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**REGULATION OF CD44 AND ITS ADHESIVE INTERACTIONS WITH THE
EXTRACELLULAR MATRIX COMPONENT, HYALURONAN, BY
CYTOKINES IN NORMAL AND TRANSFORMED HUMAN B LYMPHOCYTES**

by

Marko Andrii Kryworuchko

A thesis submitted to the Faculty of Graduate Studies
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy.

Department of Biochemistry, Microbiology and Immunology
Faculty of Medicine
University of Ottawa

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ABSTRACT

The interaction of CD44 with its ligand hyaluronan may play a vital role in many biological processes including leukocyte homing, activation and effector function, hematopoiesis as well as tumor formation and metastasis. This phenomenon may be influenced by the extracellular matrix molecules and cytokines present in the microenvironment. In this study, I investigated the regulation of CD44-HA interactions in normal human B cells and in a panel of B cell lines including: Epstein-Barr virus (EBV)-positive (⁺) and EBV-negative (⁻) Burkitt's lymphoma (BL) and lymphoblastoid B cell lines (B-LCL) generated by *in vitro* EBV-transformation of normal human B cells.

Activation of purified B cells with bacterial lipopolysaccharide (LPS), pokeweed mitogen (PWM) or anti-IgM antibodies in the presence or absence of interleukin (IL)-2 or IL-4 failed to induce HA adhesiveness. Stimulation of B cells with PMA however, induced strong HA recognition. Amongst a variety of cytokines that influence B cell activation, proliferation and differentiation, only IFN- γ and to some extent IL-4 inhibited PMA-induced CD44-HA interactions. IL-13, which shares components of the IL-4 receptor complex and exhibits many biological effects similar to that of IL-4, failed to inhibit HA recognition. Investigation of the potential molecular mechanism involved revealed that PMA-induced HA adhesion correlated with enhanced expression of CD44 H and V6-containing isoforms, as determined by flow cytometry, and the induction of numerous variable exon-containing isoforms (CD44 V), as determined by reverse transcriptase based polymerase chain reaction analysis (RT-PCR). The inhibition of PMA-induced adhesion by IFN- γ and IL-4 correlated with the downregulation of CD44 H expression and altered usage of exons V4 and V5. Changes in the electrophoretic mobility of the CD44 H protein in Western blot analysis was used as a measure of post-translational modifications. In response to PMA, a slight reduction in CD44 molecular weight, was observed when compared to unstimulated cells. However, changes were not detected upon addition of IFN- γ or IL-4. Nevertheless, this does not exclude more subtle post-translational changes to CD44 in response to these cytokines, undetectable by this method. These results suggest that most B cell mitogens, with the exception of PMA, are unable to induce HA recognition in B cells. Furthermore, PMA-induced adhesion of B cells can be inhibited by IFN- γ and partially by IL-4, implying that these cytokines may influence B cell migration through their ability to downregulate CD44-HA interactions. Control of these interactions may, in part, occur via a mechanism involving differential isoform expression and post-translational modifications (glycosylation) of CD44.

EBV infection/transformation of B cells, an alternative method of B cell activation, enhanced the expression of CD44 H, and induced a number CD44 V isoforms but abrogated their ability to bind HA in response to PMA. Stimulation with PMA induced strong HA recognition in the EBV-positive BL cell line BL-30/B95-8, whereas LPS, *Staphylococcus aureus* Cowan strain I (SAC) or PWM failed to induce HA adhesion. Investigation of the effect of a similar panel of cytokines revealed that in contrast to its inhibitory effect in normal human B cells, IL-4 was capable of inducing HA recognition in BL30/B95-8 cells. IL-13, again, failed to influence HA recognition. In contrast, the ability to recognize HA following PMA or IL-4 stimulation was not observed in B-LCL cells, highlighting the stark contrast between BL30/B95-8 and B-LCL cells. The increased HA adhesion in BL-30/B95-8 cells correlated with an

enhanced expression of CD44 H, isoforms containing V3, V6 and V9, and the increased electrophoretic mobility of the CD44 H protein. Alterations in the splicing pattern of the V4 and V5 exons were also observed in response to IL-4 and IL-5. Furthermore, treatment with tunicamycin, an inhibitor of N-linked glycosylation, was able to enhance HA recognition in BL-30/B95-8 cells. In contrast, the molecular pathways that regulate CD44 expression and CD44-mediated HA binding are inactivated at some level in B-LCL cells. These results also revealed that similar control measures to those found in normal B cells are operative in BL-30/B95-8 cells.

To understand the effect of EBV on CD44 expression and HA recognition, we investigated a panel of EBV⁺ and EBV⁻ B cell lines. EBV⁻ BL cells failed to express CD44 and hence did not adhere to HA. A remarkable heterogeneity was revealed by the study of various EBV⁺ B cell lines with respect to CD44 isoform expression and their ability to recognize HA. BL-30/B95-8, Jijoye, IM and all the B-LCLs tested expressed CD44 H and CD44 V isoforms; yet, Jijoye and B-LCLs failed to recognize HA in response to PMA. Raji, EB3 and BJAB/B95-8, although infected with EBV, neither expressed CD44 nor bound HA. To directly determine the role of EBV in CD44 isoform induction, an EBV-negative BL cell line, BL30 (negative for all CD44 isoforms), BL-30 cells infected *in vitro* with a wild type (BL-30/B95-8) and an Epstein-Barr Nuclear Antigen-2 (EBNA-2) defective mutant (BL-30/P3HR1) were examined. Infection with wild type EBV but not with the P3HR-1 strain induced the expression of CD44 H and CD44 V isoforms, associating this inductive effect with the EBNA-2 and/or Latent Membrane Protein-1 (LMP-1) genes of EBV. The variability observed between cell lines however, may be the result of genetic alterations (viral and/or chromosomal) or may reflect differences in their differentiation stage.

In conclusion, these results establish that HA recognition and CD44 isoform expression are modulated as a consequence of the mode of human B cell activation, differentiation and/or state of transformation. This modulation may reflect the changing requirements of the cell for this receptor family at different stages of its existence, for example, while propagating an immune response or in the progression of B cell malignancy.

DEDICATION

I dedicate this thesis to my beloved wife Jennifer, daughter Kalyna and son Maksym, who have been an endless source of love and support during the course of this long journey and have placed my ultimate purpose into perfect focus and perspective.

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LIST OF ABBREVIATIONS

[Ca ²⁺] _i	Intracellular calcium ions
A	Adenosine
aa	Amino acid
Ab	Antibody
anti-IgM Abs	Antibodies directed against surface immunoglobulin M
b-FGF	Basic-fibroblast growth factor
BL	Burkitt's lymphoma
B-LCL	B-lymphoblastoid cell lines; normal human B cells transformed <i>in vitro</i> with EBV
bp	Base pair
cAMP	Cyclic adenosine monophosphate
CD44 V	CD44 isoforms containing variable exons
cDNA	Complementary DNA
cGMP	Cyclic guanosine monophosphate
cpm	Counts per minute
CRG	Cytokine response gene
CS	Chondroitin sulfate
CTL	Cytotoxic T lymphocyte
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
dGTP	Deoxyguanosine triphosphate
DNFB	Dinitrofluorobenzene
DTAF	Dichloro triazinyl amino fluorescein
DTH	Delayed-type hypersensitivity reaction
dTTP	Deoxythymidine triphosphate
EBNA	Epstein-Barr nuclear antigen
EBV	Epstein-Barr virus
ECM	Extracellular matrix
GAG	Glycosaminoglycan
GM-CSF	Granulocyte macrophage – colony stimulating factor
GTP	Guanosine triphosphate
HA	Hyaluronan
HB-EGF	Heparin-binding epidermal growth factor
HEV	High endothelial venule
HS	Heparan sulfate
I.V.	Intravenous
ICAM	Intercellular adhesion molecule
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IL-2R	Interleukin-2 receptor
Kb	Kilobase
LECAM	Lymphocyte endothelial cell adhesion molecule
LFA	Lymphocyte function-associated antigen

LMP	Latent membrane protein
LPS	Lipopolysaccharide
LTBMC	Long term bone marrow culture
mAb	Monoclonal antibody
MAdCAM	Mucosal addressin cell adhesion molecule
MCP	Monocyte chemotactic protein
MHC	Major histocompatibility complex
MIP	Macrophage inflammatory protein
M-MLV	Murine-Moloney leukemia virus
mRNA	Messenger RNA
NHL	Non-Hodgkin's lymphoma
NK	Natural killer
nt	Nucleotides
PBMC	Peripheral blood mononuclear cells
PHA	Phytohemagglutinin
PK	Protein kinase
PMA	Phorbol myristate acetate
PP	Peyer's patches
PTK	Protein tyrosine kinase
PWM	Pokeweed mitogen
RANTES	Regulated on activation, normal T cell expressed and secreted
RT-PCR	Reverse transcription-polymerase chain reaction
S.C.	Sub-cutaneous
SAC	<i>Staphylococcus aureus</i> Cowan strain I
SCID	Severe combined immunodeficiency
SEB	Staphylococcal enterotoxin B
sIg	Surface immunoglobulin
T	Thymidine
TCR	T cell receptor
TGF	Transforming growth factor
T _h	T helper
TNBS	Trinitrobenzene sulfonic acid
TNF	Tumor necrosis factor
VCAM	Vascular cell adhesion molecule
VLA	Very late antigen
V β	Variable region of the T cell receptor beta chain

CHAPTER ONE

GENERAL INTRODUCTION

Cell-cell and cell-extracellular matrix (ECM) interactions play a critical role in many biological processes including the development of an immune response, inflammation, the formation of tumors and their metastasis. Factors capable of regulating these adhesive interactions can therefore exert a critical influence on the course of these processes leading to normal immune function or the development of disease. Amidst a plethora of adhesion molecules, which participate in these processes, is CD44. CD44, first described as a human brain-granulocyte-T lymphocyte antigen (1), existed under a number of designations including GP90^{HERMES}, extracellular matrix receptor III, homing cell adhesion molecule, phagocytic glycoprotein-1, glycoprotein 85, Ly-24, hyaluronate receptor, HUTCH-1, and In (Lu)-related p80 glycoprotein, based on its structural and functional features [reviewed in (2)]. A common species was revealed and the CD44 cluster of differentiation was assigned at the Third International Workshop on Leukocyte typing (3).

CD44 currently comprises a large family of transmembrane glycoproteins that exhibits extensive molecular heterogeneity and a broad tissue distribution. This heterogeneity in size is generated by alternative RNA splicing of variable exons as well as by post-translational modifications (4-8). The gene is transcribed in most mesenchymal and neuroectodermal cells, and defined epithelia (9). Importantly, it is expressed in all leukocytes and many tumor cells (9). The few tissues found to be consistently negative for CD44 expression include liver hepatocytes, kidney tubular epithelium, cardiac muscle, and portions of the skin and testis (10).

Regulation of the adhesive functions of CD44 has been implicated in altering the course of lymphocyte homing, lymphopoiesis, T cell activation and metastasis (2,9-16).

Therefore, the identification of the factors involved in regulating CD44 function and the mechanism by which this control is exerted are critical to our understanding of such important biological phenomena as cell trafficking, tumorigenesis and metastasis.

1.100 Biochemical structure of CD44

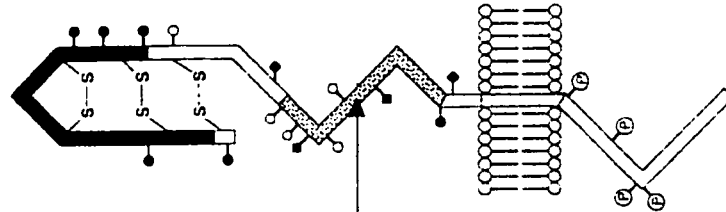
1.110 Genomic Organization

The CD44 gene is located on the short arm of chromosome 11 in humans (17) and chromosome 2 in mouse (18). It spans approximately 50 Kb of human DNA and genomic analysis has revealed a total of 20 exons, 12 of which participate in alternative splicing (4,19,20) (Fig. 1-1B). Exons 1-5 encode part of the constant region of the extracellular domain and are invariably transcribed (constant exons). Exons 6-15 (variable exons V1-V10) comprise the variable region of the extracellular domain as they can participate in alternative splicing. These exons are included in different combinations into nascent mRNAs, which explains, at least in part, the extensive CD44 molecular heterogeneity. Exons 16 and 17 are constant and encode the membrane proximal region of the extracellular domain. Constant exon 18 encodes the hydrophobic transmembrane region as well as the first 3 amino acids (aa) of the cytoplasmic tail of CD44. Exon 19 and 20 are also subject to alternative splicing. Inclusion of exon 19 containing an A+T rich untranslated region is believed to confer instability on the mRNA and results in the generation of a short cytoplasmic tail of only 3 aa. Exon 20 contributes to the long (70 aa) cytoplasmic tail of CD44 and is found in greater abundance than is the short version (19,21).

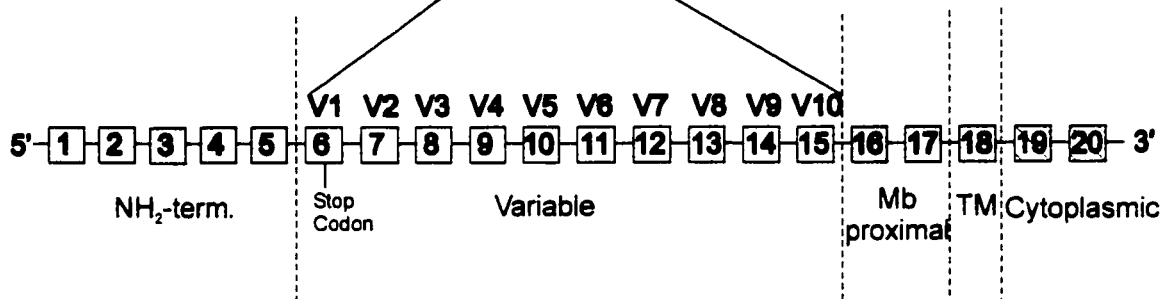
Fig. 1-1: Schematic representation of the human CD44 glycoprotein (A), its genomic organization (B), and examples of alternatively spliced CD44 variant mRNAs (C)

(A) Protein structure of the hematopoietic (H) or standard (s) CD44 isoform (reproduced in part from ref. (10)). The darkened region of the extracellular domain (aa 12-101) shows homology to the hyaluronan binding domains of cartilage link and proteoglycan core proteins (22,23). The heavily stippled area represents the membrane-proximal region. The lightly stippled area represents the transmembrane domain. ●—, potential N-linked glycosylation sites; ○—, potential O-linked glycosylation sites; ◆— potential site for addition of chondroitin sulfate; ⊕—, serine residues and potential sites for phosphorylation; S—S, probable, S---S, possible, disulfide bonds between cysteine amino acid residues; ➔, site for insertion of alternatively spliced variable exons (between amino acids 202-203) (B) Genomic organization of the CD44 gene. Shaded boxes represent constant region exons (exons 1-5, 16-18). Unfilled boxes depict extracellular domain variable exons (exons 6-15 or V1-V10). Crosshatched boxes refer to alternatively spliced cytoplasmic domain exons. The human V1 sequence contains a stop codon and therefore is not expressed. The extracellular domain exons are stratified into the NH₂-term., Amino-terminal; Variable, variable; Mb proximal, membrane-proximal regions. TM, transmembrane region exons, Cytoplasmic, cytoplasmic region exons (C) Examples of alternatively spliced CD44 transcripts. The CD44 H isoform has been predominantly found with a long cytoplasmic tail but a short-tailed isoform has been detected (19,21). The epithelial CD44 E variant is expressed on epithelial cells (6,23). CD44 V4-V7 or the metastatic variant confers metastatic behaviour to non-metastatic pancreatic tumor cells and does not contain exon 16 (5,24). CD44 V3-V10 in keratinocytes is one of the largest isoforms known (25).

A) Protein Structure

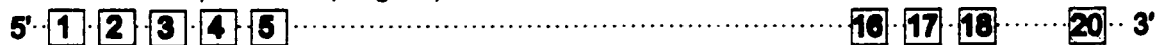


B) Genomic Organization

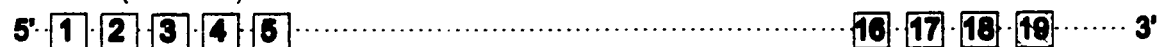


C) CD44 Isoforms

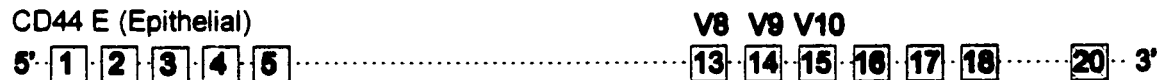
CD44 "Hematopoietic" H (long tail)



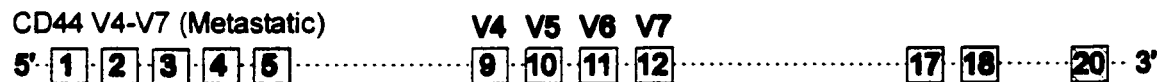
CD44 H (short tail)



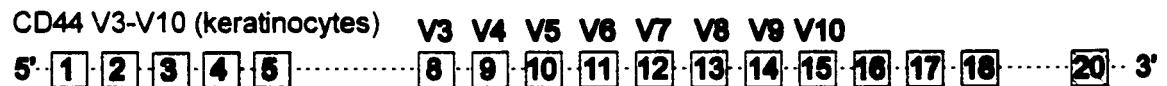
CD44 E (Epithelial)



CD44 V4-V7 (Metastatic)



CD44 V3-V10 (keratinocytes)



1.120 General protein structure

The CD44 protein contains several structural domains including the extracellular, transmembrane and cytoplasmic domains, each with their unique characteristics (2,10). The extracellular domain can be further subdivided into the amino-terminal, variable, and membrane proximal regions. The amino-terminal region contains a hydrophobic stretch of residues (aa 12-101), which is well conserved between species (80-90% sequence similarity). This stretch exhibits a 30% homology with the cartilage link and proteoglycan core proteins, which are known to make up extracellular matrices in association with the disaccharide polymer hyaluronan (HA). This homology provided the first clue that CD44 was a receptor for HA, a topic further discussed in section 1.200. There are also 6 conserved cys residues within the amino-terminal region, which are thought to function in the generation of a globular structure via disulfide bridging (see Fig. 1-1A). The membrane proximal region is less well conserved, exhibiting only 35-45% sequence similarity between species. A total of 6 N-linked, 7 O-linked glycosylation sites, and 4 ser/gly motifs (ser-gly-X-gly, where X is any aa) capable of being chondroitin sulfate (CS) or glycosaminoglycan (GAG) modified are found within the amino terminal and membrane proximal regions of the extracellular domain. The variable region consisting of different combinations of variable exons (V1-V10) is inserted between aa 202 and 203 of the extracellular domain. (Fig. 1-1A). Unlike in mouse, V1 is not expressed in humans as it contains a stop codon; exons V2-V10 contribute a total of 381 aa, showing a 64% interspecies homology (20). This region contains an additional 4 N-linked glycosylation sites and a large number of O-linked sites. Of particular note are exons V9 and V10, which contain 40% and 30% ser/thr

residues, respectively. There is also a ser/gly motif, which can be modified by GAG addition (20,26). V3 can be modified by the GAG heparan sulfate (HS) (27) while V6 can be modified by an H blood group sugar (28).

The transmembrane domain consists of 23 aa and exhibits 80-90% inter-species homology. It contains 2 cys residues at the interface between the transmembrane domain and the cytoplasmic domain, which can potentially be modified with palmitic acid (10).

The cytoplasmic domain is also highly conserved with 80-90% sequence similarity between species (2). It contains 6 potential phosphorylation sites out of which ser 303 and 305 are constitutively phosphorylated (2). Although there is little or no evidence to support their activity, consensus sites for protein kinase (PK) C, PKA, cAMP-, and cGMP-dependent kinase action have been found (2). The cytoplasmic tail of CD44 interacts with components of the cytoskeleton such as actin, ankyrin, and members of the ezrin-radixin-moesin family (2). Similarity has also been noticed between the cytoplasmic tails of CD44 and members of the G-protein superfamily in terms of ability to bind GTP and exhibition of GTPase activity *in vitro* (10,29).

1.130 CD44 isoforms

Polymerase chain reaction (PCR) analysis, cDNA cloning and Western blotting analysis have revealed that CD44 is found in a multitude of isoforms, as mentioned. They are generated by an alternative RNA splicing mechanism whereby different combinations of variable exons are included into the extracellular domain of the protein. Post-translational modifications including N-linked, O-linked glycosylation and GAG modifications (heparan sulfate, keratan sulfate, and chondroitin sulfate) are also the

source of CD44 heterogeneity. At the level of cDNA, at least 20 different isoforms have been described (2). This is likely an underestimation since in theory there are 768 possible combinations predicted (30). Cells are capable of expressing numerous isoforms simultaneously and it has become clear that their expression is regulated in a tissue and cell differentiation stage dependent manner (2,10). A brief description of some of the major isoforms studied follows.[#]

The standard or hematopoietic (CD44 H) isoform is the smallest and most common CD44 isoform. It is preferentially expressed on all blood cells including, granulocytes, erythrocytes, T and B lymphocytes, natural killer cells, macrophages, alveolar macrophages, Kupffer cells, and interdigitating and follicular dendritic cells. It does not contain any variable exons, is synthesized as a 42 kDa polypeptide, which by N-linked and O-linked glycosylation of the extracellular domain assumes its mature size of 85 kDa. Chondroitin sulfate modification has been documented to increase its size to a 180-200 kDa isoform in human peripheral blood mononuclear cells (8). It can also be modified by heparan sulfate (31,32). Northern blot analysis reveals three major transcripts of 4.8, 2.2, and 1.6 kb (22,23), which are believed to arise by use of different polyadenylation signals (33,34).

CD44 E refers to the epithelial variant originally cloned from the colon carcinoma cell line HT29, which is weakly expressed in normal epithelium but is present in abundance in many carcinomas (23). Its expression is typically increased by neoplastic transformation (23). The main difference between this isoform and CD44 H is the

[#] The convention adopted throughout the thesis in referring to CD44 isoforms containing variable exons (CD44 V) is based on whether the identity of all the variable exons included is known. For example, an isoform containing the V6 exon exclusively is referred to as CD44 V6. Reference to an isoform containing V6 in combination with other, unspecified exons, would be CD44 V6^{*}.

insertion of exons V8-10 within the extracellular domain yielding a mature protein of 473 aa residues with a predicted molecular weight of 51.4 kDa (6). Immunoprecipitation revealed a 130 kDa protein (6). Northern blot analysis revealed slightly larger transcripts, in comparison to CD44 H, of 2.0, 2.6 and 5.4 kb (6). Two similar isoforms, CD44 R1 (V8-10) and CD44 R2 (V10), were cloned from the human myelomonocytic cell lines KG1a (7,35). Both CD44 R1 and R2 expression was detected in normal PBMCs, granulocytes, and certain leukemias (7,35).

CD44 V4-V7 and V6-7 isoforms are referred to as metastasis associated variants since their overexpression in nonmetastatic rat pancreatic carcinoma and mammary adenocarcinoma confers metastatic behaviour to these cells (5). These isoforms are generally not found in normal rat tissues and do not encode the constant exon 16 in their sequence [see Fig. 1-1C] (10). Interestingly, the increased expression of variants containing exon V6* has been associated with increased aggressiveness in non-Hodgkin's lymphoma (36). V6* expression is also found to be transiently upregulated upon mitogenic or antigenic activation of human lymphocytes where it is thought to play a role in normal immunological responses (36,37). Both topics are elaborated upon in sections 1.350 and 1.360.

Another isoform, which merits mention, is CD44 V3-10. This is the largest CD44 isoform and was detected in keratinocytes as a 230 kDa protein (25). Other CD44 variants cloned from epithelial cells include CD44 V3,8-10, CD44 V6-10 and CD44V7-10 (38).

1.200 Hyaluronan is the principal ligand for CD44

The major function of CD44 lies in its ability to mediate cell-cell and cell-ECM interactions, thereby influencing the migratory behaviour of cells, primarily those of hematopoietic origin. This function is afforded mainly by the interaction of CD44 with hyaluronan (HA), a major extracellular matrix component (39-41). It is well established that the principal cell surface receptor for HA is CD44. This was initially surmised due to the homology of aa 12-101 of the CD44 protein with the cartilage link and proteoglycan core proteins known to bind HA within the extracellular matrix (22,23). Direct evidence was provided by experiments demonstrating *in vitro* that certain anti-CD44 antibodies, an excess of soluble HA, or pre-treatment of immobilized HA with hyaluronidase could inhibit binding of cells to HA-coated tissue culture plates or to fluoresceinated HA (26,40-42). Further evidence was provided by the ability of a CD44-Ig fusion protein to bind lymph node high endothelial cells in an HA dependent manner (39). It was also demonstrated that purified CD44 from placenta bound immobilized HA (43).

Hyaluronan consists of a high molecular weight, negatively charged polysaccharide (10^6 - 10^7 Da) made up of linear repeating units of the disaccharide D-glucuronic acid [1- β -3] N-acetyl-D-glucosamine (2,44). It is a major structural component of most animal ECMs and is often found surrounding proliferating and migrating cells (44). It is present in connective tissues such as the skin dermis, smooth muscle, lung, lamina propria of mucous membranes, and the adventitia surrounding blood vessels (39). It is also found in lymph and the lymph node matrix (39,45). Within the ECM, HA interacts non-covalently with proteoglycan core proteins (e.g. aggrecan of cartilage), an interaction stabilized by a link protein, forming a supramolecular ternary

complex composed of more than 100 aggrecan and link proteins bound to a single filament of HA (44,45). In addition to providing cellular support, it regulates the adhesion, spatial orientation, trafficking, growth and differentiation of cells (45). Thus, it is not surprising to find HA implicated in processes such as inflammation, wound healing, tissue remodeling, morphogenesis, as well as tumor metastasis [reviewed in (44,45)].

The isoform mainly associated with the recognition of HA is CD44 H. Insertion of variable exons may modulate the ligand binding properties of CD44, although some controversy exists in this regard. Human CD44 E, CD44 V3-10, CD44 V3,8-10, CD44 V6-10, CD44 V7-10 fail to recognize HA efficiently (6,38,46,47). In contrast, murine CD44 E and other CD44 variants exhibited significant binding to HA (48). Moreover, human CD44R1 which differs from CD44 E by 3 aa outside of the variable domain was in fact able to bind HA (49). These discrepancies may in part depend on the cell type or the intracellular milieu where analogous isoforms are synthesized and processed, giving rise to an isoform with different glycosylation and/or glycanation patterns, and thus affecting its ability to recognize HA (2). In fact, differential glycosylation is one of the possible mechanisms, which regulates HA adhesion, among others such as: the interaction with elements of the cytoskeleton via the cytoplasmic domain, phosphorylation of the cytoplasmic domain, differential CD44 isoform expression, interaction with other ligands either extracellular or at the cell surface, and shedding of CD44 (10). How CD44-HA interactions are controlled is elaborated upon in section 1.400.

Evidence has also been provided for the ability of CD44 to interact with other ECM components including collagen (50-52), fibronectin (52,53), laminin (52), chondroitin sulfate (39). Other non-ECM ligands include mucosal vascular addressin

(54), the chondroitin sulfated proteoglycan serglycin (55,56), the chemotactic cytokine osteopontin (57) and the MHC class II invariant chain (Ii) (58). Although many of these ligands bind to CD44 with reduced efficiency when compared to HA (2), in totality, their diversity emphasizes the participation of CD44 in many functions other than matrix adhesion associated with cell trafficking such as lymphocyte activation, cytotoxicity, and others. The existence of yet unidentified ligands for CD44 is likely considering the polymorphic nature of CD44 and its key role in a number of biological processes, both normal and pathological.

1.300 Role of CD44 in normal immunophysiological and pathological processes

Extensive studies have been conducted to determine the physiological roles of CD44. It has been associated with normal immunological phenomena such as lymphocyte homing, activation, and effector function, hematopoiesis, growth factor presentation, and apoptosis [reviewed in (2,10)]. The involvement of CD44 in the malignant process has received tremendous scrutiny and its association with arthritis, allergy, and human immunodeficiency virus infection has also been suggested [reviewed in (2)]. The participation of CD44 in various immunological phenomena and carcinogenesis will be addressed in this section of the thesis.

1.310 Lymphocyte homing and cell motility

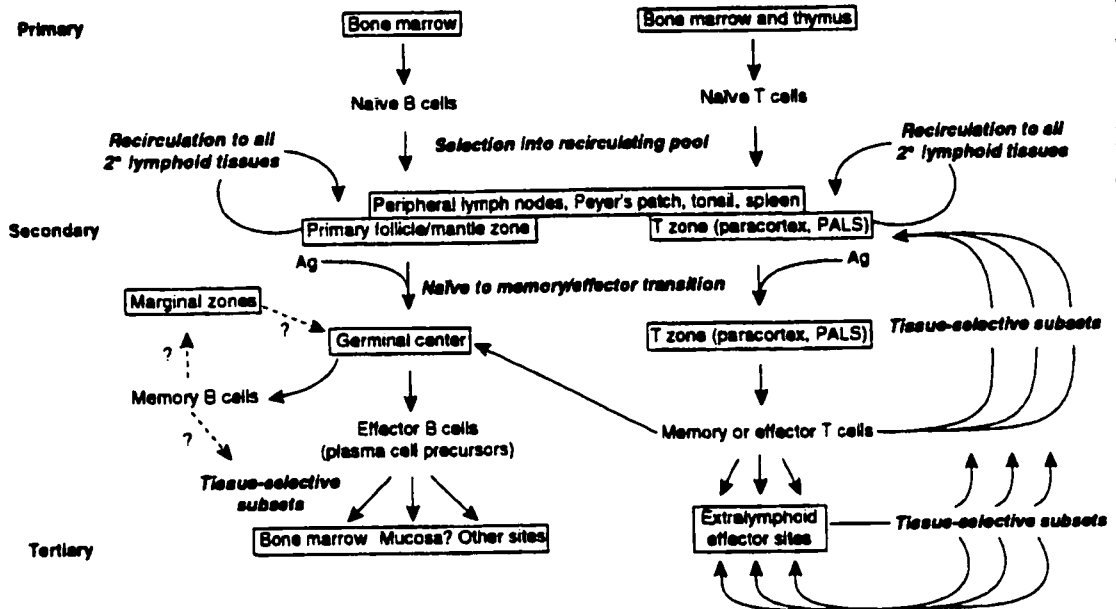
Lymphocyte homing is a specific, integrated, multistep process of regulating the traffic of lymphocytes, which is essential in the generation and control of the immune response [reviewed in (59); see Fig. 1-2]. It is intimately dependent upon a combinatorial

Fig. 1-2: Models of lymphocyte homing to lymphoid and extra-lymphoid tissues.

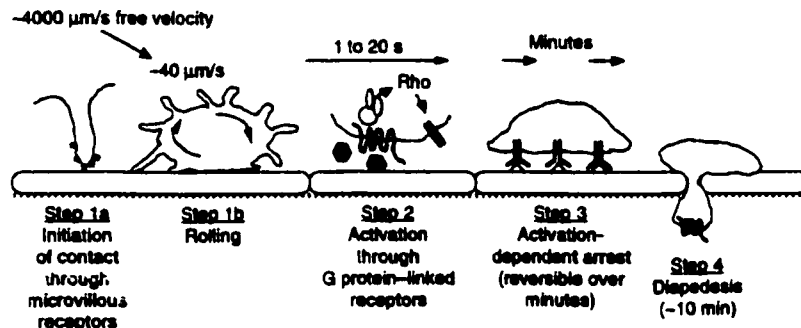
(A) The dichotomy in the homing patterns of naïve and memory lymphocytes [reproduced from ref. (59)]. Both naïve and memory lymphocytes are subject to a constant circulation (immune surveillance) from the blood, into secondary lymphoid tissues (lymph nodes, Peyer's patches, tonsils, and spleen), and back into the blood via the lymphatics. Upon encountering their specific antigen, memory and effector cells are induced to home to tertiary sites, both lymphoid and extra-lymphoid, (bone marrow, intestinal mucosa, inflamed skin, pulmonary tissues, joints, and so on) where they are needed to exert their biological actions (antibody production, cytotoxicity, and so on). PALS, periarteriolar lymphoid sheath; Ag, antigen. **(B)** The multistep model of lymphocyte-endothelial cell recognition and recruitment of lymphocytes from the blood [reproduced from ref. (59)]. This traffic of lymphocytes involves a sequential cascade of molecular interactions, which has been subdivided into four successive stages: Step 1 - primary adhesion (rolling), Step 2 - activation, Step 3 - secondary adhesion (arrest) and Step 4 - diapedesis. Primary adhesion is characterized by a rolling movement of lymphocytes along the vessel wall. It is a transient and reversible interaction occurring over seconds, which slows the lymphocytes' transit and presumably allows it to sample the vasculature for proadhesive factors. The second step is associated with a rapid (1-3s) and reversible activation of molecules known as integrins on the lymphocyte, which can bring about the arrest or firm adhesion of the lymphocyte on the vascular wall (Step 3; secondary adhesion). Step 4, under the appropriate conditions, terminates with lymphocytic extravasation (diapedesis).

A

Lymphoid tissues



B



diversity of cell-cell and cell-matrix interactions and hence influenced by the interactions of adhesion molecules with their cell surface or ECM ligands (59). There is growing evidence to suggest that the recognition of HA by CD44 forms part of the repertoire of adhesive interactions necessary for the controlled traffic of lymphocytes, and hence the regulation of these interactions is of critical importance towards an improved understanding of the homing phenomenon.

Most mature, naïve lymphocytes are subject to a constant circulation (immune surveillance) from the blood, into secondary lymphoid tissues (lymph nodes, Peyer's patches, tonsils, and spleen), and back into the blood via the lymphatics [(59); Fig. 1-2A]. A unique vascular structure, found within many lymphoid but not extra-lymphoid tissues, called the high endothelial venule (HEV), allows for intimate contacts to be made between the circulating lymphocyte and a specialized endothelial cell (high endothelium) with the capacity to sustain lymphocyte transmigration (60). In addition to undergoing normal re-circulation to secondary lymphoid organs during the propagation of an immune response however, memory and effector cells are induced to home to extra-lymphoid tissues such as inflamed skin, intestinal mucosa, pulmonary tissues, and joints where they are needed to exert their biological actions (Ab production, cytotoxicity, and so on) (59). This traffic of lymphocytes involves a sequential cascade of molecular interactions, which has been subdivided into four successive stages: 1) primary adhesion (rolling), 2) activation, 3) secondary adhesion (arrest) and 4) diapedesis [(59, 61); see Fig. 1-2B]. Primary adhesion is characterized by a rolling movement of lymphocytes along the vessel wall. It is a transient and reversible interaction occurring over seconds, which slows the lymphocytes' transit and presumably allows it to sample the vasculature for proadhesive

factors (59). Rolling interactions have been mainly attributed to the interaction of a family of C-type lectins called selectins (L, E, and P-selectins) on the lymphocyte with its ligands (sialyl lewis carbohydrate derivatives) displayed by glycoproteins on the endothelial cell surface. The second step is associated with a rapid (1-3s) and reversible activation of molecules known as integrins on the lymphocyte, which can bring about the arrest or firm adhesion of the lymphocyte on the vascular wall (3rd step; secondary adhesion). Although not as clear for lymphocytes as it is for neutrophils, the physiological triggers for this activation are believed to be the members of the chemokine (chemoattractant) family of receptors (59). Integrins are a heterodimeric family of proteins made of α and β subunits, out of which those containing β_1 , β_2 , and β_7 subunits have demonstrated importance in leukocyte-endothelial cell interactions (60). Specifically, the participation of $\alpha_4\beta_1$, $\alpha_4\beta_7$, $\alpha_L\beta_2$ (LFA-1), $\alpha_M\beta_2$ (MAC-1) in mediating firm adhesion (3rd step) is well documented and has also been proven to be a reversible process occurring within minutes. In particular, α_4 integrins are also involved in rolling interactions, but only integrins participate in secondary adhesion (59). The final step in the homing process (diapedesis), which under the appropriate conditions, terminates with lymphocytic extravasation. It too involves the participation of integrins, particularly β_2 and α_4 integrins (59).

The sequential induction and modulation of receptor expression on the lymphocyte and endothelial cell surfaces dictate the specificity, which is observed during lymphocyte homing (59). The sequence and combination of receptors/ligands expressed on the lymphocyte and endothelial cell surface determines the ultimate destination of the emigrating cell (59). The striking dichotomy between naïve vs. memory/effector cell

migration patterns can thus be explained (see Fig. 1-2A). For example, naïve lymphocytes migrate through the secondary lymphoid tissues such as Peyer's patches (PP), and lymph nodes while only memory/effector cells are targeted to specific extra-lymphoid effector sites such as the intestinal lamina propria (62,63). This tissue specific homing is achieved by the unique phenotype of receptors expressed on the lymphocyte and the endothelial cell (59). A simplified example of how this is achieved follows. The naïve cell is L-selectin⁺, $\alpha_L\beta_2$ (LFA-1)⁺, and expresses low-intermediate levels of $\alpha_4\beta_7$. The PP HEV expresses low levels of L-selectin ligand, is LFA-1 ligand⁺ (ICAM-1, ICAM-2), and exhibits high levels of $\alpha_4\beta_7$ ligand (MAdCAM-1). Only through sequential use of these receptors can the naïve cell traffic to the PP. On the other hand, the lamina propria HEV, being L-selectin ligand⁻ and MAdCAM-1⁻, does not allow access to the naïve cells. Mucosal memory cells are characteristically high expressers of $\alpha_4\beta_7$ and are therefore able to home to the lamina propria using $\alpha_4\beta_7$ alone (59).

Evidence has been provided for the role of CD44 in tissue specific homing. This was initially suggested in *in vitro* HEV binding assays (64,65). It was shown that the anti-CD44 mAb HERMES III and a polyclonal anti-CD44 serum block HEV binding in human lymphocytes. However in mice, homing to secondary lymphoid tissues was not affected by depletion of CD44 from the cell surface by anti-CD44 Abs but the onset of a cutaneous delayed-type hypersensitivity (DTH) response was delayed (66). This was unlike anti-LFA-1 treatment, which inhibited normal lymphocyte extravasation to the lymph nodes and resulted in a more protracted inhibition of the DTH response (66). In another study measuring longer term T cell re-population after depletion of mouse T cells from the peripheral blood, lymph nodes and spleen, it was shown that disruption of CD44

or L-Selectin - ligand interactions delayed reappearance of T cells in these areas (67). More recent studies have demonstrated that CD44 mediates HA-dependent primary adhesion (rolling) of murine T lymphocytes following activation, under physiological flow conditions *in vitro* (14,68). These studies were followed up in mice injected with Staphylococcal enterotoxin B (SEB) (15). SEB activates predominantly V β 8⁺ T cells (69), and this activation was shown to induce their migration into the inflamed site in a manner dependent on CD44-HA interactions. Moreover, the homing of the *in vivo* activated V β 8⁺ T cells was independent of L-selectin mediated rolling.

Another form of cell motility differs from homing behaviour by the fact that it is random in nature and occurs in the absence of vascular shear stress. Thus cell velocity is much slower [0.5-10 μ m/min; (70,71)] than seen in homing [1200 μ m/min; (14)]. Typically it has been described as cell “crawling” along ECM substrates such as collagen (72). In what is called a “wound assay”, a subconfluent monolayer of cells is scraped, thus creating a “wound”, and subsequently the migration of cells towards the wound is assayed. Melanoma cell clones expressing high levels of CD44, treated in this way, migrated towards the wound while low expressers did not. Furthermore, this migration was inhibited by anti-CD44 Abs (73). In another report, human melanoma cells over-expressing CD44 H but not E, exhibited increased motility on HA (70,74). It was also demonstrated that a CS modified form of CD44 was capable of mediating locomotion of a mouse melanoma cell line on type I collagen (75). In a recent study, a number of anti-CD44 Abs induced the formation of dendrite processes or “spreading” in murine B cells following activation with anti-IgM or anti-CD38 Abs, in the presence of IL-4 (76).

1.320 Homotypic and heterotypic cell aggregation

There have also been reports implicating CD44 in the induction of other adhesive pathways. Engagement of CD44 in resting lymphocytes triggered adhesion to an endothelial cell line that was partially mediated by VLA-4 and VCAM-1 molecules but independent of the physical participation of CD44 in binding, the presence of Ca^{2+} and Mg^{2+} , and LFA-1/ICAM interactions (77). Other studies have detailed that anti-CD44 Ab treatment can induce peripheral T cell-keratinocyte adhesion (78) as well as T cell aggregation (79) via LFA-1/ICAM-1 interactions.

1.330 Hematopoiesis and lymphocyte maturation

CD44-ligand interactions have proven to be of critical importance in the development of B cells, NK cells and cells of the myeloid lineage (2,80). Addition of anti-CD44 antibodies to Dexter-type long term bone marrow cultures (LTBMC) inhibited myelopoiesis of neutrophils and macrophages (16). Similarly, anti-CD44 but not anti-MEL-14 (L-selectin) or anti-LFA-1 (integrin) antibody treatment of Whitlock-Witte LTBMCS inhibited B lymphopoiesis (16). NK cell development in LTBMCS was also impaired when anti-CD44 Abs, soluble HA or hyaluronidase were introduced (81). In all such cases the physical interactions of the precursor cells with stroma is critical in their development. It is thought that by disrupting CD44-HA interactions in culture, precursor cell - stromal interactions are inhibited and result in developmental impairment (10).

CD44 H expression varies according to cell differentiation stage [reviewed in (2, 10)]. It is expressed on pluripotent bone marrow cells and pre-T cells prior to their population of the thymus. As thymocytes mature CD44 H is transiently expressed. It is

expressed early in ontogeny (double negative stage; CD4⁻ CD8⁻), transiently downregulated (double positive stage; CD4⁺, CD8⁺) and re-expressed in later stages of T cell development. During B cell development CD44 is expressed at high levels at very early stages, is transiently downregulated during the pre-B cell and germinal centre stages, and re-expressed at the plasma cell stage (83-85). More recently, a study following CD44 expression in relation to surface Ig and a number of other differentiation markers on human tonsillar B cells revealed an even more complex pattern (86). They showed that CD44 is highly expressed in resting (IgD⁺, IgM⁺) B cells and those at an early activation stage (IgD⁻, IgM⁺, CD23⁻). Transition to the blast (IgD⁻, IgM⁺, CD23⁺, CD38⁺, CD10⁺, CD77⁺) and centroblast stage (IgD⁻, IgM⁺, CD23⁺, CD38⁺, CD10⁺, CD77⁺) within the germinal centres is accompanied by a transient downregulation of CD44. Subsequently, at the centrocyte stage (surface IgG⁺ or IgA⁺), high and low CD44 expressing sub-populations were detected while post-germinal centre cells were high CD44 expressers.

Similarly, LECAM-1 or selectin is differentially expressed during the course of B cell development in a manner generally concordant with CD44 (87,88). It is upregulated upon B cell acquisition of CD20 expression (89) and is transiently downregulated during the follicle centre blast stage (90,91) and after *in vitro* activation (90,92). A further degree of heterogeneity in both CD44 and LECAM-1 expression, not elaborated on here, is observed when compared on B cells found within different lymphoid micro-environmental areas such as the mantle zone, or extrafollicular compartments (88).

1.340 Leukocyte activation, proliferation, differentiation and effector function

The role of CD44 in the events associated with immune cell activation and their differentiation into effector cell populations has been established mainly through the use of numerous anti-CD44 Abs and their inductive or inhibitory effect on cell proliferation, cytokine production, cytotoxic activity, Ab production, and so on *in vitro* [reviewed in (2)]. The ability of CD44 to deliver intracellular signals through the mobilization of intracellular calcium $[Ca^{2+}]_i$, the generation of cAMP, increases in intracellular protein tyrosine (tyr) phosphorylation, and the physical association with members of src family protein tyrosine kinases (PTK) have all been documented also [reviewed in (2)].

Molecules present on T cells such as CD2, LFA-1, and CD28 via interaction with their respective ligands LFA-3, ICAM-1 and B7 found on accessory cells, have well established co-stimulatory roles for T cell activation via TCR/CD3 stimulation (93,94). ECM components such as fibronectin, collagen and laminin also have co-stimulatory activities [reviewed in (95)]. The co-stimulatory role of CD44 was initially demonstrated in human T lymphocytes (12). Engagement of CD44 with certain anti-CD44 Abs, mainly pan-CD44 specific Abs was shown to enhance human T cell proliferation in response to anti-CD3 or anti-CD2 stimulation (2,12,96,97). More importantly, the ligation of CD44 with its physiological ligand, HA, was shown to be co-stimulatory with anti-CD3 Abs in human peripheral T cells and T cell clones (98). Anti-CD3 induced peripheral T cell proliferation was enhanced in the presence of HA, as was IL-2 production by helper T cell clones and granzyme release by CTL clones. Furthermore, anti-CD3 stimulated CD3⁺, CD8⁺ clones lysed HA coated targets more efficiently than targets which were not coated. There have also been reports that CD44 can deliver growth signals to T

lymphocytes on its own (99,100). Certain antibodies to CD44 are able to independently enhance T cell proliferation and IL-2 production (99). In the murine system, anti-CD44 mAbs were able to trigger CTL activity (100).

Efforts have been made to explain the above effects at the level of intracellular signal transduction pathways associated with TCR/CD3 stimulation. One anti-CD44 antibody in particular (designated 212.3) exhibited an inhibitory effect on anti-CD3 induced responses, including T cell proliferation, IL-2 production, IL-2R expression, and $[Ca^{2+}]_i$ influx (101). Interestingly, this antibody maintained a co-stimulatory activity in conjunction with CD2 ligation and had no impact on proliferation in response to PHA, PWM and a mixed lymphocyte reaction (MLR). This inhibitory effect was attributed to the ability of this antibody to induce the generation of intracellular cAMP and possibly interfere with cell cycle progression in response to CD3 stimulation (102).

Phosphorylation of proteins on tyr residues via protein tyrosine kinases is critical in the regulation of cell growth and differentiation (103). In T cells, this is critical during Ag-specific activation and proliferation (104). The role of PTKs and tyr phosphorylation in CD44-mediated signal transduction has only recently been investigated (105,106). Anti-CD44 mAbs (Hermes III, J173) were shown to induce the tyr phosphorylation of various intracellular proteins including the PTK ZAP-70 (105). Furthermore, CD44 was shown to physically associate with the src family PTK p56^{lck}, known to phosphorylate ZAP-70 (105). In an *in vitro* kinase assay, CD44 cross-linking triggered p56^{lck} activity (105). More recently, the association of CD44 with src PTKs lck and fyn within plasma membrane domains in human PBMC has been established (106). These studies suggest that the T cell activation by CD44 engagement may be transduced via these molecules,

each regarded as a major player in the TCR signal transduction cascade (107). Taken together, it is clear that CD44 plays an important role in the activation and induction of T cell effector functions.

The ability of CD44 to induce NK cell function has also been demonstrated (2,10,108-111). NK cells are the non-adherent, non-phagocytic, CD3⁺, large granular lymphocytes, which unlike CTLs, kill targets without priming in an MHC unrestricted fashion [reviewed in (112,113)]. NK cytotoxic activity is enhanced by cytokines such as IL-2, IFN- γ , and IL-12 (112,113). Antibodies to CD44 were demonstrated to trigger the cytolytic activity of NK cells, a phenomenon that may require co-stimulation with IL-2 or IL-12 (108,110,111). It was also demonstrated that engagement of the CD44 receptor results in the induction of cell surface expression of activation antigen CD69, enhancement of phorbol ester-induced TNF- α secretion and stimulates the tyrosine phosphorylation of several cellular substrates (114).

At the level of monocytes/macrophages, triggering of CD44 via Abs has been reported to induce the release of cytokines TNF- α and IL-1 β (115), both important regulators of innate immunity against viruses and the initiation of inflammation against bacterial infections (116). To place in context, the induction of these cytokines by the cross-linking of other molecules, including LFA-3 and CD45, has also been demonstrated (115). Activated macrophages also control tissue inflammation by the production of reactive oxygen and nitrogen species (117). Nitric oxide mediates host defense functions of activated macrophages such as anti-microbial and tumoricidal activity [reviewed in (118,119)]. It was recently demonstrated that fragments of the polymer HA were able to induce nitric-oxide synthase (120), as well as the chemokines MIP-1 α , MIP-1 β , CRG-2,

MCP-1, and RANTES production in activated macrophages (121). In another report, the phagocytosis of apoptotic neutrophils by macrophages, an important determinant for the resolution of inflammation, was enhanced by ligation of CD44 with certain anti-CD44 Abs *in vitro* (122). This effect may not involve hyaluronan, as other antibodies known to inhibit CD44-HA interactions had no effect. Furthermore, anti-V3, V7-8, or V10 variable exon specific mAbs also failed to have the same effect. This phenomenon appeared to be specific, as phagocytosis of apoptotic lymphocytes, or other particles such as Ig-opsonized erythrocytes, was not affected.

Relative to T cells, NK cells and cells of the monocyte/macrophage system, considerably less is known about the direct role of CD44 in immune function of B cells. One report demonstrated the ability of HA to independently trigger murine B lymphocytes (123). Incubation with HA was shown to induce B cell proliferation and differentiation resulting in increased IgM production. Most recently, the GAG chondroitin sulfate B, often secreted by activated mononuclear leukocytes and as a consequence of matrix degradation, was shown to induce the proliferation of B lymphocytes (124). This proliferation was dependent on CD44 expression and the presence of monocytes.

1.350 CD44 V associated functions

The majority of the studies described have not focussed on CD44 variant specific functions. Some progress has been made in this domain with the molecular cloning of various isoforms and the advent of variable exon specific mAbs. The participation of V6 containing isoforms has been proposed in normal immune function of B and T cells

(36,37,125-127). Their expression is induced transiently following Ag stimulation *in vivo* and *in vitro* in T cells, B cells and macrophages (36,37,126,127). Furthermore, antibodies directed against the variable region of the CD44 V4-7 isoform (1.1ASML) are able to impair both T-dependent and T-independent B cell responses as well as alloantigen-induced T cell proliferation and CTL generation (37). Results from a transgenic mouse expressing the CD44 V4-7 isoform on its thymocytes and peripheral T cells suggested that this isoform was likely involved in signal transduction during lymphocyte activation. Although the number and distribution of lymphocytes was unaffected, transgenic lymphocytes responded faster to activating stimuli, in particular, primary stimulation with T cell mitogens and T-dependent antigens (Ags), both *in vitro* and *in vivo* (125). This accelerated response could be ablated only by CD44 V6* specific antibodies. No effect was observed on the responses to the B cell mitogen LPS, T-independent Ags, or stimulation with phorbol ester (PMA) and calcium ionophore (ionomycin) (125). Antibodies to constant regions of CD44, or incubation with HA or CS had no influence, suggesting that V6 containing isoforms are co-stimulators of lymphocyte activation in response to some unknown cellular ligand (125). Other experiments have established a differential role of CD44 V6 and V7 containing isoforms in murine T_{h1}-type, and T_{h2}-type responses (128). Anti-V7* antibody treatment inhibited trinitrobenzene sulfonic acid (TNBS)-induced colitis, a murine model for T_{h1}-type responses. Both Anti-V6* and anti-V7* treatment inhibited dinitrofluorobenzene (DNFB)-induced DTH responses (T_{h1} model). However, only anti-V7* treatment interfered with FITC-induced allergic dermatitis, a T_{h2} model system. Other studies have implicated V10 containing isoforms in allergy, as anti-V10* antibodies inhibited edema

and granuloma formation in DTH responses *in vivo* (129). Cytokines, chemokines and other growth factors may be most efficiently presented to leukocytes travelling through the blood stream if anchored to a solid support such as on cell surface proteoglycans of vascular endothelial cells (32). Such a function has been proposed for CD44 isoforms containing exon V3, which are typically heparan sulfate modified. The heparin sulfate moiety may allow them to effectively present heparin binding growth factors such as MIP-1 β , b-FGF, and HB-EGF to passing or neighboring cells (27,32,38).

1.360 Role of CD44 and its isoforms in the cancerous process

It has been suggested that the processes critical for the generation of tumors and their subsequent metastasis mimic normal physiological events (2). One such process is the ability of cells to interact with other cells and with ECM molecules present in the microenvironment. As discussed, cell-cell and cell-ECM interactions are mediated by adhesion molecules such as CD44 and therefore it is not surprising to find CD44-ligand interactions intimately involved in the malignant progression of many tumors. There exists no paucity of experimental evidence for this implication *in vitro* and in animal models [reviewed in (2)]. Studies of CD44 in a clinical setting were also undertaken to investigate its potential for diagnostic, prognostic and even therapeutic applications in many different types of tumors [reviewed in (2)].

Perhaps the most striking demonstration of CD44 involvement in tumor metastasis, particularly variants V4-7 and V6-7, was accomplished in a rat model of pancreatic carcinoma (5,24). Expression of these variants was observed only in a metastatic pancreatic carcinoma cell line (BSp73ASML) but not in a non-metastatic cell

line of the same origin (BSp73AS) or normal rat tissues. When injected into the rat footpad, BSp73ASML cells rapidly colonized the lymph nodes and lung, while BSp73AS grew locally. Furthermore, introduction of either of these CD44 V, but not CD44 H, into the non-metastatic cell line was sufficient to confer metastatic behavior to these cells. In subsequent studies (130), it was demonstrated that such metastases could be prevented by antibodies to the variant sequences of these isoforms, specifically to epitopes of the V6 exon shared by both proteins. The mechanism by which lung and lymph node metastases were prevented in this system was shown not to be associated with an enhanced immune response, or a downregulation of CD44 V expression, but likely depended on the importance of these isoforms in the settlement and outgrowth of tumor cells in lymph nodes perhaps via interactions with a novel ligand. Homologous CD44 V sequences have been detected in various human tumor cell lines (25) and have been shown to participate in normal immune responses, as described above, the latter serving as an example of neoplastic cells potentially exploiting a normal physiological function (2,37). Similarly, in studies of clinical pancreatic carcinoma samples, it was found that a malignant phenotype was associated with the expression of isoforms containing the V6 exon (131,132).

Equally striking results were obtained in mouse models of Burkitt's lymphoma growth and dissemination that contrasted with those described for rat pancreatic adenocarcinoma (133-135). First, a brief introduction of Burkitt's lymphoma (BL) is in order. Burkitt's lymphomas are classified as high-grade (unfavourable prognosis) tumors of B cell origin (sIg⁻, CD19⁺, CD20⁺) cytogenetically characterized mainly by chromosomal translocations placing the *c-myc* oncogene under the regulatory control of

one of the immunoglobulin genes, resulting in constant cell cycling (136). The disease involves localized tumor formation, growth in extra-nodal sites (135) and is regarded as the most rapidly growing human tumor (136,137). Such tumors are further sub-classified into the endemic (African) and non-endemic/sporadic (American) BL, which exhibit essentially the same pathology (136). Associated particularly with the pathogenesis of endemic BLs and other pathologies including B cell lymphomas of immunosuppressed individuals, nasopharyngeal carcinomas, Hodgkin's disease, and infectious mononucleosis, is the Epstein-Barr Virus, a potent B cell transforming agent [reviewed in (138-140)]. This herpes virus latently infects and transforms normal B cells giving rise to immortalized non-neoplastic B lymphoblastoid cell lines (B-LCL) capable of indefinite growth *in vitro* (140). Study of BL and the transforming effects of EBV have been facilitated through the use of B-LCLs as well as a variety of BL cell lines. BL cell lines have also been classified based on their EBV status and based on cell surface phenotype in comparison with B-LCLs into group I, II and III (141,142). B-LCLs characteristically express the full complement of 9 latent EBV genes (EBNA-1 – 6, LMP-1, 2A, and 2B) and resemble activated B cell blasts, expressing CD23, CD30, CD39, CD70 and adhesion molecules CD44, LFA-1, ICAM-1, and LFA-3. Group I BL cell lines, like primary BL biopsies, resemble germinal centre B cells and lack all the aforementioned cell surface proteins and if EBV-positive, necessarily express solely the EBNA-1 gene. This lack of adhesion molecules is believed to explain, at least in part, the characteristic localized tumor formation and extra-nodal growth pattern of BL (143,144). Continuous *in vitro* passaging results in their phenotypic conversion to group II/III BLs with an activated B

cell or B-LCL cell surface phenotype (143,144). It is believed that the activation of latent EBV genes, particularly LMP-1, is important in this process (135).

The potentially important role of CD44 H in *in vivo* BL tumor growth and dissemination was firmly established in studies using a mouse model (46,133-135). The CD44 H or E proteins were introduced into Namalwa, a CD44⁻ BL cell line, and the impact on local tumor growth or metastatic spread was evaluated following S.C. or I.V. injection into nude mice, respectively (133). CD44 H transfectants formed local, encapsulated tumors faster and had an enhanced metastatic proclivity than the CD44⁻ parental cells. Parental cells did develop tumors, however slower, indicating that CD44 may be an important factor in BL tumor formation but is not essential *per se*. Furthermore, CD44 E transfectants had the opposite effect in this system, exhibiting reduced local growth and dissemination when compared to parental cells. In similar experiments with CD44 V6-10, V7-10, V8-10 isoforms, a similar attenuation of tumor growth was observed (46). This differential isoform effect may be attributed to the inability of these variants to bind HA, a property, which could provide a growth advantage to CD44 H-expressing tumors. In contrast, CD44 V3⁺ transfectants, although failing to adhere to HA, formed bone marrow tumors rapidly following I.V. injection. This may reveal a novel ligand binding pathway or reflect the recently identified ability of CD44 V3⁺ to present heparin binding growth factors (46). It was also demonstrated that the formation of such tumors could be inhibited by disruption of CD44 H – ligand interactions using a CD44-Ig fusion protein (134). As mentioned, one of the factors associated with BL pathogenesis, particularly endemic BL, is EBV. It has been demonstrated that EBV infection of BL cell lines *in vitro* induces the expression of CD44

(126,135,145). Furthermore, it was shown that the LMP-1 protein of EBV was responsible for this induction (145) and could enhance lymphomagenesis in a manner similar to that described for CD44 transfected Namalwa cells (135), perhaps bringing into light one way in which EBV may influence BL pathogenesis. LMP-1 transfected BLs (thus rendered CD44-positive) when injected S.C. into SCID mice acquired a disseminated tumor growth pattern, like B-LCLs, infiltrating the thymus and axillary lymph nodes, whereas parental cells exhibited only local tumor formation (135).

The involvement of CD44 was apparently dispensable for the growth and spread of a lymphosarcoma tumor (146), a lymphoid disorder clinically distinct from both leukemia and Hodgkin's disease (147). By homologous recombination, the CD44 gene in the lymphosarcoma cell line, MDA-D2, was knocked out. Subsequently, tumor formation and its hematogeneous spread were assayed following S.C. or I.V. injection into nude mice, respectively. No differences were observed in CD44 knock-outs when compared to the wild type cells in this regard, suggesting that not all malignant lymphomas require CD44 to grow and metastasize (146).

The expression of CD44 isoforms and other adhesion molecules in relation to lymphoma origin, growth characteristics, and severity has been studied to considerable extent in clinical samples (36,88,148,149). Two studies of non-Hodgkin's lymphomas (NHL), a term that represents a dozen or more separate, though related, disorders (147) have generally associated a preferential expression of V3, V6, V9 but not V4-containing isoforms with a high grade malignant phenotype (36,148). These variants were not detected in non-malignant lymph nodes and only rarely in low grade tumors. In addition, it was found that α_6 and β_4 integrins were expressed to a lower extent in several high

grade NHLs than in both low grade and normal lymph nodes (148). Other integrins tested (α_2 , α_4 , α_5 , and α_v , or β_1 , β_2 , β_3 , and β_7) showed no significant differences. Another study of NHLs described that tumors classified with an unfavourable prognosis exhibited an increased expression of CD44 H (150).

Looking more specifically at different B cell malignancies (sub-types of NHLs), it appears, to a large extent that tumor cell surface phenotype correlated with that found on the “normal counterparts” of these cells with respect to ontogeny (83,88,149). It was shown that only 1 out of 14 BL biopsies, resembling most closely germinal centre B cells, were positive for a common CD44 epitope detected by the HERMES III mAb (84). Elsewhere however, it was shown by immunohistochemical analysis that in two cases of BL, one was 10-20% positive for CD44 V6⁺ and >70% CD44 H positive, while another failed to express CD44 V6⁺ and exhibited only 10-20% CD44 H positivity (148). V3⁺, V4⁺, V9⁺ expression was undetectable in these BL samples. In diffuse large cell B cell lymphomas it was long speculated that CD44 may be necessary for hematogeneous spread to lymphoid sites, but was not in and of itself sufficient to predict such a behaviour (84). More recently, it was demonstrated that in comparison to primary nodal large cell lymphomas, those exhibiting extra-nodal and disseminated growth had higher CD44 H levels and additional variable exon containing isoforms, including CD44 V6⁺ (127). The identities of the CD44 variants expressed were however, the same as those expressed in splenic B cells activated by surface Ig cross-linking (127). Examination of primary gastro-intestinal B cell lymphomas at the level of β_1 integrins, CD44, and LECAM-1 expression revealed that although growth was confined to this extra-nodal site, there was no apparent adhesion profile shared by these tumors (149). Therefore no

organ specific homing pattern was suggested although to some extent receptor expression mimicked their putative normal cellular counterparts (149). In another study, CD44 and LECAM-1 expression was compared in normal and malignant B cell populations (88). Both molecules were expressed in B cell chronic lymphocytic leukemias, hairy cell leukemias, and mantle zone lymphomas, corresponding to their normal counterparts. Similarly, follicular centre cell lymphomas failed to express these markers. However, irrespective of their maturational state, extra-nodal B cell malignancies expressed CD44 and LECAM-1 infrequently (88). It was concluded that due to their multi-factorial nature, the expression of these adhesion molecules, or lack thereof, was alone not predictive of tumor growth pattern.

In conclusion, it can be said that CD44 and/or its variants exert a strong influence on many but not all tumors. For certain tumors such as NHLs and pancreatic adenocarcinomas, CD44 V have potential as prognostic and diagnostic markers. It has however been difficult overall to predict what facet of the tumor's growth requirements are satisfied by CD44 isoforms. It is most imperative not only to identify the isoforms expressed on particular tumor cells but how their expression and function is regulated and hence how tumor formation and metastasis may be influenced.

1.400 Control of CD44 isoform expression and interactions with hyaluronan

In light of its importance in normal and pathophysiological processes it is not surprising to find strict control measures over the ability of a CD44-positive cell to recognize HA. This was suggested initially by the observations that not all cells expressing CD44 bind HA constitutively (40,151). CD44 must be "activated" in order to recognize

HA (40,151). The phorbol ester PMA and some anti-CD44 mAbs were shown to induce CD44-HA binding originally in murine T cells and T lymphomas (40,151,152). Furthermore, *in vitro* activation of human peripheral T cells via anti-CD3 antibodies, culture of monocytes in the presence of human serum, anti-CD3 antibody or PHA stimulation of peripheral blood mononuclear cells (PBMC), all triggered transient HA recognition by these cells (98,153). Extensive studies have been conducted to determine the mechanisms involved in the regulation of CD44-HA recognition. Possible mechanisms include: interaction with elements of the cytoskeleton via the cytoplasmic domain (154-157), phosphorylation of the cytoplasmic domain (42), post-translational modification of the extracellular domain (158-161), differential CD44 isoform expression (162), interaction with other ligands either extracellular or at the cell surface (52,54), and shedding of CD44 (163) [reviewed in (10)].

Although not without controversy, it has been demonstrated that the interaction of the cytoplasmic tail of CD44 with cytoskeletal elements may be important in the induction of HA binding (154,155). However, this is apparently not an absolute requirement as it can be bypassed depending on the cell type (intracellular milieu) or by cross-linking of CD44 (156,157). In mouse, the constitutive phosphorylation of ser 325 and 327 was shown to be critical in the clustering of CD44 and essential for its adhesive interactions with HA (42). This phenomenon did not seem to hold true in the human system, however (164). Increases in the complexity of N-linked glycosides (addition of sialic acid), keratan sulfate modification and most recently inhibition of sulfation of CD44 have generally been found to reduce cell recognition of HA (158-161,165,166). In studies of HA recognition by isoforms containing exons V8, V9 and V10, it was shown

that their inclusion, particularly V9 and V10 with high ser/thr content (sites of O-glycosylation), inhibited HA recognition as a result of the extensive O-glycosylation of these sequences (26). In activated human T cells, CD44 V6* and V9* may be required for HA adhesion (162) while in a murine fibroblastoid cell line, a signal transduction event mediated through an IL-2R γ chain homologue is required (167). It has also been suggested that a threshold level of CD44 expression is required for cells to bind HA (156). Recently, PMA has been shown to induce clustering of CD44 and homodimerization of CD44 on the cell surface, a phenomenon that presumably enabled its recognition of HA (168). Shedding of CD44 has also been reported to have a regulatory role on HA recognition as soluble CD44 was able to partially inhibit cell surface CD44-HA binding (163).

It has been equally well established that CD44 expression is dependent on the activation and differentiation state of the cell (2,10). Initially this was demonstrated for standard CD44. Its expression was elevated as a result of Ag stimulation of T cells *in vivo* and *in vitro* (169-171). Memory T cells (high expressers of LFA-3, LFA-1, and CD2) were shown to express two fold higher levels of CD44 than naïve cells (172). In murine B cells, stimulation with LPS, anti-IgD-dextran, the supernatant of activated T_{h2} clones, or IL-5, upregulated CD44 expression (173,174). More recently, co-ligation of CD40 and surface IgM, both essential receptors in T-dependent B cell responses, upregulated CD44 expression (175).

CD44 V isoform expression turned out also to be elevated as a consequence of cell activation (10). As mentioned earlier, *in vivo* Ag challenge induced the expression of CD44 V6* variants in rat T cells, B cells, and monocytes (37). Their expression was

further shown to be crucial in the activation of both T and B cells (37). Variant expression has since been extensively studied in human leukocyte populations (36,126,127,176). It was shown that V9⁺ expression was detectable at very low levels in human T, B, monocytes, and granulocytes, but not thymocytes (176). Unstimulated monocytes in addition, expressed low levels of V6⁺ and V4⁺ (176). In human T cells, CD44 V6⁺ and V9⁺ expression was transiently upregulated when subjected to various stimuli *in vitro*, including PHA, PMA, anti-CD3 Abs in the presence of IL-2, Ag, or a mixed lymphocyte reaction (36,176). Stimulation of splenic B cells with anti-Ig antibodies upregulated the standard isoform CD44 H as well as CD44 V6 while at the same time apparently downregulating CD44 V6-10 (127).

The effect of cytokines present in the microenvironment on CD44 isoform expression and adhesion of cells to HA remains poorly understood. In human endothelial cells, TNF- α upregulated the expression of CD44 (177), while TNF- α and IFN- γ upregulated the expression of CD44 V6⁺ and CD44 V9⁺ on myelomonocytic cell lines, THP-1 and U-937 (176). TNF- α has also been shown to enhance binding of human monocytes to HA and IL-4 and IL-13 inhibited this binding (178). In murine B cells, IL-5 enhanced CD44-mediated binding of HA (174). The mechanism for this enhancement was suggested to be a reduction in the N-glycosylation of the CD44 H protein, since extracts from IL-5 treated cells had a lower molecular weight than unstimulated cells and digesting extracts with N-glycanase could abrogate these differences (173,174). As mentioned above, various treatments of murine B cells could enhance CD44 expression but only IL-5 generated a sub-population of cells with HA binding capacity. The adhesion of CD34⁺ hematopoietic progenitor cells to HA was enhanced by GM-CSF, IL-

3 and stem cell factor (179). Among chemotactic and proinflammatory cytokines, RANTES and IL-15 induced uropod formation in T cells and brought about the redistribution of adhesion molecules, including CD44 to the uropod (180,181).

These observations taken together with the critical role of CD44 in tumorigenesis and metastasis indicate that CD44 isoform expression, like the recognition of HA by CD44 and its modulation by cytokines is dependent on cell type and state of cellular activation, differentiation and/or transformation.

1.500 Hypothesis

The regulation of CD44-HA recognition in normal or tumorigenic human B cells may have implications with respect to trafficking of human B cells during the development of an immune response and/or localization of malignant B cells within target tissues. The hypothesis is that CD44 isoform expression and HA recognition is dependent upon the mode of B cell activation, differentiation and state of transformation. Specifically, it was surmised that cytokines, particularly those associated with B cell growth and differentiation, are responsible for regulating HA recognition by a mechanism, which may involve the differential expression of CD44 isoforms and/or post-translational modifications to the CD44 protein.

1.600 Objectives and approach

The overall objective of this thesis is to study how CD44 isoform expression and HA recognition in normal and transformed human B cells is regulated. As models, normal human B cells derived from peripheral blood, and a series of transformed B cell

lines, including EBV⁺ and EBV⁻ Burkitt's lymphomas as well as normal human B cells transformed *in vitro* with EBV, were employed. The specific aims are as follows:

1. Evaluate CD44-hyaluronan interactions in normal and transformed human B cells following stimulation with mitogens and cytokines involved in B cell activation, proliferation, and differentiation
2. Investigate the potential molecular mechanism by which the identified factors exert their effects on HA recognition
3. Study the effect of Epstein-Barr virus infection on CD44 isoform expression and HA recognition in B lymphocytes

CHAPTER TWO

MATERIALS AND METHODS

2.010 Cell lines and cultures

The B cell lines used in this study included human EBV-negative and EBV-positive BL, and a number of B-LCLs. Some of their biological characteristics are listed in Table 2-1. Unless otherwise stated all cell lines were obtained from the American Type Culture Collection (ATCC; Rockville, MD). Jijoye, EB3 and Raji are EBV-positive BL cell lines. CA46, ST486, MC116 and Ramos are EBV-negative BL cells. BL-30 is an EBV-negative BL cell line while BL-30/B95-8 and BL-30/P3HR1 are BL-30 cells infected *in vitro* with the B95-8 or the P3HR1 strain of EBV, respectively, all kindly provided by Dr. E. Kieff (Brigham and Women's Hospital, Boston, MA). BJAB is an EBV-negative B cell line while BJAB/B95-8 are BJAB cells infected *in vitro* with the B95-8 strain of EBV, both generously provided by Dr. J. Menezes (Ste. Justine Hospital, Montreal, Quebec, Canada). The EBV-negative and EBV-positive BL cells listed in Table 2-1, exhibited the characteristic *c-myc* translocation (t(8;14) (182,183). IM cells were obtained by culture of peripheral blood mononuclear cells (PBMC) from a patient diagnosed with infectious mononucleosis at the Children's Hospital of Eastern Ontario, Ottawa, Ontario, Canada. MK3.31, MK4.15, MK5.26, and MK6.1 B-LCLs, were generated by *in vitro* transformation of purified human B cells from different healthy donors with the B95-8 strain of EBV according to established procedures (184), as described below (126). The EBV status (positivity) for all B cell lines was confirmed by EBV DNA polymerase chain reaction (PCR) analysis (185). B95-8 is a marmoset derived, mixed leukocyte cell line while HUT78 is a human leukemic T cell line. L3.3 and L3.4 are human pancreatic adenocarcinoma cell lines kindly provided by Dr. M. Vezeridis (Brown University, Providence, RI).

Table 2-1: Characteristics of human B cell lines studied

Cell Line	Type	EBV Infection	<i>c-myc</i> translocation
MC116	Burkitt's Lymphoma	Negative	t(8;14)
CA46			
ST486			
Ramos			
Raji		<i>In vivo</i>	
EB3			
Jijoye			
BL30	Negative		
BL30/P3HR1	BL-30 Burkitt's lymphoma	<i>In vitro</i> with P3HR1 EBNA-2 deletion	
BL30/B95-8		<i>In vitro</i> B95-8	
BJAB	BJAB B cell lymphoma	Negative	Negative
BJAB/B95-8		<i>In vitro</i> B95-8	
IM(IM-1, 2)	B Lymphoblastoid	<i>In vivo</i>	Unknown
MK3.31		<i>In vitro</i> B95-8	Negative
MK4.15			
MK5.26			
MK6.1			

All cells were maintained at 37°C and 5% CO₂ in Iscove's Modified Dulbecco's Medium (IMDM) (Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco BRL, Burlington, Ont., Canada), penicillin (100 U/mL), gentamicin (1 µg/mL).

2.020 Isolation of human peripheral blood B cells

Human B cells were isolated from PBMC by a positive selection technique using anti-CD19 antibody conjugated magnetic beads, as recommended by the manufacturer (Dynal A.S., Oslo, Norway). Briefly, blood was obtained from the Canadian Red Cross (Ottawa, ON, Canada) from healthy volunteers and PBMCs were isolated by differential migration during centrifugation through Ficoll-Paque (Pharmacia Fine Chemicals, Piscataway, NJ). PBMCs were washed three times with phosphate buffered saline (PBS; Sigma) and resuspended in PBS at a concentration of 50×10^6 cells/mL. The B cells were purified by incubating PBMCs with the anti-human CD19 antibody-coated beads (Dynabeads M-450 Pan-B, Dynal) at a bead to target cell ratio of 4:1, for 30 mins on ice. B cells attached to beads were washed six times with PBS containing 2% FBS and subsequently resuspended in IMDM containing 1% FBS at 50×10^6 cells/mL. B cells were detached from the beads by incubating with 1 Unit of CD19-Detachabeads (a polyclonal antibody raised against the Fab fragment of the mouse immunoglobulins) per 5×10^6 cells for 1 hr at room temperature. The beads were washed three times with IMDM containing 1% FBS and the washes containing the detached B cells were collected. The B cells were subsequently assayed for the presence of T cells, monocytes, and natural killer (NK) cells by flow cytometric analysis, as described below. PerCP-conjugated mouse anti-human CD20 (pan B cell marker), phycoerythrin-conjugated mouse anti-human CD14 (monocyte marker)

and anti-human CD16, and fluorescein isothiocyanate (FITC)-conjugated anti-human CD3 antibodies (Becton Dickinson, Mountain View, CA) were used for determination of cell purity. The purified B cells were 98% CD20-positive and contained less than 2% T cells, NK cells and monocytes (126). Alternatively, B cells were isolated by negative selection. Briefly, PBMCs were treated with L-leucine methyl ester to deplete phagocytic and cytotoxic cells. CD2-positive cells were depleted by employing anti-CD2 antibody conjugated beads in a manner similar to that described for positive selection above except that a bead to target cell ratio of 10:1 was used (Dynal). The enriched B cell population contained less than 2% T cells, NK cells, and monocytes, as assessed by flow cytometric analysis. The isolation of B cells by negative selection was found not to differ from positively selected cells with respect to CD44 isoform expression after stimulation (126) and therefore the positive selection method is presented in the thesis.

2.030 EBV transformation of B cells in vitro

The EBV B95-8 virus stock was prepared according to an established technique (184). Briefly, B95-8 marmoset cells (1×10^6 cells/mL) were cultured in IMDM-10 for 3 days. Following centrifugation ($300 \times g$), the virus containing supernatant was collected and filtered through a $0.45 \mu m$ filter (Gelman Sciences, Ann Arbor, MI) and stored at $-70^\circ C$. B-LCLs were obtained by *in vitro* transformation of purified B cells with EBV, as described elsewhere (184). Briefly, purified B cells (4×10^6 cells/mL) were incubated with an equal volume of EBV viral stock for 2 hrs at $37^\circ C$, followed by the addition of an equal volume of complete IMDM medium to yield a final concentration of 1×10^6 cells/mL. EBV-infected B cells were collected at various times (from 1 day to 15 days) post-infection, and also

maintained in culture as an immortalized B-LCL. EBV infection was confirmed by EBV DNA polymerase chain reaction (PCR) analysis (185).

2.040 Reagents and cell stimulation

Purified B cells (1×10^6 cells/mL) were stimulated with phorbol 12-myristate 13-acetate (PMA; Gibco BRL) at concentrations ranging from 0.05 - 25.6 ng/mL. Rabbit anti-human IgM antibody conjugated beads (Immunobeads, Bio-Rad, Hercules, CA) were used at a final concentration of 20 μ g/mL. Cells were also stimulated with LPS from *Salmonella enteritidis* (Sigma; 1-100 μ g/mL) or pokeweed mitogen (PWM) at final dilutions between 1:10 to 1:500. Recombinant human Interleukin (IL)-1 α , IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-10, IL-13, TNF- α , TGF- α , TGF- β , and IFN- γ were purchased from R&D Systems Inc. (Minneapolis, MN) and used at concentrations of 10 ng/mL, 1 ng/mL, 100-200 U/mL, 0.12-48 ng/mL, 15 ng/mL, 4 ng/mL, 37.5 U/mL, 3-200 ng/mL, 10-20 ng/mL, 50-100 ng/mL, 0.05-5 ng/mL, and 4-108 ng/mL, respectively. IL-12 was a kind gift from Dr. Maurice Gately (Hoffman LaRoche, NJ) and was used at concentrations of 0.5-1.5 ng/mL. B cells lines were similarly stimulated with the exception of anti-IgM antibodies. In addition they were treated with *Staphylococcus aureus* Cowan strain I (SAC) (Calbiochem-Novabiochem Corp., La Jolla, CA) at a final dilution of 1:1000 and PHA-M (Gibco) at a final dilution of 1:100 and tunicamycin (Calbiochem-Novabiochem Corp.) at concentrations ranging from 0.1 - 40 μ g/mL.

2.050 RNA extraction and reverse transcription polymerase chain reaction

For detection of CD44 mRNA expression, total cellular RNA was extracted using a mono-phase solution containing guanidine thiocyanate and phenol (Tri Reagent solution, Molecular Research Center, Inc., Cincinnati, OH), as described by the manufacturer. Total RNA (1 µg) was reverse transcribed in a 20 µL reaction mixture containing 2.5 µM random hexamers, 5 mM MgCl₂, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1 mM each of dGTP, dATP, dTTP, dCTP, 20 Units of RNase inhibitor, and 500 Units of M-MLV Reverse transcriptase (Perkin Elmer, CA) at 42°C for 1 h. cDNA equivalent to 100 ng of RNA thus obtained was added to standard PCR reaction mixtures containing 100 pmol of oligonucleotide primers. Primers for CD44 amplification were synthesized by National Biosciences (Plymouth, MN) and included CD44-1 (5' sense) and CD44-2 (3' antisense) corresponding to constant region nucleotides (nt) 508-528 and 858-878 of the CD44 H cDNA sequence (see Fig. 2-1) (6). Their nucleotide sequence is listed in Table 2-2. Amplification was carried out in a Perkin Elmer DNA thermal cycler 480 for 35 cycles using the following conditions: 94°C for 1 min, 55°C for 2 min, and 72°C for 2 min. As a negative control, non-reverse transcribed RNA was added to PCR reactions.

2.060 Hybridization and DNA Probes

To identify which CD44 isoforms were expressed, PCR products were electrophoresed through 1.5% agarose gels and transferred onto a Hybond-N nylon membrane (Amersham, Buckinghamshire, UK) (186). Hybridization using a buffer containing 6 x sodium chloride/sodium citrate (SSC), 5 x Denhardt's solution, 0.5% sodium dodecyl sulfate (SDS), 50 mM sodium phosphate, and 0.25 mg/ml salmon sperm DNA and

Fig. 2-1: Schematic representation of CD44 H cDNA, variable exons (V1-V10), the RT-PCR primers and the probes used for the detection of CD44 isoforms

The variable exons V1 to V10 are inserted in different combinations at a single site into the extracellular domain after nt 790. The probes H, E and the synthetic oligonucleotides corresponding to the exons V2-V10 (pV2-pV10) are shown. PCR primers, CD44-1 (nt 508-528) and CD44-2 (nt 838-858) were used in RT-PCR experiments. Restriction enzyme sites are also indicated.

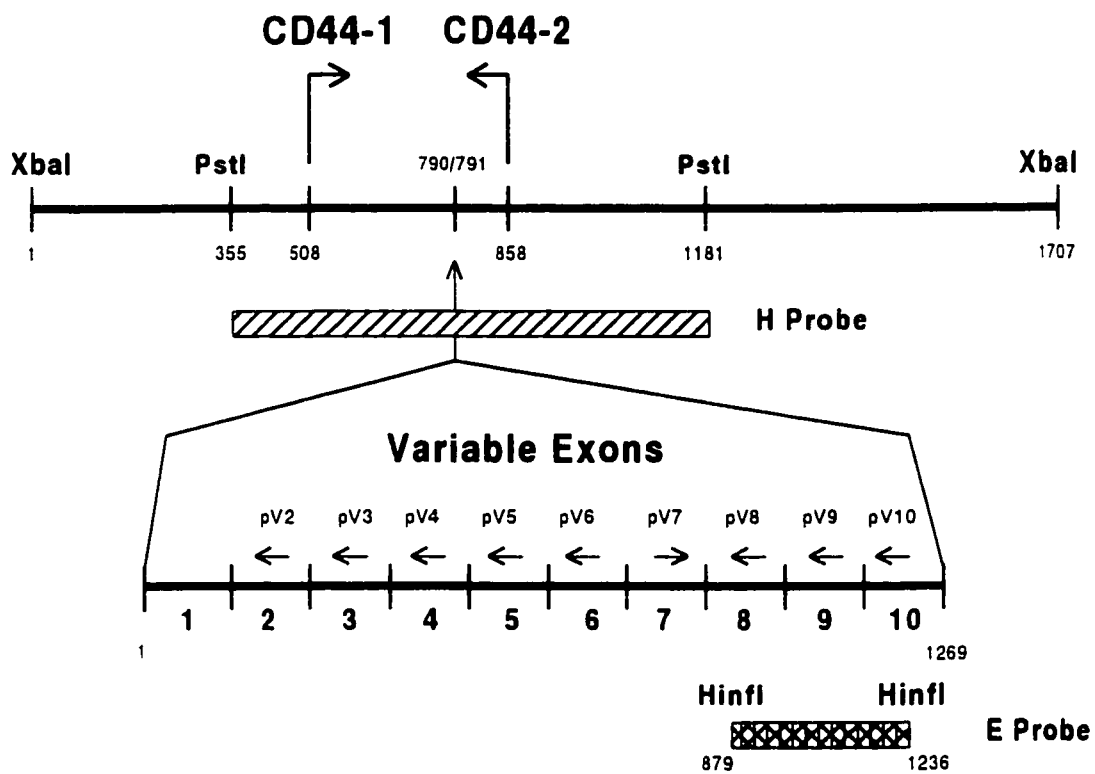


Table 2-2: Primers and probes used for CD44 isoform RT-PCR analysis

Oligonucleotide	Sequence (5' → 3')	Position* (nt)
PCR primers		
CD44-1	TGTACATCAGTCACAGACCTG	508-528
CD44-2	GTGTCCATCTGATTCAGATCC	838-858
Antisense probes		
pV2	CCCAGGTTTCTTGCCTCTTGGTTG C	162-186
pV3	CCCAGCCTGCTGAGATGGTAT TTGA	264-288
pV4	TCCGGATTTGAATGGCTTGGGTTCC	439-463
pV5	AGGGGAGGGTGTGCTTCTGGGTTCC	536-560
pV6	CATGCCATCTGTTGCCAAACCACTG	663-687
pV8	TTTGGATTTGCAGTAGGCTGAAGCG	898-922
pV9	TGGTCTTTATCTTCTTCCAAGCCTT	1012-1036
pV10	CTTCGTGTGTGGGTAATGAGAGGTA	1139-1163
Sense probe		
pV7	GCCTCAGCTCATACCAGC CAT CCA	743-766

* The position of the PCR primers corresponds to the CD44 H cDNA sequence (6). The position of the antisense and sense probes corresponds to the variable exon V2-V10 cDNAs aligned in succession (see Fig. 2-1).

performed overnight either at 50°C for ³²P-labelled, variable exon-specific oligonucleotide probes or at 65°C for CD44 H or CD44 E cDNA probes. Following extensive washing in buffers containing 0.1% SDS and varying concentrations of SSC (1 x SSC to 0.1 x SSC), the membranes were subjected to autoradiography using Hyperfilm ECL (Amersham)

CD44 H and CD44 E cDNA clones were kindly provided by Dr. I. Stamenkovic (Harvard Medical School, Boston, MA), in the form of plasmids piH3M and CDM8, respectively. These plasmids were sub-cloned into the eukaryotic expression vector pRC/CMV (InVitrogen, San Diego, CA) and served as the source for cDNA probes and as positive controls (Fig. 2-1). Digestion of the CD44H plasmid with the restriction enzyme Pst I (Gibco BRL) generated a cDNA fragment of 827 bp containing sequences common to all CD44 isoforms and served as a probe for all variants. Digestion of the CD44E plasmid with Hinf I generated a cDNA fragment of 358 bp containing sequences from exons V8-V10 (Fig. 2-1) and was used as a probe for CD44 variants containing exon V8-V10. The β -actin probe used was a 661 bp RT-PCR product obtained from PHA stimulated PBMC using β -actin specific primers obtained from Stratagene, La Jolla, CA (187). β -actin RT-PCR using the same primer set was also used as a positive control to ensure the presence of RNA in each preparation (data not shown). Complementary DNA probes were ³²P-labelled by random priming (188,189) using the Rediprime DNA labeling system (Amersham) according to the manufacturers' protocol. CD44 variable exon-specific antisense oligonucleotide probes (see Fig. 2-1) were synthesized by National Biosciences, and hybridized to the CD44 RT-PCR products obtained. ³²P-end-labelling of oligonucleotide probes was accomplished by a standard kinasing reaction using T4 polynucleotide kinase

(190) according to the manufacturers' protocol (Gibco BRL). The sequence and specificity of each oligonucleotide probe are listed in Table 2-2.

2.070 Northern blot analysis

Total RNA was extracted with Tri Reagent solution, (MRC), as described above. Poly A⁺ RNA was isolated from 200 µg aliquots of total RNA using the PolyAtract™ mRNA isolation system IV kit (Promega Corporation, Madison, WI), as described by the manufacturer. Following denaturation with 2.2 M deionized formamide (Fisher Scientific, Pittsburgh, PA), poly A⁺ RNA was electrophoresed on a 1.2% MOPS-Formaldehyde agarose gel and transferred onto a Hybond-N (Amersham) nylon membrane. Hybridization was performed overnight at 65°C with ³²P-labelled cDNA probes, as described above.

2.080 Flow cytometry

Cells were analyzed by flow cytometry for the expression of CD44 isoforms containing exons V3, V4/V5, V6, V7, V7-V8, V9 and V10 expression. Briefly, cells (2 x 10⁵) were incubated with primary anti-CD44 monoclonal antibodies (mAbs) or isotype matched control antibodies (5 µg/ml in PBS containing 0.1% sodium azide for 10 min at room temperature). These antibodies included mouse anti-human CD44 V3, CD44 V4/V5, CD44 V6 (R&D Systems Inc.), mouse anti-human CD44 V7, CD44 V7-V8, CD44 V10 (Bender MedSystems, Vienna, Austria) and mouse anti-human CD44 V9 (kindly provided by Dr. M. Zöller, Heidelberg, Germany). These mAbs recognize isoforms containing the specified variable exons namely, V3*, (V4/V5)*, V6*, V7*, (V7-V8)*, V10* and V9*, respectively, according to the convention adopted in the thesis. Mouse IgG1, IgG2a, and

IgG2b isotype matched control antibodies (Sigma) were used in parallel. Following incubation, cells were washed once in PBS containing 0.1% sodium azide and incubated with 5 $\mu\text{g/mL}$ Fluorescein-DTAF-conjugated goat anti-mouse F(ab)₂ (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) for 10 min at room temperature. Total CD44 expression was analyzed by using anti-human CD44 mAbs Leu44 (a FITC-conjugated mAb; Becton Dickinson, San Jose, CA) or Ab-1 (an unconjugated mAb; Oncogene Science Inc. Manhasset, NY). Both mAbs recognize epitopes common to all CD44 isoforms. Staining with Ab-1 was followed by incubation with the FITC-DTAF secondary Ab, as above. Prior to staining, cell surface Fc receptors were blocked by incubation with aggregated human gamma globulins (5 mg/mL). Cells were analyzed with a FACScan (Becton Dickinson) flow cytometer. Median channel fluorescence values were used for comparison of CD44 expression levels. The overlay of flow cytometry histograms and the observation of a clear population shift in log fluorescence intensity were the parameters used to determine the significance of any changes in median channel fluorescence values. It should also be mentioned that monoclonal Abs to the remaining variable exons were not available commercially. As a control in studies using tunicamycin, treated cells were similarly stained and analyzed with the fluoresceinated RCA₁₂₀ lectin (20 $\mu\text{g/mL}$), purchased from E-Y Laboratories (San Mateo, CA). A reduction of RCA₁₂₀ staining indicated that tunicamycin treatment reduced N-linked glycosylation of cellular proteins, including that of CD44 (160).

2.090 Protein extraction, quantitation and Western blot analysis

Total crude cellular protein was obtained by detergent lysis of cell pellets. Briefly, cells were resuspended in a lysis buffer containing 10 mM Tris, pH 7.5, 50 mM NaCl, 50 mM NaF, 2 mM EDTA, 1 mM EGTA, 2% Nonidet-P40, 0.75% sodium deoxycholate, 1 mM sodium orthovanadate, 1mM phenylmethylsulfonyl fluoride (PMSF), 1mM dithiothreitol, 1 µg/mL aprotinin, and 1 µg/mL leupeptin (Sigma). Protein solubilization was conducted for 30 min on ice, followed by centrifugation for 20 min, 4°C, at 14000 x g. The supernatants were collected and their protein concentration assayed using a Micro BCA Protein assay reagent kit (Pierce, Rockford, IL). Cell lysates were mixed with an equal volume of 2X loading buffer containing 125 mM Tris, pH 6.8, 10% β-mercaptoethanol, 1% SDS, 20% glycerol, and bromophenol blue (Sigma). The samples (100 µg of total protein/well) were boiled for 5 min, placed on ice for 2 min, and subjected to electrophoresis through 10% polyacrylamide gels containing 0.1% SDS. Following electrophoresis, proteins were transferred to a Hybond C-extra nitrocellulose membrane (Amersham) using a semi-dry transfer apparatus (BioRad) in a buffer containing 50 mM Tris, 400 mM glycine (BioRad), 0.04% SDS, and 20% methanol. Membranes were blocked overnight at 4°C in PBS containing 0.1% Tween 20 (PBS-T) and 5% non-fat milk then washed three times each for 10 min in PBS-T. This was followed by incubation with primary anti-CD44 mAb 2C5 (R&D systems) at a concentration of 2 µg/mL in PBS-T containing 5% milk. The 2C5 mAb recognizes an epitope common to all CD44 isoforms. The membranes were washed three times in PBS-T followed by incubation with a polyclonal goat anti-mouse antibodies conjugated to horseradish peroxidase (Amersham) at a final dilution of 1:5000 for 45 min in PBS-T containing 5% milk. The blots were again washed three times in PBS-T and

subjected to color development using ECL Western blotting detection reagents and exposure to hyperfilm-ECL (Amersham).

2.100 Hyaluronan adhesion assay

CD44-mediated HA adhesion assays were performed essentially as described by Miyake et al. (41) with minor modifications. Briefly, stimulated cells were resuspended in IMDM-10 at 2×10^7 /mL and pulsed with ^{51}Cr (Sodium chromate, Amersham) at a concentration of $300 \mu\text{Ci}/10^6$ cells for 1.5 hrs at 37°C . The cells were washed three times with IMDM-10 and $100 \mu\text{L}/\text{well}$ (2×10^6 cells/mL) was distributed into 96 well microtitre plates (NUNC, Denmark) coated overnight with either human umbilical cord hyaluronan (Sigma) at 1 mg/mL, chondroitin sulfate A (Calbiochem-Novabiochem Corp.) at 1 mg/mL, chondroitin sulfate C (Calbiochem-Novabiochem Corp.) at 1 mg/mL, or with PBS containing 10% FBS as a negative control. Adhesion of cells to coated plates was conducted for 1.5 hrs at 37°C , 5% CO_2 and was followed by stringent washing six times with pre-warmed IMDM-10 to remove non-adherent cells. Adherent cells were lysed with 1N HCl followed by counting in a Microbeta scintillation counter (Wallac, Turku, Finland). The percentage of adherent cells was calculated from the generated counts as follows: Adherent cells (% cpm input) = $\text{adherent cell cpm} \div (\text{total input cpm} - \text{spontaneous release cpm}) \times 100$. HUT78, the CD44^+ T cell line with constitutive HA binding activity ($53.9 \pm 1.8\%$) was used as a positive control (98). BL30, the CD44^+ Burkitt's lymphoma cell line was used as a negative control with a background binding level of $5.1 \pm 1.0\%$ [see Chapter 4, Fig. 4-3 and ref. (126)]. Arbitrarily, only those values at least two fold above background levels were considered as significant in all adhesion experiments. In parallel

experiments, adhesion to CSA, CSC, and PBS-10 % FBS substrates was conducted and did not differ from the negative control under any of the conditions tested (data not shown).

CHAPTER THREE

REGULATION OF CD44-HA INTERACTIONS IN NORMAL HUMAN B LYMPHOCYTES

3.100 Introduction

Cell-cell and cell-ECM adhesion is strictly regulated during the development of an immune response, inflammation, tumor formation and metastasis. The factors that regulate these adhesive interactions may play a vital role in modulating the course of a disease process and the development of a normal immune response. Regulation of CD44 interactions with its ECM ligand HA has thus been suggested to play a vital role in processes such as lymphocyte homing, lymphopoiesis, T cell activation and metastasis (2,9-13,15,16). In humans, the role of CD44 isoform expression and HA recognition has been extensively investigated in T lymphocytes (12,36,96-101,105,106,162,191), NK cells (108-111) and cells of the monocyte/macrophage system (115,120-122). However, relatively little is known about the regulation of CD44-HA interactions and CD44 isoform expression on B cells (37,123,173,174), particularly normal human B cells (126,127).

Cells expressing CD44 H do not necessarily bind HA constitutively (10,11,152). CD44 must be “activated” in order to recognize HA (152). PMA and certain anti-CD44 mAbs have been shown to induce CD44-HA binding in murine T cells and T cell lymphomas (152,157). Furthermore, *in vitro* activation of human peripheral T cells via anti-CD3 antibodies, and culture of monocytes in the presence of human serum or stimulation with anti-CD3 antibodies or PHA triggers transient HA recognition (98,153). Extracellular matrix molecules and the cytokines present in the microenvironment may modulate CD44-HA interactions. In murine B cells, IL-5, and on human hematopoietic progenitor cells, IL-3 and GM-CSF, enhanced CD44 mediated binding of HA (174,179).

CD44 isoform expression is also elevated as a consequence of cell activation (10). *In vivo* Ag challenge induced the expression of CD44 V6* variants in rat T cells, B cells, and monocytes (37). Variant expression has since been studied in human leukocyte subpopulations (36,126,127,176). It was shown that V9* expression was detectable at very low levels in human T, B, monocytes, and granulocytes, but not thymocytes (176). In human T cells, CD44 V6* and V9* expression was transiently upregulated when subjected to various stimuli *in vitro*, including PHA, PMA, anti-CD3 Abs in the presence of IL-2, Ag, or a mixed lymphocyte reaction (36,176). Recently, TNF- α has been shown to upregulate CD44 expression on human endothelial cells (177). On myelomonocytic cell lines THP-1 and U937, TNF- α and IFN- γ modulated the expression of CD44 V6* and V9* isoforms (176). Stimulation of splenic B cells with anti-Ig antibodies differentially regulated CD44 H, and CD44 V6* expression (127).

Regulation of CD44 expression and CD44-HA interactions in human B cells is not understood. The focus of this part of the study was to investigate the effects of mitogens and cytokines, particularly those, which affect B cell activation, proliferation and differentiation, on CD44 isoform expression and CD44 mediated adhesion to HA in normal human B cells.

3.200 Results

3.210 Analysis of CD44-mediated HA recognition in resting and activated human B lymphocytes

To study how CD44-HA interactions are regulated in normal human B lymphocytes, highly purified B cells were subjected to a variety of stimuli for 30, 54 and 120 hrs and analyzed for the ability to adhere to HA by the ^{51}Cr -based cell adhesion assay.

3.211 PMA induces CD44-HA recognition in normal human B cells

To test the effect of mitogenic activation on HA adhesion, B cells were stimulated with the polyclonal activators LPS (10 $\mu\text{g/mL}$), PWM (1:50) or anti-IgM antibodies (20 $\mu\text{g/mL}$) in the presence or absence of IL-2 (100 U/mL) or IL-4 (40 ng/mL). Such stimulation failed to induce significant binding at any time point or concentration (Fig. 3-1 and data not shown). However, stimulation of B cells with PMA (20 ng/mL) resulted in strong adherence to HA ($3.8 \pm 0.6\%$ in unstimulated vs. $49.2 \pm 2.4\%$ in PMA stimulated cells; Fig.3-1). Fig. 3-1 and all subsequent figures, where indicated in the legend, show a representative experiment from at least three conducted, all from different donors ($n \geq 3$). The binding of PMA activated B cells to HA was dependent on the concentration of PMA used. At the highest concentration of PMA (25.6 ng/mL), $49.7 \pm 6.6\%$ of B cells bound to HA (Fig. 3-2A). Kinetics experiments revealed that adhesion of PMA stimulated B cells to HA was detectable by 6 hrs ($24.1 \pm 1.2\%$), reached a plateau by 12 hrs ($50.4 \pm 0.2\%$) and returned gradually towards background levels by 120 hrs post-stimulation ($10.8 \pm 1.6\%$;

Fig. 3-1: Adhesion of normal human B cells to hyaluronan

Highly purified B cells were stimulated with LPS (10 $\mu\text{g/mL}$), PMA (20 ng/mL), PWM (1:50) or anti-IgM antibodies (20 $\mu\text{g/mL}$) in the presence or absence of IL-2 (100 U/mL) or IL-4 (40 ng/mL) for 30 hrs. This was followed by measuring their adhesion to HA-coated plates in 96 well plates by the ^{51}Cr -based cell adhesion assay, as described in the Materials and Methods. B cell adhesion is reported as percentage of cpm input. The mean of triplicate adherent cell values $\pm$ standard deviation (S.D.) of one representative experiment from at least three conducted is shown.

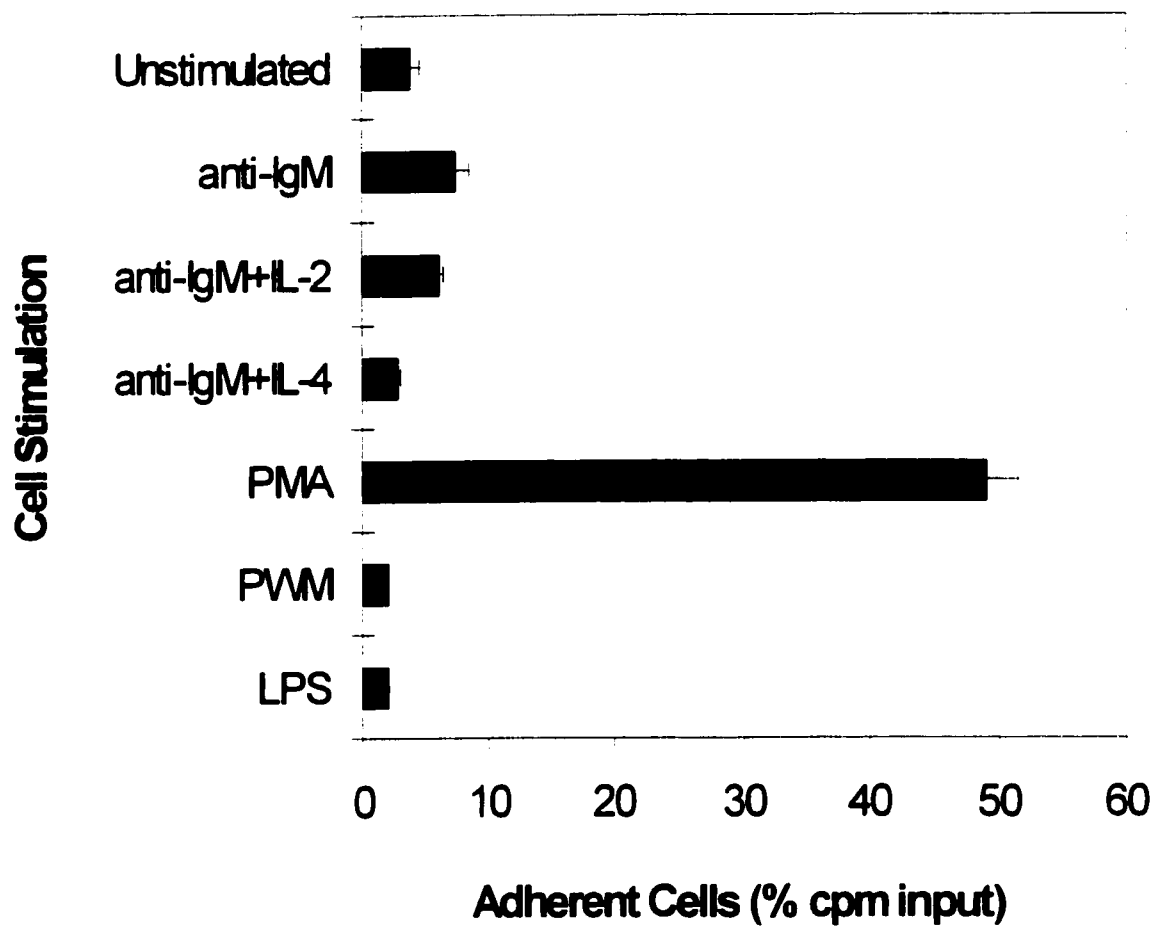


Fig. 3-2: Temporal and concentration dependent HA adhesion of human B cells in response to PMA

(A) HA adhesion in response to increasing concentration of PMA. Purified human B cells were stimulated with two fold increasing concentrations of PMA for 30 hrs. (B) Kinetics of PMA induced hyaluronan adhesion in B cells. Purified B cells were stimulated with optimal (6.4 ng/mL) concentrations of PMA for 2-120 hrs. (C) Neutralization of PMA-induced hyaluronan adhesion of B cells by anti-CD44 monoclonal antibodies (Ab-1). Purified B cells were stimulated with 6.4 ng/mL of PMA in the presence or absence of 20 µg/mL of anti-CD44 antibodies. Isotype control antibodies were used in parallel. B cell adhesion experiments were performed as described in the legend to Fig. 3-1 and in Materials and Methods. B cell adhesion is reported as percentage of cpm input. The mean of triplicate adherent cell values $\pm$ S.D. of one representative experiment from at least three conducted is shown.

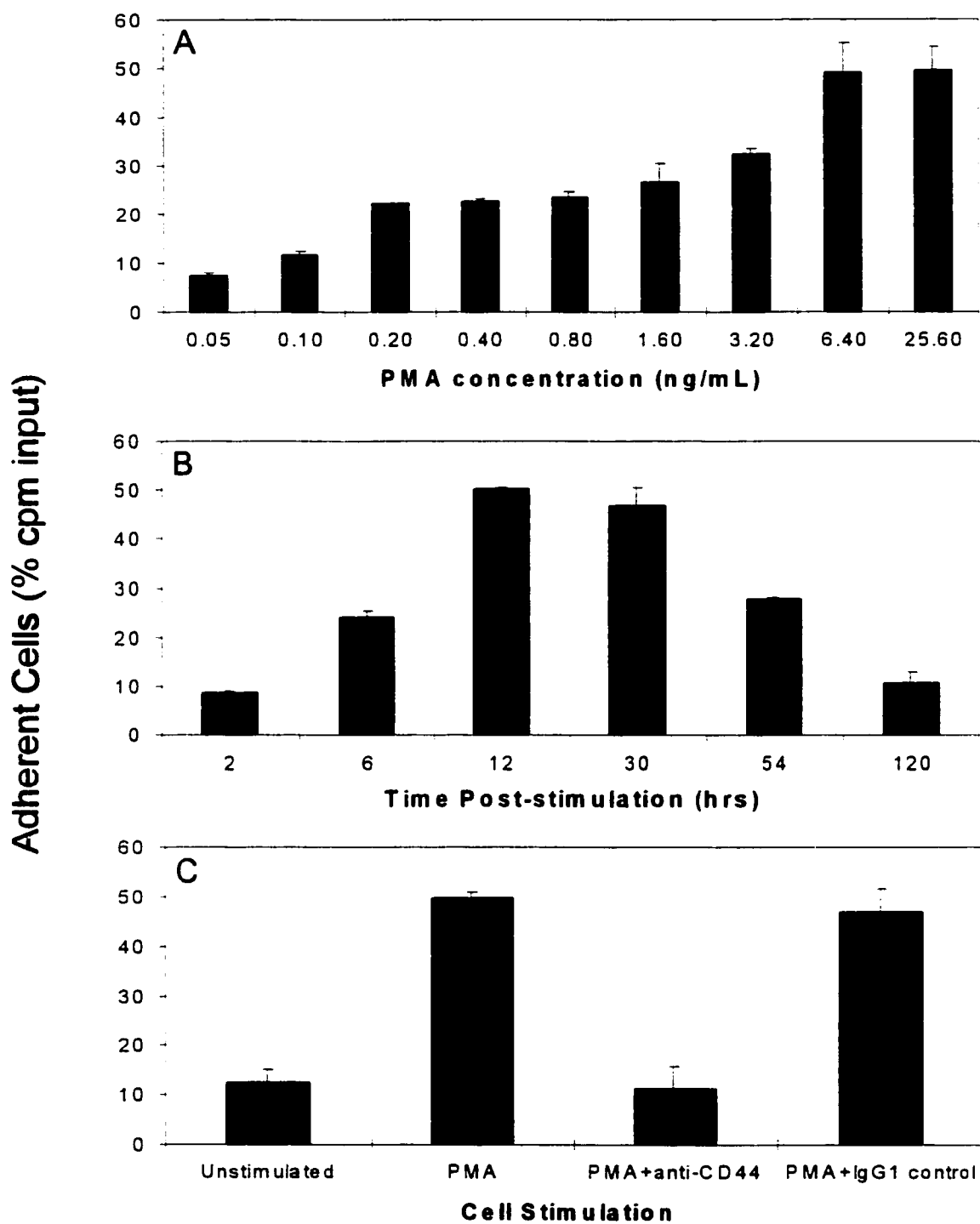


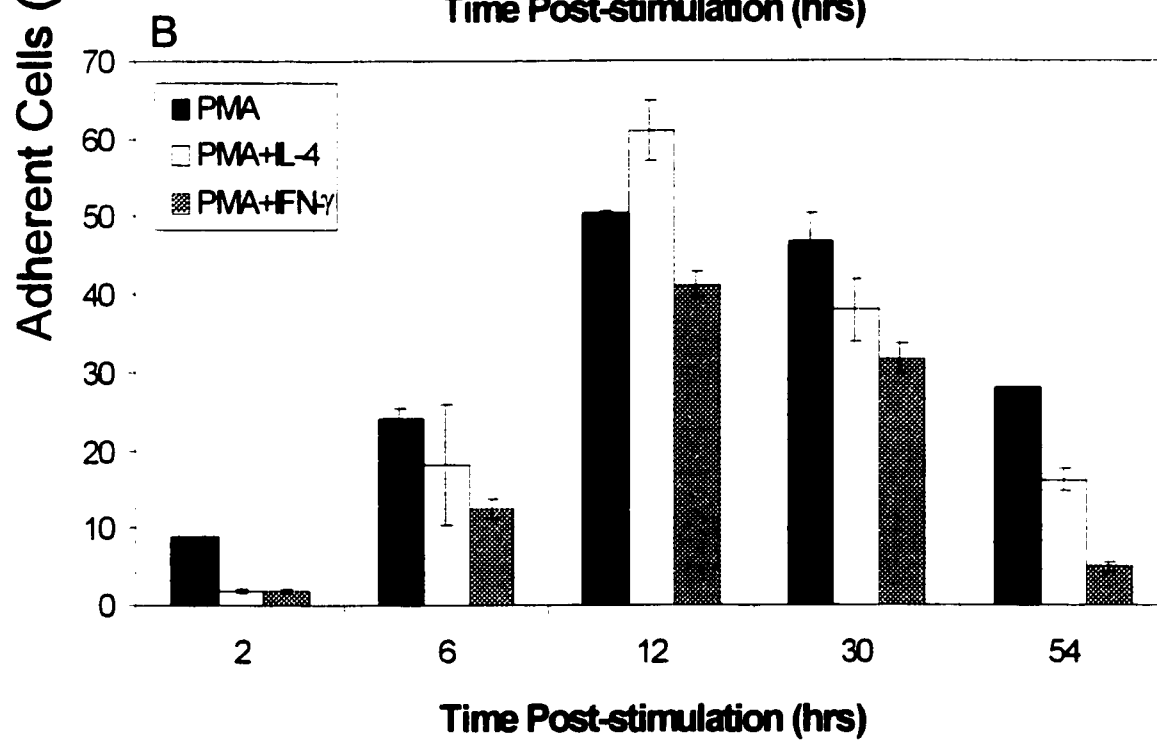
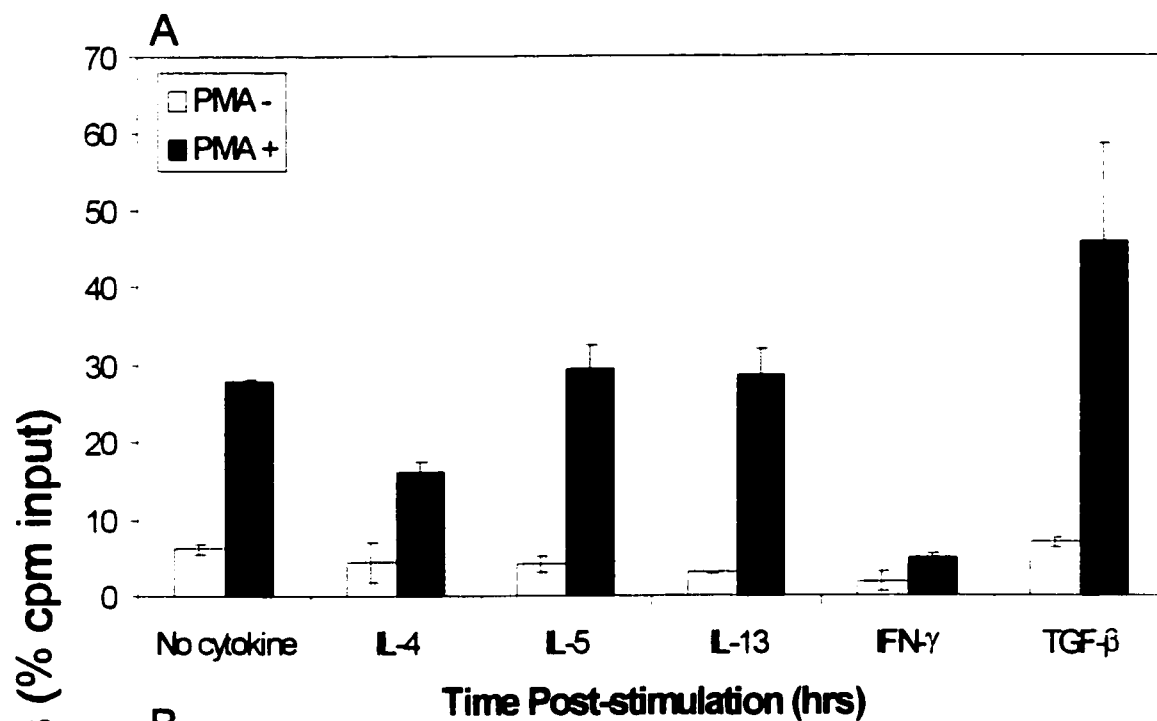
Fig. 3-2B). To confirm that the binding of B cells was mediated through the CD44 molecule, PMA stimulated B cells were incubated with anti-CD44 antibodies prior to incubation with HA. Treatment of B cells with anti-CD44 antibodies abrogated binding of B cells to HA whereas isotype matched control antibodies did not affect binding (Fig 3-2C). These results suggest that amongst the mitogens, PMA alone induced CD44-mediated HA binding.

3.212 IFN- γ and IL-4 inhibit PMA-induced HA recognition in B cells

To study the regulation of CD44-mediated HA adhesion in B cells by cytokines, cells were stimulated with a panel of cytokines that influence B cell activation, proliferation or differentiation, in the presence or absence of PMA, for 30, 54 and 120 hrs. The cytokines tested included IL-1 α , IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IL-13, TNF- α , IFN- γ , TGF- α and TGF- β and were used at the concentrations ranges listed in Materials and Methods. Amongst these cytokines, only IL-4 and IFN- γ , both B cell differentiation factors, significantly affected HA recognition in response to PMA (Fig. 3-3A). PMA (6.4 ng/mL) alone induced 27.9 ± 0.1 % of cells to adhere to HA while simultaneous treatment with PMA and IL-4 or IFN- γ reduced the percentage of adherent cells to 16.1 ± 1.4 % and 4.8 ± 0.6 %, respectively. Inhibition of HA recognition by IFN- γ was striking as it reduced HA binding to the levels observed with unstimulated cells (6.2 ± 0.6 %; Fig. 3-3A). However, in the case of IL-4, only a partial inhibition was observed. The same trend was observed when sub-optimal concentrations of PMA (1.6 ng/mL) were used (data not shown). Furthermore, significant inhibitory effects of IL-4 were only observed after 54 hrs

Fig. 3-3 Effect of cytokines on B cell-HA adhesion

(A) Inhibition of PMA-induced hyaluronan adhesion of B cells by IFN- γ and IL-4. Purified B cells were stimulated with 6.4 ng/mL of PMA for 54 hrs in the presence of IL-4 (40 ng/mL), IL-13 (40 ng/mL), IL-5 (15 ng/mL), IFN- γ (50 ng/mL) and TGF- β (5 ng/mL). The cells were also cultured in the presence of other cytokines including IL-1 α (10 ng/mL), IL-1 β (1 ng/mL), IL-2 (100 U/mL), IL-6 (4 ng/mL), IL-10 (37.5 U/mL), IL-12 (1.5 ng/mL), TNF- α (10 ng/mL) and TGF- α (100 ng/mL), all of which failed to influence HA binding (data not shown). **(B)** Kinetics of IFN- γ and IL-4 mediated inhibition of PMA induced B cell binding to HA. Purified B cells stimulated with 6.4 ng/mL of PMA were cultured in the presence of IFN- γ (50 ng/mL) or IL-4 (40 ng/mL) for 2, 6, 12, 30 and 54 hrs. B cell adhesion experiments were performed as described in the legend to Fig. 3-1 and in Materials and Methods. B cell adhesion is reported as the percentage of cpm input. The mean of triplicate adherent cell values $\pm$ S.D. of one representative experiment from at least three conducted is shown.



of stimulation. Contrarily, the inhibitory effects of IFN- γ were discernible as early as 6 hrs post-stimulation and by 54 hrs, the levels of binding to HA were comparable to unstimulated cells (Fig. 3-3B).

IL-5, another B cell differentiation cytokine previously shown to induce HA recognition in murine B cells (174), did not influence HA recognition either in unstimulated or PMA stimulated human B cells (Fig. 3-3A). Of particular note, IL-13, which shares the IL-4 α and the common γ_c components of its receptor with IL-4 (192,193), also failed to show the same inhibitory effects on HA recognition as that observed with IL-4 (Fig. 3-3A). Furthermore, stimulation of B cells with the cytokines mentioned above including IL-4 and IFN- γ in the absence of PMA did not affect adhesion to HA when compared to unstimulated cells (Fig. 3-3A). These results suggest that IFN- γ and IL-4 are not only potent B cell differentiation cytokines, but are also strong inhibitors of PMA-induced CD44-mediated HA recognition in human B cells.

3.220 Analysis of CD44 isoform expression in human B lymphocytes

Multiple mechanisms have been proposed to explain the induction of CD44 mediated binding to HA such as the induction of novel CD44 isoform(s), enhanced CD44 isoform expression levels, and/or alterations in the glycosylation pattern of CD44 (10,159,160,162,174). Therefore, first of all I investigated the expression of CD44 isoforms in normal human B cells under various conditions, focusing particularly on those stimuli that induced alterations in CD44-HA adhesion (PMA in the presence or absence of IL-4 or IFN- γ).

CD44 protein expression levels were evaluated by flow cytometry using antibodies specific for particular variable exons and antibodies recognizing an epitope common to all isoforms. One of the limitations of flow cytometric analysis however, was that the specific combination of V exons contained within a particular isoform could not be distinguished. It could only be ascertained whether or not and to what extent a given exon was expressed at the protein level. Therefore, RT-PCR analysis was undertaken in an attempt to resolve the identity of the variant mRNAs expressed. Total RNA extracted from stimulated B cells was reverse transcribed and amplified by RT-PCR using primers CD44-1 and CD44-2 (see Table 2-2). These primers, spanning the variable region of the CD44 extracellular domain (Fig. 2-1), give rise to amplicons of different lengths, based on the isoform present in the reaction mixture such as CD44 H (351 bp), and CD44 E (747 bp). The detection of products of different sizes indicated the insertion of a different set of variable exons into the extracellular domain. PCR products were subjected to Southern transfer and hybridization to CD44 specific probes (see Fig. 2-1).

3.221 Stimulation by cross-linking surface immunoglobulin or with PMA induces CD44 isoform expression at the protein and mRNA levels

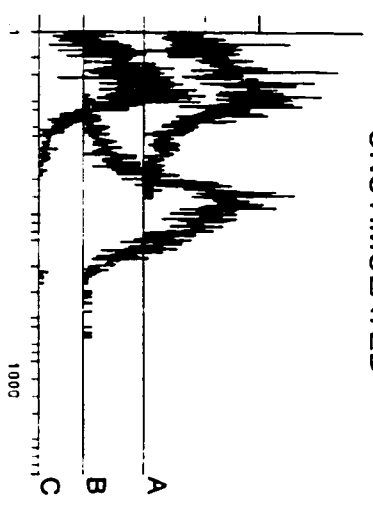
Investigation of CD44 isoform expression in human B cells (126) by flow cytometry (Fig. 3-4) showed that unstimulated B cells express an abundance of CD44, as revealed by strong reactivity with mAb Ab-1. This mAb recognizes an epitope common to all CD44 isoforms but reflects CD44 H expression mainly, the predominant isoform found in leukocytes (10). Since CD44 V6,V7^{*} isoforms have been implicated in B and T cell activation while V6^{*} and V9^{*} may be important in the adhesion of activated T cells to HA

Fig. 3-4: Measurement of total CD44 and CD44 V6⁺ on activated human B cells by flow cytometric analysis

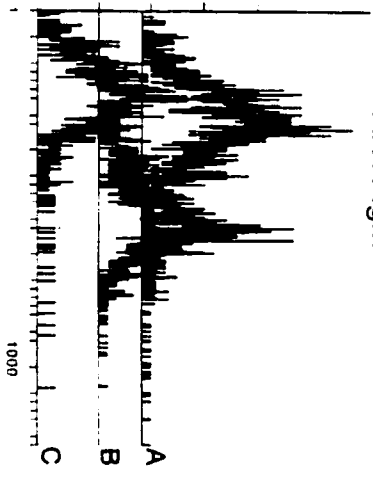
Purified B cells were stimulated with anti-IgM beads (20 µg/mL), anti-IgM beads and IL-2 (100 U/mL) or PMA (20 ng/mL) for 30 hrs, as described in Materials and Methods. The cells were subsequently analyzed for Total CD44 and V6⁺ expression. The cells were stained by indirect immunofluorescence using (A) mAb VFF-7 (specific for exon V6), (B) Ab-1 (specific for a CD44 epitope common to all CD44 isoforms and (C) an isotype control mAb. Pancreatic adenocarcinoma cells, L3.4, were used as positive controls.

Relative Cell Number

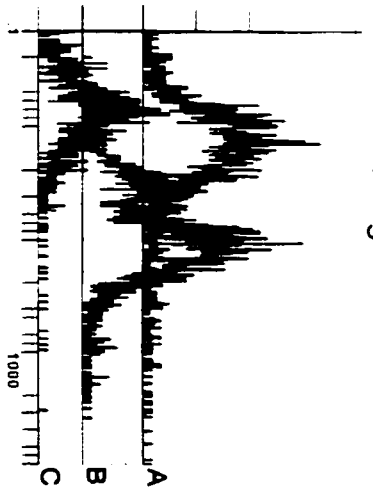
UNSTIMULATED



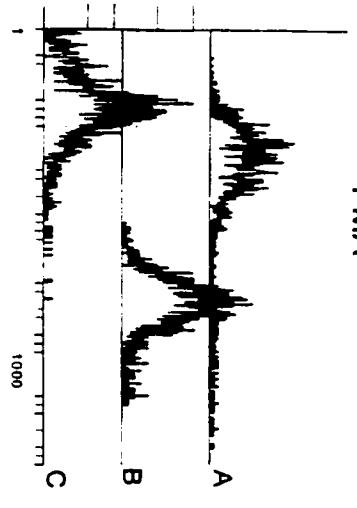
ANTI-IgM



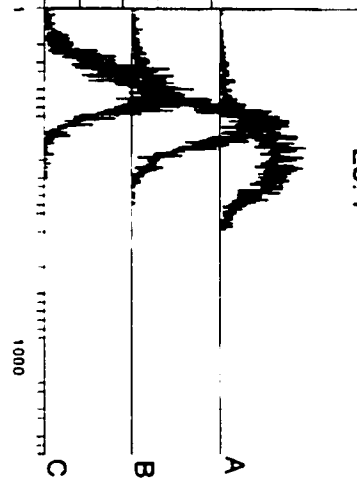
ANTI-IgM + IL-2



PMA



L3.4



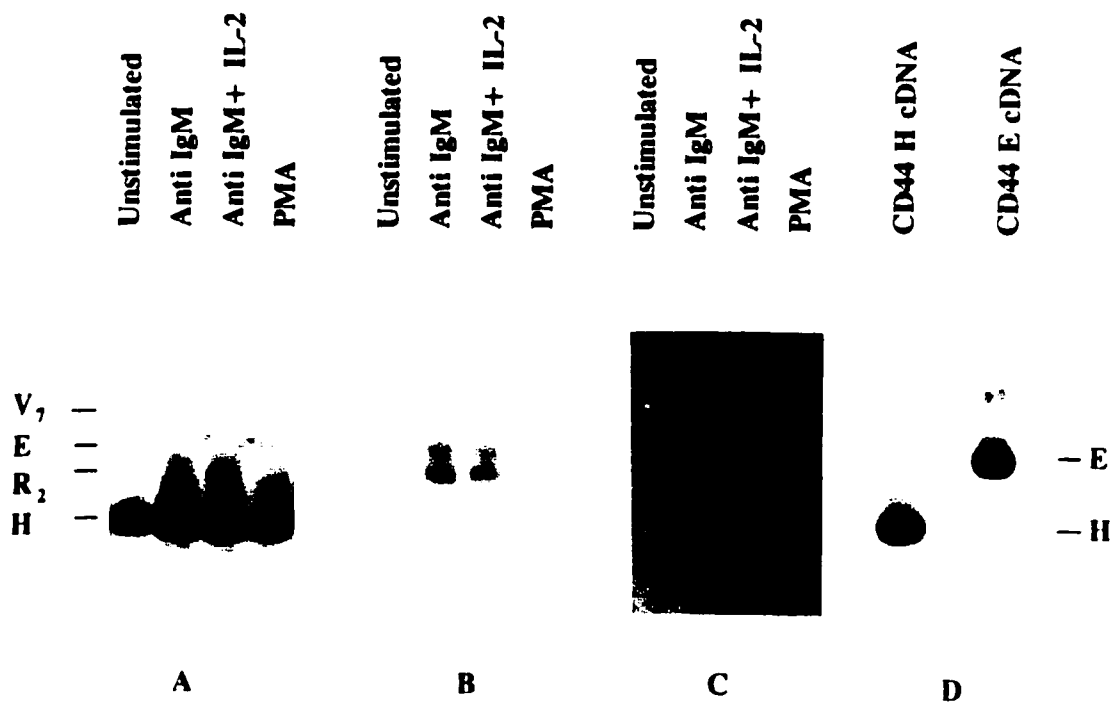
Log Fluorescence Intensity

(37,162), initial experiments focussed on investigating their expression. CD44 V6^{*} isoforms were undetectable by the V6^{*} specific mAb VFF7 in unstimulated cells. Stimulation for 30 hrs with anti-IgM antibodies (in the presence or absence of IL-2), or with PMA induced the expression of CD44 V6^{*} and enhanced the reactivity with mAb Ab-1.

To examine CD44 isoform expression in more detail RT-PCR analysis was carried out and demonstrated that activated B cells express a number of CD44 variants (Fig. 3-5). Probing with the H cDNA revealed the predominance of the CD44 H isoform (350 bp; Fig. 3-5A) in both resting and activated B cells. Hybridization with the E cDNA (V8-V10; Fig. 3-5B) detected two bands of approximately 720 and 550 bp and which were estimated to correspond to CD44 E (V8-10) and R2 (V10) isoforms. V7 (Fig. 3-5C) probing revealed two main species of approximately 800 and 600 bp in size. CD44 H and E cDNAs were used as controls. The isoforms described were detected following stimulation with PMA or anti-IgM (in the presence or absence of IL-2) and not in unstimulated cells. The signals were however of lower intensity in PMA-stimulated cells. This may be due to variations in the kinetics of CD44 V isoform induction upon stimulation with these two mitogens, but the fact that this assay is not quantitative suggests that an interpretation of the level of expression is tenuous at best. These results suggested that the expression of CD44 V7^{*}, CD44 V8-10 (E) and CD44 V10 (R2) is induced following mitogenic activation of B cells (126).

Fig. 3-5: Measurement of CD44 isoform expression on activated human B cells by RT-PCR

Resting human B cells were stimulated with anti-IgM beads (20 µg/mL), anti-IgM beads and IL-2 (100 U/mL) or PMA (20 ng/mL) for 30 hrs as described in the Materials and Methods. Total RNA was amplified with primers CD44-1 and CD44-2 followed by Southern blotting. The same filter was sequentially hybridized with pV7 (C), the E probe (B) and the H probe (A). CD44 H and CD44 E cDNAs were used as positive controls (D).



3.222 Effect of PMA, IL-4 and IFN- γ on protein expression of CD44 isoforms

In order to test whether CD44 isoform expression was indeed associated with HA binding, I conducted a detailed analysis of their expression in B cells stimulated with PMA in the presence or absence of IL-4 or IFN- γ , as only these factors affected adhesion of B cells to HA. Unstimulated and PMA stimulated B cells expressed low to undetectable levels of CD44 V^{*} isoforms. Therefore, superimposition of flow cytometry histograms and the observation of a cell population shift in log fluorescence (see Fig. 3-7) were the determinants of significance in these cases. Stimulation of B cells with PMA significantly upregulated total CD44 expression (MCF numbers increased from 89.8 in unstimulated cells to 294.3 in PMA stimulated B cells; Fig. 3-6A). In parallel with adhesion data, IFN- γ and IL-4 significantly inhibited PMA-induced total CD44 or CD44 H expression (Fig. 3-6A; MCF values of 294.3 in PMA stimulated cells versus 171.5 and 212.9 in IFN- γ and IL-4 treated cells, respectively). Similarly, as seen in Fig. 3-4, as compared to unstimulated cells, a clear upregulation of CD44 V6^{*} isoform expression was observed in PMA stimulated cells (Fig. 3-6B, 3-7). Although a similar upregulatory trend was observed for CD44 V3^{*}, V7-8^{*}, and V9^{*} expression (Fig. 3-6B), a clear population shift was not readily detectable (Fig. 3-7 and data not shown) and therefore was not deemed significant. Differences at the level of CD44 V4/5^{*}, V7^{*}, or V10^{*} expression were also insignificant (Fig. 3-6B). Analysis of CD44 isoform expression on PMA stimulated B cells following exposure to IL-4 and IFN- γ yielded contrasting results, however. Contrary to the inhibition of total CD44 expression, the expression of CD44 V6^{*} increased according to MCF values (Fig. 3-6C). A clear population shift was not easily discernible in this case and therefore not deemed significant (Fig. 3-7). Changes in the expression of other isoforms were also insignificant. Contrarily,

Fig. 3-6: Effect of IFN- γ and IL-4 on PMA-induced expression of CD44 H and CD44 V' isoforms on B cells by flow cytometric analysis

Purified B cells stimulated with 6.4 ng/mL of PMA were cultured in the presence or absence of IFN- γ (50 ng/mL) or IL-4 (40 ng/mL) for 54 hrs. The cells were analyzed for the expression of (A) Total CD44; (B) CD44 V' isoforms following stimulation of B cells with PMA alone. (C) CD44 V' isoforms following stimulation of B cells with PMA in the presence or absence of IL-4 or IFN- γ . As indicated in Materials and Methods, CD44 V' exon specific mAbs or isotype matched control mAbs were used. Median channel fluorescence (MCF) was used to compare the expression of CD44 isoforms. The results shown are a representative of at least three experiments performed.

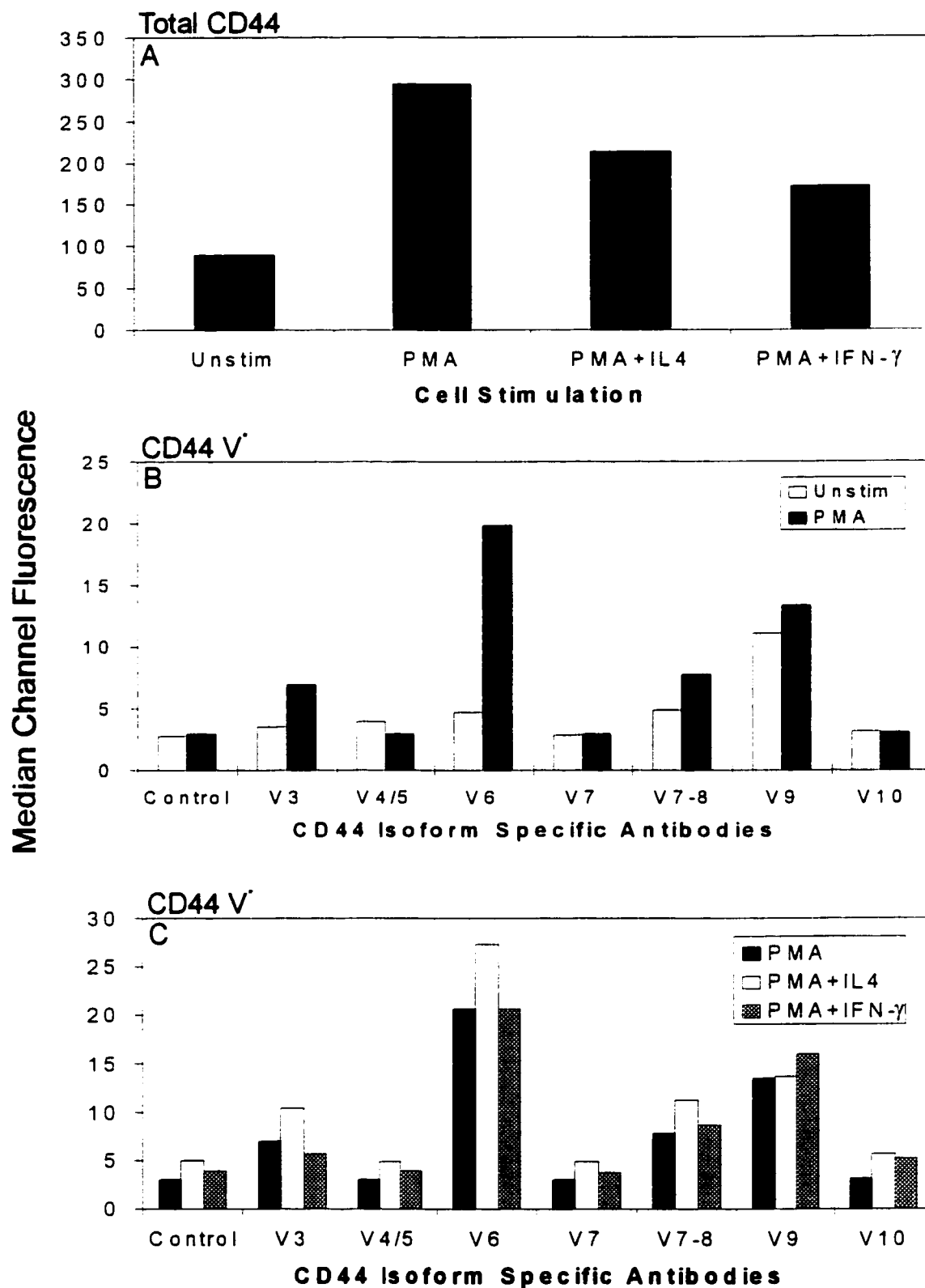
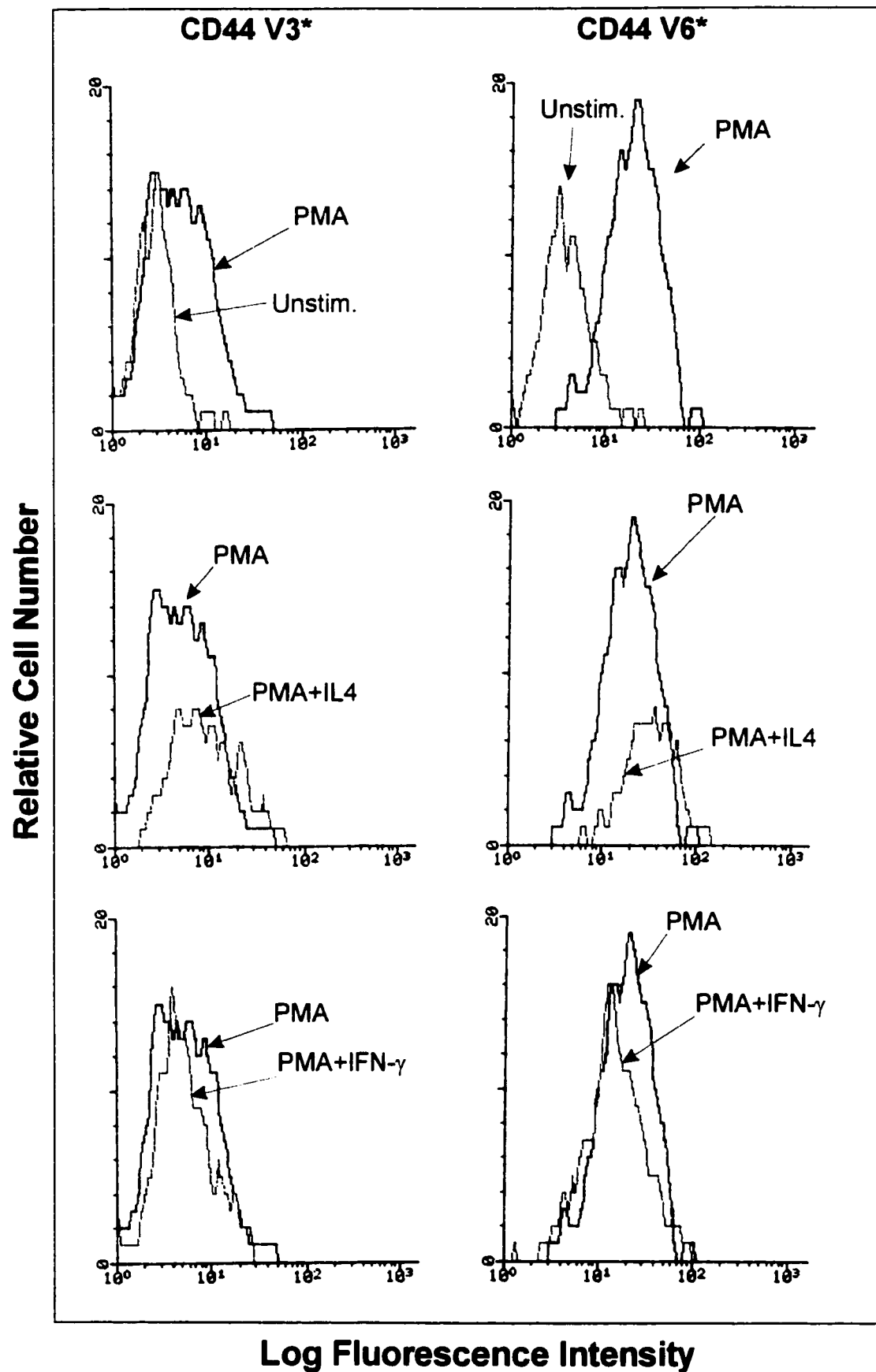


Fig. 3-7: Modulation of CD44 isoform expression in normal human B cells by PMA, IL-4, and IFN- γ (representative histogram overlays)

B cells were stimulated with PMA (6.4 ng/mL) in the presence or absence of IL-4 (40 ng/mL), or IFN- γ (50 ng/mL). Using mAbs specific for the respective variable exons, CD44 V3' and V6' expression was compared by flow cytometry, as described in the legend to Fig. 3-6. The results shown are a representative of at least three separate experiments.



a downregulatory trend was observed for CD44 V6⁺ expression following stimulation with IFN- γ but again, based on our criteria, was not deemed significant. IFN- γ did not influence the expression of any of the other CD44 V⁺ isoforms (Fig. 3-6C, 3-7). Interestingly, IL-13 neither alone nor with PMA was able to significantly affect the expression of any CD44 isoform in B cells (data not shown). These results suggest that IL-4 and IFN- γ inhibit the expression of CD44 H on PMA stimulated B cells. These cytokines seemed to exert a contrasting influence on the expression of CD44 V6⁺ but this effect was not considered significant or at best could be scored as a redistribution of the isoform(s) on the cell surface.

3.223 Differential CD44 V mRNA induction by PMA, IL-4, and IFN- γ

The results described above suggested that stimulation of B cells with mitogens and cytokines induced a complex pattern of CD44 isoforms both at the protein and RNA levels. In order to establish their identity and verify whether differential utilization of a particular V exon could be correlated with HA binding, or its inhibition, it was necessary to evaluate the expression of each variable exon using the V2-V10 exon specific oligonucleotide probes depicted in Fig. 2-1. Therefore, in most cases, based on the size of the PCR product and its detection with a particular set of V exon probes, the identity of the isoform expressed could be resolved. As only PMA in the presence or absence of IL-4 or IFN- γ affected adhesion of B cells to HA, further investigation of CD44 isoform expression by RT-PCR concentrated on these stimuli. IL-5, which had no effect on adhesion, was used as a control. CD44 RT-PCR products from normal human B cells activated with PMA in the presence or absence of IL-4, IL-5, or IFN- γ for 4 hrs were subjected to sequential hybridization with

oligonucleotide probes specific for individual exons V2-V10. The results shown in Fig. 3-8 reveal that normal human B cells express an extensive pattern of CD44 V isoforms.

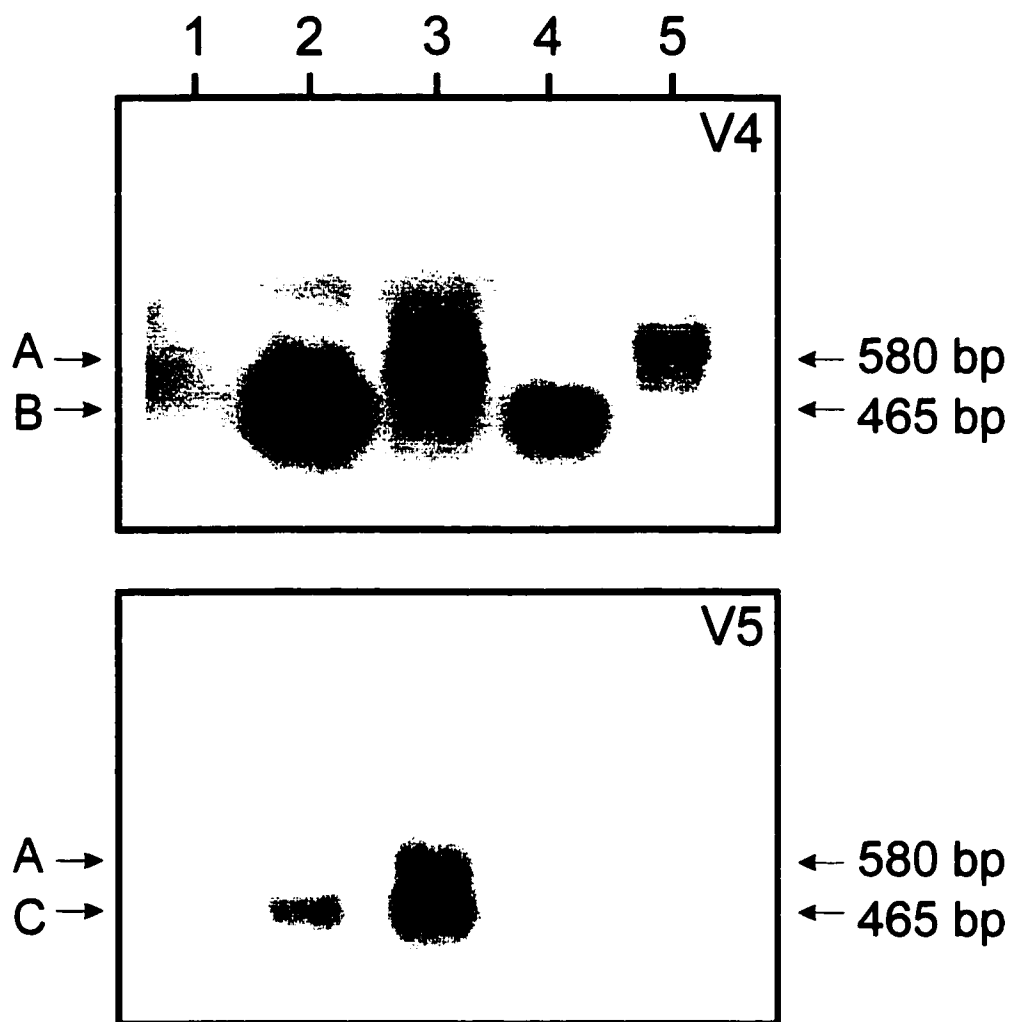
Probing of PCR amplified products with V4 and V5 specific oligonucleotides revealed that PMA stimulation alone induced the expression of CD44 V4 (465 bp, band B) and V5 (465 bp, band C) containing isoforms, respectively (lane 2, Fig. 3-8, Panel A). Addition of IL-4 (lane 3) or IFN- γ (lane 5) resulted in the induction of a higher molecular weight isoform containing V4 and V5 exons (band A, 580 bp). The molecular weight of bands B and C (465 bp) indicate that they may represent CD44 V4 and CD44 V5 single exon-containing isoforms, whereas band A (580 bp) may represent an isoform containing both V4 and V5 exons (V4-5). Induction of the CD44 V4-5 isoform was not observed in response to IL-5 (lane 4) and the presence of V4 or V5 exons was not detected in unstimulated B cells (lane 1).

However, probing with V2, V3, V6, V7, V8, V9 or V10 specific probes did not reveal similar fluctuations in the splicing pattern of these exons. Probing with V3 and V10 oligomers (Fig. 3-8, Panel B) revealed that the expression of CD44 V3 (band D, 480 bp) and CD44 V10 (band E, 540 bp) was strongly induced by PMA and maintained in the presence of IL-4, IL-5 and IFN- γ (lanes 2-5). V10 and V3 expression in unstimulated cells was low or undetectable, respectively (lane 1).

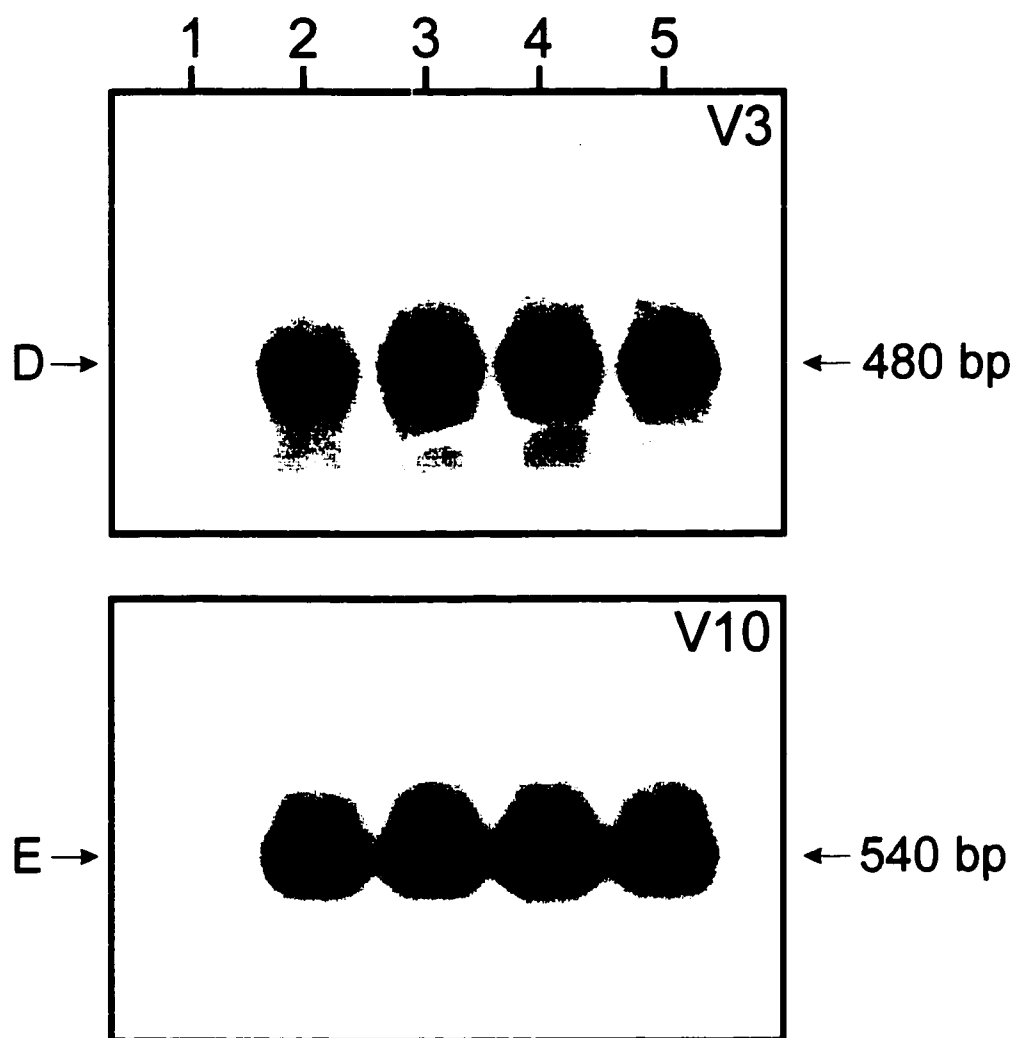
A V2 containing isoform was detected as a 560 bp product (band F, lanes 2-5) only in stimulated cells (Fig. 3-8, Panel C). Based on its molecular weight this corresponded to an isoform with V2 and one additional exon, whose identity could not be ascertained in these experiments. Two main species were identified in stimulated cells only following probing with V7 oligomers at 800 and 600 bp, bands G and J, respectively. Band J appeared

Fig. 3-8: RT-PCR analysis of CD44 isoforms containing variable exons induced in normal human B cells by PMA, IL-4, IL-5, and IFN- γ

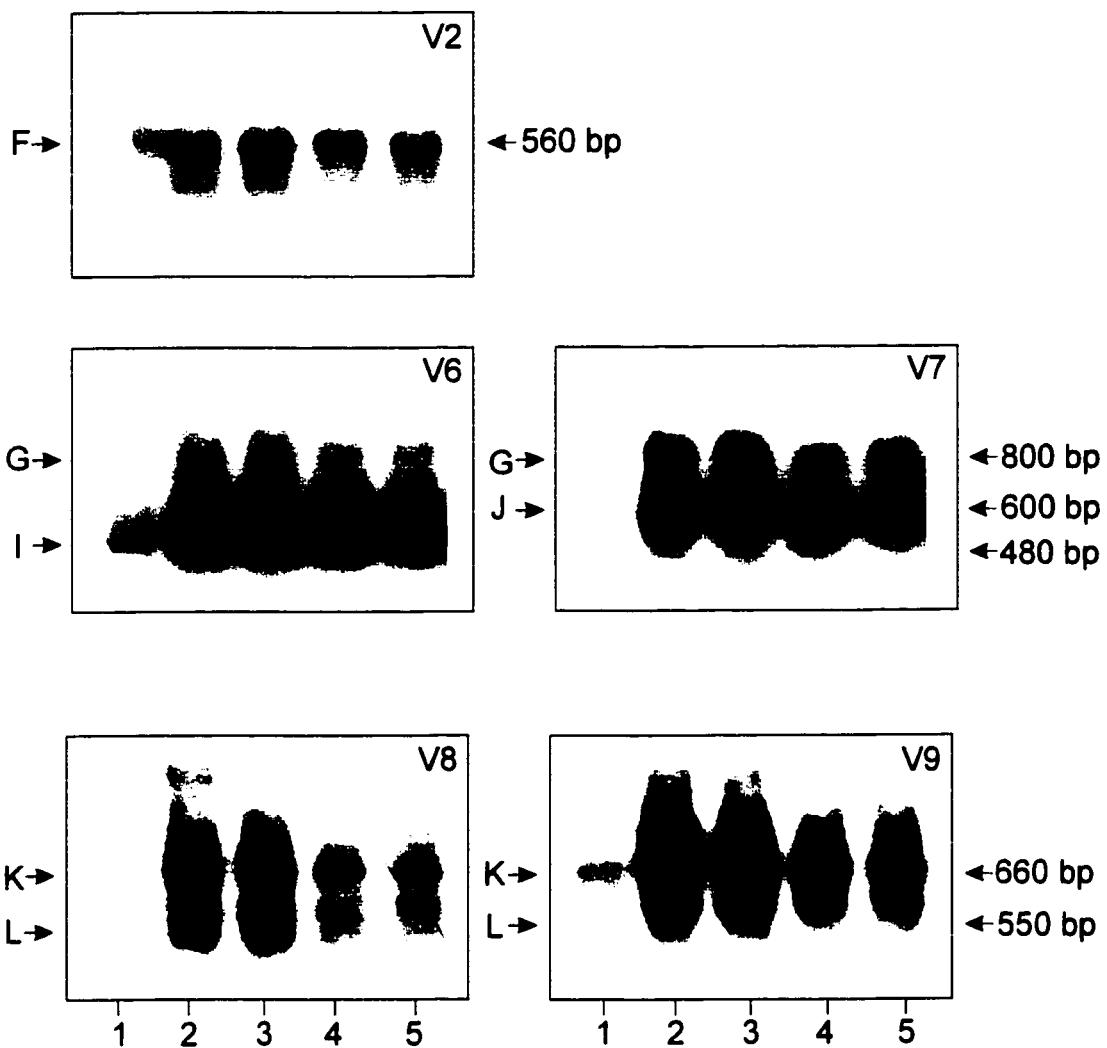
Purified B cells were stimulated with PMA (6.4 ng/mL) in the presence or absence of IL-4 (40 ng/mL), IL-5 (15 ng/mL) or IFN- γ (50 ng/mL). Total RNA was amplified by RT-PCR using primers CD44-1 and CD44-2 and probed with V4 and V5 (**Panel A**), V3, V10 (**Panel B**) and V2, V6, V7, V8, and V9 (**Panel C**) specific oligonucleotide probes. Lanes 1-5 are as follows: 1, unstimulated cells; 2, PMA stimulated cells; 3, PMA and IL-4 stimulated cells; 4, PMA and IL-5 stimulated cells; 5, PMA and IFN- γ stimulated cells.



Panel A



Panel B



Panel C

to represent an isoform containing exon V7 and one other unascertained exon. A product of the same size as band G (800 bp) was also detected with the V6 probe indicating that this exon may contain exon V6, V7 and two other unrevealed exons. Band I of 480 bp corresponded to CD44 V6 and was expressed at low levels in unstimulated cells and was strongly upregulated upon stimulation. A product of approximately 660 bp (band K, lanes 1-5) was detected following hybridization with both V8 and V9 probes, potentially identifying an isoform containing V8, V9 and one other exon that did not resolve in these experiments. In addition, a smaller product (band L, 550bp) appeared to correspond, based on its size, to the CD44 V8-9 isoform. Both V8 and V9 containing isoforms were detected at a low level in unstimulated cells and were strongly induced following stimulation.

In summary, it is clear that activated B cells express a large number of CD44 V isoforms. They include variants containing only one variable exon namely, CD44 V3, CD44 V4, CD44 V5, CD44 V6, and CD44 V10. Variants containing two variable exons are also induced and potentially include CD44 V4-5, and CD44 V8-9. In addition, variants not completely resolved in these experiments include CD44 V2,w and those containing 3 and 4 V exons include CD44 x,V8-9 and CD44 V6-7,y,z, respectively, where w, x, y, and z represent variable exons of unknown identity. Their identification could be achieved using a nested primer and/or sequencing approach but was not pursued here. It should be noted that the isoform initially thought to be CD44 E (V8-10) (see Fig. 3-5, section 3.221) turned out not to contain V10 and identified as CD44 x,V8-9. In contrast to the other exons, V4 and V5 are differentially included into CD44 mRNAs in response to IL-4 and IFN- γ . This differential splicing paralleled the ability of these cytokines to inhibit PMA-induced HA

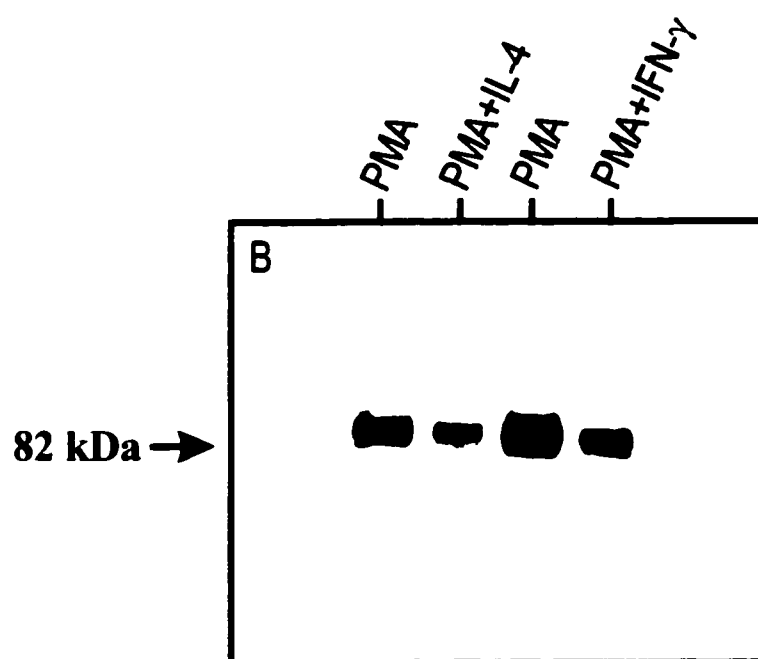
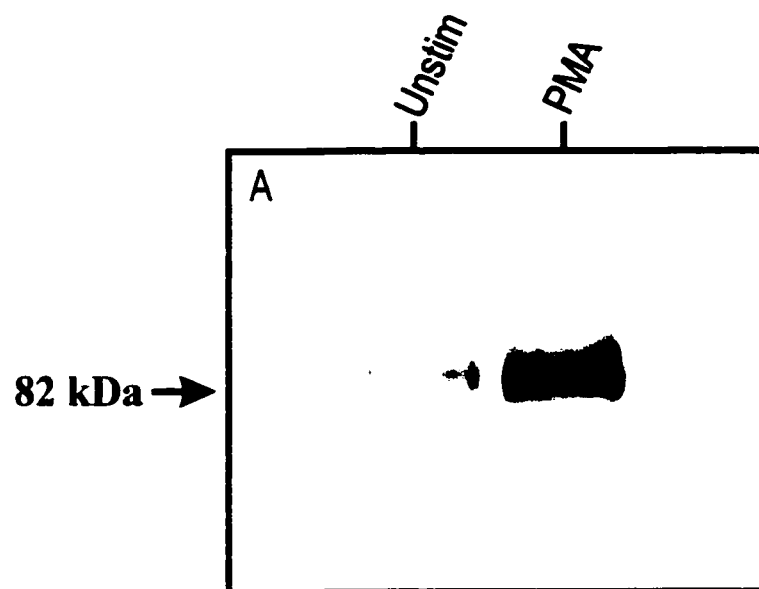
recognition in these cells. Finally, unstimulated cells generally expressed little or no V isoforms.

3.230 Evaluation of potential post-translational modifications to CD44 H in response to PMA, IL-4 and IFN- γ stimulation

As mentioned, the mechanism by which PMA, IL-4 and IFN- γ exert their effect on HA recognition may, at least in part, involve post-translational modification of CD44. This was investigated by comparing electrophoretic mobilities of CD44 proteins obtained from unstimulated B cells and those stimulated with PMA in the presence of either IL-4 or IFN- γ for 54 hrs by Western blot analysis. In addition to enhancing CD44 protein expression, PMA stimulation of B cells generated CD44 proteins with slightly lower molecular weights in comparison to unstimulated cells (Fig. 3-9A). These differences in molecular weight may result from a reduction in the glycosylation of CD44 proteins in response to PMA and correlates with the induction of CD44-HA recognition. IL-4 and IFN- γ however, did not induce detectable changes in the molecular weight of CD44 but did reduce overall expression levels as also observed by flow cytometry (Fig. 3-9B, 3-6A).

Fig. 3-9: Western blot analysis of the effect of PMA, IFN- γ , and IL-4 on the electrophoretic mobility of CD44 on B cells

Purified B cells were stimulated with PMA (6.4 ng/mL) in the presence or absence of IFN- γ (50 ng/mL) or IL-4 (40 ng/mL) for 54 hrs. Equal amounts of total cellular protein (100 μ g/well) were analyzed by Western blot analysis for CD44 expression and molecular size using mAb 2C5. The 82 kDa molecular weight marker is indicated by an arrow and lane identification is as follows: **(A)** *Unstim*, Unstimulated B cells; *PMA*, PMA (6.4 ng/mL)-stimulated B cells. **(B)** *PMA*, PMA-stimulated B cells; *PMA + IL-4*, B cells stimulated with PMA and IL-4. (Experiment # 1); *PMA*, PMA-stimulated B cells; *PMA + IFN- γ* , B cells stimulated with PMA and IFN- γ (Experiment # 2).



CHAPTER FOUR

REGULATION OF CD44-HA INTERACTIONS IN BURKITT'S LYMPHOMA AND EPSTEIN BARR VIRUS-TRANSFORMED LYMPHOBLASTOID B CELLS

4.100 Introduction

As mentioned, CD44, in many instances must be “activated” in order to recognize its ECM ligand HA (2,152). Mitogenic activation of cells or stimulation with cytokines can induce or inhibit CD44 activation. A role for the mitogen PMA and cytokines IFN- γ and IL-4 in this was established in normal peripheral blood B cells (chapter 3). TNF- α has been shown to enhance binding of human monocytes to HA and this binding was inhibited by IL-4 and IL-13 (178). In murine B cells, IL-5 enhanced CD44-mediated binding of HA (174). These observations indicate that recognition of HA by CD44 and its modulation by cytokines is dependent on cell type and mode of cellular activation.

CD44 isoform expression is also subject to regulation by cell activation and differentiation phenomena (10). As mentioned earlier, *in vivo* and *in vitro* activation induced the expression of CD44 V6* and V9* variants in rat and human lymphocytes (36,37,176). Their expression was further shown to be crucial in the activation of both T and B cells (37). In studies described in chapter 3, it was demonstrated that activation of peripheral blood B cells upregulated a large number of CD44 V* isoforms in addition to CD44 H. In contrast to the other exons however, V4 and V5 are differentially included into CD44 mRNAs in response to IL-4 and IFN- γ . This differential splicing and a reduction in CD44 H expression paralleled the ability of these cytokines to inhibit PMA-induced HA recognition in these cells. Unstimulated B cells were found to express CD44 H but little or no CD44 V* isoforms and failed to bind HA.

Enhanced expression of various CD44 isoforms in different tumors has been correlated with malignancy but their specific role in tumor formation and metastasis remains largely undefined. CD44 V6* has been associated with rat pancreatic carcinoma, human

mammary adenocarcinoma and non-Hodgkin's lymphoma (5,36,194). Overexpression of the CD44 H but not CD44E or other isoforms in the Burkitt's lymphoma cell line Namalwa, enhanced tumor formation and metastatic behaviour in these cells (46,133,134).

The control of CD44 isoform expression and CD44-HA recognition in transformed human B cells is not understood. Modulation of CD44-HA interactions especially by cytokines may have implications with respect to the localization of leukemic B cells in target tissues and formation of tumors. Therefore, in this part of the study, I examined the effect of mitogens and cytokines (especially those involved in B cell activation and differentiation), on CD44-HA interactions using a series of transformed B cell lines, including EBV⁺ and EBV⁻ BL cell lines and normal human B cells transformed *in vitro* with EBV (B-LCLs).

4.200 Results

4.210 Study of CD44-HA interactions in Burkitt's lymphoma and normal human B cells transformed *in vitro* with EBV

To study the regulation CD44-HA interactions in transformed human B cells, a panel of EBV⁺ and EBV⁻ Burkitt's lymphoma (BL) cells and B cells transformed *in vitro* with EBV (B-LCLs) were subjected to a variety of stimuli and analyzed for the ability to bind HA by the ⁵¹Cr-based cell adhesion assay.

4.211 Activation with PMA induced CD44-mediated HA recognition in BL-30/B95-8 but not in B-LCLs

EBV-positive BL-30/B95-8 BL cells and MK3.31 B-LCL cells were stimulated with a series of mitogens for 1, 2, and 4 days and analyzed for their ability to recognize HA. Unstimulated cells of either type did not exhibit binding to HA in either cell line. Stimulation of both cell types with LPS (10 $\mu\text{g/mL}$), SAC (1:1000) or PWM (1:50) had no significant effect on the HA recognition of these cells (Fig. 4-1). In contrast, PMA (20 ng/mL) stimulation induced strong HA recognition in BL-30/B95-8 cells ($6.8 \pm 0.4\%$ in unstimulated vs. $46.2 \pm 8.4\%$ in PMA stimulated cells) but not in MK3.31 cells ($5.0 \pm 1.2\%$ in unstimulated vs. $2.8 \pm 0.3\%$ in PMA stimulated cells). PHA (1:100) stimulation also increased HA binding in only BL-30/B95-8 cells ($6.8 \pm 0.4\%$ in unstimulated vs. $17.0 \pm 0.9\%$ in PHA stimulated cells, Fig. 4-1). PMA-induced adhesion of BL-30/B95-8 cells was dose dependent, reaching a plateau at concentrations of 0.8 ng/mL of PMA (Fig. 4-2A). Kinetics experiments revealed that optimal binding occurred after 24 hrs of stimulation ($67.0 \pm 0.3\%$) and was reduced by 120 hrs post-stimulation to nearly background levels ($8.0 \pm 1.2\%$, Fig. 4-2B).

To determine if the differential CD44-HA interactions observed with this set of cell lines were applicable to other BL and B-LCL cell lines, a panel of such cells were tested for HA binding in response to PMA stimulation (Fig. 4-3). For comparison, normal purified B cells isolated from peripheral blood following stimulation with PMA exhibited strong HA binding, as seen in chapter 3. In contrast, upon *in vitro* transformation with EBV (B-LCLs), B cells did not acquire HA binding in response to PMA stimulation. This was observed in all the B-LCLs tested, including MK3.31, MK4.15, MK5.26, and MK6.1, all generated

Fig. 4-1: Differential adhesion of BL-30/B95-8 BL and MK3.31 B-LCL cells to hyaluronan following mitogenic stimulation

Cells ($3 \times 10^5/\text{mL}$) were stimulated for 24, 48 and 120 hrs with LPS ($10 \mu\text{g}/\text{mL}$), SAC (1:1000), PMA (20 ng/mL), PHA (1:100) and PWM (1:50) followed by analysis of cell adhesion to HA-coated plates as described in Materials and Methods. Adhesion results from 24 hrs of stimulation are shown and do not differ from the other time points tested for all mitogens (data not shown) except PMA (see Fig. 4-2). Adherent cells (% cpm input) are presented as the mean of triplicate samples $\pm$ S.D. The results shown are a representative of at least three separate experiments.

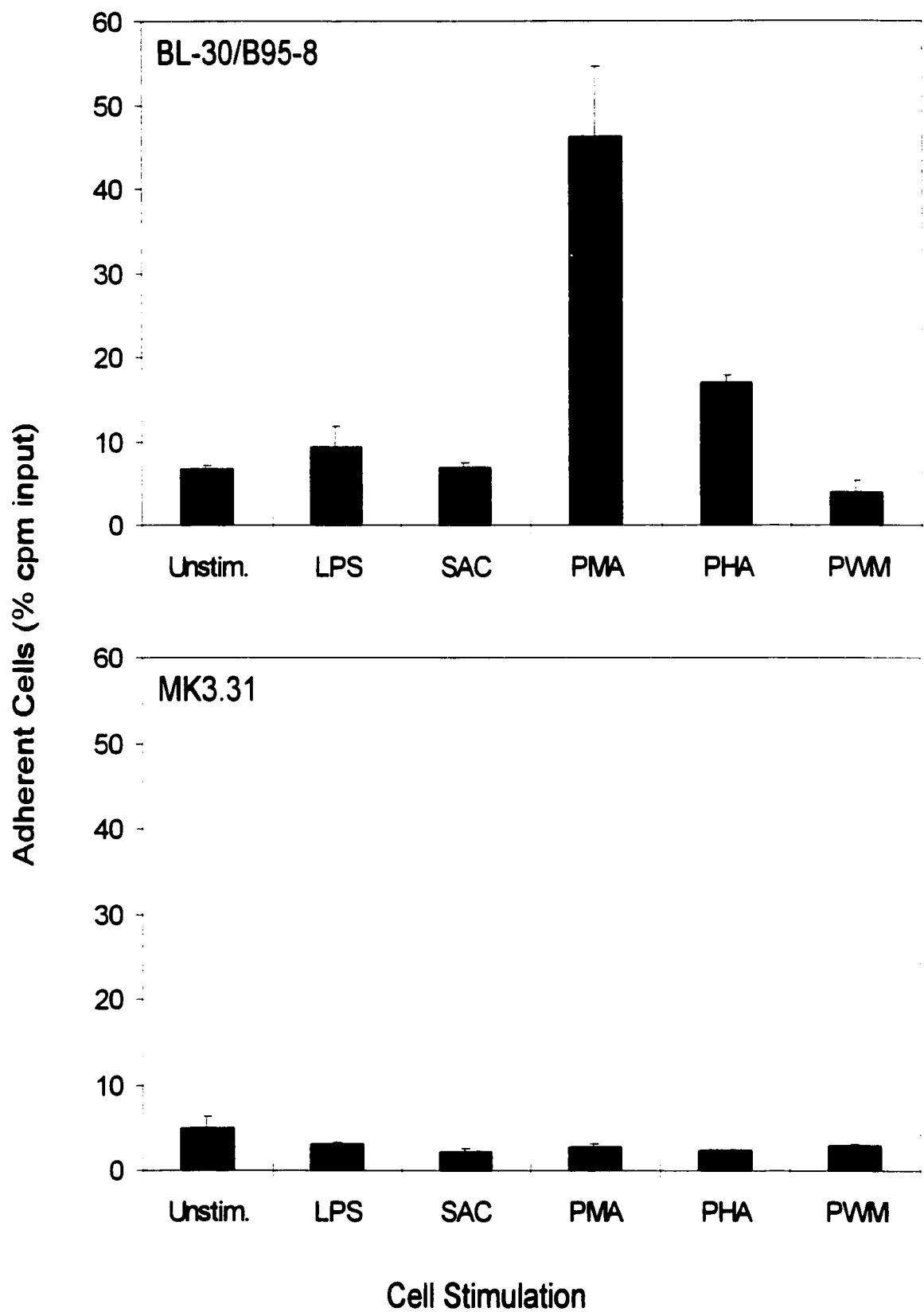


Fig. 4-2: Concentration and temporal dependence of BL-30/B95-8 cell adhesion to HA in response to PMA

Adhesion of BL30/B95-8 to HA is dependent on the concentration of PMA (**A**) and the time post-stimulation (**B**). Cells ($3 \times 10^5/\text{mL}$) were stimulated for 24 hrs with increasing concentrations of PMA (0.05-51.2 ng/mL). For time course experiments (**B**), cells were stimulated with 20 ng/mL of PMA. Cell adhesion to HA was performed as described in the legend to Fig. 4-1. Adherent cells (% cpm input) are presented as the mean of triplicate samples $\pm$ S.D. The results shown are a representative of at least three separate experiments.

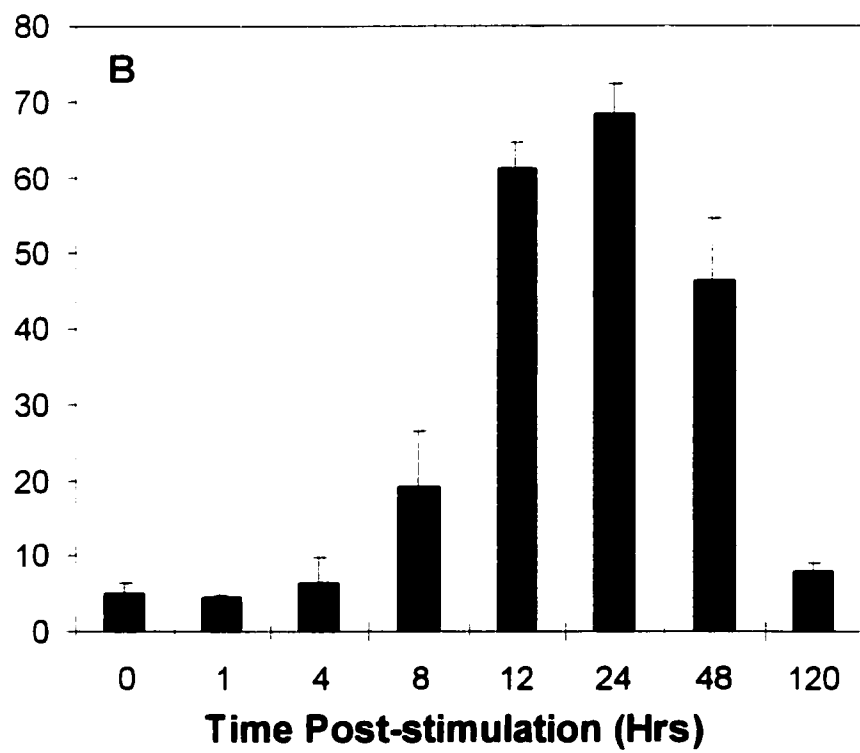
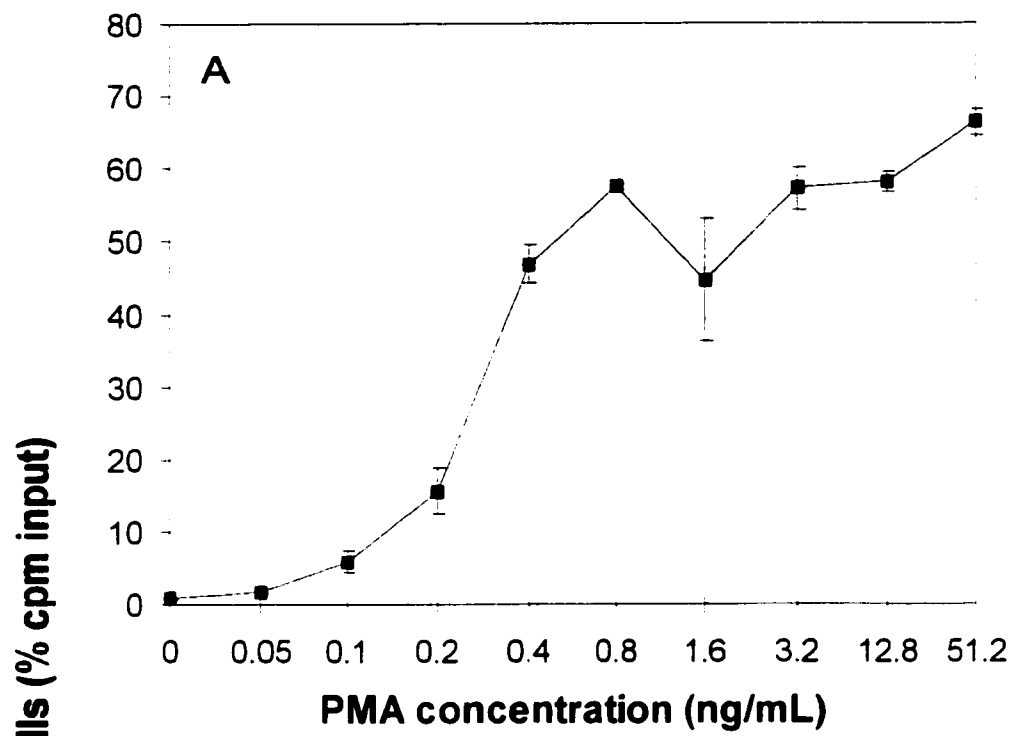
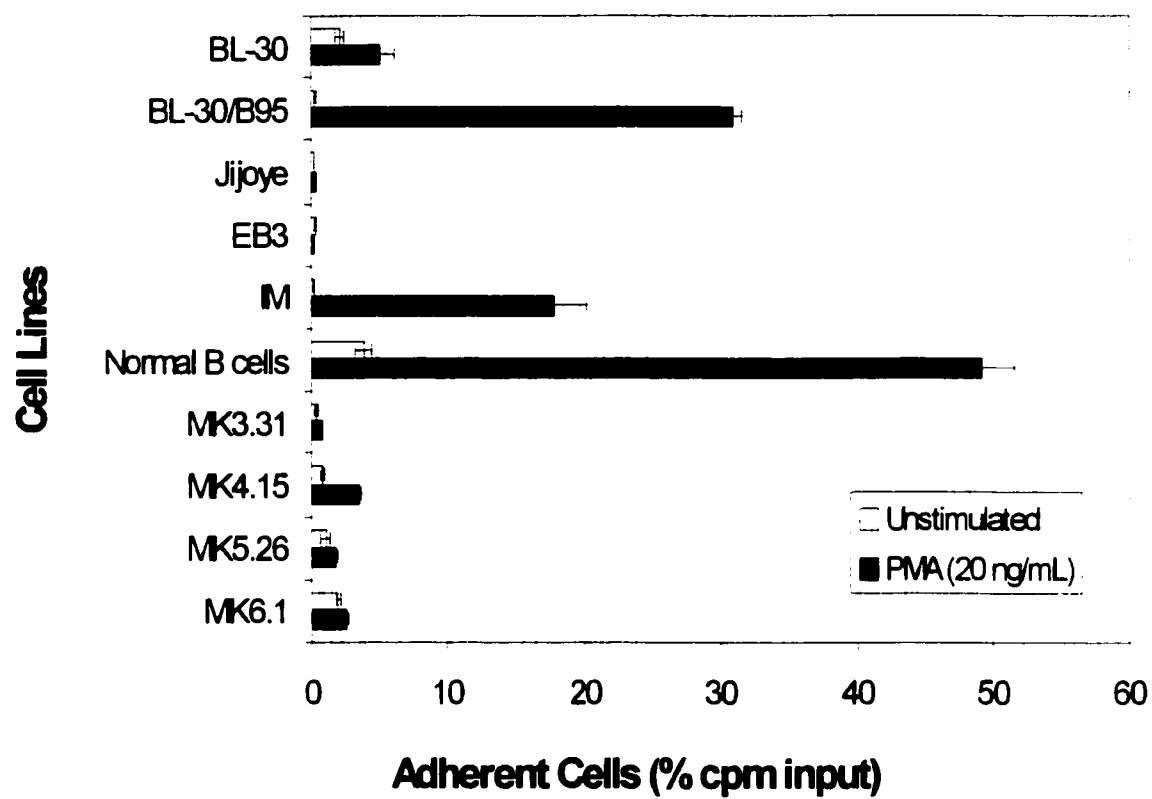


Fig. 4-3: Adhesion of a panel of BL and B-LCL cells to HA in response to PMA stimulation

Cells ($3 \times 10^5/\text{mL}$) were stimulated for 24 hrs with 20 ng/mL of PMA. Cell adhesion to HA was performed as described in the legend to Fig. 4-1. Adherent cells (% cpm input) are presented as the mean of triplicate samples $\pm$ S.D. The results shown are a representative of at least three separate experiments.



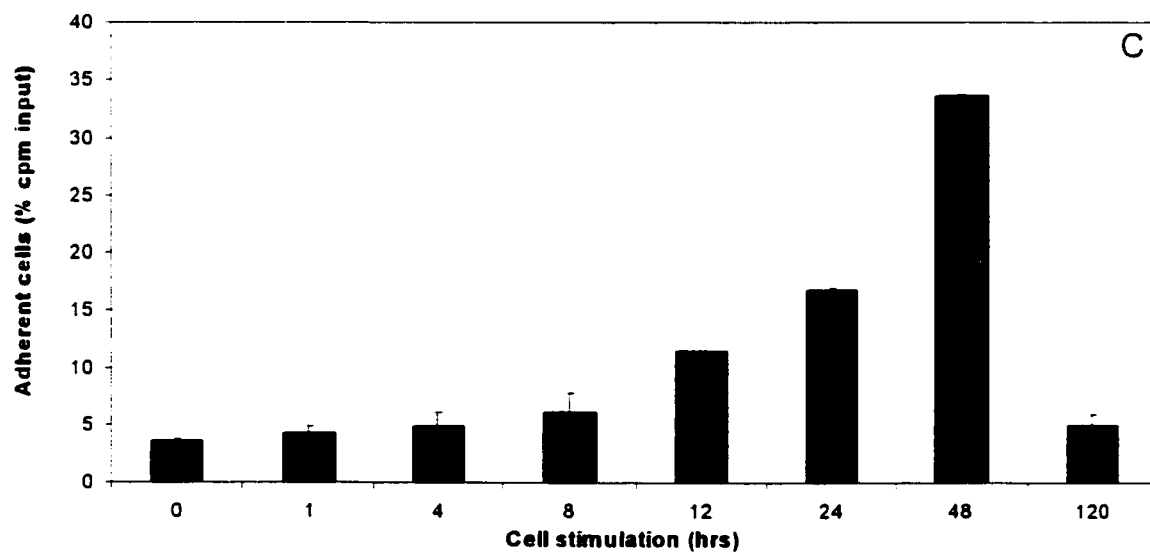
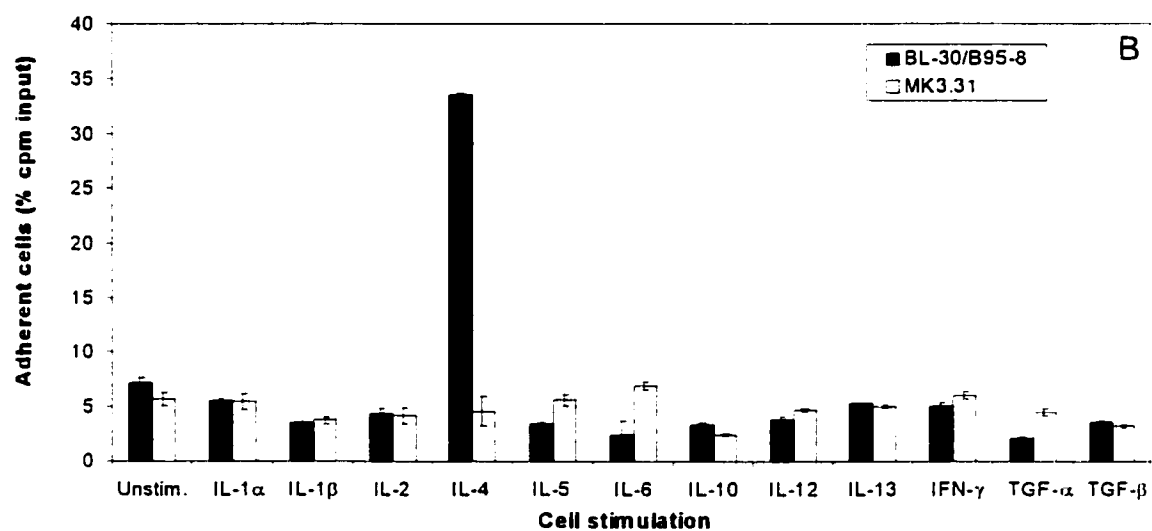
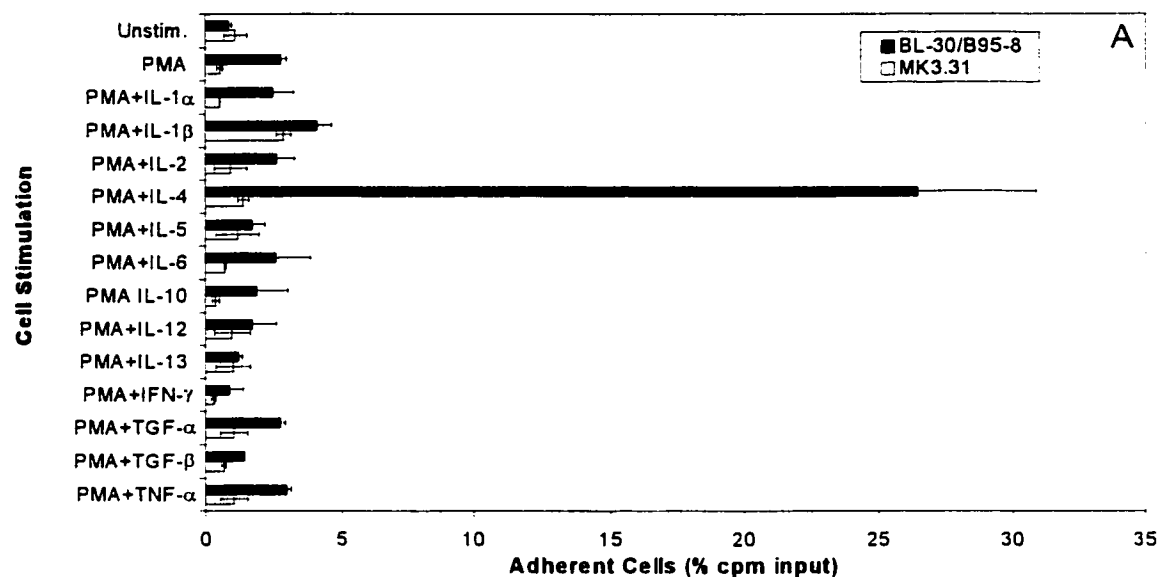
from different donors (Fig. 4-3). However, variable results were obtained in BL cell lines. Unlike in BL-30/B95-8 cells, PMA stimulation of EBV⁺ BL cells, Jijoye and EB3, failed to induce HA recognition when compared to unstimulated cells. EBV⁺ IM cells obtained from the PBMCs of a patient with infectious mononucleosis, which had been infected with EBV *in vivo*, strongly recognized HA in response to PMA ($0.2 \pm 0.01\%$ in unstimulated vs. $17.8 \pm 2.4\%$ in stimulated cells). These results suggest that *in vitro* transformation of normal human B cells with EBV abrogated their ability to bind HA following PMA stimulation even though they expressed high levels of CD44 (126). In contrast, BL-30/B95-8 cells, which, like B-LCLs, are infected with wild type EBV, bind HA following PMA stimulation. Furthermore, *in vitro* infection with EBV (B-LCLs) appears to be distinct from infection of B cells *in vivo* (IM) with respect to the induction of HA recognition by PMA.

4.212 IL-4 induced CD44-HA interactions in BL-30/B95-8 cells but not in B-LCLs

Further studies investigating the effect of cytokines involved in B cell activation, proliferation or differentiation on the regulation of HA recognition in these two cell types were pursued. BL-30/B95-8 BL and MK3.31 B-LCL cells were stimulated with a panel of cytokines, in the presence or absence of sub-optimal concentrations (0.05 ng/mL) of PMA, as defined in Fig. 4-2. Amongst all of the cytokines tested, which included IL-1 α , IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IL-13, TNF- α , TGF- α , TGF- β and IFN- γ , only IL-4 was able to induce strong HA binding in BL-30/B95-8 cells (Fig. 4-4). Cytokines were tested over the range of concentrations listed in Materials and Methods. The effect of IL-4 was observed in the presence ($26.5 \pm 4.4\%$) or absence ($33.6 \pm 0.1\%$) of sub-optimal concentrations of PMA compared to cells sub-optimally stimulated with PMA alone ($2.8 \pm$

Fig. 4-4: Effect of cytokines on the adhesion of BL-30/B95-8 BL and MK3.31 B-LCL to HA

Modulation of adhesion to HA by cytokines in the presence (A) or absence (B) of sub-optimal doses (0.05 ng/mL) of PMA. Cells (3×10^5 /mL) were stimulated for 48 hrs with a variety of cytokines [IL-1 α (10 ng/mL), IL-1 β (1 ng/mL), IL-2 (100 U/mL), IL-4 (40 ng/mL), IL-5 (15 ng/mL), IL-6 (4 ng/mL), IL-10 (37.5 U/mL), IL-12 (1.5 ng/mL), IL-13 (40 ng/mL), TNF- α (10 ng/mL), TGF- α (100 ng/mL), TGF- β (5 ng/mL), and IFN- γ (50 ng/mL)] in the presence or absence of PMA. (C) Time course (0-120 hrs) for the adhesion of BL30/B95-8 cells to HA following stimulation with IL-4 alone. Cell adhesion to HA was performed as described in the legend to Fig. 4-1. Adherent cells (% cpm input) are presented as the mean of triplicate samples $\pm$ S.D. The results shown are a representative of at least three separate experiments.



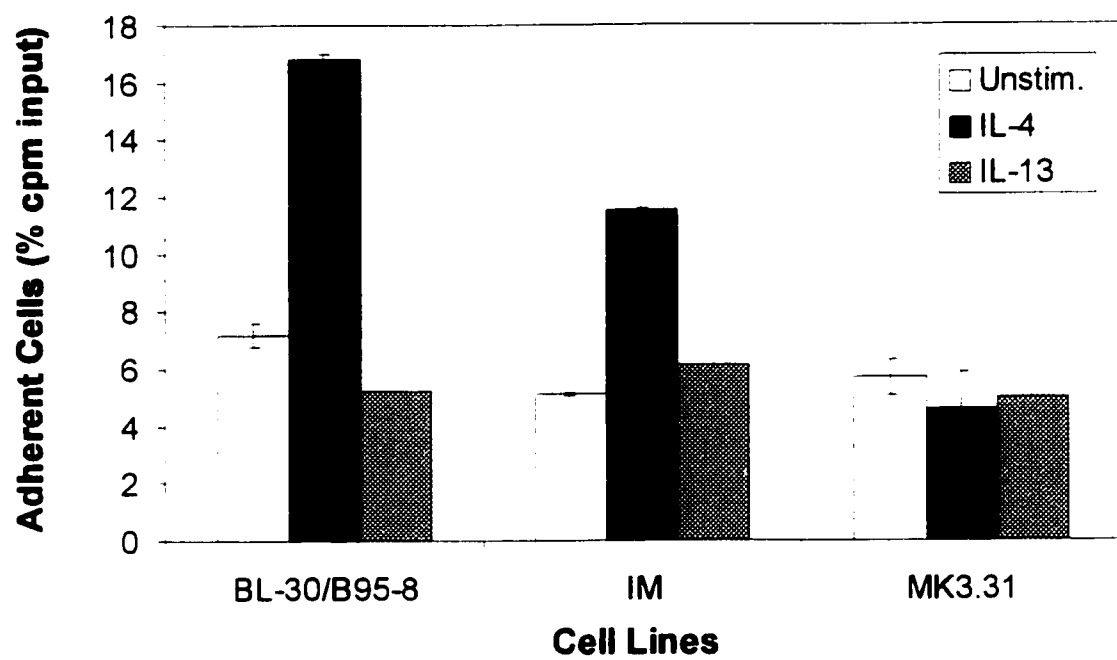
0.2%) or unstimulated cells ($7.2 \pm 0.4\%$), as illustrated in Fig. 4-4A and 4-4B, respectively. The binding observed in BL-30/B95-8 cells was mediated by CD44, as anti-CD44 antibodies inhibited this effect (37.1% adhesion in untreated versus 9.41% in treated cells). IL-4-induced binding to HA in BL-30/B95-8 cells was detectable as early as 12 hrs following stimulation (Fig. 4-4C). IM cells exhibited responsiveness similar to that of BL-30/B95-8 cells with respect to HA recognition following stimulation with either IL-4 alone (Fig. 4-5) or in concert with sub-optimal concentrations of PMA (data not shown). Of particular note again, IL-13, which shares the IL-4 α and the common γ_c components of its receptor with IL-4 (192,193), was not able to induce CD44 mediated adhesiveness to HA in any of the cell lines tested (Fig. 4-5). None of the cytokines tested induced CD44-mediated binding to HA in any of the other B-LCLs tested (data not shown).

4.220 Analysis of CD44 isoform expression in BL-30/B95-8 BL and MK3.31 B-LCLs

As mentioned, a number of potential mechanisms explaining how CD44-HA interactions are controlled have been proposed. They may involve alterations in: CD44 RNA splicing patterns, isoform expression levels, and glycosylation pattern (10,159,160,162,174). Therefore, I elected to investigate the expression of CD44 isoforms mainly in BL-30/B95-8 BL and MK3.31 B-LCL cells under conditions where the contrasting effects on HA recognition were observed. CD44 protein expression levels were evaluated by flow cytometry using antibodies specific for particular variable exons and antibodies recognizing an epitope common to all isoforms. Analysis of CD44 isoform expression was subsequently investigated by RT-PCR and Southern blotting in an attempt to

Fig. 4-5: Adhesion of BL-30/B95-8, IM and MK3.31 cells to HA in response to IL-4 and IL-13

Cells (3×10^5 /mL) were stimulated for 48 hrs with IL-4 (40 ng/mL) or IL-13 (40 ng/mL) and analyzed for adhesion to HA, as described in the legend to Fig. 4-1. Adherent cells (% cpm input) are presented as the mean of triplicate samples $\pm$ S.D. The results shown are a representative of at least three separate experiments.



resolve the identity of the isoforms expressed and verify whether differential utilization of a particular V exon could be correlated with HA binding.

4.221 PMA and IL-4 upregulated protein expression of CD44 isoforms in BL-30/B95-8 cells but not in B-LCL cells

Flow cytometry experiments evaluated the expression of CD44 isoforms on BL30/B95-8 and MK3.31 cells following stimulation with either PMA or IL-4, and IL-4 in concert with sub-optimal concentrations of PMA (Fig. 4-6). Unstimulated BL and B-LCL cells expressed low levels of CD44 isoforms as detected by exon specific monoclonal antibodies [<5 median channel fluorescence (MCF) values]. Therefore, similar to chapter 3 superimposition of flow cytometry histograms and the observation of a cell population shift in log fluorescence intensity with a change of greater than 3 MCF values was regarded as significant in these cases. This is illustrated in the representative histograms of Fig. 4-7. PMA stimulation of BL-30/B95-8 cells significantly enhanced the expression of total CD44 (Fig. 4-6A) as well as that of isoforms containing exons V4/5^{*}, V3^{*}, V6^{*} and V9^{*} compared to unstimulated cells (Fig. 4-6C). However, differences in the levels of expression of CD44 isoforms containing exons V7^{*}, V7-8^{*}, and V10^{*} in stimulated and unstimulated cells were not considered significant (Fig. 4-6C). In the presence of sub-optimal doses of PMA, IL-4 stimulation of BL-30/B95-8 cells also significantly upregulated total CD44 (Fig 4-6B). Similarly, IL-4 induced the expression of CD44 V3^{*}, V6^{*}, and V9^{*} isoforms on BL30/B95-8 cells stimulated with sub-optimal concentrations of PMA (data not shown). Stimulation of BL30/B95-8 cells with IL-4 alone also significantly upregulated total CD44 (Fig 4-6B) and CD44 V3^{*} and V6^{*} expression

Fig. 4-6: Modulation of CD44 isoform expression on BL-30/B95-8 and MK3.31 cells by flow cytometric analysis

(A) Total CD44 expression was determined on BL30/B95-8 and MK3.31 cells following stimulation for 24 hrs with sub-optimal (0.2 ng/mL) and optimal doses (20 ng/mL) of PMA. (B) Modulation of total CD44 expression on BL-30/B95-8 cells stimulated with sub-optimal doses of PMA (0.2 ng/mL) for 48 hrs in the presence or absence of IL-4 (40 ng/mL) or with IL-13 (40 ng/mL) alone. (C) Modulation of CD44 V' isoform (V3, V4/5, V6, V7, V7-8, V9, V10) expression in BL-30/B95-8 cells following stimulation with PMA (20 ng/mL) for 48 hrs and (D) with IL-4 (40 ng/mL) or IL-13 (40 ng/mL). As indicated, CD44 exon specific or isotype matched control mAbs (control) were used. MCF values were used for comparison of CD44 expression levels and the results shown are a representative of at least three separate experiments.

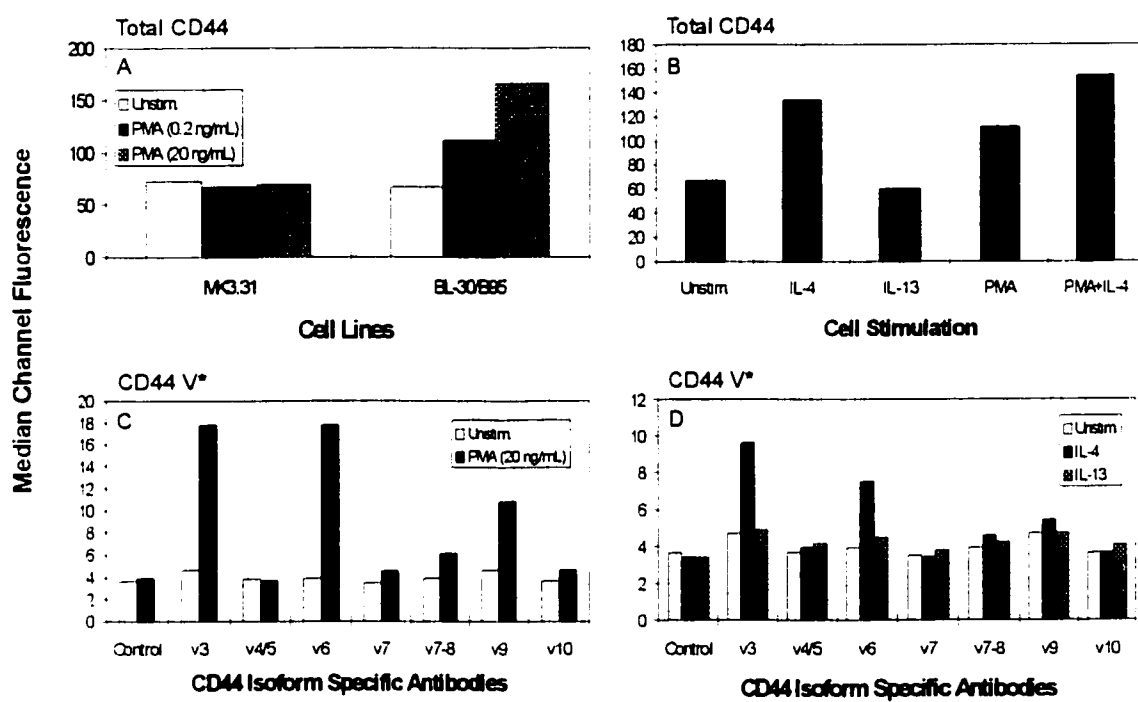
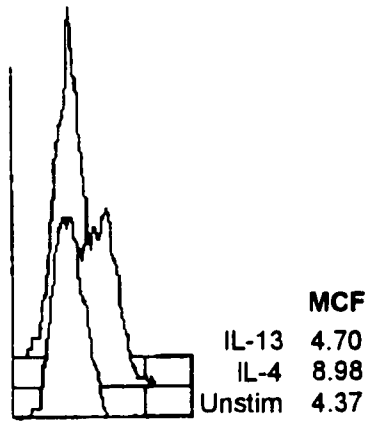


Fig. 4-7: Effect of IL-4 and IL-13 on CD44 isoform expression in BL-30/B95-8 cells (representative histogram overlays)

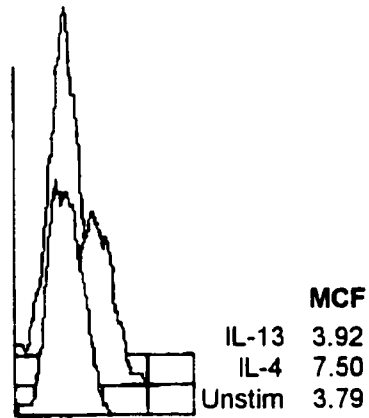
BL-30/B95-8 cells were stimulated with IL-4 (40 ng/mL) and IL-13 (40 ng/mL). Using mAbs specific for the respective variable exons, CD44 V3*, V6*, and V9* expression was compared to unstimulated cells by flow cytometry, as described in the legend to Fig. 4-6. The MCF values corresponding to the stimulation condition are listed for comparison. The results shown are a representative of at least three separate experiments.

Relative Cell Number

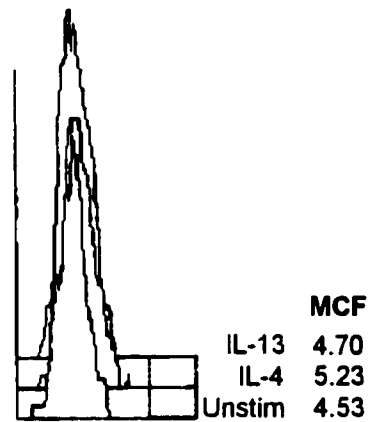
CD44 V3*



CD44 V6*



CD44 V9*



Log Fluorescence Intensity

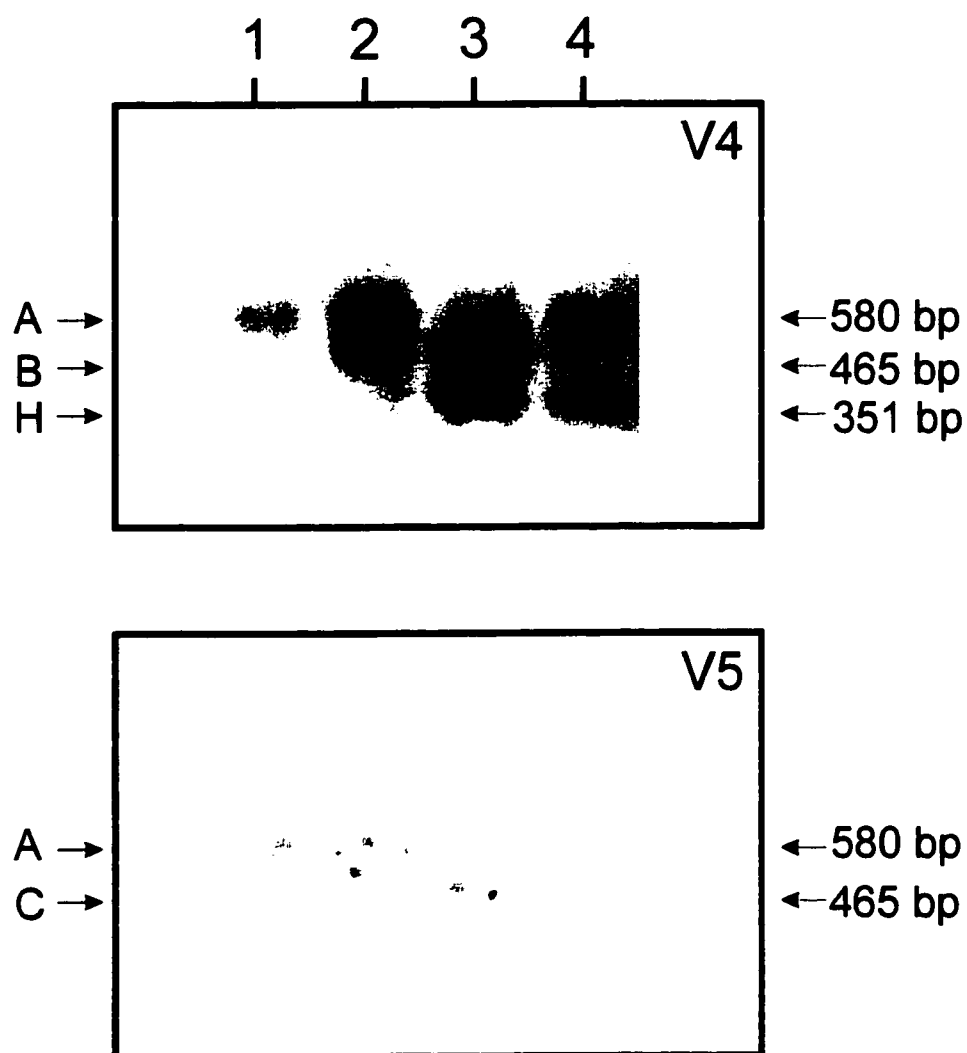
(Figs. 4-6D, 4-7). Interestingly, IL-13 neither alone (Figs. 4-6B, 4-6D, 4-7) nor with PMA (data not shown) was able to upregulate CD44 expression in BL30/B95-8 cells. Furthermore, neither PMA nor IL-4 was able to upregulate CD44 isoform expression on any of the B-LCLs tested, as detected by flow cytometry (Fig. 4-6A and data not shown). Therefore, the upregulated CD44 isoform expression observed correlated with the induction of HA recognition by PMA and/or IL-4 in BL-30/B95-8 cells, further illustrating the stark contrast between this cell line and those generated by *in vitro* EBV-transformation.

4.222 Differential induction of CD44 V mRNA in activated BL-30/B95-8 cells

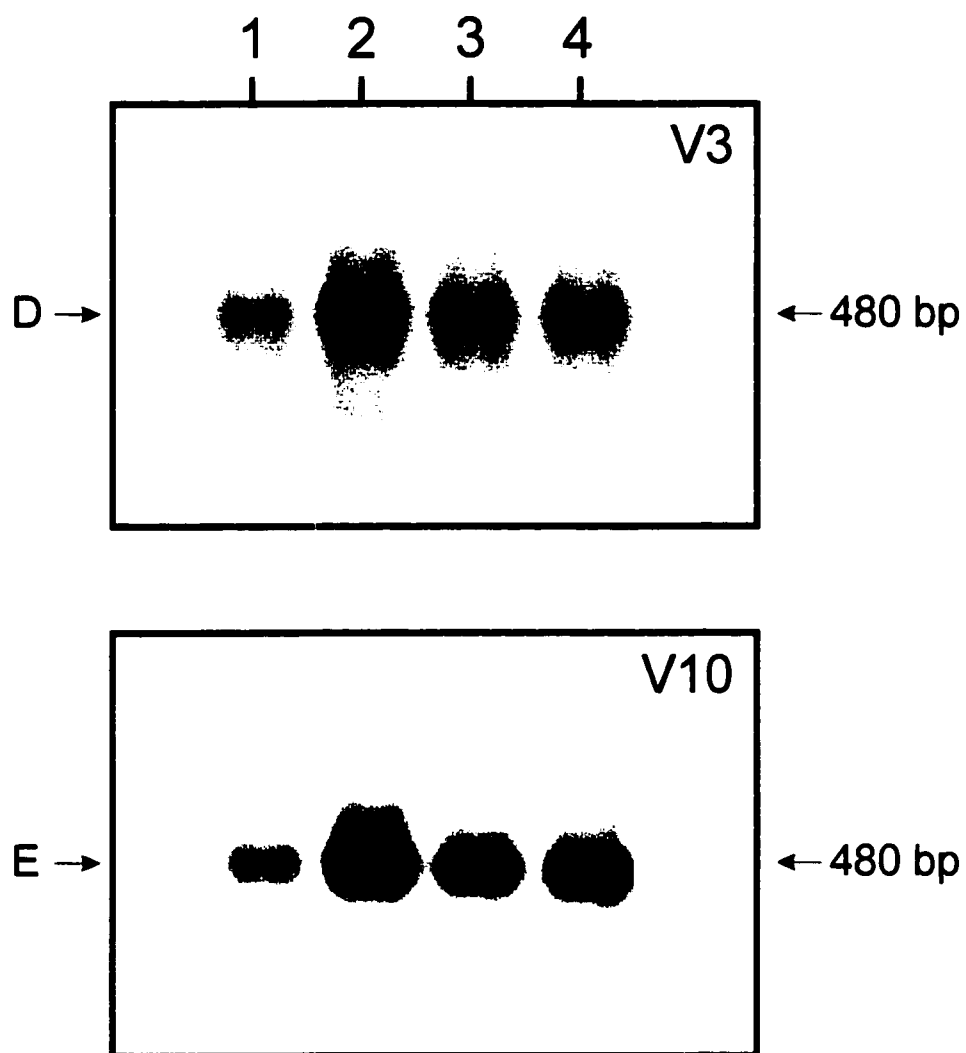
Due to the limitations of flow cytometry already mentioned, the induction of variable exon-containing CD44 isoforms was also investigated by RT-PCR analysis, essentially as described in chapter 3, section 3.223. CD44 RT-PCR products from BL-30/B95-8 cells stimulated for 4 hrs with IL-4, IL-5 or IFN- γ in the presence (data not shown) or absence (Fig. 4-8) of sub-optimal concentrations of PMA were subjected to sequential hybridization with oligonucleotide probes specific for individual exons V2-V10. IL-5 and IFN- γ were used as controls since they had no effect on HA adhesion in these cells. The results shown in Figs. 4-8 reveal that BL-30/B95-8 cells also express an extensive pattern of CD44 V isoforms. In contrast to other variable exons, V4 and V5 usage appeared to be differentially regulated by IL-4, and IL-5, but not IFN- γ (Fig. 4-8A). Based on PCR product molecular weight, it was surmised that unstimulated cells expressed CD44 V4-5 (band A, 580 bp). IL-4 stimulation (lane 2) induced the additional expression of CD44 V4 (band B, 465 bp). Stimulation with IL-5 (lane 3) resulted in the expression of CD44 V4 and CD44 V5 (band C, 465 bp). The fact that fluctuations in splice pattern were only observed

Fig. 4-8: RT-PCR analysis of CD44 isoforms containing variable exons induced in BL-30/B95-8 BL cells by IL-4, IL-5, and IFN- γ

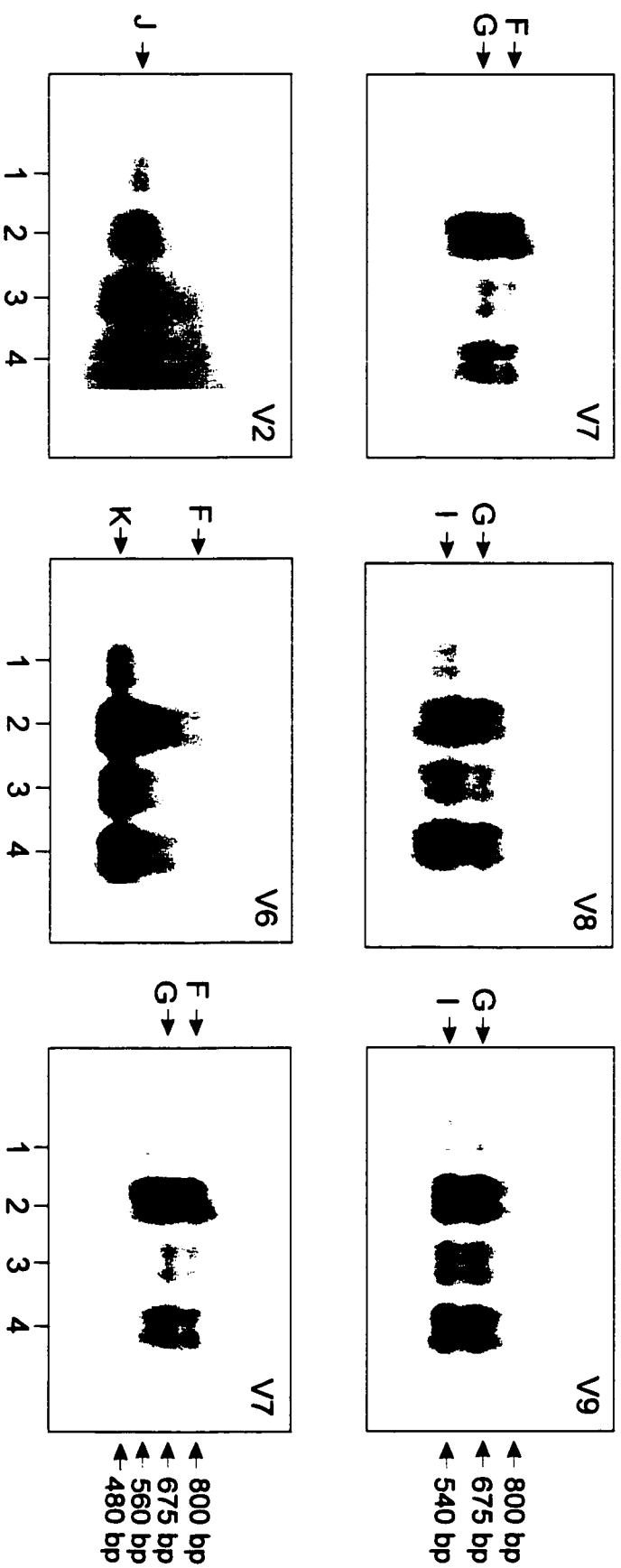
Cells were stimulated with IL-4 (40 ng/mL), IL-5 (15 ng/mL) or IFN- γ (50 ng/mL). Total RNA was amplified by RT-PCR using primers CD44-1 and CD44-2 and probed with V4 and V5 (**Panel A**), V3, V10 (**Panel B**), and V2, V6, V7, V8, and V9 (**Panel C**) specific oligonucleotide probes. Lanes 1-4 are as follows: 1, unstimulated cells; 2, IL-4 stimulated cells; 3, IL-5 stimulated cells; 4, IFN- γ stimulated cells.



Panel A



Panel B



Panel C

following V4 and V5 hybridization (Fig. 4-8), supported this interpretation. Band H (351 bp) corresponded to the CD44H isoform that was detected non-specifically due to its high abundance in relation to variable exon containing isoforms (126).

Similar fluctuations in splicing pattern were not observed for exons V2, V3, V6, V7, V8, V9 or V10. Nevertheless, isoforms carrying these exons were expressed. Probing with V3 and V10 oligomers (Fig. 4-8B) revealed that CD44 V3 (band D, 480bp) and CD44 V10 (band E, 540 bp) isoforms were present under all conditions (lanes 1-5). Two main species were identified following probing with V7 oligomers in Fig. 4-8C (lanes 1-4) at 800 bp (band F) and 675 bp (band G). Band G was also identified clearly with the V8 and V9 probes indicating that this isoform may correspond to CD44 V7-9. A smaller band I (540 bp) was also identified by V8 and V9 probing potentially as CD44 V8-9. A V2' PCR product was detected at 560 bp (band J, lanes 1-4, Fig. 4-8C) identifying an isoform containing V2 and one other exon whose identity was not resolved in these experiments. Hybridization with the V6 probe (Fig. 4-8C) clearly identified band K of 480 bp as CD44 V6. A larger product of approximately 800 bp was also faintly visible following V6 probing which corresponded to band F, also detected by the V7 probe. Therefore, this implied the existence of an isoform containing V6 and V7 along with two other exons, which were not ascertained in these experiments. It should be noted that a similar pattern of CD44 isoform expression was observed when sub-optimal doses of PMA were used in stimulations of BL-30/B95-8 cells (data not shown). Furthermore, MK3.31 cells stimulated with PMA expressed the same set of CD44 isoforms as BL-30/B95-8 cells (data not shown).

In summary, the CD44 isoform expression pattern in BL-30/B95-8, as in normal human B cells, is quite complex. It includes variants containing only one variable exon

namely, CD44 V3, CD44 V4, CD44 V5, CD44 V6, and CD44 V10. Variants containing two variable exons include CD44 V4-5, CD44 V8-9, and those containing 3 variable exons include CD44 V7-9. In addition two potential variants not completely resolved in these experiments include CD44 V2,x and CD44 V6,V7,y,z, where x, y, z represent variable exons of unknown identity. In contrast to the other exons, V4 and V5 are differentially included in CD44 mRNAs upon stimulation of BL-30/B95-8 cells with IL-4 and IL-5 but not IFN- γ . Unlike in normal human B cells, this differential regulation did not parallel the upregulated HA adhesion observed in these cells in response to IL-4. Furthermore, no correlation could be made between the PMA-induced HA binding in BL-30/B95-8 cells and lack thereof in MK3.31 cells at the level of CD44 splice variant mRNA expression since these cells expressed the same set of isoforms in response to PMA.

4.230 Evaluation of potential post-translational modifications to CD44 in BL-30/B95-8 and MK3.31 B-LCLs

Post-translational modifications of the CD44 protein, such as a reduction in the complexity of N-linked glycosylation, have been demonstrated to enhance CD44-HA interactions (160,161,174). This possibility was investigated in BL-30/B95-8 and B-LCL cells by Western blot analysis, comparing CD44 protein electrophoretic mobility following stimulation of these cells with PMA and IL-4. Also, tunicamycin, an inhibitor of N-linked glycosylation (195), was used to test whether the inhibition of such glycosylation, could alter HA recognition in both BL-30/B95-8 and B-LCL cells.

4.231 PMA and IL-4 induced CD44 proteins with slightly lower molecular weights in BL-30/B95-8 but not in B-LCLs

B-LCLs and BL-30/B95-8 cells were stimulated with PMA or IL-4 for 48 hrs and CD44 proteins analyzed by Western blot analysis (Fig. 4-9). A number of species centering around 85-90 kDa were detected in both cell lines. In addition to enhancing CD44 protein expression levels, it was noticed that PMA stimulation of BL-30/B95-8 cells generated CD44 proteins with slightly lower molecular weights in comparison to unstimulated cells (Fig. 4-9A). Similarly, IL-4 stimulation alone not only increased CD44 protein expression considerably but generated species with slightly lower molecular weights than unstimulated BL-30/B95-8 cells (Fig. 4-9B). In contrast, neither PMA nor IL-4 affected the electrophoretic mobilities of CD44 proteins obtained from B-LCLs (Fig. 4-9A and data not shown). The observed increase in electrophoretic mobility in BL-30/B95-8 cells was not due simply to increases in expression levels. This is based on the observation that dilution of protein extracts from PMA or IL-4 stimulated BL-30/B95-8 cells did not alter this increased electrophoretic mobility (Fig. 4-10). Therefore, these differences in molecular weight may result from post-translational modifications to CD44 proteins in response to these factors and correlate with the induction of CD44-HA recognition.

4.232 Tunicamycin treatment induced HA recognition in BL-30/B95-8 cells

To determine whether a reduction in N-glycosylation could induce HA recognition in this system, BL-30/B95-8 and MK3.31 cells were treated with tunicamycin. A number of experimental conditions were tested to assay the effect of inhibiting N-linked glycosylation on HA recognition in these cells. A reproducible change in HA recognition was observed

Fig. 4-9: Western blot analysis of CD44 in BL-30/B95-8 and MK3.31 cells

Cells were stimulated with PMA (20 ng/mL) or IL-4 (40 ng/mL) for 48 hrs. Equal amounts of total cellular protein (100 µg/well) were analyzed for alterations in molecular size by western blot analysis using mAb 2C5. The 82 kDa molecular weight marker is indicated by an arrow and lane identification is as follows: **(A)** *BL-30*, CD44-negative cell line (negative control); *BL-30/B95-8-Unst.*, BL-30/B95-8 cells unstimulated; *BL-30/B95-8-PMA*, BL-30/B95-8 cells stimulated with PMA; *MK3.31-Unst.*, MK3.31 cells unstimulated; *MK3.31-PMA*, MK3.31 cells stimulated with PMA. **(B)** *BL-30/B95-8-Unst.*, BL-30/B95-8 cells unstimulated; *BL-30/B95-8-IL-4*, BL-30/B95-8 cells stimulated with IL-4; *BL-30*, CD44-negative cell line (negative control).

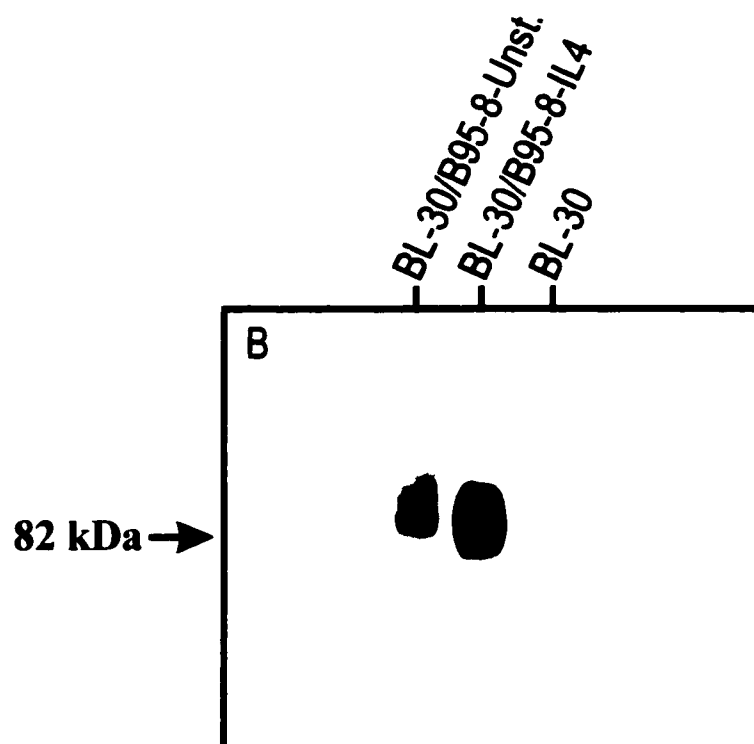
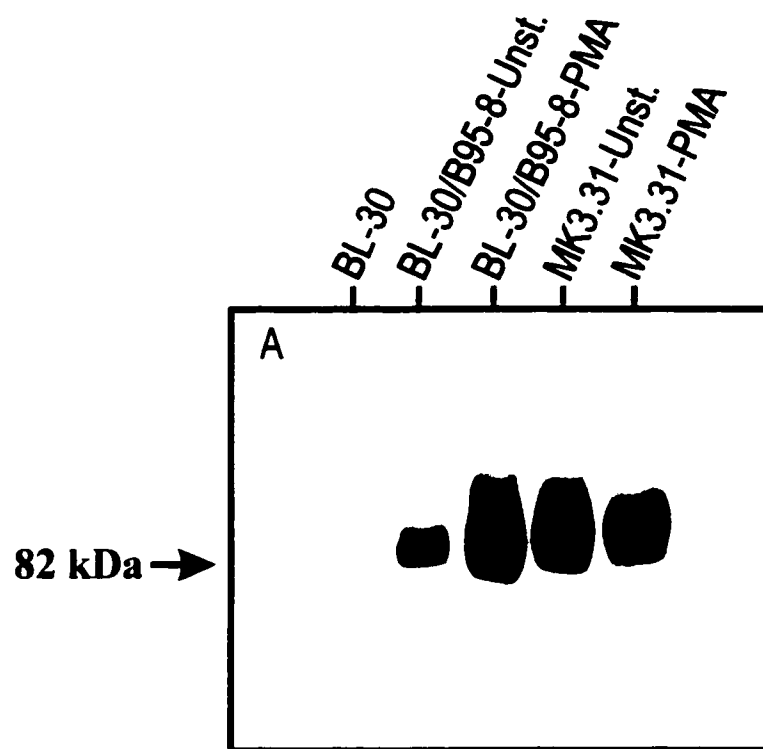
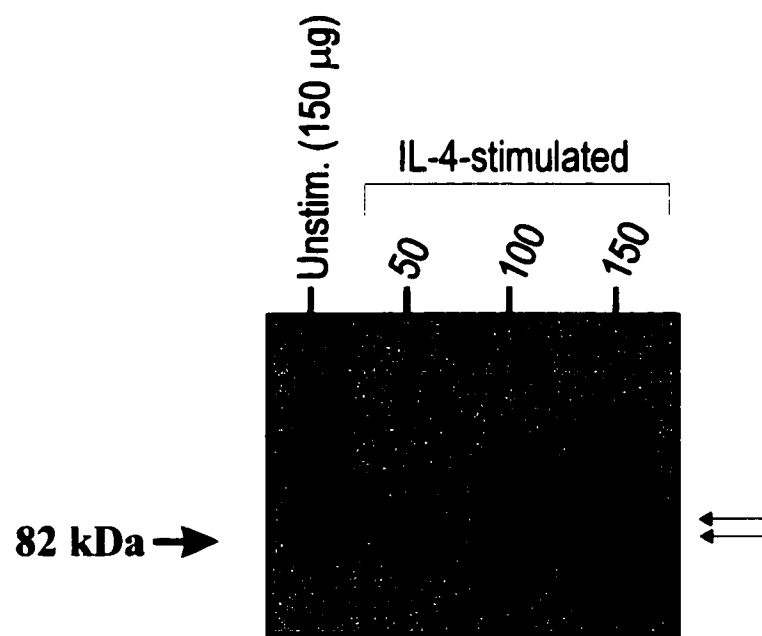


Fig. 4-10: Western blot analysis control for overexpression of CD44 in BL-30/B95-8 cells in response to IL-4

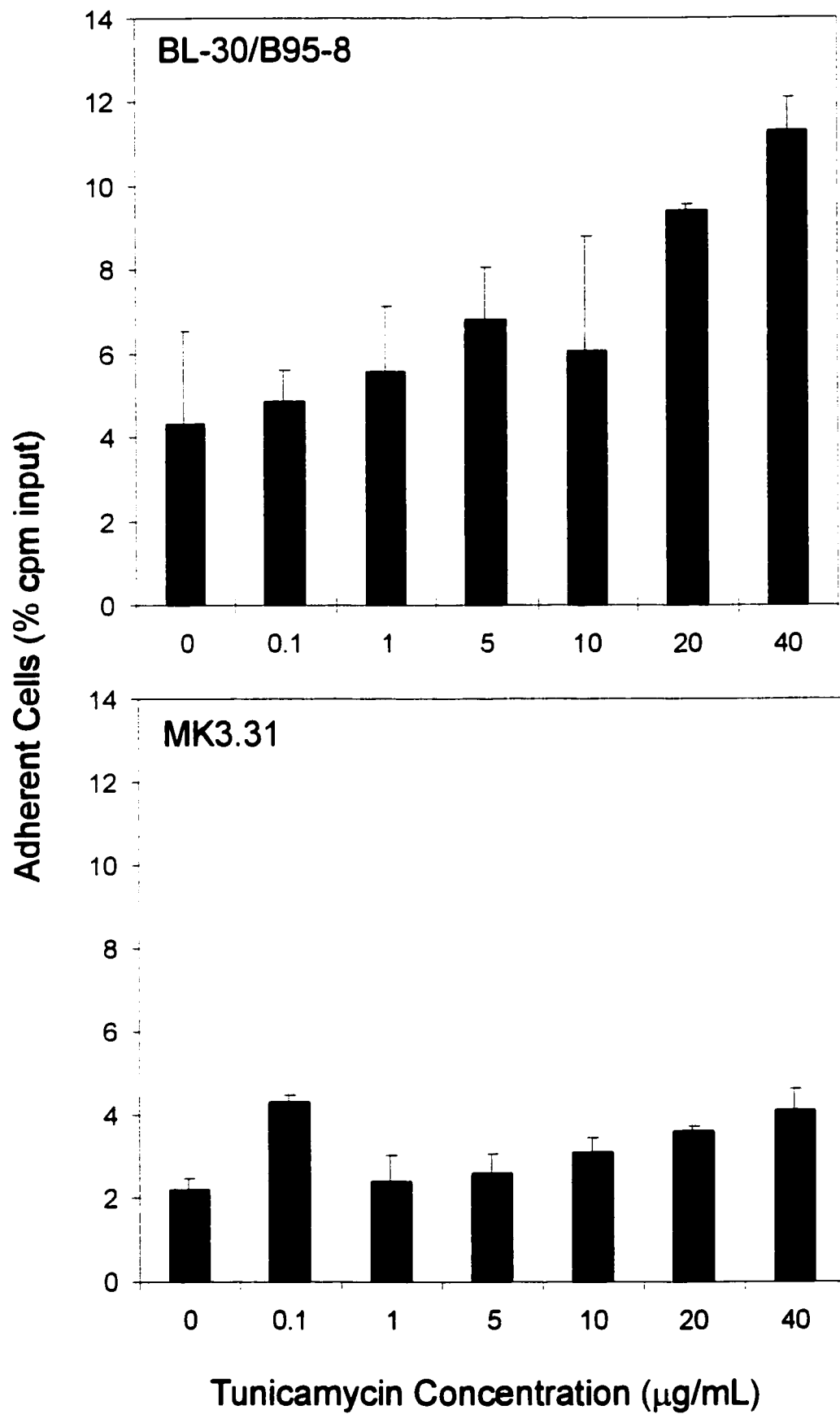
To discount that the increased migration of CD44 proteins after IL-4 treatment (Fig. 4-9) was only the result of its enhanced expression level, protein extracts from IL-4 (40 ng/mL) stimulated cells (IL-4/CD44) were substituted with increasing amounts of CD44⁺ extracts (CD44⁻) derived from BL-30 cells. The 82 kDa molecular weight marker and the approximate centre of the CD44 signals detected with mAb 2C5 in response to IL-4 and in unstimulated cells are indicated by arrows. Lane identification is as follows: *Unstim.* (150 µg), 150 µg of protein from unstimulated BL-30/B95-8 cells; *50*, 50 µg of IL-4/CD44 and 100 µg of CD44⁻ proteins; *100*, 100 µg of IL-4/CD44 and 50 µg of CD44⁻ proteins, *150*, 150 µg of IL-4/CD44 proteins.



when the cells were pre-activated (12 hrs) with sub-optimal concentrations of PMA (0.05 ng/mL) and subsequently incubated with tunicamycin for 48 hrs. Fig. 4-11 shows that with increasing concentrations of tunicamycin (0.1 - 40 μ g/mL) HA adhesion is increased significantly up to $11.3 \pm 0.8\%$ from $4.3 \pm 2.2\%$ in untreated BL-30/B95-8 cells. Similar increases were not observed in MK3.31 cells. A reduction in N-linked glycosylation was confirmed by a decreased staining with fluorescent lectin RCA₁₂₀ (160) by flow cytometry (data not shown). Total CD44 expression levels were also tested following tunicamycin treatment (data not shown) and confirmed that CD44 continued to be expressed under these conditions. These results suggest that the inhibition of the N-linked glycosylation of CD44 can induce HA recognition in BL-30/B95-8 cells. Taken together with the Western blot data, a reduction in N-linked glycosylation, as shown by others (160,161), may be one mechanism invoked by IL-4 and PMA to induce CD44 recognition in BL-30/B95-8 cells, a mechanism not operative in B-LCLs.

Fig. 4-11: Effect of tunicamycin on the adhesion of BL-30/B95-8 BL and MK3.31 B-LCL cells to HA

Cells ($3 \times 10^5/\text{mL}$) were pre-activated for 12 hrs with PMA (0.05 ng/mL) and subsequently treated with increasing concentrations of tunicamycin in culture for 48 hrs. Cell adhesion to HA was performed as described in the legend to Fig. 4-1. Adherent cells (% cpm input) are represented as the mean of triplicate samples $\pm$ S.D. The results shown are a representative of at least three separate experiments.



CHAPTER 5

ROLE OF EPSTEIN-BARR VIRUS INFECTION IN THE INDUCTION OF CD44 ISOFORM EXPRESSION AND HA RECOGNITION IN B LYMPHOCYTES

5.100 **Introduction**

Epstein-Barr virus is a member of the γ sub-family of herpes viruses (196) and etiologically associated with infectious mononucleosis, endemic Burkitt's lymphoma, nasopharyngeal carcinoma, and lymphomas arising in immunosuppressed individuals [reviewed in (138-140)]. With a characteristic tropism for the C3d complement receptor, it infects primary human B lymphocytes latently, transforming them into immortalized lymphoblastoid B cell lines (B-LCL) capable of indefinite growth in culture (197). EBV infection results in the induction of various cellular genes such as CD21, CD23, bcl-2, IL-10, and adhesion molecules LFA-1, LFA-3, and ICAM-1 (198-201). Through the study of B-LCLs, as well as EBV⁺ and EBV⁻ BL cell lines *in vitro*, it has been established that the latent genes of EBV, particularly EBNA-2 and LMP-1, play a critical role in these inductive pathways (145,199,201,202).

BL tumors and the cell lines derived from them are of B cell origin and characterized by the chromosomal translocation placing *c-myc* expression under the control of Ig regulatory elements. The result is a constitutively high level of *c-myc* expression and maintenance of the cells in a constant proliferative state. Hence, this gene is responsible for the majority of the malignant features of BL (203,204). Two types of BL have been distinguished. Endemic (African) BL, found in areas of equatorial Africa and New Guinea, are usually (96%) EBV⁺ (147). Sporadic (American) BLs with identical pathology have been described in the U.S.A. and other non-tropical countries (147). In contrast, only 15% of sporadic BL cases are reported to be EBV⁺ (147). BL cell lines have also been classified, based on EBV status and cell surface phenotype in comparison with B-LCLs, as group I, II and III (141,142). B-LCLs characteristically

express the full complement of 9 latent EBV genes (EBNA-1, -2, -3A, -3B, -3C, -LP, LMP-1, -2A, and -2B) and resemble activated B cell blasts, expressing CD23, CD30, CD39, CD70 and adhesion molecules CD44, LFA-1, ICAM-1, and LFA-3. BL cells with this phenotype are referred to as Group III. Group I BL cell lines, like primary BL biopsies, resemble germinal centre B cells and lack all the aforementioned cell surface proteins but express CD10, CD38 and if EBV⁺, express only the EBNA-1 gene, which is indispensable in maintenance of the viral episome (140,143,144). Continuous *in vitro* passaging results in their phenotypic conversion towards group II/III, with an activated B cell or B-LCL cell surface phenotype, and downregulation of CD10 and CD38 (143,144). The Group II BL phenotype is intermediate between Group I and III. It is believed that the activation of latent EBV genes, particularly LMP-1, is important in this process of phenotypic conversion (135). Both EBV infection and *c-myc* translocation have been implicated in the pathogenesis of BL, a subject elaborated upon in chapter 7 (140,143).

The inductive effect of EBV on CD44 expression in B cells remains unclear (145). In chapter 4, it was shown that the ability of EBV⁺ BL cells and B-LCLs to adhere to HA via CD44 was differentially regulated, via a mechanism that likely involved a combination of differential CD44 isoform expression levels and post-translational modifications of the CD44 protein. These findings in combination with the established trans-regulatory role of EBV prompted the investigation of how EBV infection itself, could influence CD44 isoform expression and HA recognition. These aspects were investigated using normal human B cells, following EBV infection, and a panel of EBV⁻ and EBV⁺ B cell lines.

5.200 Results

5.210 Analysis of CD44 expression in EBV⁺ and EBV⁻ B cell lines by flow cytometry

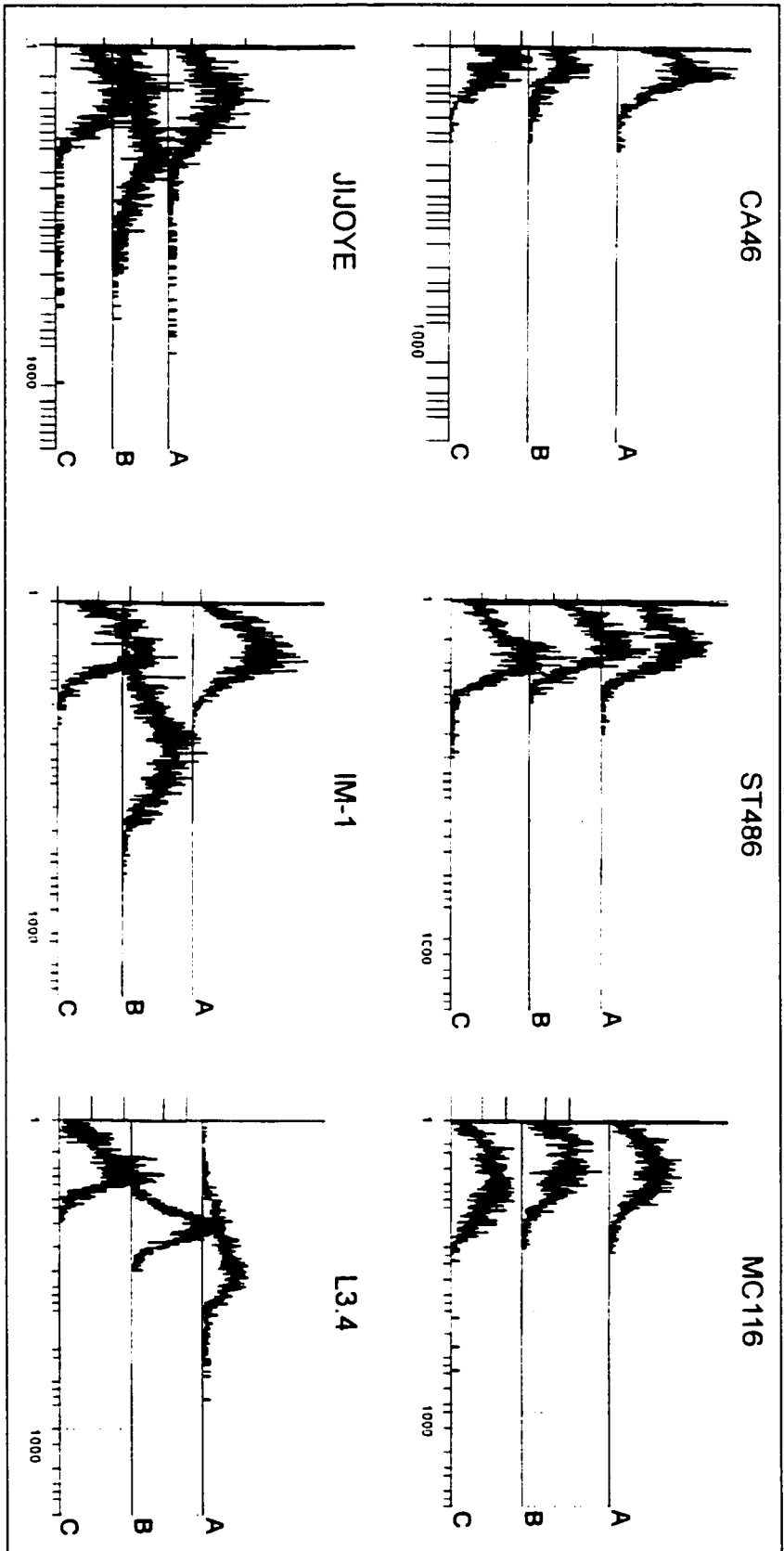
To study the impact of EBV on CD44 isoform expression in transformed human B cells, a number of EBV⁻ and EBV⁺ B cell lines were analyzed for CD44 expression. When these studies were initiated, CD44 V6,V7^{*} isoforms had been implicated in B and T cell activation (37), therefore I focussed on investigating their expression. Anti-CD44 mAbs VFF7 (V6^{*} exon specific) as well as Ab-1 or Leu-44 (specific for a common CD44 epitope) were used in flow cytometry experiments. EBV⁻ BL cell lines, namely CA46, ST486, MC116, did not express CD44 (Fig. 5-1). The EBV⁺ BL cell line Jijoye was CD44⁺, however (Fig. 5-1). The B cell line infected *in vivo* with EBV, IM-1, derived from a patient with infectious mononucleosis, also expressed CD44 (Fig. 5-1). None of the cells tested expressed CD44 V6^{*}, as revealed by the absence of staining with the mAb VFF-7 (Fig. 5-1). The L3.4 cell line was again used as positive control in these experiments. These results suggested that EBV⁻ BL cell lines do not express CD44, while EBV⁺ B cell lines are positive.

CD44 expression studies were extended to include other B cell lines, both EBV⁺ and EBV⁻ (Fig. 5-2). BJAB and Ramos, both EBV⁺ B cell lines did not express CD44. Like in HA binding studies, variability in CD44 expression was obtained in EBV⁺ B cell lines. As already described (chapter 4), BL-30/B95-8 expressed substantial levels of CD44 (Fig. 5-2). In contrast, EB3 and RAJI, both EBV⁺ BL cells, failed to express CD44 (Fig. 5-2). For comparison, normal B cells transformed *in vitro* with EBV

Fig. 5-1: Flow cytometric analysis of total CD44 and CD44 V6⁺ isoform expression in human EBV⁻ and EBV⁺ B cell lines

EBV⁻ (CA46, ST486, and MC116) and EBV⁺ (IM-1, and Jijoye) B cell lines were stained by indirect immunofluorescence using mAb VFF-7 (V6⁺ expression) (**A**), Ab-1 (total CD44 expression) (**B**), and an isotype control antibody (**C**).

Relative Cell Number

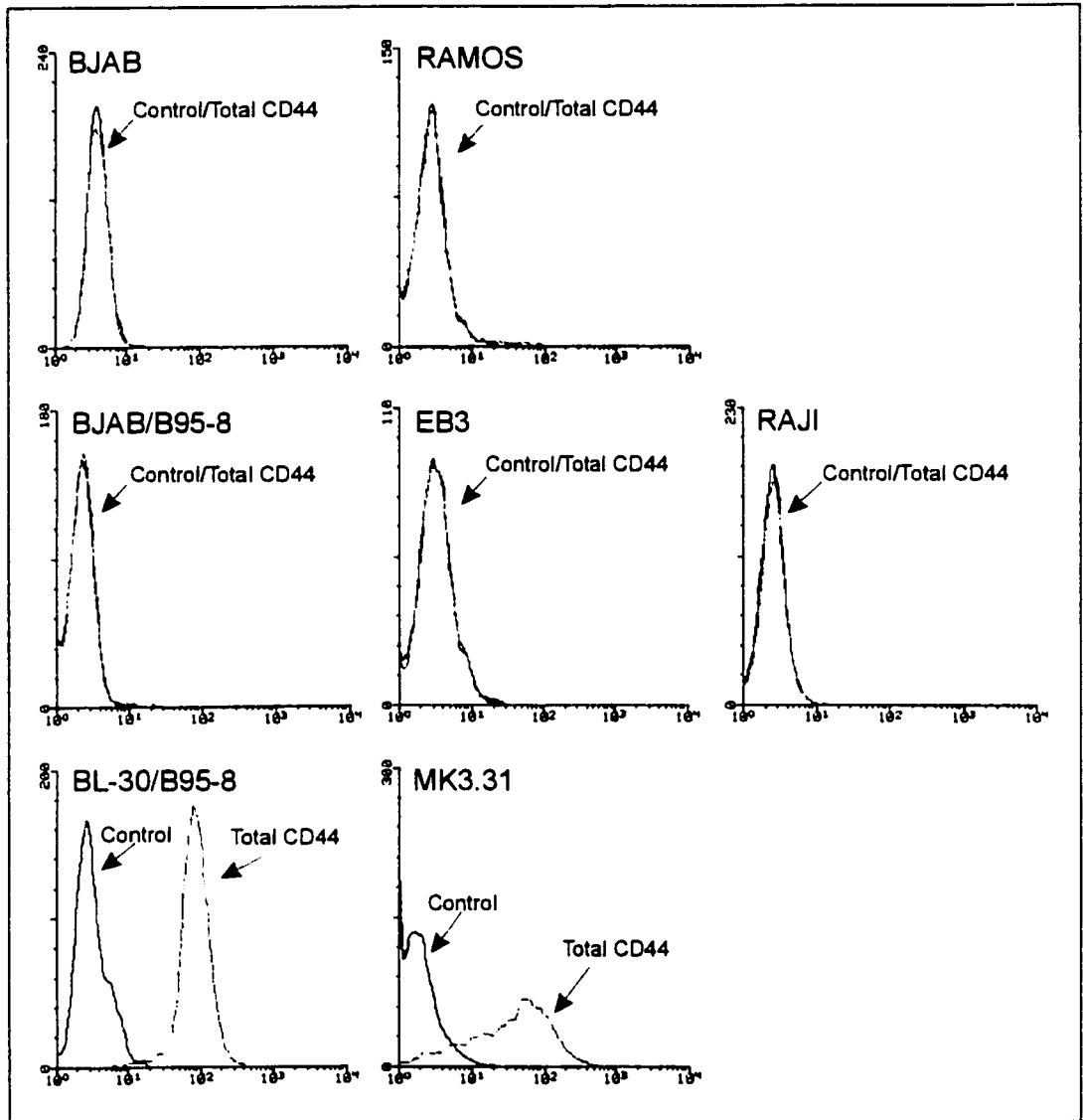


Log Fluorescence Intensity

Fig. 5-2: Flow cytometric analysis of total CD44 expression in a panel of human B cell lines

EBV⁻ (BJAB, RAMOS) and EBV⁺ (BJAB/B95-8, EB3, RAJI, BL-30/B95-8, and MK3.31) B cell lines were stained by direct immunofluorescence using a fluoresceinated mAb specific for a common epitope of CD44 (Total CD44) or an isotype control mAb (Control).

Relative Cell Number



Log Fluorescence Intensity

(MK3.31 B-LCLs), as seen in chapter 4, also expressed an abundance of total CD44 (Fig. 5-2). These results maintain that EBV⁻ BL cell lines do not express CD44. While CD44 expression was observed in some EBV⁺ B cell lines, this was not the case for all such cell lines.

5.220 Presence of the EBNA-2 gene in the EBV genome may be required for the induction of CD44 isoform expression in BL cells

The role of EBV infection in inducing CD44 isoform expression was more precisely investigated in a unique panel of BL cell lines, which differed in EBV status but shared the same genetic background. These included BL30 (an EBV-negative BL cell line), BL30/P3HR1 (BL30 cells infected with an EBNA-2 defective strain of EBV-P3HR1) and BL30/B95-8 (BL30 cells infected with a wild type EBV-B95-8 strain). Flow cytometric analysis with mAb Ab-1 demonstrated CD44 expression in BL30/B95-8 cells, but not in BL30/P3HR1 or BL30 cells (Fig. 5-3). However, CD44 V6⁺ variants were not detected by mAb VFF7 (Fig. 5-3). Northern blot analysis confirmed CD44 expression only in BL30/B95-8 cells (not BL-30 or BL-30/P3HR1), showing three major RNA species of 5.0, 2.2 and 1.6 Kb detected with the CD44 H probe (Fig. 5-4B). These transcripts were also detected in the EBV⁺ IM-1 cell line, but not in the EBV⁻ cell line CA46. No signal was detected with the E probe (Fig. 5-4C) and β -actin message was detected in all the cell lines tested (Fig. 5-4A). RT-PCR analysis further indicated the presence of CD44 H, and isoforms containing exons V8⁺, V9⁺ and V10⁺ in BL30/B95-8 cells only (Fig. 5-5A, B). BL30 and BL30/P3HR1 cells did not show any CD44 expression by RT-PCR. The same pattern of expression as in BL-30/B95-8 cells

Fig. 5-3: Flow cytometric analysis indicating that the presence of the EBNA-2 gene may be required for the induction of CD44 expression

Flow cytometric analysis of total CD44 and CD44 V6⁺ expression on human EBV⁻ BL30 cells, and BL30 cells infected with EBNA-2 defective P3HR1 (BL30-P3HR1) and the wild type B95-8 strain of EBV (BL30-B95/8). The cells were stained by indirect immunofluorescence using mAb VFF-7 (CD44 V6⁺) (**A**), mAb Ab-1 (total CD44) (**B**), and an isotype control antibody (**C**).

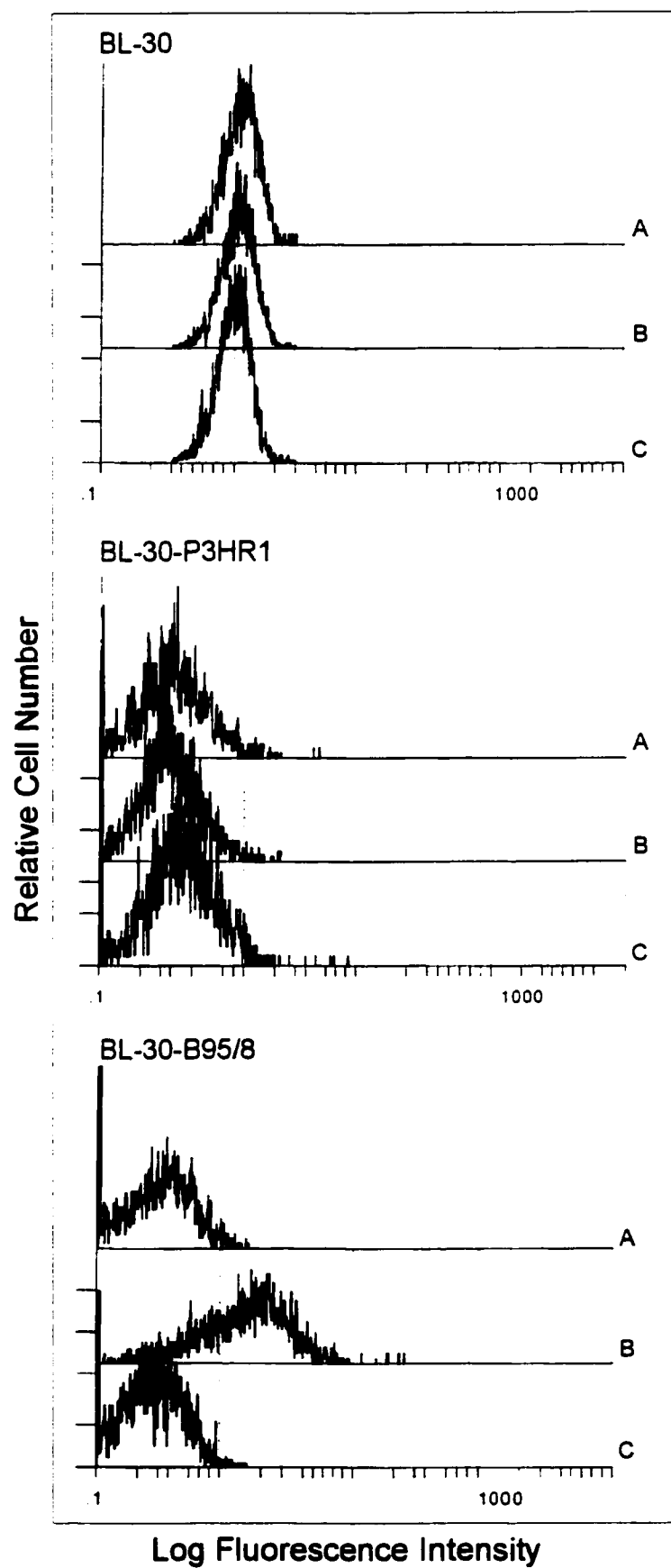
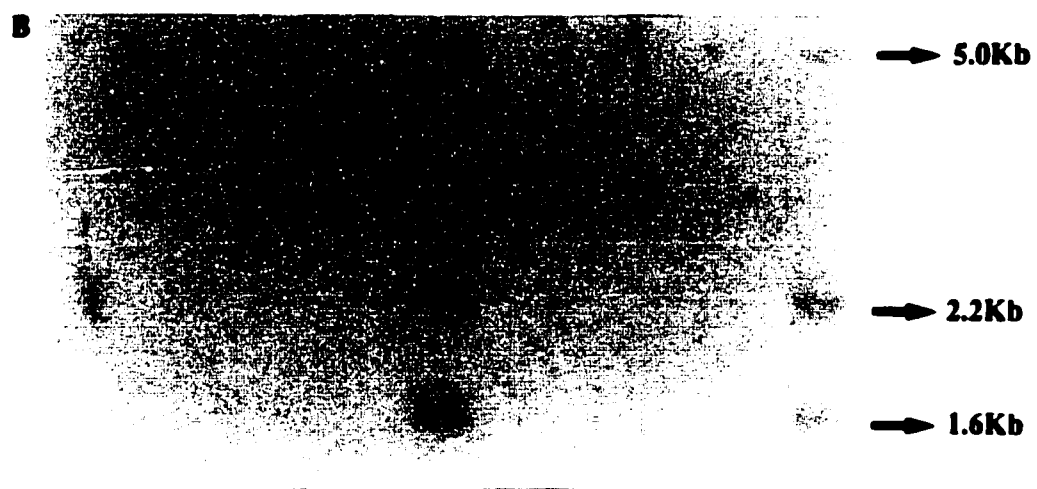
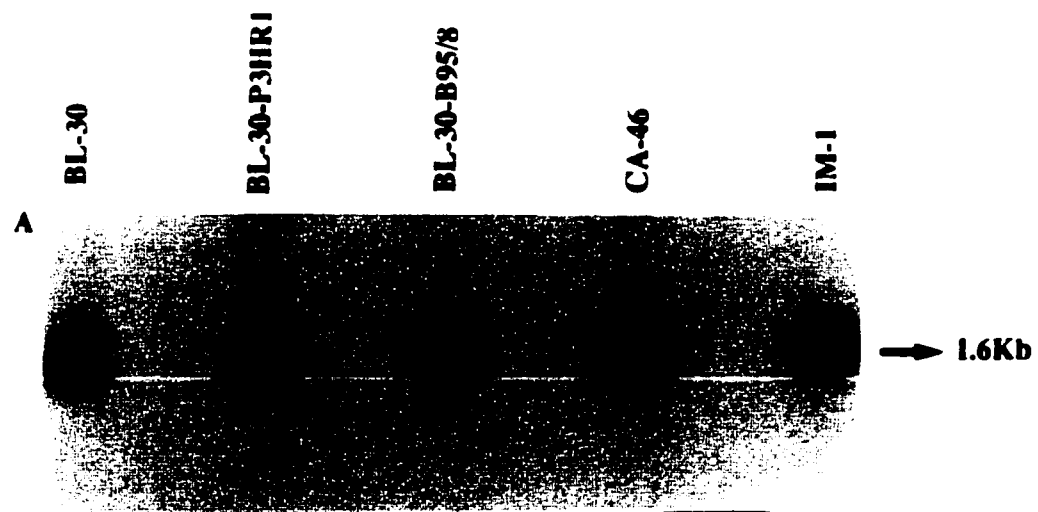


Fig. 5-4: Northern blot analysis of CD44 expression in human B cell lines

Northern blot analysis of RNA extracted from the indicated B cell lines was performed as described in Materials and Methods. The same filter was hybridized with the β actin probe (**A**), the H probe (**B**), and the E probe (**C**).



C

Fig. 5-5: RT-PCR analysis of CD44 isoform expression in human B cell lines

Total RNA from BL30, BL30-P3HR1, BL30-B95/8, IM-1, IM-2, and marmoset derived B95/8 cells was analyzed by CD44 RT-PCR and Southern blotting, as described in Materials and Methods. The same filter was hybridized with the H probe (**A**), the E probe (**B**) and V7-specific oligonucleotide (**C**). CD44 H and CD44 E cDNA and the pancreatic adenocarcinoma cell line, L3.4, were used as positive controls.

E
R,
H

1 1 1



M-B95/8

IM-1

IM-2

A



BL30-B95/8

BL30-P3HR1

BL30

L3.4

CD44 H cDNA

CD44 E cDNA



M-B95/8

IM-1

IM-2



B

BL30-B95/8

BL30-P3HR1

BL30

L3.4

CD44 H cDNA

CD44 E cDNA



M-B95/8

IM-1

IM-2

.

BL30-B95/8

BL30-P3HR1

BL30

L3.4

CD44 H cDNA

CD44 E cDNA

C

/

1
2

was detected in the *in vivo* EBV-infected IM-1 and IM-2 B cell lines. The cell line L3.4, and CD44 H and E cDNAs served as positive controls. The marmoset-derived leukocyte cell line, M-B95/8 was also positive for CD44 H (Fig. 5-5A). No signal was detected in any of the cell lines with the V7 probe (Fig. 5-5C). These findings suggest that infection of the EBV⁻ BL-30 cells with wild type EBV can rescue CD44 isoform expression. Furthermore, the presence of EBNA-2 in the viral genome appears to be required for CD44 induction. In contrast, BJAB/B95-8 cells, although similarly infected with EBV, did not express CD44 (Fig. 5-3). Taken together these results indicate that the control over CD44 isoform induction, like HA recognition, in B cell lines is more complex than the simple introduction of an intact EBV genome.

5.230 EBV-transformation of normal human B cells induces the expression of CD44 variants containing exons V8', V9' and V10'.

As mentioned, EBV modulates the expression of a variety of adhesion molecules, growth factors, growth factor receptors and oncogenes (145,198-202,205,206). The effect of EBV on the expression of CD44 variants in normal human B cells, purified as described in Materials and Methods, was assessed after infection with EBV. CD44 isoform expression was determined by RT-PCR at sequential time points (0-15 days) following EBV infection (Fig. 5-6). RNA samples obtained following EBV infection were amplified by RT-PCR employing CD44-1 and CD44-2 primers, subjected to Southern transfer and hybridized with CD44 specific cDNAs (H and E-probes) or exon V7 specific oligonucleotides, pV7, (see Fig. 2-1, Table 2-2). Hybridization with the H-probe detected band H at 351 bp, corresponding to CD44 H and confirmed the predominant expression of

Fig. 5-6: RT-PCR analysis of CD44 isoforms expression on human B cells infected *in vitro* with EBV

Human B cells were infected with the B95-8 strain of EBV as described in Materials and Methods. Total RNA was amplified by CD44 RT-PCR followed by Southern blotting, as described in Materials and Methods, at sequential times (0-15 days) post-infection. The same filter was hybridized with the H probe (A), the E probe (B), and V7 specific oligonucleotides (C). CD44 H and CD44 E cDNAs, PHA-PBL and the pancreatic adenocarcinoma cell line, L3.3, served as positive controls.



this isoform in human B cells; and whose expression was maintained during the course of infection (Fig. 5-6A). CD44 RT-PCR products from PHA-stimulated peripheral blood lymphocytes (PHA-PBL) and the L3.3 cell line as well as CD44 H and E cDNAs were used as positive controls. Hybridization to the E probe revealed two species, one faint band E (700 bp) and a prominent band R2 (550 bp) by 1 day following infection. These isoforms were not detected prior to EBV infection. Likely due to its low expression overall, band E was discernible only on days 1, 3, 4, and 5 while R2 was detected at each time point post-infection (Fig. 5-6B). The cross-reaction of the E probe with CD44 H was probably due to the abundance of this isoform in relation to all other isoforms expressed (126). Probing with V7 specific oligonucleotides failed to detect any signal (Fig. 5-6C). These results suggested that EBV infection of normal human B cells, followed over a 15 day time course, induces isoforms CD44 V8⁺, V9⁺ and V10⁺ within 1 day post-infection.

5.240 The impact of EBV infection on HA recognition

Based on the results presented above, it can be said that EBV infection of normal B lymphocytes and BL cell lines (Jijoye, BL-30/B95-8) is able to induce CD44 isoform expression in these cells. However, this inductive effect is not observed in all the B cell lines studied (EB3, Raji, BJAB/B95-8). Furthermore, it should be stressed that infection with EBV and the resultant induction of CD44 isoform expression is not sufficient, on its own, to enable the CD44⁺ B cells to bind HA. Stimulation with PMA and in the case of BL-30/B95-8 BL cells, IL-4, is required for this (chapter 4). Furthermore, even though *in vitro* EBV infection of normal human B cells (B-LCLs) induced CD44 isoform expression (Fig. 5-1, 5-3), it concomitantly abrogated the ability of these cells

to bind HA in response to PMA (Fig. 4-3). In contrast, the B cell lines infected *in vivo* with EBV, IM, derived from a patient with infectious mononucleosis, expressed CD44 and bound HA in response to PMA or IL-4. As expected, EBV⁻ B cell lines CA46, ST486, MC116, BL-30, Ramos, and BJAB, which did not express CD44 (Fig. 5-2, 5-3), also failed to recognize HA (Fig. 4-3, data not shown).

CHAPTER SIX

DISCUSSION

6.100 Regulation of CD44-HA interactions in normal human B cells

The results provide evidence that the ability of CD44 to interact with HA is dependent on the mode of activation of normal human B cells and is subject to regulation by cytokines. It was shown that amongst the B cell mitogens tested, PMA, induced strong CD44-mediated HA recognition while the potent B cell differentiation cytokines, IFN- γ and IL-4, inhibited this phenomenon. To date, this is the first report implicating IFN- γ and IL-4 in the inhibition of CD44 mediated HA adhesion in normal human B cells. Studies conducted to investigate the molecular mechanisms involved, suggested that PMA-induced HA adhesion correlated with enhanced expression of CD44 H and V6-containing isoforms, and the differential induction of V4 and/or V5 containing variant mRNAs. Furthermore, post-translational modifications of CD44 implied by a slight reduction in its molecular weight may also, in part, explain the induction of CD44-mediated HA adhesion by PMA. The inhibition of PMA-induced adhesion by IFN- γ and IL-4 was associated with CD44 H downregulation and the induction of CD44 V4-5 mRNA.

The ability of CD44-positive cells to recognize HA is a strictly regulated phenomenon that may be critical for the homing of lymphocytes to inflammatory sites and in the development and spread of tumors (15,66,133,135). As mentioned, not all cells expressing CD44 are able to bind HA constitutively (40). CD44 must be “activated” in order to recognize HA (40,151,152). *In vitro* activation of human peripheral T cells via anti-CD3 antibodies, culture of monocytes in the presence of human serum, anti-CD3 antibody or PHA stimulation of peripheral blood mononuclear cells (PBMC), all triggered transient HA recognition by these cells (98,153). Recently,

the effect of cytokines on CD44 expression and CD44-HA interactions has been investigated in different cell types (174,179-181,207). It has been shown that HA adhesion of CD34⁺ hematopoietic progenitor cells could be enhanced by GM-CSF, IL-3 and stem cell factor (179). TNF- α -induced adhesion of primary human monocytes to HA and this was inhibited by IL-4 and IL-13 (178). Among chemotactic and pro-inflammatory cytokines, RANTES and IL-15 induced uropod formation in T cells and brought about the redistribution of adhesion molecules, including CD44 to the uropod (180). It has also been demonstrated that IL-5 enhanced CD44-HA recognition in murine B cells (174). My results show that PMA was the only mitogen to induce CD44-mediated HA recognition in normal human B cells (Fig. 3-1). PMA-induced HA adhesion in these cells could be inhibited by IFN- γ and to some extent by IL-4 (Fig. 3-3). None of the other cytokines tested, including IL-5, TGF- β and IL-13, either alone or in conjunction with PMA, influenced CD44-HA recognition. The incompatibility between murine and human B cells with respect to the effect of IL-5 may be due to inter-species differences.

IL-4 is a pleuripotent cytokine that plays a central role in B cell activation, growth and differentiation [reviewed in (116,208)]. IFN- γ , in addition to its role in the activation of inflammatory cells, also promotes the differentiation of B cells and stimulates antibody secretion [reviewed in (116,209)]. IFN- γ is known to induce the expression of MHC class II antigens on many cell types including B cells and other cell surface molecules such as Fc ϵ R on the myelomonocytic cell line U937, the adhesion molecule ICAM-1 on endothelial cells and epidermal keratinocytes, and so on (209). Similarly, IL-4 induces the expression of a number of cell surface molecules including CD23 and MHC class II

molecules on B cells (208), and a VCAM-1 like adhesion molecule on endothelial cells (210,211). Interestingly, IL-4 and IL-13 exhibited distinct effects with respect to CD44-HA recognition even though both cytokines share the IL-4 α and common γ_c receptor subunits (192,193) and manifest similar biological effects on B cells such as enhanced proliferative responses to IgM and CD40 crosslinking, upregulation of MHC class II and CD23 expression and anti-CD40 dependent IgE class switching (82,212-214). Unlike IL-4, IL-13 failed to influence HA adhesion in normal human B cells.

The observed effects on CD44-mediated HA binding may be attributed, in part, to the expression level of CD44 H (10). Furthermore, a minimum threshold level of CD44 H expression that is necessary for cells to bind HA has been suggested (156). It was found that PMA upregulated the expression of CD44 H in normal B cells and this effect could be reversed by IFN- γ and to a lesser extent by IL-4 (Fig. 3-6). Therefore, alterations in CD44 H expression levels by these factors correlated with their effect on HA binding.

The expression of CD44 isoforms containing specific variable exons may also be a determinant for binding to HA. For example, CD44 V6* and V9* expression may be required for HA recognition by activated human T cells (162). Whether a similar situation came into play in human B cells was also investigated (Fig. 3-6, 3-8). PMA significantly upregulated CD44 V6* isoform expression, as determined by flow cytometry. Changes in the levels of other CD44 V* isoforms were not regarded as significant. In contrast to their inhibition of CD44 H expression, a similar inhibitory effect on the expression of CD44 V* isoforms in response to IFN- γ and IL-4 was not observed. Similar findings have also been reported by Mackay et al. (176) while

studying the effect of cytokines on CD44 expression in myelomonocytic and epithelial cell lines. The expression of CD44 V6* and CD44 V9* isoforms was markedly upregulated by TNF- α and IFN- γ whereas the expression of CD44 H remained unaffected by these cytokines on THP-1 and U937 cells. Similarly, IFN- γ downregulated the expression of CD44 H and at the same time markedly enhanced the expression of CD44 V6* isoforms on LCLC97 lung carcinoma, HT29 colon carcinoma and HPKII keratinocyte cell lines. These findings suggest that the expression of CD44 H and CD44 V isoforms is regulated by distinct mechanisms.

To more precisely study which CD44 isoforms were expressed and to test whether a particular isoform may be associated with the induction of HA binding, RT-PCR analyses were conducted. Generally, in normal B cells, it was found that unstimulated cells express little or no CD44 V isoforms. Stimulation resulted in the upregulation of a variety of isoforms. Of particular interest was the induction of the CD44 V4-5 isoform by IL-4 and IFN- γ , which correlated with the ability of these cytokines to inhibit PMA-induced HA recognition. Such alterations in the splicing of the V4 and V5 exons were not detected at the protein level, however. This discrepancy was probably due to the fact that the level of CD44 V4-5 isoform expression may have been below the threshold of detection with this particular commercially available antibody by flow cytometry. Moreover, CD44 V4 and CD44 V5 single exon containing isoforms are not recognized by this antibody. Further studies are necessary to determine the role of CD44 V4-5 in the binding of normal human B cells to HA. The specific role of the numerous isoforms expressed in normal human B cells are not clear at present. Considering the emergence of novel CD44 V* functions such as the ability of CD44 V3* to act as heparin sulfate binding growth

factor reservoirs (27,38), it is conceivable that a similar function, perhaps not directly related to HA adhesion, is carried out by these isoforms.

The effects on HA adhesion observed may not exclusively depend upon the level of CD44 H expression as unstimulated B cells constitutively express substantial levels of CD44 (Fig. 3-6) yet do not exhibit spontaneous binding to HA (Fig. 3-1). Moreover, the fact that both surface Ig cross-linking and PMA stimulation of normal human B cells enhanced CD44 H and induced CD44 V6^{*} expression, while only PMA was shown to induce HA adhesion, suggested that this enhancement may not on its own explain the induction of HA binding, or lack thereof in this system. In fact, CD44 has been described to exist in three states: inactive, inducible and constitutively active, with respect to the ability to bind HA (161). It is possible that CD44-mediated binding of B cells to HA required the induction of activated CD44 molecules on the cell surface. PMA-induced CD44-mediated HA binding was detected at 6 hrs post-stimulation. Similarly, the inhibitory effect of IFN- γ on PMA-induced CD44-HA recognition was detected as early as 6 hrs post-stimulation. This time frame would suggest that the modulation of binding to HA requires the expression of newly synthesized CD44 molecules on the cell surface, as proposed by others who demonstrated that new protein synthesis in response to PMA was required to induce HA recognition (215). This was partially confirmed by enhanced synthesis of CD44 mRNA detected by RT-PCR analysis (Fig. 3-8) and slight alterations in the molecular size of CD44 (Fig. 3-9). In contrast, some reports suggest that alterations in the tertiary structure of the CD44 molecule can induce binding to HA, a statement supported by the ability of certain anti-CD44 antibodies (10,157), and

cytokines such as IL-3 and GM-CSF (179) to induce HA binding within as little as 15 minutes of exposure.

A reduction in the complexity of N-linked glycosylation (removal of sialic acid), reduced keratan sulfate modification and most recently, the increased sulfation of CD44 have been found to induce cell recognition of HA (158-161,166). Alterations in the glycosylation pattern of other molecules in response to IL-4 and PMA have been demonstrated. One report established that IL-4 reduces the complexity of N-linked glycosylation of secretory IgA when used in conjunction with IL-5 (216). PMA was also shown to reduce N-linked glycosylation of fibronectin receptors in erythroleukemic cells (217). The generation of CD44 proteins with slightly reduced molecular weight was correlated with the enhanced HA recognition by normal B cells in response to PMA. However, a change in the electrophoretic mobility of CD44 in PMA stimulated B cells following treatment with either IFN- γ or IL-4, was not observed. The absence of any gross alterations in CD44 protein molecular weight in response to IFN- γ and IL-4 does not preclude the possibility of more subtle changes in the composition or structure of the carbohydrate moieties decorating CD44. Therefore, further studies are necessary to investigate the potential effect of IL-4 and IFN- γ on specific alterations in N-linked glycosylation (not detectable by crude Western analysis), that may be responsible for the observed downregulatory effects on CD44 mediated HA adhesion. Recently, PMA has been shown to induce clustering and homodimerization of CD44 on the cell surface, a phenomenon which presumably enabled its recognition of HA (168). However, I can not exclude in this assay system the possibility of other mechanisms postulated to mediate CD44-HA recognition such as the interaction of CD44 with elements of the cytoskeleton

via the cytoplasmic domain, phosphorylation of the cytoplasmic domain, interaction with other ligands either extracellular or at the cell surface, and shedding of CD44 (10).

The molecular mechanisms responsible for the differential regulation of CD44 expression and CD44-HA recognition in PMA stimulated human B cells by IL-4, IL-13, and IFN- γ are not understood. The contrasting effects of IL-4 and IL-13 with respect to CD44-HA recognition on human B cells may be due to the transduction of distinct signals at the level of the Janus kinase activation pathway (218) or the induction of distinct transcription factors. EGR1, a transcription factor associated with B cell activation, has been implicated in the regulation of CD44 transcription in murine B cells stimulated with PMA or anti-IgM antibodies (219). Whether IFN- γ , IL-4 and IL-13 differentially regulate CD44 transcription via EGR1 remains to be investigated.

6.200 Regulation of hyaluronan interactions in Burkitt's lymphoma and EBV-transformed lymphoblastoid B cells

The study of CD44-HA interactions in transformed human B cells demonstrated that amongst the mitogens, PMA, and amongst the cytokines, IL-4, induced strong HA recognition in the EBV⁺ BL cells, BL-30/B95-8 and in the *in vivo* EBV-infected B cells, IM. This phenomenon was not observed in B-LCL cells, highlighting the stark contrast between BL30/B95-8 and B-LCL B cells. It appears that *in vitro* EBV-transformation of normal human B cells abrogates their ability to recognize HA following PMA stimulation. Attempts to delineate the molecular mechanism suggested that the increased HA adhesion observed in BL-30/B95-8 cells following PMA and/or IL-4 stimulation correlated with enhanced expression levels of CD44 H, V3^{*}, V6^{*} and V9^{*} isoforms and

post-translational modifications to CD44. Differential utilization of the V4 and V5 exons was also observed in BL-30/B95-8 cells following stimulation with PMA, IL-4 and IL-5.

As described, cytokines have been reported to modulate CD44-mediated HA binding and CD44 expression in various cell types (174,176,178,179). Originally in murine T cells and T cell lymphomas, PMA and certain anti-CD44 mAbs have also been shown to induce CD44-HA binding (152,157). The observed upregulation of HA binding in response to PMA or IL-4 in BL-30/B95-8 and lack of this effect in B-LCLs may be attributed to the level of CD44 H expression (10,156) and/or the differential expression of variable exon containing isoforms (162). PMA and IL-4 exerted upregulatory effects on CD44 H expression in BL-30/B95-8 and IM cells (Fig. 4-6). However, B-LCLs, as seen in binding experiments, failed to respond. In BL-30/B95-8 cells, PMA also enhanced CD44 V3*, V6* and V9* while IL-4 upregulated CD44 V3* and V6* expression. This was not observed in any of the B-LCLs studied. Therefore, the induction of HA recognition in response to these factors correlated with the enhanced expression of the indicated CD44 isoforms. CD44 H expression levels may also explain, at least in part, the variability in binding of BL cells to HA. Although infected with EBV, the BL cells EB3, Raji, and BJAB/B95-8 did not express CD44 (Fig. 5-2) and hence failed to bind HA. RT-PCR analysis of CD44 V isoform expression in BL-30/B95-8 cells demonstrated that in contrast to the other variable exons, V4 and V5 exon usage appeared to be differentially regulated in response to IL-4 and IL-5. Alterations in CD44 V4-5* were not detected at the protein level. This may be due to the same reasons suggested for normal human B cells. In addition to the fact that CD44 V4 and CD44 V5 are not recognized by the antibody used, the expression level of CD44 V4-5 may be too low for

detection by this method. It should also be noted that although the levels of isoform expression vary between BL-30/B95-8 cells and B-LCLs, their identity, as determined by RT-PCR, was the same. Overall, no clear correlation could be made between the induction of HA adhesion and a specific CD44 V isoform in BL-30/B95-8 cells. The specific role of the various isoforms expressed in these B cell lines and in particular the significance of the differential inclusion of exons V4 and V5 observed are not clear at present.

It is probable that the effect on HA adhesion observed may not exclusively depend upon the level of CD44 H expression as unstimulated BL-30/B95-8 and B-LCL cells constitutively express substantial levels of CD44 (Fig. 4-6) yet do not exhibit spontaneous binding to HA (Fig. 4-3). This would indicate that these cells might express a predominantly inactive form of CD44. Furthermore, PMA or IL-4-induced CD44 expression and CD44-mediated HA binding in BL30/B95-8 cells was detectable by 8 or 12 hrs after stimulation, respectively, a time sufficient to allow cell surface expression of newly synthesized CD44 molecules. This is evident by the detection of new CD44 mRNA synthesis by RT-PCR analysis (Fig. 4-8) and slight alterations in the molecular size of CD44 (Fig. 4-9).

As mentioned, post-translational modification of the CD44 protein has surfaced as a critical mechanism by which HA recognition is controlled (158-161,166). In BL-30/B95-8 cells, it was shown for the first time that IL-4 as well as PMA induced CD44 proteins with slightly reduced molecular weight relative to unstimulated cells, indicative of such alterations. These observations taken together with the enhanced binding to HA following treatment of BL30/B95-8 cells with the N-linked glycosylation inhibitor

tunicamycin, strengthen the contention that such alterations in CD44 glycosylation status may be important in the induction of HA binding in these cells by IL-4 and PMA. These factors failed to induce similar changes in B-LCLs. A distinction should be made here between a reduction in the complexity of glycosylation and its absence. Treatment with tunicamycin would have the effect of inhibiting N-linked glycosylation altogether. Recently, a study using N-linked glycosylation site-directed mutants of murine CD44 provided evidence that the elimination of particular sites of CD44 N-linked glycosylation could induce HA recognition (220). This would explain how the inhibition of N-linked glycosylation by tunicamycin in BL-30/B95-8 cells translated into enhanced HA recognition. These results lay the foundation for future work investigating what specific alterations in CD44 glycosylation are important for the induction of HA recognition in these cells. Also, it should be recognized that the inhibition of N-linked glycosylation in B-LCLs was not sufficient to alter HA adhesion in these cells. This likely reflects the requirement of a combination of effects in order to alter binding in this system including alterations in: N-linked glycosylation, CD44 H expression levels and CD44 V* isoform expression.

In EBV-positive BL and B-LCL cells, IL-4 induces the expression of IgE germ line transcripts without inducing IgE synthesis (221), and expression of CD23, class II molecules and adhesion molecules LFA-1 and LFA-3 (222,223). Unlike IL-4, IL-13 failed to influence CD44 expression or HA adhesion in BL30/B95-8 cells, even at concentrations of up to 200 ng/mL. Even though BL30/B95-8 and B-LCL cells are infected with wild type EBV, and express the same set of CD44 isoforms, PMA or IL-4 did not enhance CD44 protein expression or CD44-mediated HA binding in B-LCL cells.

Furthermore, EBV transformation of normal human B cells abrogates their ability to bind HA following PMA stimulation. The signal transduction pathway leading to the induction of HA binding by PMA or IL-4, the distinction between IL-4 and IL-13 in this regard observed for BL-30/B95-8 cells, and the lack of these effects in B-LCL cells, is not understood at present. Furthermore, the biological effects mediated by IL-13 on BL cells have not been studied. It is possible that *in vitro* EBV transformation of B cells inactivates a vital element in the intracellular signaling pathway such as the EGR1 transcription factor mentioned above. The contrasting effects of IL-4 and IL-13 in BL-30/B95-8 may also be due to the transduction of distinct signals at the level of the Janus kinase activation pathway (218) or the differential induction of transcription factors such as EGR1. Furthermore, the *c-myc* translocation not found in B-LCLs may have a contribution in CD44-mediated HA adhesion. Transfection of an activated *c-myc* gene into B-LCL cell lines has been shown to yield cells with many of the phenotypes attributed to BL cells including CD10 and CD38 expression and the propensity to undergo apoptosis (224). However, PMA and IL-4 were able to induce CD44 expression and CD44-HA binding in IM cells, the *in vivo* infected human B cells. The status of IM cells with respect to *c-myc* translocation and other genetic alterations is the subject of further investigation.

6.300 Role of Epstein-Barr virus infection in the induction of CD44 isoform expression and HA recognition in B lymphocytes

The effect of EBV infection on CD44 isoform expression in normal and transformed human B cells was investigated. It was shown that although EBV possesses the ability to induce CD44 isoform expression, this does not hold true in all EBV⁺ B cell lines and may reflect a difference at the level of cell differentiation stage. Furthermore, it should be stressed that infection with EBV and the resultant induction of CD44 isoform expression is not sufficient, on its own, to enable the CD44⁺ B cells to bind HA. Stimulation with PMA and in the case of BL-30/B95-8 BL cells, IL-4, is required for this (chapter 4). Of particular interest was that even though *in vitro* EBV infected normal human B cells (B-LCLs) express CD44 V8⁺, V9⁺, V10⁺ and substantial levels of CD44 H (Fig. 5-6), their ability to bind HA in response to PMA was concomitantly abrogated (Fig. 4-3). In contrast, the B cell lines infected *in vivo* with EBV, IM, derived from a patient with infectious mononucleosis, expressed CD44 and bound HA in response to PMA or IL-4. It was further demonstrated that the EBNA-2 gene of EBV is required for the induction of CD44 isoform expression. As expected, EBV⁻ B cell lines CA46, ST486, MC116, BL-30, Ramos, and BJAB, which did not express CD44 (Fig. 5-1, 5-2), also failed to recognize HA (Fig. 4-3, data not shown).

Epstein-Barr virus infection of B lymphocytes is known to induce the expression of activation and differentiation antigens (CD21, CD23, CD30, CD39, Bcl-2, Bcl-1, vimentin, surface IgD, etc.), adhesion molecules (LFA-1, ICAM-1), and drives immunoglobulin synthesis as seen in antigen activated B cells (145,199,201,202,205,206). EBV also promotes cellular growth by inducing the loss of responsiveness to the growth inhibitory

signals of TGF β (225). Although EBV positive BL have been previously shown to express CD44 (145,206), the results from chapter 5 show in addition that EBV induces alternatively spliced CD44 V8^{*}, V9^{*} and V10^{*} isoforms in normal human B cells and in certain, but not all, BL cell lines. This was more precisely studied by employing EBV negative BL cells: BL-30, and BL-30 cells infected with the Epstein-Bar virus Nuclear Antigen-2 (EBNA-2) defective EBV strain P3HR1 (BL-30/P3HR1) or the marmoset derived wild type B95-8 strain of EBV (BL-30/B95-8). This panel of cell lines provided an isogenic background and minimized the effects of genes other than EBV in influencing this phenomenon. The fact that the B95-8 strain and not P3HR1 induced CD44 expression in BL30 cells, suggested that the presence of EBNA-2 may be required for this induction.

The specific role of EBNA-2 was not addressed in these experiments but studies conducted by others sheds some light on the mechanism at work. Wang et al. (145), introduced individual EBV latent genes into a panel of B cell lines (BJAB, BL41, Louckes and Daudi) and assayed for the expression of a number of cellular genes including CD44. Not unlike my results, considerable variability between cell lines was observed with respect to CD44 induction and that of other genes tested such as CD21, CD23, CD39, and CD40. Specifically, EBNA-2 did not influence total CD44 expression, as revealed by flow cytometry, in any of the cell lines. However, LMP-1 was shown to induce total CD44 expression in Daudi transfectants, which also carry an EBNA-2-deleted EBV genome, but not in the other cell lines tested. Therefore, it appears that the LMP-1 protein and not EBNA-2 has the capacity of inducing CD44. The reason underlying why a deletion in the EBV genome encoding EBNA-2 abrogated the ability of the defective virus to induce CD44 in BL-30/P3HR1 cells, is likely the function of EBNA-2 as a transcriptional regulator of

LMP-1 (226). During infection with the EBNA-2 deleted P3HR1 strain then, LMP-1 is not expressed and therefore is unable to upregulate CD44 expression. Interestingly, the fact that LMP-1 transfection induced CD44 in only Daudi (EBNA-2 deleted) cells suggests that there may exist some form of cooperation between LMP-1 and other latent EBV gene(s). The inductive effect of EBV on CD44 expression did not hold true in all of the EBV⁺ B cell lines tested, namely, RAJI, EB3 and BJAB/B95-8. The reason for this is not clear at present. Alterations in the EBV genome and hence expression of latency genes, in combination with other chromosomal abnormalities accumulated by these cells may explain this, at least in part. It is known for example that EBV from Raji cells contains deleted portions in EBNA-3C and therefore, this gene is not expressed and may render the virus non-transforming (227-229). BJAB cells do not contain a translocated *c-myc* gene and exhibit a different cell surface phenotype from typical group I BLs, such as the constitutively high expression of the adhesion molecules LFA-1 and ICAM-1 (145). As mentioned, in agreement with our results, their transfection with individual EBV proteins failed to induce CD44 expression (145). Such variations, as far as the BL cells are concerned, may have initiated a partial block in their phenotypic conversion towards a group III, CD44⁺ phenotype *in vitro*. BJAB cells on the other hand may be at a different stage of differentiation and hence varied in their response to EBV infection or the introduction of individual EBV genes.

The present studies also confirmed previous observations that EBV negative BL cells do not express CD44 (145) and hence do not adhere to HA [data not shown and ref. (206)]. Both EBV negative and EBV-positive BL cells with a group I phenotype, as mentioned, resemble the proliferating pool of centroblasts within germinal centres, which

characteristically express CD10, CD38 and CD77 antigens but do not express CD44 (85,86). The absence of CD44 in these cells may thus be mapped to their reputed centroblast origin (85,86). Alternatively, these cells may have lost CD44 expression through the course of their malignant transformation. The emergence of the *c-myc* translocation in these cells may have some contribution here. As already mentioned, it was found that transfection of B-LCL cells with an activated *c-myc* gene generated cells with many of the phenotypes attributed to group I BL cells including EBNA-2 and LMP-1 independent proliferation, CD10 and CD38 expression and the propensity to undergo apoptosis (224,230).

6.400 Summary

I show that normal human B cells can be induced to bind HA following stimulation with PMA and this binding can be effectively reversed with IFN- γ and to some extent by IL-4. IFN- γ and IL-4 are thus not only potent B cell differentiation cytokines but may play a vital role in regulating the CD44-mediated binding of B cells to the extracellular matrix molecule, HA. However, the physiological significance of CD44-mediated HA adhesion by PMA is not clear at present. It has been suggested that PMA induces B cells to differentiate into cells resembling the large (monocytoid) form of extrafollicular B cells (231). The acquisition of such a B cell phenotype may be necessary for the binding and retention of these cells in the extrafollicular microenvironment of lymphoid tissues. The inhibition of CD44-mediated binding by IFN- γ and IL-4 may thus have implications with respect to B cell trafficking during the development of an immune response. CD44-mediated binding to HA in BL30/B95-8 BL

cells is upregulated by IL-4 and PMA. The molecular mechanism responsible for these effects on binding may include a combination of: altered CD44 H expression levels, differential CD44 V isoform expression and post-translational changes to the CD44 H protein. This mechanism is inactivated in B-LCLs. Although we have not observed this phenomenon in all BL cell lines, the results may have implications in the establishment and dissemination of B cell tumors. Furthermore, the novel effect of IL-4 on CD44-HA interactions in BL30/B95-8 cells provides a model system to further study the molecular mechanisms responsible for the regulation of CD44 expression and CD44-mediated HA binding in human B cells. Taken together, these findings suggest that IL-4 in addition to its role in B cell activation, proliferation and differentiation may also influence the adhesive interactions between transformed B cells and ECM molecules, which may affect their trafficking and localization within different tissues and organs. Finally, EBV clearly possesses an inductive influence on CD44 isoform expression in human B cells. Nevertheless, it appears that there is a complex interplay between the virus, the B cell, and the microenvironment, which directs the pathway leading to CD44 isoform expression and HA recognition. The loss of control over this pathway may potentially enhance the proclivity of B cells to form tumors and/or metastasize.

CHAPTER 7

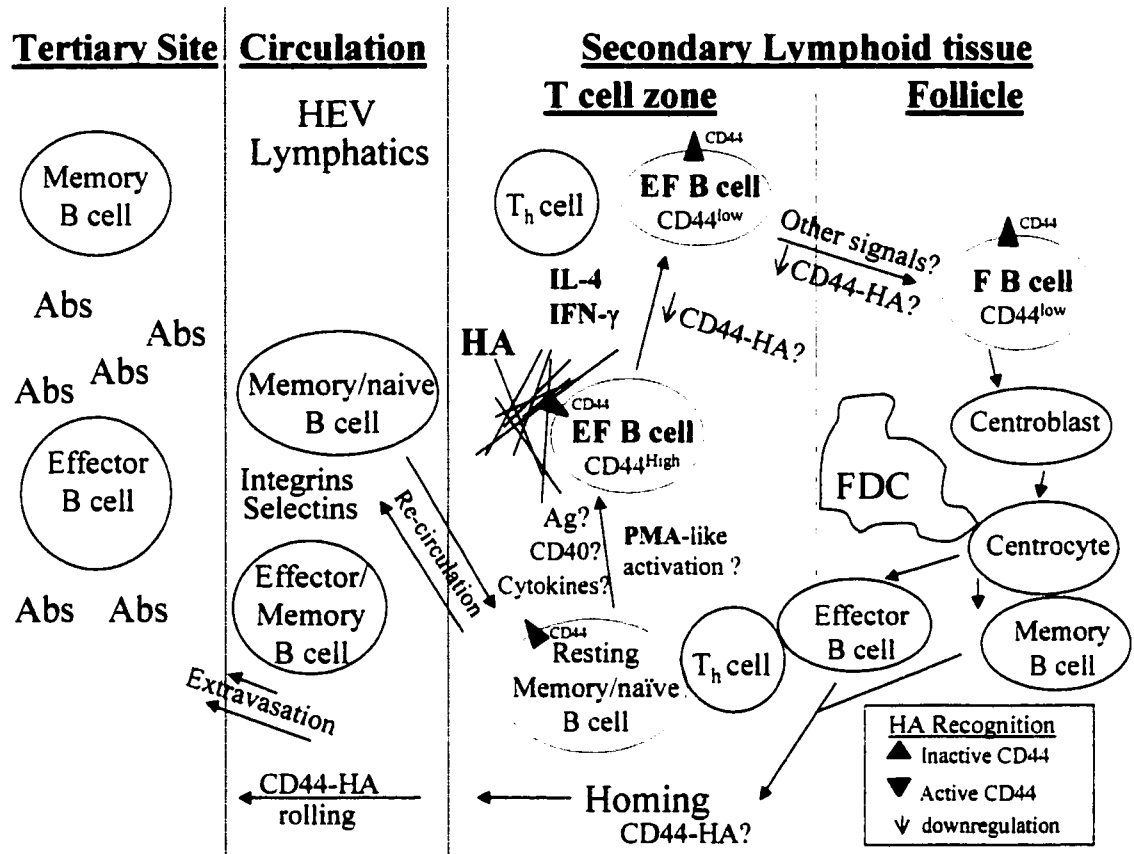
CONCLUDING REMARKS

The main findings of the thesis establish that the CD44-mediated hyaluronan adhesion in normal and transformed human B cells is dependent on mode of activation and subject to regulation by a restricted set of cytokines known to be critical in B cell differentiation, namely, IFN- γ and IL-4. The potential mechanism at work in this regulation may involve a combination of differential CD44 isoform expression and post-translational modification of the CD44 protein. It is befitting to speculate that such a regulation may affect B cell trafficking *in vivo* during the generation of immune responses, formation of tumors and/or metastasis. The models presented in Fig. 7-1 and 7-2 expand upon how the phenomena brought to light in this body of work may be physiologically relevant, based on the results presented and pertinent information available in the literature. It should be stressed however that their establishment demands further investigation and proof.

Although the physiological significance of PMA-induced HA recognition is not clear at present, it has been suggested that PMA brings about the differentiation of B cells into cells resembling the large (monocytoid) form of extrafollicular B cells (EF B cell, Fig. 7-1) (231). Another study has suggested that co-ligation of sIgM and CD40 on the surface of B cells induces a similar B cell population with elevated CD44 levels (175). The acquisition of such a B cell phenotype may be necessary for the retention of these cells in the extrafollicular microenvironment of lymphoid tissue. The provision of other signals from Th cells such as IL-4 or IFN- γ , for example, which downregulate HA adhesion, may enable these cells to enter lymphoid follicles and progress further along their differentiation pathway, as depicted in Fig. 7-1 (F B cell). Integrins are thought to be of fundamental importance in this process, referred to as microenvironmental homing,

Fig. 7-1: Model depicting the maturation of a B cell immune response and the potential role of CD44-HA interactions herein

Schematic representation of how the regulation of CD44-HA recognition in normal human B lymphocytes may influence its differentiation and trafficking within lymphoid tissues, the circulatory system, and to tertiary sites such as the bone marrow, mucosa, and others. The stimulation of resting B cells with Ag in combination with T-B cell cognate interactions is critical in the propagation of B cell immune responses. Activation of B cells with PMA may mimic such interactions and induce cells resembling extrafollicular B cells [EF B cells; ref. (231)], capable of binding HA. Under the influence of IFN- γ or IL-4, provided by T cells, EF B cells may lose their HA binding capacity, which in turn may allow their entry into follicles as follicular B cells (F B cells) and continue their differentiation towards plasma and memory cells. Effector and memory B cells are believed to enter the circulation and home to tertiary tissues. Naïve B cells, although unable to traffic to tertiary sites, re-circulate between the blood, lymph and secondary lymphoid tissues, as do memory B cells, a process mediated primarily by integrins and selectins.



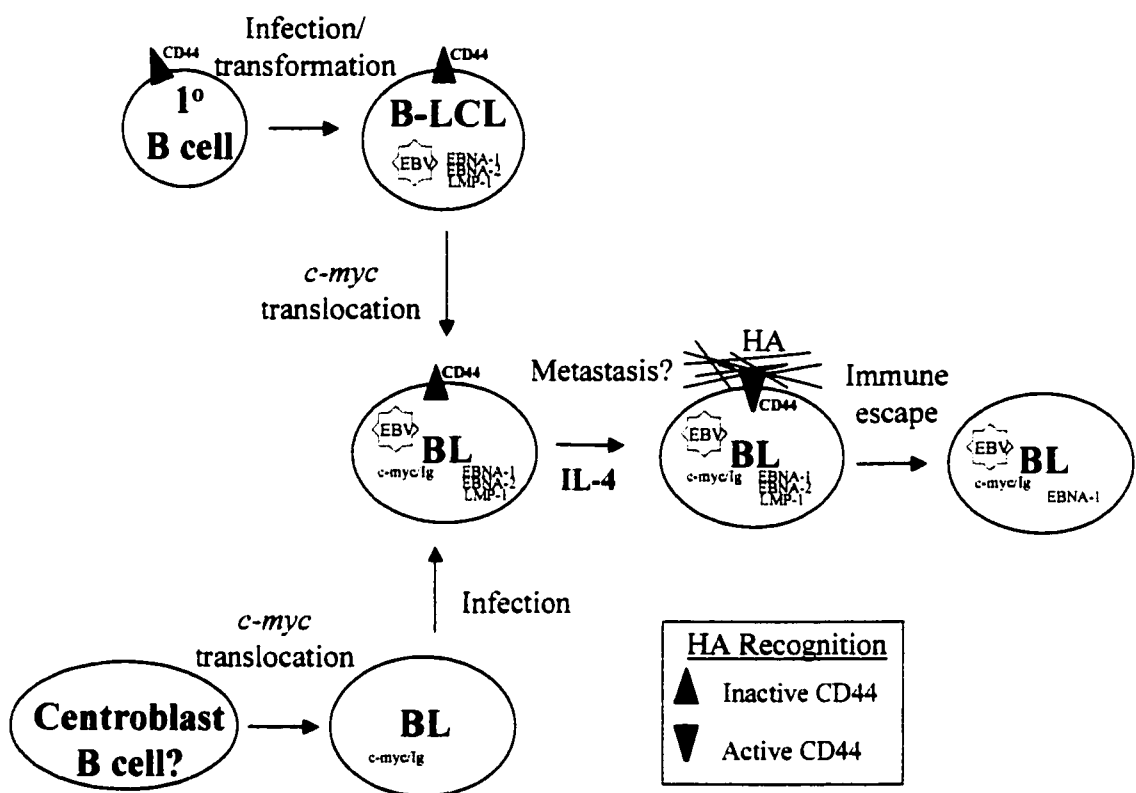
a domain, which remains largely uninvestigated (59). The role of CD44-HA interactions in microenvironmental homing is only speculative at this point. However, some circumstantial support for this hypothesis comes from the fact that CD44 is downregulated in follicular B cells, particularly the rapidly dividing centroblasts, and is re-expressed in a sub-population of centrocytes and post-germinal centre cells (effector and memory B cells) (83-86). Post-germinal centre B cells are then thought to home to tertiary sites such as the intestinal lamina propria where their action (Ab production) is required (59). The importance of HA recognition in the ability of these cells to exit lymphoid tissue and enter circulation is again speculative. CD44-HA interactions may also come into play as the cells traffic to the tertiary site. Evidence for this has been provided in activated murine T cells, which have been shown to home to inflammatory sites in a manner dependent upon their CD44-mediated rolling (1^o adhesion) on HA (14,15,68). It should be emphasized at this point that CD44 is one molecule among many, which participate in the direction of tissue specific homing. Unlike its relative importance in homing to tertiary sites, it appears that CD44 may be dispensable for “immune surveillance” in which lymphocytes constantly circulate between lymphoid tissue and the blood, a process mediated primarily by selectins and integrins (59,66).

Turning to the results obtained in transformed B cells, it is also obligatory to speculate on the physiological implications in the context of BL pathogenesis (see Fig. 7-2). Two possible scenarios have been proposed with respect to how BL tumors may arise and the role of EBV and the *c-myc* translocation in this process (140,143). A model proposed by Polack et al. (230) involves the initial infection/immortalization of primary

Fig. 7-2: Model of Burkitt's lymphoma pathogenesis and the potential role of CD44-HA interactions

One possible scenario involves the initial EBV infection and transformation of a primary B cell (1° B cell) and the expansion of a B-LCL-like population, expressing the full complement of EBV antigens. Subsequent *c-myc* translocation results in a tumorigenic BL cell, which under the influence of IL-4 could acquire HA binding capacity and metastasize. The *c-myc* translocation also enables EBV Ag-independent cell proliferation and thus the selection pressure for the shut-off of viral gene expression (230), downregulation of CD44 expression and evasion of the anti-EBV immune response. The EBNA-1 gene is necessary for viral genome maintenance and therefore continues to be expressed but does not elicit a significant immune response (140). Alternatively, the *c-myc* translocation could occur in a centroblast cell, the reputed origin of BL cells (85,86) and precede infection with EBV (143). EBV infection induces CD44 expression and under the influence of IL-4 a behaviour similar to that described above could ensue.

Burkitt's Lymphoma Pathogenesis



B cells, which allows their expansion until a T cell response is mounted. The subsequent occurrence of the *c-myc* translocation results in EBNA-2- and LMP-1-independent B cell proliferation and provides selection pressure for the shut off of viral antigen expression, downregulation of adhesion molecule expression (including CD44, LFA-1, ICAM-1, and LFA-3) and thus, immune escape. It is also possible that EBV infects a rare cell in which the *c-myc* translocation has already occurred (143). Fig. 7-2 presents how my results may fit into the BL pathogenesis models. The fact that IL-4 was able to upregulate CD44-HA interactions in BL-30/B95-8 cells may provide an advantage to these cells with regard to their ability to form tumors and/or metastasize. Support for this hypothesis comes from two prior studies conducted in murine experimental models (133,135), demonstrating that the overexpression of CD44 H in BL cells gives them the ability to form tumors and disseminate more readily than parental (CD44-negative) cells. Furthermore, it was shown that the LMP-1 gene of EBV was able to mediate this CD44 expression in BL cells and similarly influence tumor formation (135). I believe that a similar situation may hold true here. EBV infection of these cells was capable of inducing strong CD44 expression. The added complexity revealed by my work was that this strong CD44 expression, on its own, was unable to confer HA recognition to these cells. IL-4 stimulation was required to upregulate HA recognition. This cytokine, provided in the microenvironment, could thus alter the homing/growth pattern of the cells such that they not only form local tumors more readily but are able to disseminate to distant sites and form metastases, in a manner perhaps similar to the way effector/memory B cells target to immunological effector sites.

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Curriculum Vitae

Marko Andrii Kryworuchko

Institut Pasteur
Unite d'Immunogenetique Cellulaire
28 rue du Dr. Roux
Paris, France
Cedex 15

Fax: 011-33-1-45-68-88-38

Email: jennko@hotmail.com

Education

Post-secondary

Ph.D. Microbiology and Immunology

University of Ottawa
Dept. of Biochemistry, Microbiology and Immunology
Ottawa, Ontario, Canada
April 1999

B.Sc. Honours Biochemistry

University of Ottawa
Department of Biochemistry
Ottawa, Ontario, Canada
April 1992

Scholarships

Medical Research Council of Canada

Ottawa, Ontario, Canada 1-613-954-1964
Post-doctoral fellowship (3yrs) May 1999 - April 2002

Natural Sciences and Engineering Research Council of Canada

Ottawa, Ontario, Canada 1-613-996-3796
Post-graduate scholarship held May 1995 - May 1997

University of Ottawa

School of Graduate Studies and Research
Ottawa, Ontario, Canada 1-613-562-5742
Excellence scholarship held May 1995-May 1997
Travel Grants awarded March 1996, June 1994

Dept. of Biochemistry, Microbiology and Immunology
Ottawa, Ontario, Canada 1-613-562-5800 ext. 8164
Travel Grant awarded March 1996

Graduate Students Association
Ottawa, Ontario, Canada 1-613-592-5935
Travel Grant awarded February 1999

National Cancer Institute of Canada
Toronto, Ontario, Canada 1-416-961-7223 ext. 356
Travel award awarded October 1998

Cancer Research Society of Canada
Montreal, Quebec, Canada 1-514-861-9227
Post-graduate studentship awarded May 1995

Secondary Sir Robert Borden High School
Nepean, Ontario, Canada
Ontario Secondary School Honours
Graduation Diploma (June 1988)
Certificate of French Immersion (June 1987)

Other Royal Conservatory of Music
University of Toronto
Toronto, Ontario, Canada
Grade Eight Practical Piano (1986)

Lifeguarding qualifications:
National Lifeguard Service
Red Cross Instructor
Royal Life Saving Society Canada Swim Instructor
Cardio-pulmonary resuscitation-Basic Rescuer
Standard First Aid

Martial arts training:
World Taekwondo Federation black stripe belt

Research Experience

Graduate student (January 1993 – April 1999)
University of Ottawa
Department of Biochemistry, Microbiology, and Immunology
Supervisors: Dr. A. Kumar
Dr. F. Diaz-Mitoma
CD44-hyaluronan interactions in normal and transformed human B cells.

Technical experience: leukocyte sub-population purification, proliferation assays, cell adhesion to extracellular matrix, microscopy, transfection, flow cytometry, immunoprecipitation, Western, Northern and Southern blotting, RT-PCR, molecular cloning

Summer student (May - August 1992)

University of Ottawa

Department of Biochemistry

Supervisor: Dr. R. Haché

Molecular endocrinology research

Technical experience: basic RNA and DNA manipulation, molecular cloning, sequencing, electrophoretic mobility shift assays, DNA footprinting (KMnO₄), chromatography

B.Sc. Honours and volunteer summer student (June 1991 - April 1992)

University of Ottawa

Department of Biochemistry

Supervisor: Dr. M. Chevette

Activation of ras and myc oncogenes in prostate cancer

Technical experience: basic molecular biology (DNA purification, quantitation, digestion, electrophoresis, Northern blotting) and cell culture techniques

Volunteer summer student (June - August 1990)

University of Ottawa

Department of Physiology

Supervisor: Dr. B. Korecky

Cardiac physiology research

Technical experience: Rat heart transplants and related experiments

Teaching experience

Guest Lecturer

Basic Immunology course (1996)

University of Ottawa

Department of Microbiology, Immunology

course co-ordinator: Dr. A. Kumar

Elected positions

Treasurer

University of Ottawa

Department of Microbiology and Immunology

Graduate Students Council (1994)

Vice-president

University of Ottawa
Department of Microbiology and Immunology
Graduate Students Council (1995)

Publications

Papers

1. **Kryworuchko, M., Diaz-Mitoma, F., and Kumar, A. 1999. IFN- γ Inhibits CD44-Hyaluronan Interactions in Normal Human B-Lymphocytes. *Exp. Cell Res.* (In Press).**
2. **Kryworuchko, M., Gee, K., Diaz-Mitoma, F., and Kumar, A. 1999. Regulation of CD44-Hyaluronan Interactions in Burkitt's Lymphoma and Epstein-Barr Virus Transformed Lymphoblastoid B Cells by PMA and Interleukin-4. *Cell. Immunol.* (In press).**
3. **Kryworuchko, M., Diaz-Mitoma, F., and Kumar, A. 1998. Interferon- γ and IL-4 Regulate CD44-Hyaluronan Interactions in Normal Human B Lymphocytes and B cell lines. *Proc. 10th Intl. Congress Immunol.* G.P. Talwar, I. Nath, N.K. Ganguly, K.V.S. Rao, eds. Monduzzi Editore, Bologna, Italy. 1: 361-365.**
4. **Daftarian, M.P., Kumar, A., Kryworuchko, M., and Diaz-Mitoma, F. 1996. IL-10 Production is Enhanced in Human T cells by IL-12 and IL-6 and in Monocytes by Tumor Necrosis Factor- α . *J. Immunol.* 157: 12-20.**
5. **Singh, B., Merchant, P., Walker, C.R., Kryworuchko, M., and Diaz-Mitoma, F. 1996. IL-6 Expression in Cord Blood of Patients with Clinical Chorioamnionitis. *Ped. Res.* 39(6): 976-979.**
6. **Kryworuchko, M., Diaz-Mitoma, F., and Kumar, A. 1995. CD44 Isoforms Containing Exons V6 and V7 are Differentially Expressed on Mitogenically Stimulated Normal and Epstein-Barr Virus-Transformed Human B Cells. *Immunol.* 86: 41-48.**
7. **Diaz-Mitoma, F., Kumar, A., Karimi, S., Creery, D., Kryworuchko, M., Daftarian, M.P., Fillion, L.G., and Cameron, W. 1995. Expression of Interleukin (IL)-10, IL-4 and Interferon- γ in Unstimulated and Mitogen Stimulated Peripheral Blood Lymphocytes from HIV Seropositive Patients. *Clin. & Exp. Immunol.* 102:31-39.**
8. **Kumar, A., Kurl, R.N., Kryworuchko, M., Diaz-Mitoma, F., and Sharma, S. 1995. Differential Effect of Heat Shock on RNA Metabolism in Human Burkitt's Lymphoma B-Cell Lines. *Leuk. Res.* 19(11): 831-840.**

Abstracts

1. **Kryworuchko, M.,** Gee, K., Diaz-Mitoma, F., and Kumar, A. IL-4 Differentially Regulates CD44-Hyaluronan Interactions in Burkitt's Lymphoma BL-30/B95-8 and EBV-Transformed Lymphoblastoid B Cells. Nov. 1998. *The 10th Intl. Congress Immunol.* New Delhi, India.
2. **Kryworuchko, M.,** Diaz-Mitoma, F., and Kumar, A. IFN- γ and IL-4 Inhibit CD44-Hyaluronan (HA) interactions in Normal Human B Lymphocytes. Nov. 1998. *The 10th Intl. Congress Immunol.* New Delhi, India.
3. **Kryworuchko, M.,** Diaz-Mitoma, F., and Kumar, A. Regulation of CD44-Hyaluronan Interactions in Normal and EBV-Transformed Human B-Lymphocytes by IL-4 and IFN- γ . 1997. *J. Allergy Clin. Immunol.* Vol. 99, No. 1, part 2, pp. S456, Abstract #1854.
4. **Kryworuchko, M.,** Diaz-Mitoma, F., and Kumar, A. Regulation of CD44-Hyaluronan Interactions in Normal and EBV-Transformed Human B-Lymphocytes by IL-4 and IFN- γ . 1996. *FASEB J.* 10(6): A1462, Abstract #2662.
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9. Singh, B., Diaz-Mitoma, F., **Kryworuchko, M.,** Walker, C.R., and Merchant, P. IL-6 Expression by Cord Blood T-Lymphocytes in Perinatally Acquired Infection. 1994. *The Amer. Ped. Soc.*

References

Ashok Kumar D.V.M., Ph.D.
Assistant Professor
Children's Hospital of Eastern Ontario
Division of Virology
401 Smyth Rd. Ottawa, Ontario, Canada
K1H 8L1
1-613-738-3920

Francisco Diaz-Mitoma M.D., F.R.C.P.C., Ph.D.
Medical Director of Laboratory Medicine
Children's Hospital of Eastern Ontario
401 Smyth Rd. Ottawa, Ontario, Canada
K1H 8L1
1-613-737-2736