expression can block adipocyte specific gene expression in the above system (Lin and Lane 1992).

The CRP2 and CRP3 genes are LPS inducible (Akira et al 1990, Kinoshita et al 1992). This information along with the LPS-inducibility of many cytokines in macrophages and the presence of CRP binding sites in the promoter regions of these cytokine genes, suggests that CRP2 and CRP3 may be essential for inducibility of cytokines by LPS (see Chapter 3). The expression of CRPs in macrophages, coupled with induction of their expression at early times in M1 and 32Dc13 differentiation suggests that they play a role in myeloid differentiation (see Chapter 4). It has already been shown that CRPs play a role in another differentiative process, adipogenesis (Cao et al 1991).

C/EBP and CRP2 have also been shown to play a central role in the control of expression of liver genes both during normal liver metabolism as well as in response to a diseased state by expression of acute-phase proteins (Akira and Kishimoto 1992, Poli et al 1989).

Acute-phase response

The acute-phase response accompanies the inflammatory response by altering the level of several plasma proteins known as acute-phase proteins (APPs) (Akira and Kishimoto 1992). The APPs are thought to act to protect against general tissue destruction by inflammation (Kuby 1992). Several cytokines as well as LPS can induce a full acute-phase response when injected into animals. But in cultured hepatocytes, IL-1, TNF, IFNγ, and TGFβ induce only a subset of APPs. IL-6, which can be induced by both IL-1 and TNF, has been shown to be a principal mediatior of APP induction along with oncostatin M, and leukemia inhibitory factor (LIF) (Akira and Kishimoto 1992).

Several APP genes possess CRP binding sites in their promoters (Table 2). The sites from the C-reactive protein, hemopexin, and haptoglobin genes have been shown to confer IL-6 inducibility when co-transfected with either CRP2 or CRP3 expression vectors (Ramji et al 1993). CRP2 has also been shown to transactivate the α1-acid glycoprotein gene and can synergize with the glucocorticoid receptor (Nishio et al 1993). Jun B expression can down-regulate this gene (Baumann et al 1991).

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Lineage Switch Macrophages Can Present Antigen

JAMES D. BRETZ, SHU-CHIH CHEN, DIANE REDENIUS, HSUN-LANG CHANG, WALTER J. ESSELMAN, and RICHARD C. SCHWARTZ*

Department of Microbiology, Michigan State University, East Lansing, Michigan 48824-1101

Recent reports of "lineage switching" from a lymphoid to macrophage phenotype have left unresolved the question of whether such cells are functional macrophages or nonfunctional products of differentiation gone awry. This study demonstrates that several "macrophage-like" cell lines derived from v-Ha-*ras*-transformed pre-B cells have gained the capacity to effectively present antigen in MHC-restricted fashion. Using an assay involving the cocultivation of putative antigen-presenting cells with chicken ovalbumin (cOVA) and a cOVA-specific T-cell hybridoma, "lineage switch" cell lines were found to present antigen as effectively as macrophage-containing peritoneal exudates. Neither the original pre-B-cell precursors nor B-cell lymphomas derived from them present antigen. Thus, we have demonstrated that these "lineage switch" macrophages are capable of antigen presentation, a mature differentiated function.

While gaining macrophage characteristics, these cells have also rearranged their kappa light-chain immunoglobulin locus, suggesting that macrophage differentiation and immunoglobulin rearrangement are not mutually exclusive processes. The existence of both lymphoid and myeloid characteristics in a cell fully capable of antigen presentation suggests greater plasticity in hematopoietic lineage commitment than conventionally thought to be the case.

KEYWORDS: Lineage switch, macrophage, antigen presentation.

INTRODUCTION

The concept that hematopoietic differentiation involves an early and irreversible lineage commitment is brought into question by numerous observations of leukemias and lymphomas that express myeloid or lymphoid markers outside their respective lineages. The coexpression of differentiation markers has been interpreted as being either an aberrant phenomenon caused by leukemogenesis (McCulloch, 1983) or a reflection of the normal but transient existence of bipotential progenitors in hematopoiesis (Greaves et al., 1986). In particular, the existence of a number of transformed cell lines with both lymphoid and macrophage characteristics has suggested a close relationship between these lineages. Murine macrophage cell lines have been derived from lymphoid tumors and from in vitro transformants induced either by murine leukemia viruses or chemical carcinogens (Boyd and Schrader, 1982; Holmes et al., 1986; Hanecak et al., 1989). Three groups have studied systems in which a transition from a lymphoid to a macrophage phenotype could be induced. Klinken et al. (1988) demonstrated that B lymphoid cells from transgenic mice that express c-*myc* using the immunoglobulin mu enhancer could be induced to take on macrophage-like characteristics when infected with a retrovirus expressing v-*raf*. Davidson et al. (1988) showed that a v-Ha-*ras*-transformed lymphoid cell line could be stimulated by lipopolysaccharides (LPS) to differentiate along either the lymphoid pathway into pre–B-like cells or along the myeloid pathway into macrophage-like cells. Recently, Borzillo et al. (1990) reported the CSF-1-dependent macrophage lineage transition of a pre-B-cell line expressing the human CSF-1 receptor.

The macrophage-like cell lines that have been derived from B lymphoid cells have been classified as macrophage on the basis of their morphology, expression of MAC-1, MAC-2, α-naphthyl acetate esterase and lysozyme, and their

*Corresponding author.

ability to phagocytose latex beads. More functional assays for antigen presentation and tumoricidal activity that would establish whether these cells could act in vivo similarly to authentic macrophages have not been presented. In this paper, we demonstrate the ability of several macrophage cell lines derived from v-Ha-*ras*-transformed pre-B cells to present antigen to a T-helper cell hybridoma.

RESULTS

A tumor consisting of adherent cells with a macrophage morphology was identified during our studies on the tumor progression of a pre-B lymphoid cell line expressing v-H-*ras* (Chen et al., 1991). This tumor, designated tumor 4, was derived from a clonal cell line, designated R2, that was generated by infection of fresh murine bone marrow with a mixture of a v-Ha-*ras*-expressing retrovirus and Moloney murine leukemia virus (MoMuLV) (Schwartz et al., 1986b). The R2 cell line was classified as being a pre-B cell on the basis of several criteria. It possessed a blast-cell morphology with a large nucleus and scant cytoplasm. It expressed the B lineage-specific marker, B220 (Coffman and Weissman, 1981). Though not expressing a detectable immunoglobulin mu chain, R2 showed a rearrangement in the DNA of that locus. The immunoglobulin kappa-chain locus was in a germline configuration.

Tumor 4 is Derived from the R2 Cell Line

In order to ascertain whether we had identified a probable instance of lineage switching, it was necessary to demonstrate that tumor 4 was derived from R2. To that end, the sites of integration of the v-Ha-*ras*-expressing retrovirus and MoMuLV were compared between the tumor and the cell line. Southern hybridization analysis of EcoRI-digested DNA with a v-Ha-*ras* probe showed that tumor 4 contained the same 5.3-kb proviral integration fragment as R2 (Fig. 1A). This proviral integration fragment is defined by a 3′ EcoRI site internal to the viral genome and a 5′ EcoRI site peculiar to the site of integration. In addition to the 5.3-kb fragment, there is a 23-kb fragment representing the endogenous c-Ha-*ras* in all the DNAs. Southern hybridization analysis of BglII-digested DNA, using a probe for the ecotropic MuLV *env* gene, revealed similar MoMuLV integration fragments in R2 and tumor 4 (Fig. 1B). The MoMuLV genome possesses a BglII site within *env*, such that the foregoing hybridization would detect a fragment extending from that BglII site to a BglII site in the host-cell genome flanking the 3′ terminus of the provirus. These data demonstrate that the putative macrophage tumor was derived from the pre-B-cell line.

Tumor 4 Cells Possess Macrophage Characteristics

Tumor 4 was initially suspected to be a macrophage because of the large size of its cells and its adherent growth in cell culture. Microscopic examination of Wright-Giemsa-stained cells confirmed their large size and revealed the cells of tumor 4 (Fig. 2B) to have a much more extensive and granular cytoplasm than R2 (Fig. 2A). An immunoperoxidase detection procedure found tumor-4 cells to have retained some expression of B220, and to have gained expression of high levels of MAC-1 (data not shown). MAC-1 is generally considered to be a marker for cells of the myeloid lineage (Springer et al., 1979). Histo-

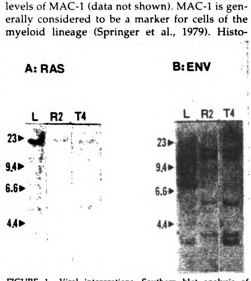


FIGURE 1. Viral intergrations. Southern blot analysis of DNAs from liver (L), R2, and tumor 4 (T4). (A) DNA was digested with EcoRI and 10 μg of each sample was electrophoresed through 0.8% agarose. The blot was probed for v-Ha-*ras*. (B) DNA was digested with BglII. The blot was probed for murine ectotropic *env* sequences. Size markers are the positions of an ethidium bromide-stained HindIII digest of bacteriophage λ and are denoted in kilobases.

chemical procedures revealed a high level of α-naphthyl acetate esterase activity in tumor-4 cells, which is not found in R2 cells (data not shown). This is an enzyme activity generally associated with cells of the monocyte-macrophage lineage (Rogers et al., 1980). Tumor-4 cells (Fig. 2B) were positive for the nonspecific phagocytosis of latex beads, whereas R2 cells (Fig. 2A) were not. Nonspecific phagocytosis is another marker of the monocyte-macrophage lineage (Raschke et al., 1978). These data strongly suggest a macrophage phenotype for tumor-4 cells.

At late stages of myeloid differentiation, the levels of *c-myc* and *c-myb* mRNA decrease, whereas the level of *c-fms* mRNA increases (Gonda and Metcalf, 1984; Sheng-Ong et al., 1987). The levels of mRNA from these proto-oncogenes detected in tumor-4 cells were consistent with tumor 4 having advanced to a late stage of myeloid differentiation. Cytoplasmic poly A⁺ RNAs of the parental R2 cell line, tumor 4, and six other tumors derived from R2 that had lymphoid characteristics were examined by

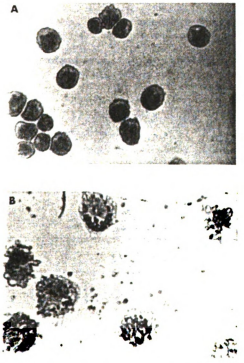


FIGURE 2. Nonspecific phagocytosis of latex beads. The cells were Wright-Giemsa stained and photographed at 200× magnification. (A) R2, and (B) tumor 4.

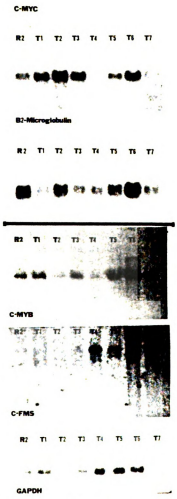


FIGURE 3. RNA analyses of *c-myc*, *c-myb*, and *c-fms*. Northern blot analyses were performed on polyA⁺ RNA from R2 and seven tumors (T1–T7). Each sample of RNA was the polyA⁺ fraction selected from 150 μg of total cytoplasmic RNA. One blot (upper panel) was probed successively for both *c-myc* and β₂-microglobulin, and the other blot (lower panel) was probed successively for *c-myb*, *c-fms*, and rGAPDH.

Northern hybridization analysis (Fig. 3). One blot was hybridized successively with c-*myc* and β_2-microglobulin probes. Another blot was hybridized successively with c-*myb*, c-*fms*, and rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probes. Hybridization to the β_2-microglobulin and GAPDH probes provided a control for gel loading. Tumor-4 cells clearly show reduced levels of c-*myc* and c-*myb* expression in comparison to R2 cells and lymphoid tumors. In contrast, c-*fms* expression is elevated in tumor-4 cells. A cell line with macrophage characteristics has also been isolated from tumor-5 cells, which show elevated c-*fms* expression (Fig. 3).

Another aspect of macrophage function is the ability to release cytokines in response to LPS stimulation. We examined the presence of IL-1, IL-6, and TNF in the media of cells cultured in the presence or absence of 10 μg/ml of LPS for 24 hr. Cellular proliferation assays for IL-1 and IL-6, and a cytotoxicity assay for TNF revealed varying levels of cytokine release for six subclones of tumor 4, whereas the parental R2 pre-B-cell line did not elaborate any of these cytokines except low levels of IL-1 (Table 1). The LPS-inducible release of cytokines was again consistent with a macrophage phenotype for tumor-4 cells.

Tumor 4 Cells Also Show Differentiated Lymphoid Characteristics

Davidson et al. (1988) found that a v-Ha-*ras*-transformed lymphoid cell line could be stimulated to differentiate along either the myeloid or lymphoid pathways. Because tumor-4 cells showed a variety of DNA rearrangements in the kappa light-chain locus (data not shown), it was of interest to determine whether the cells that had gone on to rearrange the kappa locus were the same cells that had progressed toward a macrophage phenotype or whether the tumor-4 cells were a mixed population of B cells and macrophages. To that end, tumor-4 cells were plated in soft agar medium and six subclones were recovered. Southern hybridization analysis of EcoRI-digested DNA isolated from the subclones showed that they all contained the same 5.3-kb v-Ha-*ras* proviral integration fragments as R2 and tumor-4 cells (data not shown; see Fig. 1A). The six subclones of tumor-4 possessed the same myeloid characteristics described before for the uncloned tumor, but varied in their pattern of kappa light-chain gene rearrangement. Southern hybridization analysis of BamHI-digested DNAs with a kappa probe revealed that subclones 3 and 5 possessed one germline and one rearranged kappa allele, and subclones 1, 2, 4, and 6 possessed rearrangements in both alleles (Fig. 4). All the subclones possessed a rearranged BamHI fragment of approximately 7 kb. Tumor 4 was apparently derived from an outgrowth of R2 that had undergone this rearrangement. Some subclones then proceeded to rearrange their other kappa allele. Clearly, tumor 4 contained cells that individually had differentiated along both the lymphoid and myeloid pathways.

Having observed kappa light-chain rearrangements in macrophage subclones of tumor 4, we next examined the status of immunoglobulin expression by Northern blot analysis. Kappa light-chain transcript could not be detected (data

TABLE 1
LPS-Induced Cytokine Release by Tumor-4 Macrophage Subclones*

	IL-1 (U/ml)		IL-6 (U/ml)		TNF (U/ml)	
	-LPS	+LPS	-LPS	+LPS	-LPS	+LPS
R2	0	2	0	0	0	0
T4.1	0	2	0	1	0	0
T4.2	0	6	0	100	0	40
T4.3	0	6	0	1500	0	85
T4.4	0	19	0	39	0	34
T4.5	1	4	0	0	0	0
T4.6	0	4	0	35	0	0

*The capacity of cell lines to release the cytokines IL-1, IL-6, and tumor necrosis factor (TNF) was determined by assaying culture supernatants. For this purpose, cell lines were incubated for 24 h at 2.5×10^5 cells/ml with 10-μg/ml LPS in RPMI 1640 supplemented with 10% fetal calf serum and 5×10^{-5} M 2-mercaptoethanol. Culture supernatants were collected, passed through a 0.2-micron filter, and stored at −70°C until assayed. IL-1 activity was assayed by its ability to induce proliferation of D10.G4.1 cells in the presence of Concanavalin A as described by Ayala et al. (1988b). IL-6 activity was determined by its ability to induce the proliferation of the 7TD1 B-cell hybridoma as previously described by Hultner et al. (1989). TNF activity was assessed by its cytotoxicity to WEHI-164 clone 13 cells as previously described by Ayala et al. (1988a). The relative units of cytokine activity were determined by comparison of the activity of dilution series of experimental supernatants to the activities of dilution series of purified human IL-1 (Genzyme), recombinant human IL-6 (Amgen Corp.) or murine TNF-alpha (Amgen Corp.) standards.

not shown). A mu heavy-chain probe revealed a diverse range of RNAs in the macrophage subclones (Fig. 5) that correspond in size to 1.9-, 2.1-, 2.3-, and 2.9-kb transcripts reported to be initiated in the mu-switch region of myeloid cell lines (Kemp et al., 1980). The R2 pre-B-cell line possesses predominantly larger RNA species that include those that correspond in size to mature mu mRNAs of 2.4 and 2.7 kb. These species are diminished upon lineage switch. Comparison to a hybridization of the same blot with a probe for GAPDH (Fig. 5) shows the R2 RNA to be underloaded and thus the diminution of mu transcription in the macrophage is even more dramatic than apparent from casual inspection of the data. Apparently, the macrophage subclones of tumor 4 lose the capacity to transcribe functional mu mRNA, even though the rearrangement of the kappa locus suggests progress in lymphoid differentition.

Since CD45 isoforms have been reported to be lineage-specific (Ralph et al., 1987; Saga et al., 1987; Streuli et al., 1987), the expression of this surface marker was examined among the subclones of tumor 4. An immunoperoxidase-detection procedure detected B220, the B lymphoid isoform of CD45, in tumor-4 cells. The expression of CD45 was further examined among the tumor-4 subclones in order to determine the relative expression of the B220 isoform in comparison to the isoform that predominates in myeloid cells. Recently, Chang et al. (1989) described the use of a reverse transcription-polymerase chain reaction (RT-PCR) technique to determine the pattern of alternate exon use in CD45 expression of hematopoietic cells. They found that B lymphoid cell lines uniquely expressed a form of CD45 mRNA possessing three optional exons, whereas two myeloid cell lines (a macrophage and a mast cell) predominantly expressed a form lacking these exons. We utilized RT-PCR to examine CD45 expression among R2 and the subclones of tumor 4 (Fig. 6). All of the cell lines expressed multiple species of CD45 mRNA. Subclones 2, 3, 4, and 6 expressed a CD45 mRNA containing three optional exons, typical of B lymphoid cells, whereas subclones 1 and 5 predominantly expressed mRNA lacking these exons, typical of myeloid cells. R2 expressed the expected three exon B lymphoid isoform. Thus, the pattern of CD45 expression is heterogeneous among macrophage subclones of the same tumor.

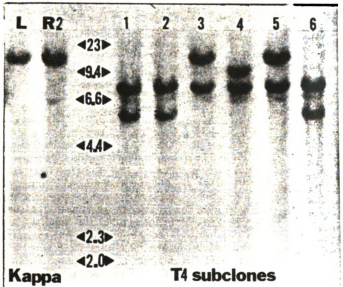


FIGURE 4. Kappa light-chain rearrangements. Southern blot analysis of DNAs from liver (L), R2, and six subclones of tumor 4 (1-6). DNA was digested with BamHI and 10 μg of each sample was electrophoresed through a 0.8% agarose. The blot was probed for kappa light-chain constant region sequences. Size markers are the positions of an ethidium bromide-stained HindIII digest of bacteriophage λ and are denoted in kilobases.

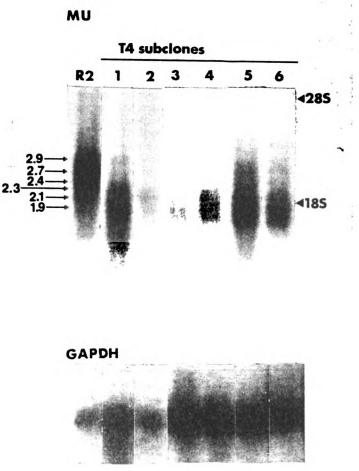


FIGURE 5. Expression of mu heavy-chain RNA. Northern blot analysis was performed by poly A+ RNA from R2 and six subclones of tumor 4 (1–6). Each sample of RNA was the poly A+ fraction selected from 100 μg of total cytoplasmic RNA. The blot was probed for mu heavy chain. The positions of ethidium bromide-stained rRNAs are noted on the right. The positions of mu RNA species are marked on the left and denoted in kilobases. The lower panel shows the same blot probed for GAPDH as a control for loading.

The Subclones of Tumor 4 Can Function Effectively in Antigen Presentation

In order to test the ability of tumor-4 subclones to present antigen, an assay system required an antigen-specific T-helper cell line that could be stimulated to produce interleukin-2 (IL-2) upon presentation. For these experiments, the putative antigen-presenting cells were cocultivated with a T-cell hybridoma specific for chicken ovalbumin (cOVA) and restricted for I-A^d (the haplotype for BALB/c), DO.11.10/54.4. In the presence of cOVA, authentic macrophages such as those in a peritoneal exudate stimulate the hybridoma to produce IL-2 (Fig. 7A). IL-2 production was assayed by the application of media supernatants from cocultivations to an IL-2-dependent cell line, CTLL-2. All of the macrophagelike tumor-4 subclones displayed antigen-presentation capacities comparable to peritoneal exudates (Fig. 7A). Furthermore, all of the tumor-4 subclones showed antigen-presentation capacities dramatically greater than either the parental R2 pre-B-cell line or tumor 1, a B-cell tumor derived from R2 (Fig. 7A). IL-2 production was dependent on the presence of cOVA during cocultivation of presenting cells with cells of the helper T-cell hybridoma. Supernatants produced in the

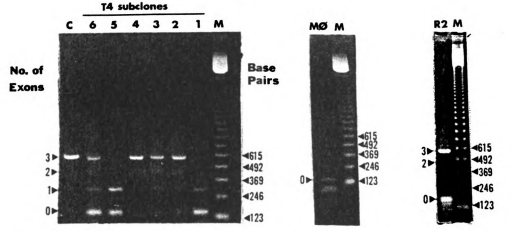


FIGURE 6. RT-PCR analysis of CD45. RT-PCR was performed on the polyA+ RNAs of R2, the six subclones of tumor 4, and P388D1 (a myeloid control). The products were electrophoresed through 2% agarose and stained with ethidium bromide. (C) 3-exon plasmid control; (1–6) T4 subclones; (M) 123-bp ladder; (MØ). P388D. PCR products smaller than the 0 exon product may represent an RNA species lacking an additional exon (Chang and Esselman, unpublished results).

absence of cOVA were analyzed for all the cell lines and the values for IL-2 production were found to be near zero (data not shown). These control values were subtracted from those determined for supernatants produced in the presence of cOVA to generate the data presented in Figs. 7A, 7B, and 7C. IL-2 production was also dependent on the presence of T-cell hybridoma cells. Supernatants produced by incubations of putative presenting cells with cOVA in the absence of T-cell hybridoma cells had no detectable IL-2 (data not shown). The ability to present antigen was not stimulated by exposure to LPS for any of these cell lines (data not shown). A macrophage-like outgrowth from tumor 5 (also derived from R2) and the cells of a macrophagelike tumor derived from the pre-B-cell line R1 (9) showed levels of antigen presentation similar to those observed for the tumor-4 subclones (Fig. 7B). The ability to present antigen, therefore, may be a common phenomenon among v-Ha-*ras*-transformed B lymphoid cells that acquire macrophage-like characteristics.

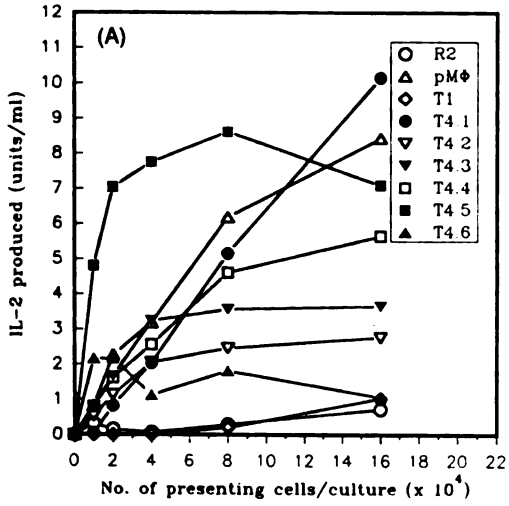
Authentic antigenic presentation should be MHC-restricted, so all of the putative antigen-presenting cells were also cocultivated with a T-cell hybridoma specific for cOVA and restricted for I-A^k, 3Q023-24.4. As exemplified by subclone 4 of tumor 4 (T4.4) and the macrophage tumor derived from R1 (R1T), the antigen presentation observed is MHC-restricted (Fig. 7C).

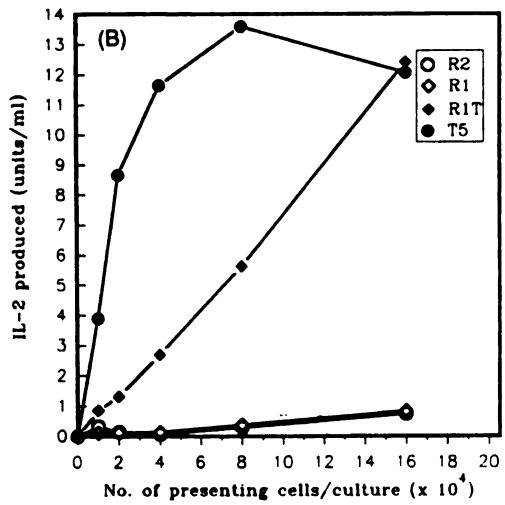
I-A Expression

Antigen presentation to T cells requires Ia expression and the observation of I-A^d-restricted presentation (Fig. 7C) indicates that these "lineage switch" macrophages express I-A^d. In order to assess whether the acquisition of presentation capacity correlated with acquisition of Ia expression, in particular I-A^d, we performed flow cytometry with FITC-conjugated antimouse I-A^d on the macrophage cell lines and their pre-B-cell precursors. Although both R1 and R2 (pre-B cells) displayed no detectable I-A^d, the macrophage cell lines represented by R1T and T4.4 showed a low expression of I-A^d (Fig. 8).

DISCUSSION

This study demonstrates the capacity of several macrophage-like tumor cell lines derived from v-Ha-*ras*-transformed pre-B-cell lines to present antigen with MHC-restriction. This finding establishes that cells having undergone "lineage switching" can perform a function normally associated with a fully differentiated macrophage





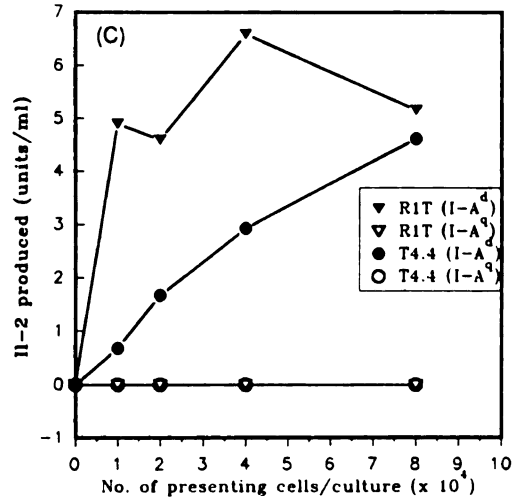


FIGURE 7. Antigen presentation. (A) Antigen-presentation assays were performed on R2, the tumor-4 subclones (4.1–4.6), tumor 1 (a B-cell tumor derived from R2), and peritoneal exudates (pMø). (B) Antigen-presentation assays were performed on R2, a macrophage outgrowth of tumor 5 (T5; derived from R2 in a different animal), R1 (another pre-B-cell line transformed by v-Ha-*ras*), and R1T (a macrophage tumor derived from R1). (C) Antigen-presentation assays were performed on a tumor-4 subclone (T4.4) and R1T with DO-11.10/54.4 (I-A^d-restricted) or 3Q023-24.4 (I-A^q-restricted). Each point represents an average value obtained from a dilution series for each presentation supernatant, testing the response of CTLL-2 cells to IL-2 in those supernatants. The results shown are representative of at least two experiments with each cell line.

FIGURE 7

or B cell. Although numerous examples exist of B lymphomas with the capacity to present antigen (Chesnut et al., 1982; Walker et al., 1982), neither the pre-B-cell precursors of the macrophage-like cell lines nor B-cell lymphoma cell lines derived from those precursors could present antigen. Thus, the capacity to present antigen appears to correlate with the differentiation of these cells along the macrophage lineage. Indeed, two cell lines with the most dramatic level of antigen presentation had lost expression of the B-cell isoform of CD45 and displayed a pattern of CD45 more typical of a myeloid cell (subclones 1 and 5, Fig. 6; T4.1 and T4.5, Fig. 7A). Perhaps, loss of the B-cell isoform of CD45 is indicative of further maturation along the myeloid lineage. It may be worthwhile to investigate the role of CD45 in macrophage function. The fact that similar antigen-presentation abilities were found in macrophage derivatives of two completely independent cell lines (R1 and R2) suggests the generality of this phenomenon.

Because it is well established that IL-1 along with antigen presentation is an important coactivator of T cells, it is surprising that the inducibility of cytokine release by LPS (Table 1) does not correlate with the effectiveness of antigen presentation by the T4 subclones (Fig. 7A).

Apparently the low levels of IL-1 that some of these macrophages are capable of elaborating is sufficient for T-cell activation. The observation that two of the best lines for antigen presentation (T4.1 and T4.5) have a weak response to LPS suggests that LPS-induced cytokine release may not be an adequate measure in itself for evaluating macrophage function.

The "lineage switch" macrophages reported here express a low level of Ia (Fig. 8). This is consistent with the previous report of Davidson et al. (1988). The precursor pre-B cells lack detectable Ia. Perhaps Ia expression is the critical property determining the capacity to present antigen among these cells. Certainly, Ia expression is necessary for antigen presentation, but its sufficiency for antigen presentation among the cell lines we have studied will require further experimentation.

The six macrophagelike subclones of tumor 4, while possessing a common rearranged kappa allele, displayed a variety of kappa light-chain gene rearrangements at their other kappa allele. Compared to their parental cell line, these cells have progressed along the B as well as the monocyte/macrophage lineage. The varying rearrangements of one kappa allele suggest rearrangement subsequent to macrophage conversion and that at least certain elements of lymphoid and macrophage differentiation programs are not mutually exclusive. The fact that the R2 cell line can also generate a lymphoma (T1, Fig. 7A) that expresses both mu and kappa chains (data not shown) demonstrates the potential of this cell line to differentiate quite far along either the lymphoid or macrophage pathways. The relationship between lymphoid and macrophage differentiation revealed in these cells differs somewhat from that seen in cases of "lineage switch" previously reported. Klinken et al. (1988) found "lineage switch" macrophages at both the pre-B- and B-cell stages of immunoglobulin rearrangement. However, they did not find macrophages that had progressed in their immunoglobulin rearrangement compared to their lymphoid cell precursors, as we have. Davidson et al. (1988), examining v-Ha-*ras*-transformants similar to those reported here, could induce those cells to differentiate into either lymphoid or macrophage cells upon exposure to LPS. The lymphoid derivatives they reported did not progress beyond the pre-B-cell stage, whereas we have identified an immunoglobulin-producing tumor derived from a pre-B-cell line that also gave rise to a macrophage tumor. Perhaps the more complex environment provided during tumor chal-

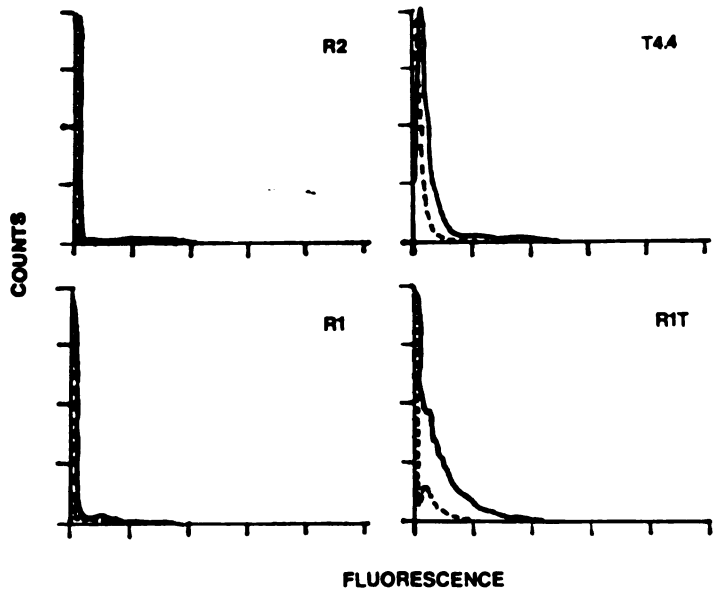


FIGURE 8. I-A^d expression. Flow cytometry was performed on R1, R2, T4.4, and R1T after reactions with FITC-conjugated anti-I-A^d (solid line) or, as a control, FITC-conjugated mouse $IgG_{2b}K$ (dashed line). For R1 and R2, the plots of experimental and control are virtually coincidental.

lenge allowed the cells described here to more fully develop along the lymphoid lineage when that pathway was selected. At any rate, the v-Ha-*ras*-transformed pre-B cells described here seem truly bipotential.

The ability of these cells that undergo an apparent "lineage switch" to perform a fully differentiated function presents the possibility that they may represent an unusual but normal subset of hematopoetic cells rather than an oddity induced by transformation. The existence of both lymphoid and macrophage characteristics in a cell fully capable of antigen presentation suggests greater plasticity in hematopoietic lineage commitment than conventionally thought to be the case.

MATERIALS AND METHODS

Cell Lines

R1 and R2 are v-Ha-*ras*-transformed murine pre-B-cell lines described in Schwartz et al. (1986b). Tumors derived from R1 and R2 were generated as described in Schwartz et al. (1986a, 1986b) in syngeneic BALB/c mice and in BALB/c athymic nude mice. Briefly, cells were washed twice in RPMI 1640 and were then resuspended in the same at 8×10^6 cells per ml. Five-week-old mice were injected intraperitoneally with 0.25 ml of the cellular suspension. Tumor 4, in particular, was isolated from an inguinal lymph node at 74 days postinjection. Tumor cell lines were readily produced from explanted tumors by dispersal and transfer to feeder cultures of adherent bone marrow cells (Whitlock et al., 1983). All of these cell lines were cultured over feeder cells in RPMI 1640 supplemented with 5% fetal calf serum and 5×10^{-5} M 2-mercaptoethanol.

The subclones of tumor 4 were generated from single colonies grown in soft agar medium as described by Whitlock et al. (1983). The T-cell hybridoma, DO-11.10/54.4, was a generous gift of Drs. Philippa Marrack and John Kappler (University of Colorado, Denver) (White et al., 1983). This hybridoma is specific for chicken ovalbumin in the context of $I\text{-}A^d$ and cross reacts weakly with chicken ovalbumin in the context of $I\text{-}A^b$. 3Q023-24.4, another T-cell hybridoma, was also a gift of Drs. Marrack and Kappler. This hybridoma is specific for chicken ovalbumin in the context of either $I\text{-}A^q$ or I-E. CTLL-2 is a T-cell line responsive to IL-2 and was obtained from the ATCC. All of these cell line were cultured in RPMI 1640 supplemented with 10% fetal calf serum and 5×10^{-5} M 2-mercaptoethanol in the absence of any feeder cells.

Peritoneal exudates containing macrophages were produced from BALB/c mice treated 1 week previously with a 0.5-ml intraperitoneal injection of pristane.

Nucleic Acid Analysis

Cytoplasmic RNA was isolated from actively growing cells by a sodium dodecyl sulfate-urea procedure as described by Schwartz et al. (1981). Poly A^+ RNA was selected by oligo-dT cellulose chromatography (Aviv and Leder, 1975). RNA was denatured, electrophoresed in a formaldehyde-1% agarose gel (Rave et al., 1979), and transferred to Nytran (Schleicher and Schuell) (Thomas, 1980).

High molecular weight DNA was isolated from nuclei collected in the preceding RNA isolation procedure as described in Schwartz et al. (1986b). DNA was digested with restriction enzymes as noted in the figure legends, electrophoresed through 0.8% agarose, and transferred to Nytran (Southern, 1975).

Hybridization probes were prepared by nick translation (Rigby et al., 1979) through the incorporation of [α-^{32}P] dATP (3000 Ci/mmol; ICN). The v-Ha-*ras* probe was the replicative form of phage M13mp10 containing a 0.46-kb EcoRI fragment corresponding to v-Ha-*ras* encoding sequences (Ellis et al., 1980). The *env* probe was a 0.8-kb BamHI fragment from the *env* region of Friend murine leukemia virus and is specific for the *env* sequences of murine ecotropic retroviruses (Silver and Kozak, 1986). The c-*myc* probe was the 4.7-kb genomic HindIII fragment of murine c-*myc* (Stanton et al., 1984). The murine c-*myb* probe was a cloned 2.4-kb cDNA (a generous gift of Dr. Timothy Bender, University of Virginia, Charlottesville). The *fms* probe was a cloned 2.7-kb ClaI-BamHI fragment of the McDonough strain of feline sarcoma virus (Donner et al., 1982). The murine β_2-microglobulin probe was a cloned 0.5-kb cDNA (Parnes et al., 1981). The rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe was a cloned 1.3-kb cDNA (Fort et al., 1985). The murine

kappa light-chain probe was the replicative form of phage M13mp10 containing a genomic 0.48 kb HpaI-BglII fragment extending from a point about 50 base pairs within the 5' terminus of the kappa light-chain constant region gene to the poly A addition site (Seidman and Leder, 1978). The murine mu heavy-chain probe was a cloned cDNA (μ12) that extends from C_H2 to the 3'-untranslated region of the secreted form of mu mRNA (Rogers et al., 1980). All hybridizations were performed under aqueous conditions in 5× SSC at 65 °C and washed to a stringency of 0.1× SSC at 65 °C.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

RT-PCR was performed according to the procedure of Chang et al. (1989, 1991) using poly A^+ RNA as substrate. The primers were a sense primer specific to exon 2 (GCCCTTCTGGACACAGAAGT, base positions 167–186) and an antisense primer specific to exon 9 (AATTCACAGTAATGTTCCCAAACAT; base positions 764–740) of the cDNA of murine CD45 (Thomas et al., 1987). cDNA was prepared by incubating 1 μg of poly A^+ RNA for 60 min at 37 °C with 200 units of MoMuLV reverse transcriptase in a 20-μl reaction volume containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 5 mM DTT, 100 μg/ml BSA, 40 units RNasin, 500 μM dNTP, and 200 ng of antisense primer. A 5-μl aliquot was used directly for PCR amplification in a 50-μl reaction volume containing 50 mM KCl, 10 mM Tris-HCl (pH 9.3), 3 mM $MgCl_2$, 0.1% w/v gelatin, 500 μM dNTP, 400 ng of sense and antisense primers, and 2.5 units of Taq polymerase. PCR was performed in a DNA Thermal Cycler (Perkin-Elmer-Cetus, Inc.) for 24 cycles. Each cycle consisted of 40 s at 94 °C for denaturation, 15 s at 55 °C for annealing, and 30 s at 72 °C for elongation. The first cycle was preceded by a 5-min incubation at 94 °C and the last cycle followed by a 4-min incubation at 72 °C.

Cytological Analyses

Cells were cytocentrifuged onto a microscope slide and allowed to air dry overnight. The cells were then incubated with either rat anti-B220 (monoclonal 14.8) or rat anti-MAC-1 (Boehringer Mannheim). Goat antirat immunoglobulin-horseradish peroxidase (Boehringer Mannheim) was used in a secondary incubation for detection. The presence of α-naphthyl acetate esterase was determined by cytochemical staining (Yam et al., 1971) with a Sigma research kit. Nonspecific phagocytosis of latex beads was assayed by the method of Raschke et al. (1978).

Antigen Presentation

Assays for antigen presentation were performed in a manner similar to that described by Marrack et al. (1989). Briefly, the cell lines to be assayed for antigen presentation were titrated into 200-μl microcultures containing 10^5 cells of either the T-cell hybridomas DO-11.10/54.4 or 3Q023-24.4, both of which produce IL-2 in response to the presentation of chicken ovalbumin (cOVA) in the context of $I\text{-}A^d$ or $I\text{-}A^q$, respectively. These assays were carried out in RPMI 1640 supplemented with 10% fetal calf serum, 5×10^{-5} M 2-mercaptoethanol and, where required, cOVA at 1 mg/ml. After 24 hr, incubation supernatants from these cultures were assayed for IL-2 using CTLL-2, an IL-2-dependent cytotoxic T-cell line. Twofold serial dilutions of supernatants were added to 5×10^3 CTLL-2 cells in 100-μl microcultures and incubated for 48 hr at 37 °C. MTT (Sigma), a substrate for production of a colored product indicative of cell survival (Mosmann, 1983), was added at 0.5 mg/ml and the cultures incubated for an additional 4 hr at 37 °C. Acid-isopropanol (40-mM HCl) was then added to dissolve the MTT formazan reaction product. The optical density of each well was quantitated by an ELISA reader at a wavelength of 540 nm. The specific activity of IL-2 in the supernatants was determined by comparison to a standard curve produced through the use of purified recombinant IL-2 (Cetus Inc.).

Flow Cytometry

Cells were stained in PBS, 2% FCS with either FITC-conjugated monoclonal antibody AMS-32.1 (antimouse $I\text{-}A^d$) (Phar Mingen) or FITC-conjugated mouse IgG_{2b}, K (Phar Mingen) as an isotype-matched control. Cells were then fixed in PBS, 2% FCS, 0.5% formaldehyde, and stored at 4 °C until analysis. Flow cytometry was performed using an Ortho Diagnostics Cytofluorograph 50-H.

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Chapter 3

C/EBP-related proteins confer LPS-inducible expression of IL-6 and MCP-1 to a lymphoblastic cell line[1]

Abstract

C/EBP-related proteins 2 and 3 (CRP2 and CRP3) are differentially expressed by P388 lymphoblasts and their derivative P388D1(IL-1) macrophages. We have ectopically expressed CRP2 and CRP3, either singly or together, in P388 lymphoblasts. The expression of either CRP2 or CRP3 is sufficient to confer the LPS-inducible expresson of IL-6 and MCP-1 (macrophage chemoattractant protein) to a cell type not typically displaying LPS induction of inflammatory cytokines. Consistent with these findings, the expression of CRP2 antisense RNA blocks the LPS-induction of IL-6 expression in P388D1(IL-1) macrophages. This work clearly establishes the essential role of C/EBP-related proteins in the induction of cytokine genes by LPS. Additionally, these data add MCP-1 to the list of cytokines showing an involvement of either CRP2 or CRP3 in their expression.

Introduction

C/EBP-related proteins (CRPs) are a family of basic region-leucine zipper (bZIP) transcription factors. These dimerize through a leucine zipper and bind to DNA through an

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[1] Adapted from JD Bretz, S Williams, PF Johnson, and RC Schwartz. Proc. Natl. Acad. Sci. (submitted).

adjacent basic region (Johnson et al 1987, Landschulz et al 1988, and Vinson et al 1989). Several lines of evidence implicate CRPs in the regulation of inflammatory cytokines. The promoter regions for the IL-6, IL-1α, IL-1β, IL-8, TNFα and G-CSF genes contain sequences that bind CRPs (Akira et al 1990, Furatani et al 1986, Shirakawa et al 1993, and Zhang and Rom 1993). The best fit consensus CRP binding site is T(T/G)NNGNAA(T/G) (Akira et al 1990). Both CRP2 (Williams et al 1991) (also known as NF-IL6 [Akira et al 1990] and C/EBPβ [Cao et al 1991]) and CRP3 (Williams et al 1991) (also known as NF-IL6β [Kinoshita et al 1992] and C/EBPδ [Cao et al 1991]) can transactivate a reporter gene driven by the IL-6 promoter in transient expression assays (Akira et al 1990 and Kinoshita et al 1992). Additionally, the LPS-induced expression of IL-1β (Shirakawa et al 1993 and Zhang and Rom 1993) and G-CSF (Akira et al 1990, Nishizawa et al 1990 and Nishizawa and Nagata 1990) requires one or more elements that bind a CRP-like activity.

Among the hematopoietic lineages, mature macrophages and granulocytes are specific in their expression of high levels of CRP2 and CRP3 (Natsuka et al 1992 and Scott et al 1992). Macrophages are also notable for their lipopolysaccharide (LPS)-induced trancription of the genes for various inflammatory cytokines, the same genes that posses CRP binding sites in their promoters. Indeed, CRP2 and CRP3 are LPS-inducible (Akira et al 1990 and Kinoshita et al 1992) and the CRP binding sites of the IL-1β and G-CSF genes have been

characterized as LPS-responsive elements (Shirakawa et al 1993, Zhang and Rom 1993, Nishizwa et al 1990 and Nishizawa and Nagata 1990). These data suggest that CRP2 and CRP3 may be necessary for the LPS-induced cytokine response in macrophages.

The evidence for the function of CRP2 and CRP3 in the regulation of IL-6 and other cytokines has rested on the transient transactivation of reporter genes rather than the activation of endogenous cytokine genes with intact promoter regions. Additionally, the relative activities of these two co-expressed and structurally related transcription factors have not been tested in vivo. In this paper, we have directly assessed the capacities of CRP2 and CRP3 for conferring LPS-induced cytokine expression to a lymphoblastic cell line normally lacking these activities. We have found that either singly or together, the ectopic expression of CRP2 and CRP3 in the lymphoblastic P388 cell line (Bauer et al 1986) confers LPS-inducible expression of the genes encoding IL-6 and MCP-1 (macrophage chemoattractant protein). Consistent with this, the expression of CRP2 antisense RNA blocks the induction by LPS of IL-6 in P388D1(IL-1) macrophages. The roles of CRP2 and CRP3 in the LPS-induction of IL-6 and MCP-1 are clearly established.

Materials and methods

Cells and cell culture. P388 lymphoblasts are $P388D_1$ cells (ATCC CCL 46). We have denoted these cells as P388 to

void confusion with their macrophage derivative which is usually referred to as P388D1 (Bauer et al 1986). P388 cells ($P388D_1$ ATCC CCL 46) are lymphoblastic in morphology and lack appreciable expression of Mac 1, Mac 2, and Mac 3 as determined by flourescent activated cell sorting (FACS). P388D1(IL-1) macrophages are $P388D_1$ (IL-1) cells (ATTC TIB 63). These cells are macrophage-like in morphology and express Mac 1, Mac 2, and Mac 3 at high levels as determined by FACS. Cells were cultured in RPMI 1640 medium supplemented with 5% fetal calf serum and 50μM 2-mercaptoethanol.

Transfections. Transfections of G418-resistant vectors were carried out with 10^6 cells, 5μg of plasmid DNA and 40μg of lipofectin Reagent (Gibco BRL) in 3 ml of Opti-MEM I reduced-serum medium (Gibco BRL). Cells were incubated in the transfection cocktail for 16 hours followed by the addition of RPMI 1640 supplemented with 20% fetal calf serum. After 72 hours the medium was replaced with standard growth medium supplemented with 0.67 mg/ml G418 (Gibco BRL). Transfections of puromycin-resistant vectors were carried out similarly except that 10 μl of Transfectam Reagent (Promega) was used and selections performed with 3.75 μg/ml puromycin (Sigma).

Expression vectors. pSV(X)Neo is pZIP-NEO SV(X)1 (Cepko et al 1984). This vector contains Moloney murine leukemia virus (MoMLV) long terminal repeats (LTRs) and expresses the Tn5 neo gene through a subgenomic mRNA from the 5′ LTR. pSV(X)CRP2 and pSV(X)CRP2AS were constructed by insertion of the 1.55 kb NcoI/EcoRI genomic fragment encoding rat CRP2

(Williams et al 1991) into the BamHI site of pSV(X)Neo in both sense and antisense orientations. pSV(X)CRP3 was similarly constructed by insertion of the 0.8kb NcoI/HindIII genomic fragment encoding murine CRP3 (Williams et al 1991) into the BamHI site of pSV(X)Neo. pBABE-Puro (Morgenstern and Land 1990) contains MoMLV LTRs and expresses the pac gene for puromycin resistance from the SV40 early promoter. pBABE-CRP2 was constructed by insertion of the 1.55 kb NcoI/EcoRI CRP2 fragment into the BamHI site of pBABE-Puro.

Nucleic acid isolation and analysis. Cytoplasmic RNA was isolated by an SDS-urea procedure as described by Schwartz et al (1981). Genomic DNA was isolated from nuclei collected in the preceding procedure by a method described in Schwartz et al (1986).

Restriction enzyme-digested DNAs were electrophoresed through 0.8% agarose. RNAs were electrophoresed through 1% agarose-formaldehyde gels. Transfers to membranes were hybridized and washed to a stringency of 0.1xSSPE in 0.1% SDS.

Hybridization probes were prepared by random priming using a kit from United States Biochemical Corp. with the incorporation of 5′-[α-^{32}P]dATP (3,000 Ci/mmol; Dupont/NEN). The probes for CRP2 and CRP3 were genomic fragments described above for expression vectors. The glyceraldehyde phosphate dehydrogenase (GAPDH) probe was a 1.3 kb rat cDNA (Fort et al 1985). The IL-6 probe was a 0.65 kb murine cDNA (from Drs. N. Jenkins and N. Copeland, NCI-FCRDC). The MCP-1 probe was a 0.58 kb murine cDNA (Rollins et al 1988). The IL-1α probe was

a 1.7 kb murine cDNA (Lomedico et al 1984). The IL-1β probe was a 1.0 kb murine cDNA (Tannenbaum et al 1988).

Western analysis. Nuclear extracts were prepared as described below. These extracts (50μg) were suspended in Laemmli buffer and electrophoresed through 12% SDS-polyacrylamide gels. The gels were electrophoresed onto Immobilon-P membranes (Millipore), and antibody-antigen complexes were visualized with the Enhanced Chemiluminescence detection kit (Amersham) exactly as recommended by the supplier.

Electrophoretic mobility shift assays (EMSAs). Nuclear extracts were prepared as described by Lee et al except that the samples were not dialyzed into buffer D. Extracts were incubated with a double-stranded oligonucleotide probe homologous to nucleotides -107 to 90 of the rat albumin promoter (the DE1 site) or to a probe containing an optimal C/EBP binding site (Johnson et al 1993). Reactions were carried out by the method of Fried and Crothers (1981), as modified by Nye and Graves (1990). Samples were electrophoresed through 6% polyacrylamide gels in 0.5 x TBE at 150 volts. For "supershifts", antisera were preincubated with nuclear extracts for 30 minutes at 4°C prior to the binding reaction.

Metabolic labeling and immunoprecipitations. Cells were washed twice in methionine-deficient DMEM (Gibco BRL) and then labeled in the same medium containing 200 μCi of EXPRE^{35}S^{35}S protein labeling mix (Dupont/NEN) and 5% dialyzed fetal calf

serum. After 3 hours, cells were collected and lysed, and immunoprecipitation was performed and analyzed as described by Whitlock et al (1983).

Antisera. Rabbit anti-CRP2 was generated by immunization with a peptide corresponding to amino acids 1-12 of CRP2 (Williams et al 1991). Rabbit anti-CRP3 antiserum was generated by immunization with a peptide corresponding to amino acids 255-266 of the murine CRP3 protein.

Results

Differential CRP2 and CRP3 expression between P388 lymphoblasts and their macrophage derivitive P388D1(IL-1). Upon screening a panel of hematopoietic cell lines for differential CRP2 and CRP3 expression by Northern blot analysis, we found that the P388 B lymphoblastic cell line lacked CRP2 and CRP3 transcripts, while its macrophage derivative P388D1(IL-1) expressed these transcripts abundantly (Figure 1A). C/EBP transcripts were not detected in either cell line (data not shown). These findings suggested to us that these cell lines could provide a model system in which to test the capacity of CRP2 and CRP3 to confer the characteristics of macrophages to a lymphoid cell line not normally expressing CRP2 and CRP3. In particular, we sought to examine the LPS-induced transcription of genes encoding inflammatory cytokines.

Having observed differential expression at the RNA level we sought to confirm that P388D1(IL-1) expressed active CRP2

Figure 1. Analysis of differential CRP2 and CRP3 expression between P388 and P388D1(IL-1). (A) Northern analysis of 20μg of cytoplasmic RNA. The same blot was successively hybridized to probes for CRP2, CRP3, and GAPDH. Hybridization to GAPDH served as a control for loading. The positions of ethidium bromide-stained 28S and 18S rRNAs are marked. (B) Western analysis of proteins derived form nuclear extracts. CRP2-specific antiserum was used for detection. (C) EMSA analysis of nuclear extracts. Samples were treated in the absence of antiserum, and in the presence of normal rabbit serum (NRS), CRP2 antiserum (CRP2), and CRP3 antiserum (CRP3). Recombinant bacterially-produced CRP2 (bact CRP2) and recombinant bacterially-produced CRP3 (bact CRP3) were analyzed as positive controls for DNA binding and "supershift". Species a represents CRP2 homodimer, while b and c probably contain heterodimeric forms of CRP2; all are "supershifted" with CRP2 antiserum. Species d is not "supershifted" and may represent LIP (Descombes and Schibler 1991; see text). The positions of species supershifted by antisera are indicated by arrows.

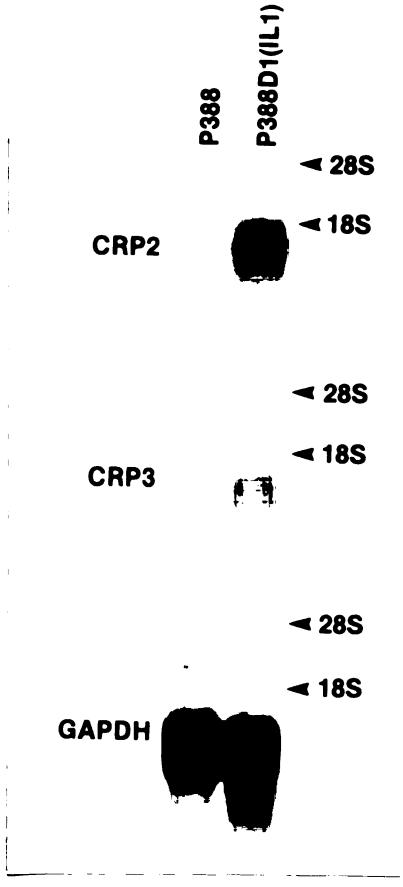

Figure 1A

Figure 1B

Antiserum:
-
NRS
CRP2
CRP3
P388
P388D1(IL1)
bact CRP2
bact CRP3
a
b
c
d

Figure 1C

and CRP3 proteins while P388 did not. A western blot analysis of nuclear extracts for the two cell lines revealed CRP2 protein to be present in P388D1(IL-1) cells, but not in P388 cells (Figure 1B). An EMSA using an oligonucleotide probe containing an optimal CRP binding site found that the nuclear extract of P388D1(IL-1) cells formed several species (a, b and c) that could be "supershifted" by incubation with antiserum specific for CRP2 protein (Figure 1C). Species a, b, and c may represent various modified or heterodimeric CRP binding forms. Species d was not supershifted and it may represent the LIP product, an amino terminal-truncated form of CRP2 (Descombes and Schibler 1991) that would not be reactive with our amino terminal-specific antiserum. CRP3 protein was not observable (data not shown) but a "supershift" species was detectable with antiserum specific for CRP3 (Figure 1C). This may be explained by the low abundance of CRP3 protein coupled with an antiserum of relatively low titer. Nonetheless, it is clear that P388D1(IL-1) macrophages display CRP binding site activity which is absent in P388 B lymphoblasts.

Ectopic expression of CRP2 and CRP3 in P388 B lymphoblasts. Three murine retrovirus vectors were utilized to ectopically express the CRP2 and CRP3 genes in P388 cells. pSV(X)CRP2 and pSV(X)CRP3 express CRP2 and CRP3, respectively, from the MoMLV LTR. Both vectors also express the gene for G418-resistance from a subgenomic mRNA. pBABE-CRP2 expresses CRP2 from the MoMLV LTR and the gene for puromycin-resistance from the SV40 early promoter. Populations of P388 cells were

transfected with pSV(X)CRP2, pSV(X)CRP3 or their parental vector lacking an expressed insert, pSV(X)Neo. Populations transfected with pSV(X)CRP3 were transfected,in turn, with pBABE-CRP2 or its parental vector lacking an expression insert, pBABE-Puro. Stably transfected populations were obtained after selection with the appropriate drug.

CRP2 and CRP3 expression were initially analyzed in the transfected populations by Northern blot analysis (Figure 2A). P388-C2 (transfected with pSV(X)CRP2) expressed a ~5.6 kb CRP2 RNA corresponding to the expected genome length retroviral transcript. P388-C3/C2 (transfected with pSV(X)CRP3and pBABE-CRP2) expressed a ~1.8 kb CRP2 RNA corresponding to a transcript extending from the 5' LTR to the genomic CRP2 poly A addition site. P388-C3, P388-C3/Puro, and P388-C3/C2 (transfected with pSV(X)CRP3, pSV(X)CRP3 and pBABE-Puro, and pSV(X)CRP3 and pBABE-CRP2, respectively) all expressed a ~4.8 kb CRP3 RNA corresponding to the expected genome length retroviral trancript. A Western blot analysis of nuclear extracts from the transfected populations confirmed the ectopic expression of the transfected CRP2 gene at the protein level (Figure 2B). An EMSA detected protein-DNA complexes that could be "supershifted" by antiserum specific for CRP2 protein in nuclear extracts of the P388-C2 and P388-C3/C2 populations (Figure 2C). Neither CRP3 protein nor a "supershift" species was observable with antiserum specific for CRP3 protein, perhaps indicating a rather low level of CRP3 protein expression.

Figure 2. Analyses of P388 cells transfected for CRP2 and CRP3 expression: P388 + pSV(X)Neo (P388-Neo); P388 + pSV(X)CRP2 (P388-C2); P388 + pSV(X)CRP3 (P388-C3); P388 + pSV(X)CRP3 + pBABE-Puro (P388-C3/Puro); P388 + pSV(X)CRP3 + pBABE-CRP2 (P388-C3/C2); P388D1(IL-1) + pSV(X)Neo (P388D1(IL-1)-Neo). (A) Northern analysis of 20 μg of cytoplasmic RNA. Blots were successively hybridized to probes for CRP2 and GAPDH, or CRP3 and GAPDH. Hybridization to GAPDH served as a control for loading. The positions of ethidium bromide-stained 28S rRNAs are marked. (B) Western analysis of protein derived from nuclear extracts. CRP2-specific antiserum was used for detection. Recombinant bacterially-produced CRP2 and CRP3 (bact CRP2; bact CRP3) are included as controls for specificity of the antiserum. The position of CRP2 protein is indicated. (C) EMSA analysis of nuclear extracts. Samples were treated in the absence of antiserum, and in the presence of normal rabbit serum (NRS), CRP2 antiserum (CRP2), and CRP3 antiserum (CRP3). Recombinant bacterially-produced CRP2 (bact CRP2) and CRP3 (bact CRP3) were analyzed as positive controls for DNA binding and "supershift." The positions of CRP2 EMSA species are indicated with asterisks and those species supershifted by antisera are indicated by arrows. (D) southern analysis of 10μg of genomic DNAs. P388-C2-2 is an independent transfectant similar to P388-C2. DNAs were digested with BglII for CRP2 vector integraton sites and BamHI for CRP3 vector integration sites. DNAs were digested with XbaI for internal vector fragments. Blots were hybridized to probes for either CRP2 or CRP3. Size markers are the positions of an ethidium bromide-stained HindIII digest of bacteriophage lambda DNA.

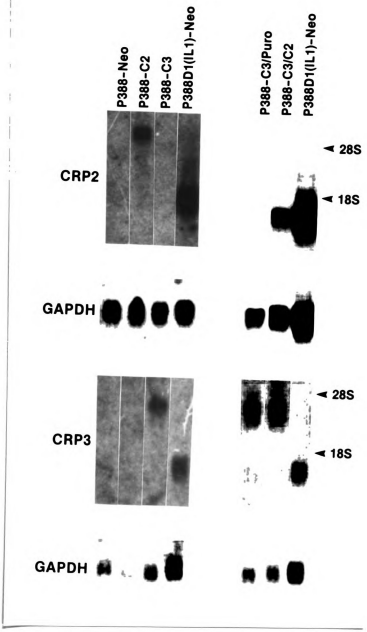
P388-Neo
P388-C2
P388-C3
P388D1(IL1)-Neo
P388-C3/Puro
P388-C3/C2
P388D1(IL-1)-Neo
28S
18S
CRP2
GAPDH
28S
18S
CRP3
GAPDH

Figure 2A

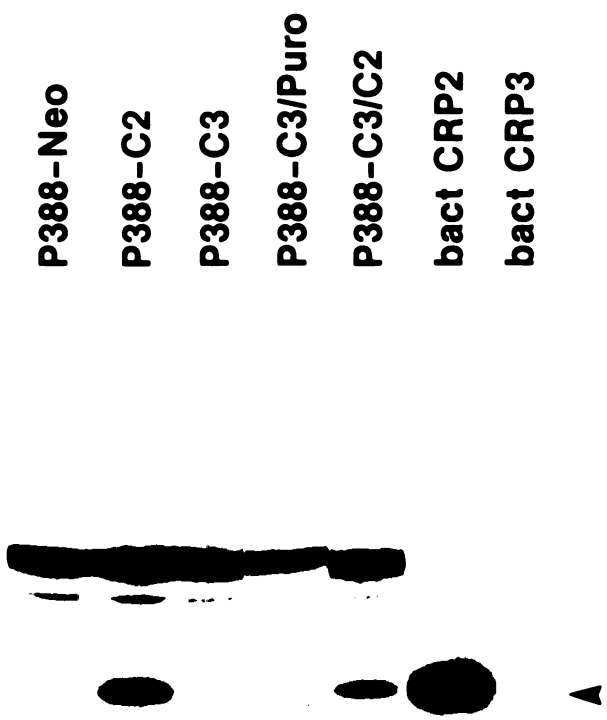
P388-Neo
P388-C2
P388-C3
P388-C3/Puro
P388-C3/C2
bact CRP2
bact CRP3

Figure 2B

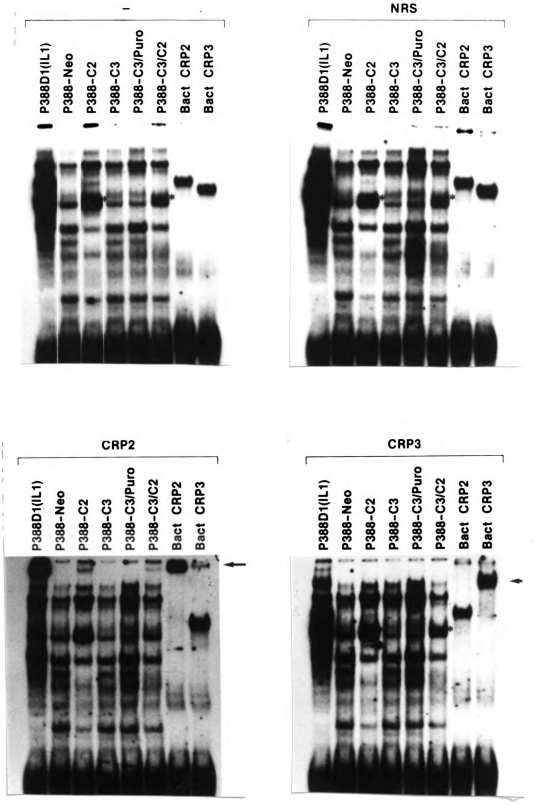
-
NRS
CRP2
CRP3
P388D1(IL1)
P388-Neo
P388-C2
P388-C3
P388-C3/Puro
P388-C3/C2
Bact CRP2
Bact CRP3

Figure 2C

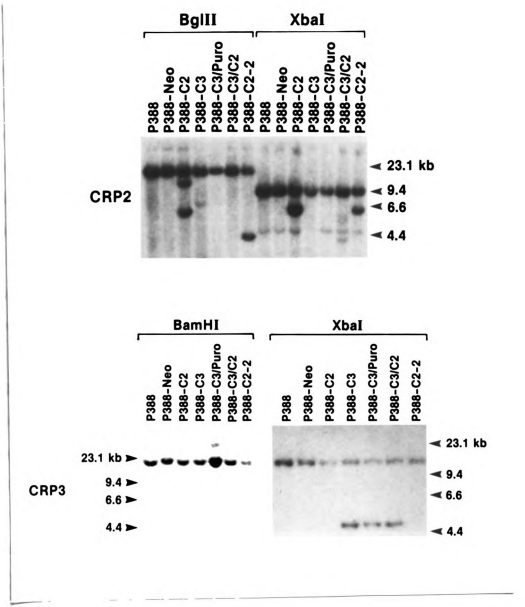
BglII
XbaI
P388
P388-Neo
P388-C2
P388-C3
P388-C3/Puro
P388-C3/C2
P388-C2-2
CRP2
23.1 kb
9.4
6.6
4.4
BamHI
XbaI
CRP3

Figure 2D

In order to assess whether the transfected cells were comprised of single clonal outgrowths or complex populations of many transfectants, a Southern blot analysis was performed (Figure 2D). For the detection of CRP2 vector integration sites, genomic DNAs from the transfected populations were digested with BglII, which cleaves only once within the pSV(X) vectors and not at all within the pBABE vectors. For the detection of CRP3 vector integration sites, genomic DNAs from the transfected populations were digested with BamHI, which does not cleave at all within the pSV(X)CRP3 vector. For evaluation of the integrity and abundance of the transfected genomes, the DNAs were digested with XbaI, which cuts once in each vector LTR to produce restriction fragments of a predicted size diagnostic of the transfected vectors.

The predicted 5.6 kb CRP2-specific XbaI fragment was detected with at least haploid abundance in DNAs from P388-C2 and P388-C2-2, an independent population of cells produced in a manner identical to that of P388-C2. The DNA of P388-C3/C2 displayed the predicted 4.0 kb CRP2-specific XbaI fragment as well as several larger fragments, all at subhaploid abundance. Perhaps this indicates instability of the transfected pBABE-CRP2 vector. Nonetheless, P388-C3/C2 shows ectopic expression of CRP2 RNA, protein and EMSA activity. All of the DNAs possess identical endogenous CRP2-specific fragments of about 9 and 4 kb. The predicted 4.9 kb CRP3-specific XbaI fragment was detected with at least haploid abundance in DNAs from P388-C3, P388-C3/Puro, and P388-C3/C2. All of the DNA possess

an identical endogenous CRP3-specific fragment at about 20 kb.

Digestion of the transfected populations with BglII revealed two unique CRP2-specific restriction fragments for P388-C2 and one unique CRP2-specific restriction fragment for P388-C2-2. All of the DNAs possessed an identical endogenous CRP2-specific fragment of about 20 kb. P388-C2 and P388-C2-2 are clearly clonal or biclonal. Unique CRP2-specific fragments were not observed for P388-C3/C2, but this may be attributable to the instability of the transfected vector rather than the presence of a multitude of independent transfectants. A unique CRP3-specific restriction fragment of about 30 kb was observed for P388-C3, P388-C3/Puro and P388-C3/C2 upon digestion with BamHI. These populations are clonal for ectopic CRP3 integration. All of the DNAs possessed an identical endogenous CRP3-specific fragment of about 20 kb.

LPS-induced cytokine expression occurs in the transfectants that ectopically express CRP2 and/or CRP3. Cultures of cells ectopically expressing CRP2 and/or CRP3 were treated with LPS (20 μg/ml) over a time course and RNA was isolated from each of several time points. Control populations of P388 lymphoblasts and P388D1(IL-1) macrophages, each transfected with pSV(X)Neo, were also subjected to time courses of LPS treatment and RNA was isolated. Northern analyses were performed to detect transcripts for IL-6, MCP-1, IL-1α, and IL-1β. Transcripts for GAPDH were also examined as a normaliztion control. LPS was found to induce transcripts for IL-6 and MCP-1 in P388-C2 and P388-C3 (Figure 3A) as well

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P38
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PSV
to

as in P388-C3/Puro and P388-C3/C2 (Figure 3B). However, EMSA analysis did not show any increase in the DNA binding activity of nuclear extracts prepared in parallel to the RNA preparations (data not shown). These inductions were generally comparable to those seen in the P388D1(IL-1)-Neo macrophage control population. P388-C3 did show a less sustained induction (Figure 3A), but the similar P388-C3/Puro showed a sustained induction of IL-6 and MCP-1 transcripts (Figure 3B). Both transfectants display similar levels of CRP3 mRNA expression and, as evidenced by identical sites of integration for the CRP3 expression vector, P388-C3/Puro is derived from P388-C3. The basis for this difference in the kinetics of cytokine mRNA induction is not known. Transcripts for IL-1β were weakly induced in the cell populations ectopically expressing CRP2 and CRP3; the inductions that occur are best observed with P388-C3/C2 (Figure 3B), where it is still far below the levels observed for the P388D1(IL-1)-Neo macrophages. Transcripts for IL-1α were not observed to be induced by LPS in any of the populations expressing CRP2 and CRP3, while a vigorous induction was observable in the P388D1(IL-1)-Neo macrophages. All four cytokines showed either no induction or a weak induction (MCP-1) upon LPS-treatment of P388-Neo lymphoblasts.

Since the P388-C2 population is a clonal or biclonal outgrowth, we examined an independently derived population of pSV(X)CRP2 transfectants, P388-C2-2 (See Figure 2D), in order to assure ourselves that the acquisition of LPS-inducibility

Figure 3. Northern analyses for cytokine expression in P388 cells transfected for CRP2 and CRP3 expression. Cytoplasmic RNA was isolated over time courses of LPS teatment as indicated. 20 μg of RNA was analyzed. Blots were successively hybridized to probes for IL-6, MCP-1, IL-1α and IL-1β. Hybridization to GAPDH served as a control for loading. (A) Single transfectants were analyzed: P388 + pSV(X)Neo (P388-Neo); P388 + pSV(X)CRP2 (P388-C2); P388 + pSV(X)CRP3 (P388-C3); P388D1(IL-1) + pSV(X)Neo (P388D1(IL-1)Neo). (B) Double transfectants were analyzed: P388 + pSV(X)CRP3 + pBABE-puro (P388-C3/Puro); P388 + pSV(X)CRP3 +pBABE-CRP2 (P388-C3/C2); P388D1 + pSV(X)Neo (P388D1(IL-1)-Neo).

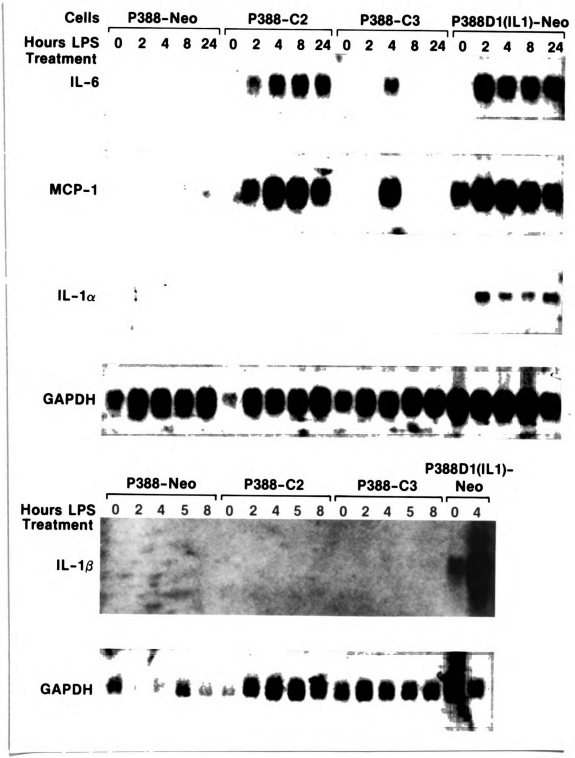

Cells
P388-Neo
P388-C2
P388-C3
P388D1(IL1)-Neo
Hours LPS Treatment
0 2 4 8 24 0 2 4 8 24 0 2 4 8 24 0 2 4 8 24
IL-6
MCP-1
IL-1α
GAPDH
P388-Neo
P388-C2
P388-C3
P388D1(IL1)-Neo
Hours LPS Treatment
0 2 4 5 8 0 2 4 5 8 0 2 4 5 8 0 4
IL-1β
GAPDH

Figure 3A

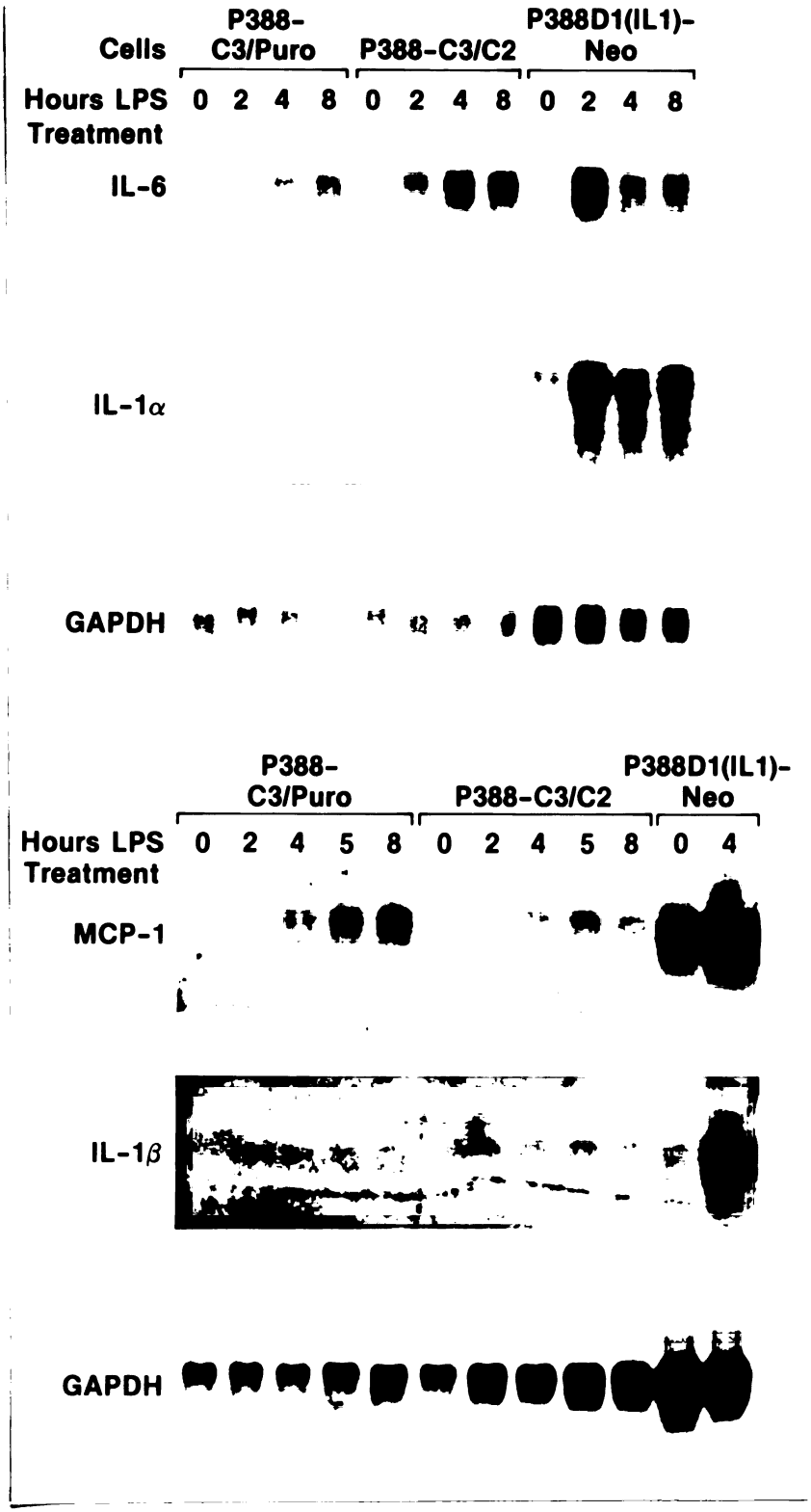

Cells
P388-C3/Puro
P388-C3/C2
P388D1(IL1)-Neo
Hours LPS Treatment
0 2 4 8 0 2 4 8 0 2 4 8
IL-6
IL-1α
GAPDH
P388-C3/Puro
P388-C3/C2
P388D1(IL1)-Neo
Hours LPS Treatment
0 2 4 5 8 0 2 4 5 8 0 4
MCP-1
IL-1β
GAPDH

Figure 3B

was a consequence of ectopicc CRP2 expression rather than clonal variation. P388-C2-2 cells were treated with LPS over a time course and RNA was isolated from each time point. A Northern analysis (Figure 4) confirmed that transcripts for IL-6 were induced by LPS-treatment in these CRP2-expressing cells.

IL-1 and IL-6 have been reported to induce transcription of their own genes. Experiments parallel to those described above that examine the abilities of IL-1 and IL-6 to induce transcription of their own genes were performed. No induction of IL-6, MCP-1, IL-1α or IL-1β were observed (data not shown).

Inhibition of LPS-induced cytokine expression by CRP2 antisense RNA. The ectopic expression experiments show that CRP2 or CRP3 expression is sufficient to confer LPS-inducible IL-6 and MCP-1 expression to P388 lymphoblasts. To approach the question of whether CRP2 is necessary for the LPS-inducible expression of cytokines, we sought to block the expression of CRP2 in P388D1(IL-1), a P388-derived macrophage. This cell line expresses CRP2 and CRP3 (Figure 1) and displays LPS-inducible expression of IL-6, MCP-1, IL-1α, and IL-1β (Figure 3). In order to block expression of CRP2, pSV(X)CRP2AS (carrying the CRP2 gene in an antisense orientation) was stably transfected into P388D1(IL-1) cells. As a control, P388D1(IL-1) cells were also transfected with pSV(X)Neo. RNAs were prepared from populations of these transfected cells in the presence or absence of a 4 hour treatment with LPS (20μg/ml). A Northern analysis (Figure 5)

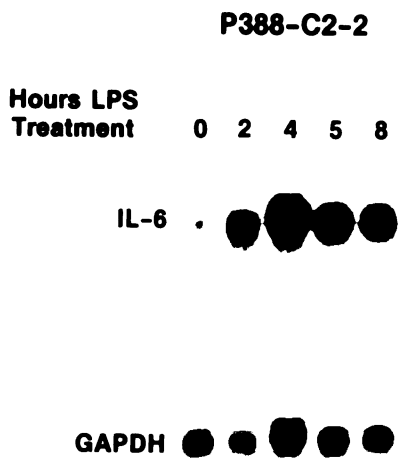


Figure 4. Analysis of a second population of P388 cells transfected for CRP2 expression. Cytoplasmic RNA was isolated over a time course of LPS treatment. 20μg of RNA were analyzed by successive hybridization to probes for IL-6 and GAPDH. Hybridization to GAPDH served as a control for loading.

was performed to examine transcripts for IL-6 (highly induced in P388 cells ectopically expressing CRP2 and CRP3) and IL-1β (weakly induced in P388 cells ectopically expressing CRP2 and CRP3). Both IL-6 and IL-1β transcripts failed to be induced in P388D1(IL-1)-C2AS, a transfectant population carrying pSV(X)CRP2AS. In comparison, a vigorous induction of IL-6 and IL-1β transcripts is observed for P388D1(IL-1)-Neo. It is noteworthy that IL-1β transcripts are little affected by CRP expression in P388 cells, while being dramatically affected by antisense CRP2 RNA in P388D1(IL-1) cells. This most likely indicates that high level transcription of IL-1β requires activation of a transcription factor(s) not present in P388 cells that acts together with CRP2 to elicit an LPS-response.

In order to confirm that the expression of antisense RNA actually reduced the level of CRP2 expressed, the transfected populations were metabolically labeled and their proteins immunoprecipitated with antiserum specific to CRP2 protein. The expression of CRP2 protein was reduced in populations expressing antisense CRP2 RNA in comparison to the control P388D1(IL-1)-Neo population (Figure 5). These results indicate that the antisense suppression of LPS-induction is indeed mediated through blockage of CRP2 expression.

Discussion

The data presented in this paper demonstrate that CRP2 and CRP3, either singly or together, are sufficient to confer LPS-inducible expression of IL-6 and MCP-1 to P388, a

Figure 5. Inhibition of IL-6 and IL-1β expression by antisense CRP2 RNA in P388D1(IL-1) cells: P388D1(IL-1) + pSV(X)Neo (P388D1(IL-1)-Neo); P388D1(IL-1) + pSV(X)CRP2AS (P388D1(IL-1)-C2AS). Cells were either untreated or exposed to a 4 hour LPS treatment. A Northern analysis of 20μg of cytoplasmic RNA was successively hybridized to probes for IL-6, IL-1β and GAPDH. Hybridization to GAPDH served as a control for loading. Metabolically labeled protein from identically treated cells was immunoprecipitated with CRP2-specific antiserum and analyzed by PAGE in the lower panel. The positions of CRP2 and marker protein are indicated.

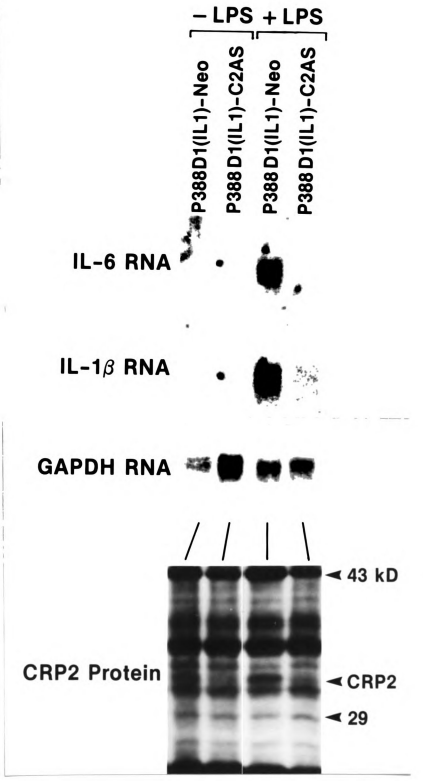

– LPS
+ LPS
P388D1(IL1)-Neo
P388 D1(IL1)-C2AS
P388 D1(IL1)-Neo
P388 D1(IL1)-C2AS
IL-6 RNA
IL-1β RNA
GAPDH RNA
43 kD
CRP2 Protein
CRP2
29

Figure 5

lymphoblastic cell line lacking these activities. Furthermore, the expression of CRP2 antisense RNA in P388D1(IL-1), a macrophage cell line derived form P388, blocks this cell line's ability to induce IL-6 transcripts in response to LPS. These data definitively establish the critical role of the CRP2 and CRP3 transcription factors in the LPS-induction of endogenous IL-6 and MCP-1 genes. This is the first indication that CRPs may be involved in the regulation of MCP-1. The MCP-1 promoter does contain a sequence -14 to -6 of translational initiation that is an inverted consensus CRP-binding site, as well as seven other sequences that are nonhomologous at only one base to the consensus CRP-binding site (Timmers et al 1990).

Although the promoters for the IL-1α and the IL-1β genes possess CRP-binding elements, ectopic expression of CRP2 and/or CRP3 was not sufficient to confer responsiveness of these genes to LPS in P388 lymphoblasts. On the other hand, antisense RNA for CRP2 blocked the LPS induction of IL-1β mRNA in P388D1(IL-1) macrophages. While CRP2 may be essential for IL-1β expression, other factors are likely necessary for the LPS induction of IL-1β. Indeed, other investigators have found that while both MCP-1 and IL-1 are induced by LPS, there are critical differences in their regulation (Tannenbaum and Hamiliton 1989 and Ohmori et al 1990). Agents that elevate intracellular levels of cAMP suppress the LPS induction of MCP-1, but do not affect the induction of IL-1α and actually enhance the induction of IL-1β.

We have found both CRP2 and CRP3 expression to be similarly effective in conferring LPS-inducibility for IL-6 and MCP-1 expression. In addition, there was no obvious synergy in the population of cells expressing both CRP2 and CRP3. This result is consistent with the experiments of Cao et al (1991), which examined the relative abilities of C/EBP, CRP2 and CRP3 to transactivate a reporter construct in transient transfections. On the other hand, Kinoshita et al (1992) found CRP3 to be more effective than CRP2 in driving expression of a reporter gene in transient transfections. A mild synergy between the two factors was also reported. All of these assays are relatively crude in their ability to accurately compare specific activities of the CRP2 and CRP3 proteins, since the relative abundance of the two proteins was not directly measured. It may be, however, that the relative activities vary between cell lines and depend on the presence or absence of other CRPs that allow differing spectra of heterodimers (Williams et al 1991) or the presence or absence of cooperating transcription factors of other families such as NFκB (Stein et al 1993), the glucocorticoid receptor (Nishio et al 1993), AP-1 (Sterneck et al 1992) and c-myb (Ness et al 1993, Burk et al 1993).

We found that IL-1 and IL-6, in contrast to LPS, did not elicit the induction of IL-6 and MCP-1 in populations of P388 lymphoblasts ectopically expressing CRP2 and CRP3 (data not shown). IL-1 has previously been found to stimulate the production of IL-6 (Walther et al 1988 and Zhang et al 1988)

and MCP-1 (Colotta et al 1992). IL-6 has been found to stimulate its own synthesis (Miyaura et al 1989 and Shabo et al 1989). The lack of response to IL-1 and IL-6 in the P388 transfectants described here might be explained trivially by the lack of receptor for these cytokines. On the other hand, it may be that the primary role of IL-1 and IL-6 in the stimulation of IL-6 and MCP-1 synthesis is the induction of CRP2 and CRP3. Indeed, both IL-1 and IL-6 have been observed to induce the transcription of CRP2 and CRP3 in a number of cell lines (Akira et al 1990 and Kinoshita et al 1992). If CRP2 and CRP3 are being expressed at sufficient levels, then the roles of IL-1 and IL-6 may be minimal. IL-1 and IL-6 have been observed to have post-transcriptional effects on CRP2 and CRP3 activity as well (Akira et al 1990, Poli et al 1990, and Ramji et al 1993), but this varies between different cell lines.

It is clear that the LPS induction of IL-6 and MCP-1 in our system operates through either the post transcriptional alteration of CRP2 and CRP3 or the induction of a necessary cooperating transcription factor. While we did not observe any alteration in the species observed upon EMSA before and after LPS treatment (data not shown), it has recently been shown that 12-O-tetradecanoyl-phorbol-13-acetate (TPA) increases the site-specific phosphorylation of CRP2 and enhances its activity (Trautwein et al 1993). Many of the activities of LPS are mimicked by phorbol esters (Ray et al 1988). In addition, LPS can induce NFκB activity (Sen and

Baltimore 1986). NFκB can not only functionally associate with CRPs (Descombes and Schibler 1991), but the IL-6 promoter contains both an NFκB and a CRP binding site (Isshiki et al 1990 and Libermann and Baltimore 1990).

The abilities of CRP2 and CRP3 to confer LPS-inducible expression of the inflammatory cytokines, IL-6 and MCP-1, to a B lymphoblast cell line has some parallels to the ability of NF-M (avian CRP2) and c-myb to activate myeloid genes such as mim-1 and lysozyme in heterologous cell types such as erythroid and fibroblastic cells (Ness et al 1993 and Burk et al 1993). CRPs may have a role in determining the differentiated function and the differentiation itself of myelomonocytic cells. The examination of myeloid-specific gene expression in the CRP2 and CRP3-transfected B lymphoblasts of this study will further illuminate this issue.

Chapter 4

CRP2 and CRP3: Implications for differentiation and discussion

Abstract

P388 B lymphoblasts do not express CRP2 or CRP3. Their lineage switched (lymphoid to myeloid) derivative P388D1(IL-1), a macrophage cell line, expresses both CRP2 and CRP3 in abundance. I demonstrate here that ectopic expression of CRP2 and/or CRP3 in P388 B lymphoblasts induces morphological and cytochemical changes characteristic of myeloid cells. Implications for the lineage switch model of myelomonocytic differentiation will be discussed.

Introduction

C/EBP-related proteins (CRPs) are a family of basic region-leucine zipper (bZIP) transcription factors (Williams et al 1991). Two members of this family are CRP2 and CRP3. Two lines of evidence suggest that CRPs may be involved in differentiation of myeloid cells. First, among the hematopoietic lineages, abundant expression of CRP2 and CRP3 is exclusive to mature macrophages and granulocytes (Scott et al 1992 and Natsuka et al 1992). Second, ectopic expression of CRPs in heterologous cells can lead to the atypical expression of genes often associated with the myeloid lineage such as IL-6 and MCP-1 in lymphoid cells (Chapter 3 of this

thesis), and mim-1 and lysozyme in chicken erythroid cells and fibroblasts (Ness et al 1993).

Lymphoid-to-myeloid lineage switched cell lines have been shown to be capable of normal macrophage function (Bretz et al 1992). 50% of B-cell precursors of day-12 mouse fetal liver are bipotential for both myeloid and lymphoid differentiation (Cumano et al 1992). These data suggest that lineage switch may reflect normal differentiative processes and the study of factors regulated during lineage switch may shed light on the regulation of myelomonocytic differentiation. Here, I show that the ectopic expression of CRP2 and/or CRP3 in a non-myeloid cell type can confer morphological and cytochemical changes characteristic of myeloid cells.

Experimental procedures

Cell lines and cell culture. P388 (ATCC #CCL 46), P388D1(IL-1) (ATCC #TIB 63), T4.3 (Bretz et al 1992), T4.5 (Bretz et al 1992), DO-11.10/54.4 (Marrack et al 1989), 70Z (ATCC # TIB 58), IC21 (ATCC # TIB 156), and M1 (ATCC # TIB 152) were cultured in RPMI 1640 media supplemented with 10% fetal calf serum (FCS) and 50μM 2-mercaptoethanol (2-ME). WEHI 274.1 (ATCC # CRL 1679), and P815 (ATCC # TIB 64) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS. 32DCl.3 and 32DCl.3GR (both gifts from J. Greenberger) were cultured in RPMI 1640, 10% FCS, 2-ME with 10% WEHI 3 conditioned medium and Iscove's media supplemented with 20% FCS and 10% WEHI 3 conditioned media,

respectively. BB88 (ATCC # TIB 55) was cultured in RPMI 1640 with 10% heat inactivated FCS and 2-ME. CTLL2 (ATCC # TIB 214) was cultured in RPMI 1640, 10% FCS, 2-ME and 7 units/ml recombinant IL-2. 14.8 (ATCC # TIB 164) was cultured in DMEM, 10% FCS and 2-ME. R2 was cultured using the Whitlock-Witte long term B lymphocyte culture method as described in chapter 2. Transfected cell lines were generated as described in chapter 3 of this thesis.

RNA isolation and northern analysis. RNA was isolated as previously described (Schwartz et al 1981) or with Trizol (Gibco/BRL) using procedures recommended by the manufacturer. RNA was size fractionated by formaldehyde agarose gel electrophoresis and blotted by standard methods (Sambrook et al 1989). Nucleic acid hybridization probes for CRP2, CRP3, and GAPDH were as previously described in chapter 3 of this thesis. The C/EBP probe was a 0.4 kb. NcoI/EcoRI genomic fragment.

Immunoprecipitations and protein analysis. Immunoprecipitations and analysis of proteins were carried out as described previously in chapter 3 of this thesis.

Cell staining. Cells were stained for morphology with Wright-Giemsa stain (Sigma, catalog no. WG-16) as described by the manufacturer. Leukocyte alkaline phosphatase cytochemical staining was performed with a kit from Sigma using procedures recommended by the manufacturer (procedure number 86). Hematoxylin was used as a counterstain.

Results

Survey of hematopoietic cell lines for CRP expression. Various cell lines spanning the major hematopoietic lineages and several stages of myelomonocytic differentiation were examined by northern analysis to determine whether the expression of CRPs was lineage specific (Figure 1A). Abundant mRNA expression of CRP2 was found exclusively in myelomonocytic cell types near terminal stages of differentiation. The monocyte/macrophage cell lines IC21, WEHI274.1, and P388D1(IL-1) showed the most abundant expression along with M1 myeloblasts that had been stimulated with LPS. LPS induces M1 cells to express many MyD genes (immediate early genes of induced terminal macrophage differentiation-see chapter 1), but the cells fail to fully differentiate (Lord et al 1990). 32DC13GR, a committed neutrophilic precursor, was also a strong expresser of CRP2. Low levels of CRP2 mRNA were detected in M1 (unstimulated myeloblast), 32DCl.3 (a multipotential myeloid precursor), P815 (mastocytoma), BB88 (an erythroid precursor cell line that can be induced to differentiate with DMSO into hemoglobin producing cells), and 14.8 (a B cell hybridoma). CRP2 was undetectable in 70Z (pre B cell), CTLL2 (an IL-2 dependant cytotoxic T cell), and DO-11.10/54.4 (a T-cell hybridoma). CRP3 mRNA expression essentially paralleled CRP2 expression except in 14.8 where it was undetectable. C/EBP mRNA was expressed abundantly in the WEHI 274.1, M1, 32DCl.3, and 32DCl.3GR myeloid cell lines but at low (M1 stimulated with

Figure 1: (A) Survey of hematopoietic lineages for CRP expression. Northern analysis of 20 μg of cytoplasmic RNA from various cell lines (as listed in figure) for expression of CRP2, CRP3, and C/EBP. (B) Northern analysis of R2 and T4.3 for CRP2 and CRP3 expression. GAPDH was used as an RNA loading control for both A and B. (C) Immunoprecipitation of 35-S metabolically labeled protein extracts from NIH3T3, R2, RT5.F41 (T4.5), P388, AND P388D1(IL-1). Anti sera specific for CRP2 was used. Lanes were equalized for protein loading by TCA precipitable counts. (D) Immunoprecipitation of metabolically labeled protein extracts from T4.3. NRS is the normal rabbit serum negative control, CRP2N (specific for amino-terminus), pan CRP (specific for CRP DNA binding domain), CRP2C (specific for carboxy-terminus), CRP3C (specific for carboxy-terminus), and CRP3N (specific for amino-terminus).

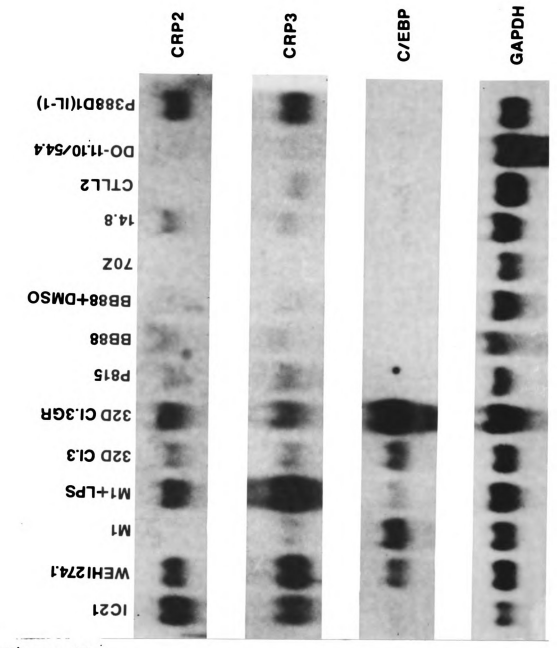


Figure 1A

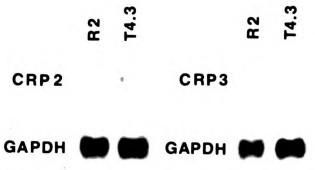


Figure 1B

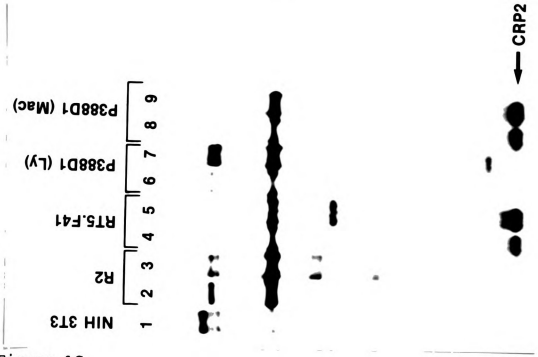


Figure 1C

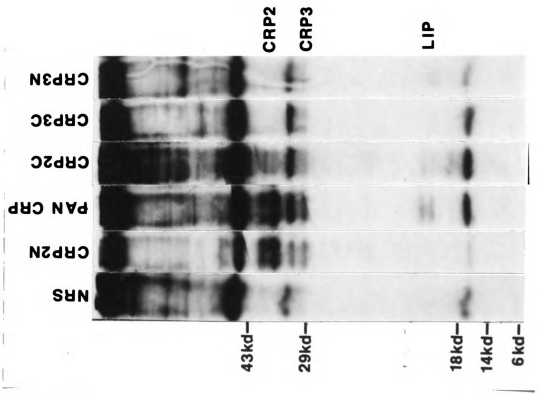


Figure 1D

LPS) or undetectable levels in the other cell lines, including terminally differentiated macrophages IC21 and P388D1(IL-1). The P388 B lymphoblast (precursor to P388D1(IL-1)) did not express CRP2 or CRP3 mRNA (see chapter 3). In the lineage switch pair described in chapter 2, the lymphoid R2 (pre B lymphocyte) cell line did not express either CRP2 or CRP3, but the myeloid T4.3 (macrophage) cell line expressed abundant CRP2 and CRP3 mRNA (Figure 1B). C/EBP was not detectable in either lineage switch pair (data not shown).

In general CRP2 and CRP3 were abundantly expressed only in myelomonocytic cell lines of an advanced stage of differentiation. Less differentiated myelomonocytic cell lines expressed CRP2 and CRP3 at a low level. C/EBP was expressed only in cell lines of myelomonocytic lineage that had not reached a terminally differentiated state. It is evident that CRP2 and CRP3 are upregulated during lineage switch.

Immunoprecipitations were performed to show that the CRP2 protein was also expressed. In the two lymphoid/myeloid lineage switch pairs, R2/T4 and P388/P388D1(IL-1), neither lymphoid cell line expressed CRP2, while the myeloid derivative of each expressed a protein of the predicted molecular wieght that was precipitated by antisera specific for CRP2 (Figure 1C). CRP3 protein proved to be more elusive due, perhaps, to its low abundance and possible conditional expression coupled with a low titer antibody. CRP3 was indirectly detected in macrophage extracts due to its ability

to dimerize and coprecipitate with CRP2 (see appendix Figure 1). CRP3 protein expression appears to coincide with CRP2 expression in lineage switch pairs.

Morphological and cytochemical analysis of P388 B lymphoblasts ectopically expressing CRP2 and/or CRP3. P388 cells were transfected with CRP2 and CRP3 genes expressed from a retroviral LTR in order to test whether these genes could induce myeloid characteristics in a lymphoid cell line previously shown to be capable of a lineage switch. Generation of these cell lines was described in chapter 3 of this thesis. The morphologies of these transfected cell lines was observed by Wright-Giemsa staining. The control cell line P388-Neo, transfected with the vector only, retained the characteristics of a pre B lymphocyte (relatively small size, single round nucleus, round, smooth shaped cell) (Figure 2). Suprisingly, the CRP transfectants acquired the morphological characteristics of neutrophilic granulocytes in many of the cells. These characteristics are multi-lobed nuclei (sometimes appearing as more than one nucleus), an irregularly shaped cytoplasmic membrane and a relatively large cell size compared to the pre B lymphocyte from which they are derived.

A leukocyte alkaline phosphatase (LAP) assay was performed to determine if these cells acquired cytochemical characteristics of granulocytes. This assay is specific for neutrophils and a positive result is observed as blue-black granules in the cytoplasm of stained cells. The double transfectant expressing CRP2 and CRP3 shows positive staining

Figure 2: Morphological analysis by Wright-Giemsa staining of CRP transfectants (400X magnification).

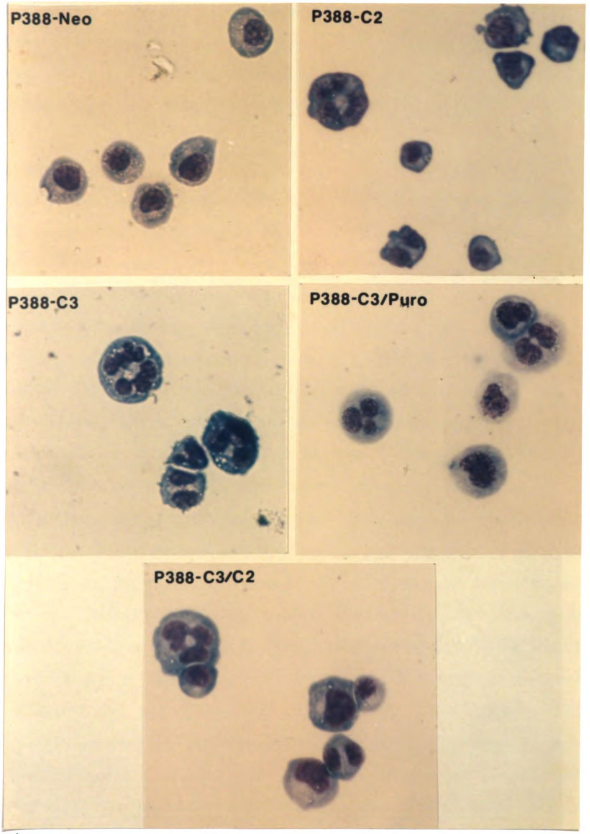


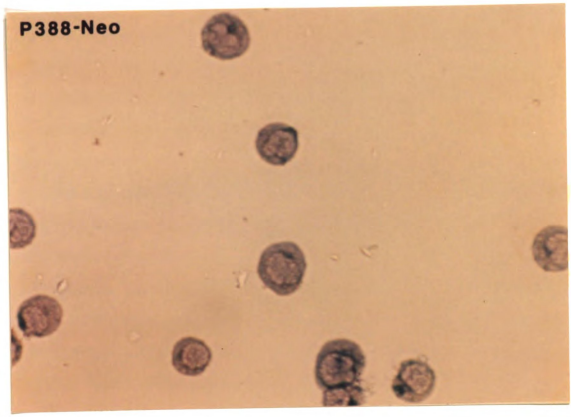
Figure 2

for LAP (Figure 3). It is important to note the location of the staining. Granulocytes often stain in areas surrounded by the nucleus or within an indentation of the nucleus while lymphocytes may show weak staining in the cytoplasm.

Discussion

The P388 B lymphoblast cell line was chosen for these transfection experiments for a number of reasons. Its previously mentioned capacity to switch lineages was important. The R2 cell line developed in our lab was also capable of a lineage switch (see chapter 2). The phenomena of differential expression of CRP2 and CRP3 during a lineage switch was first shown in the R2/T4 lineage switch pair. R2's dependence on a feeder layer of stromal cells and its possession of a drug resistance marker (neo) made it difficult to use for multiple transfections. Attempts to transfect R2 with CRP2 using a selection other than neo resistance were unsuccessful. M1 and 32DC13, cell lines capable of induced myeloid differentiation, also proved difficult to transfect by several different methods. This suggested to us that CRP2 expression was deleterious when ectopically expressed. CRP2 lethality was further confirmed by the low co-transfection frequency of the fibroblast NIH3T3 cell line with a CRP2 expression vector and neo resistance on a seperate vector. Fewer resistant clones were obtained in the cotransfection than with the neo resistant vector alone. CRP3 transfections were more efficient than CRP2. But our desire to transfect

Figure 3: Leukocyte alkaline phosphatase staining of P388-Neo and P388C3/C2 (400X magnification).



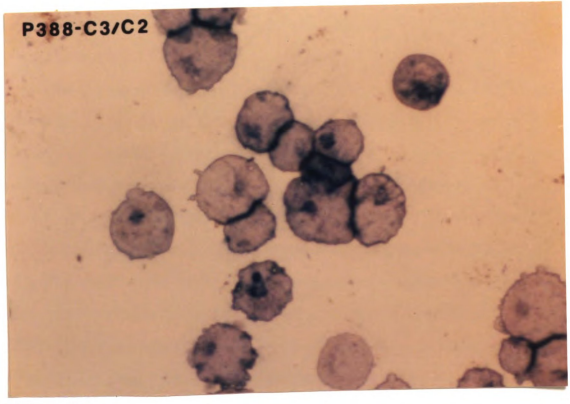


Figure 3

both CRP2 and CRP3 narrowed our choice of a recipient to P388. P388 did not require special handling nor did it possess any drug resistance markers.

The above mentioned CRP2 lethality may be due to the abnormally high expression from the retroviral promoter or from expression in a genetic background incapable of tolerating CRP2 protein. Alternatively, the expression of CRP2 may induce immediate terminal differentiation which leads to a loss of immortalizing elements that allow the cell line to be cultured indefinitely. This problem would be especially acute for granulocytic differentiation due to the short lifespan of normal terminally differentiated neutrophils (~3 days). This cell death by terminal differentiation would be undetectable in the stable transfections that we performed, but might be observed by expressing CRP2 from an inducible promoter.

It is apparent that expression of CRP2 and CRP3 have a dramatic effect on the morphology of P388 B lymphoblasts. Several lines of evidence suggested that a macrophage morphology would be the expected result of expression of these genes in this particular background. The lymphoid P388 cell line was chosen due to its previously described lineage switch capability to a macrophage morphology with expression of markers such as F_c receptor, Mac 1, Mac 2 and Mac 3, and the ability for phagocytosis (Bauer et al 1986). Also, in chapter 3 of this thesis we showed that CRP2 and CRP3 transfectants of P388 expressed IL-6 and MCP-1 in response to LPS stimulation.

LPS induction of cytokines IL-6 and MCP-1 is a common function in macrophages but not granulocytes. It was entirely unexpected that the CRP transfectants of P388 would acquire a morphology and LAP staining more similar to that of neutrophilic granulocytes.

Elements of exclusivity in lineage commitment. The monocyte/macrophage and neutrophilic granulocyte lineages are closely related and derive from the same myeloid precursor which is incapable of lymphoid differentiation (see chapter 1, figure 1). It is possible that CRP2 and CRP3 are involved in differentiation of both lineages (refer to Scott et al 1992). CRP2 and CRP3 may have different functions depending on which lineage background they are expressed within. The P388 B lymphoblast background may be deficient of elements required for exclusive commitment to either of the two lineages. This may account for the acquisition of both macrophage and granulocyte characteristics by P388. Candidates for such an element might be expression of the id (Benezra et al 1990) or evi-1 (Moroshita et al 1992) genes which can inhibit GCSF induced granulocytic differentiation of 32DC13 cells, or the egr-1 gene (Nguyen et al 1993) which inhibits HL-60 differentiation along the granulocyte but not the macrophage lineage. Both evi-1 and egr-1 are transcription factors and id inhibits certain bHLH transcription factors. The status of the expression of these genes in the P388 transfectants is unknown.

Evidence for incomplete differentiation. A number of

experiments were performed on the P388 transfectants to further substantiate their putative lineage switch to a myelomonocytic cell type and to determine at what point along that lineage they had proceeded. These included cytochemical assays for both specific and non-specific esterases, and myeloperoxidase (data not shown). As a functional assay, the ability to phagocytose latex beads was tested (data not shown). Molecular markers such as GCSF receptor, lactoferrin, and myeloperoxidase were assayed by northern analysis (data not shown). All of the above experiments were negative in showing myeloid markers for the P388 transfectants. Many markers during differentiation, such as lactoferrin and MPO, are expressed only transiently and this may account for the inability to detect these markers. Also, the P388 transfectants may still be relatively immature, not having yet acquired these markers through terminal differentiation. However, the polymorphonuclear appearance and leukocyte alkaline phosphatase expression are both late markers occurring at or near terminal granulocytic differentiation. This data suggests that P388 differentiation in response to CRP2 and CRP3 expression may be partial or incomplete.

While probing northern blots for transcripts of myeloperoxidase, GCSF receptor and lactoferrin, we noticed that although a discrete band could not be seen for P388-C2, P388-C3, or P388C3/C2, a smear was evident. This only occurred with genes specific for the granulocytic lineage (data not shown). Other probes used on the same blot showed

discrete bands demonstrating the intact quality of the RNA. This may have been due to the possible accumulation of degradative enzymes in terminally differentiated granulocytes of the population. These cells might degrade mRNA locally (within one cell) during RNA isolation. This may indicate the occurence of differentiating granulocytes among the CRP expressing lymphoblasts. These cells may be the ones that are more granulocytic in appearance and stain more intensely with LAP.

Positive elements of myelomonocytic differentiation. In addition to the possible lack of lineage suppressive regulatory elements mentioned above, the P388 B lymphoblasts may also lack certain positive elements required for full myelomonocytic differentiation. The expression of CRP2 and/or CRP3 in P388 B lymphoblasts in combination other factors may promote complete myelomonocytic differentiation in these cells. The genes mentioned in the following discussion may be involved in terminal myelomonocytic differentiation. Expressing them in our P388 B lymphoblasts may provide insight into the mechanisms of myelomonocytic differentiation.

The transcription factors c-fos, c-jun, jun B, and jun D are all upregulated during M1 myeloblast differentiation (Abdollahi et al 1991). C-fos is induced during normal and leukemic myelomonocytic differentiation (Gonda and Metcalf 1984, Liebermann and Hoffman-Liebermann 1989). Forced expression of c-jun and c-fos enhanced inducible differentiation of M1 myeloblasts. C-fos is slightly

upregulated in P388 lymphblasts expressing CRP2 (data not shown); the expression status of c-jun is unknown.

The tyrosine kinase genes c-fgr and hck are expressed predominantly in myelomonocytic cells and are associated with early commitment and differentiation (Ziegler et al 1991, Willman et al 1991). Expression of hck was undetectable by northern analysis in P388 lymphoblasts expressing CRP2 and/or CRP3. The status of c-fgr expression in these cells is unknown.

V-raf expression has been shown to be involved in lymphoid to myeloid lineage switch (Klinken et al 1988). The expression of v-Ha-ras can activate c-raf. This may explain the involvement of v-Ha-ras expression in our lineage switch model (see chapter 2). Perhaps activation of c-raf may be required for myelomonocytic differentiation. The status of c-raf and c-ras activation is unknown in P388 lymphoblasts expressing CRP2 and/or CRP3.

Of course, temporal gene regulation may be important during myelomonocytic differentiation. Any of the above mentioned genes that influence differentiation may only need to be expressed transiently to contribute to differentiation. Two genes are known to be expressed early in myelomonocytic differentiation, but are not expressed at terminal differentiation. C/EBP (this chapter, Scott et al 1992) and c-myb (Gonda and Metcalf 1984) are such genes. C/EBP is not expressed in P388 lymphoblasts (Figure 1). C-myb expression is detectable in P388 lymphoblasts and is not influenced by

ectopic CRP expression.

Elements that block myelomonocytic differentiation. Alternatively, the P388 lymphoblasts may express genes that block differentiation. CRP2 and/or CRP3 may only be able to overcome part of this block. Removal of these blocking elements may lead to full differentiation. Candidates for such blocking elements are the c-myb and c-myc protooncogenes. Suppression of these transcription factors has been shown to be linked to terminal differentiaton of M1 myeloblasts (Hoffman-Liebermann and Liebermann 1991a). C-myb and c-myc inhibit induced differentiation when ectopically expressed in M1 myeloblasts (Hoffman-Liebermann and Liebermann 1991b, Selvakumaran et al 1992). C-myb mRNA expression remains unchanged while c-myc mRNA expression is slightly reduced in P388 lymphoblasts when CRP2 and/or CRP3 is expressed (data not shown). Inhibiting the expression of these genes with antisense constructs may possibly remove the block on complete differentiation. The SCL transcription factor (expressed in M1 myeloblasts, but down-regulated during differentiation) and the Hox-2.4 gene (not expressed in M1 myeloblasts) can both inhibit differentiation of M1 when ectopically expressed (Tanigawa et al 1993, Blatt et al 1992). The status of these genes is unknown in P388 B lymphoblasts. SCL and Hox-2.4 could possibly be involved in blocking differentiation in P388 B lymphoblasts.

Combinatorial gene regulation in myelomonocytic cells by CRPs in cooperation with other transcription factors. Until

this point in the discussion, I have referred to CRP induced myelomonocytic differentiation in a general way. But as transcription factors, the CRPs act to promote transcription of specific genes. In chapter 3 we demonstrated CRP involvement in expression of genes normally associated with myelomonocytic cell types. Cooperative interactions with other transcription factors may provide an additional level of transcriptional control, including lineage specificity and temporal specificity, of gene expression during differentiation. Direct protein:protein interactions of CRPs with other transcription factors have been demonstrated with the NFκB family (Stein et al 1993), glucocorticoid receptor (a transcription factor) (Nishio et al 1993), as well as Sp1 (P. Johnson personal communication).

The Rel homology domain, common to NFκB p65, p50, and C-rel, physically interacts with C/EBP, CRP2, and CRP3. These transcription factors also synergistically stimulate promoters with C/EBP binding sites, while inhibiting promoters with κB binding sites. CRP and NFκB binding sites have been found closely juxtaposed in several promoters including those of the IL-6, IL-8, angiotensinogen, and GCSF (Himes et al 1993) genes and expression of CRP2 and NFκB synergizes to activate transcription of the IL-6 and IL-8 genes (Stein et al 1993).

The glucocorticoid response element (GRE) and the CRP binding site are found adjacent in the promoter of the α1-acid-glycoprotein gene. Physical interaction of the glucocorticoid receptor with CRP2 has been shown to be

responsible for transcriptional synergy of these two factors (Nishio et al 1993).

Several studies focusing on the chicken homologue of CRP2 (NF-M) (Katz et al 1993) have shown it to cooperate with other factors involved in transcription of myelomonocytic lineage specific genes. Chicken myelomonocytic growth factor (related to mammalian GCSF and IL-6) promoter activity requires both NF-M and AP-1 binding to adjacent regions (Sterneck et al 1992). The myeloid specific mim-1 gene promoter contains binding sites for both c-myb and NF-M that are required for full activity (Burk et al 1993). Expression of NF-M and c-myb in heterologous cell types was able to confer mim-1 expression (Ness et al 1993). This also induced expression of the lysozyme gene that does not contain a known c-myb binding site but does have a NF-M site. This implies that this combination of transcription factors may also induce a more generalized switch to the myelomonocytic differentiation pathway than either factor alone. These findings suggest our P388 B lymphoblasts expressing CRPs may be used to find combinations of factors involved in myelomonocytic-specific gene expression. Both specific gene regulation and general differentiation may be studied.

Conclusions. The expression of CRP2 and/or CRP3 in P388 B lymphoblasts is 1) capable of inducing incomplete myelomonocytic differentiation, 2) capable of confering LPS induciblity to several cytokines normally expressed by cells in the monocytic but not lymphoid lineage, and 3) may provide

a useful system to study the genetic mechanisms of myelomonocytic differentiation.

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