EXPRESSION AND FUNCTIONAL ANALYSIS OF MURINE INTERCELLULAR ADHESION MOLECULE 1 (ICAM-1)

BY

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ABSTRACT

Cell adhesion molecules enhance interactions between adjacent cells in order to mediate a large variety of functions of the immune system. An antibody against the murine lymphocyte surface antigen MALA-2 has previously been shown to inhibit mixed lymphocyte response. A λgt10 cDNA library from NS-1 cells was screened and a cDNA clone, K3-1.1, was previously isolated. It had significant homology to the human ICAM-1 gene. This thesis covers the isolation of a second cDNA clone, K4-1.1, and its comparison to K3-1.1 in terms of expression, function and distribution.

The two clones are identical in sequence with the exception of the 5' ends. Expression of these two clones was examined using a transient expression system of COS cell transfection. Cell surface expression of the K3-1.1 clone could not be detected by FACS analysis. Even when the 5' untranslated region of the K3-1.1 clone (which has 10 potential translation start sites) was removed, protein could not be detected at the cell surface, intracellularly, or extracellularly. However, K4-1.1 expression was detected at the cell surface. Northern blot analysis reveals that there are two distinct messages which are likely to be represented by the two clones. When the northern blot was probed with the 5' end of the K3-1.1 clone, only one of the messages was detected. This together with the result of Southern blot analysis suggests that the two messages are likely the result of alternate splicing.

In order to examine the interactions of the murine ICAM-1 with the surface of other cells, an expression system which would produce large amounts of a secreted soluble form was established. The soluble protein was purified from the supernatant of transfected cells by an antibody-affinity column and used in preliminary binding assays.

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LIST OF ABBREVIATIONS

AMP	ampicillin
APC	antigen-presenting cell
bp	base pair
BSA	bovine serum albumin
c-abl	cellular abl protooncogene
CD	cluster of differentiation
cDNA	complementary DNA
CML	chronic myelogenous leukemia
ConA	Concanavalin A
cpm	counts per minute
CTL	cytolytic T lymphocyte
DEAE	diethylaminoethyl
DMEM	Dulbecco's modified minimum essential media
EDTA	ethylenediamine tetraacetic acid
EtBr	ethidium bromide
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
GTE	glucose-Tris-EDTA buffer
HBSS	Hanks' balanced salt solution
HEPES	N-2-hydroxyethylpiperazine
HEV	high endothelial venule
HEV_{LN}	peripheral lymph node-specific HEV
HEV_{PP}	Peyer's patch-specific HEV
HIV	human immunodeficiency virus
ICAM	intercellular adhesion molecule
IFN	interferon
Ig	immunoglobulin
IL	interleukin
IPTG	Isopropylthio-β-D-galactoside
kb	kilobases
kd	kilodalton
LAD	leukocyte adhesion deficiency
LAK	lymphokine-activated killer cells
LB	Lennox broth base
LFA	lymphocyte function-associated antigen
LPS	bacterial liposaccarides
MAb	monoclonal antibody
MAd	mucosal addressin
MAG	myelin-associated glycoprotein
MALA-2	murine activation lymphocyte antigen
MHC	major histocompatibility complex
MLR	mixed lymphocyte reaction
mRNA	messenger RNA
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NCAM	neural cell adhesion molecule
NK	natural killer cell
NP-40	nonidet P-40
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PEG	polyethylene glycol

PMA	phorbol myristic acetate
SDS	sodium dodecyl sulphate
SSC	saline sodium citrate buffer
SSPE	saline sodium phosphate EDTA buffer
TAE	Tris acetate EDTA buffer
TBE	Tris borate EDTA buffer
TcR	T-cell receptor
TE	Tris EDTA buffer
TET	tetracycline
TNE	Tris sodium chloride EDTA buffer
TNF	tumor necrosis factor
Tris	tris(hydroxymethyl)aminomethane
TWEEN-20	polyoxyethylene-sorbitan monolaurate
VCAM	vascular cell adhesion molecule
VLA	very late antigen
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

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"Most losers never realize how truly close to achieving their goals they were when they decided to quit. This is the greatest tragedy I know of."

Mike Keenan, Chicago Black Hawks' Coach
1989 Stanley Cup Playoffs

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CHAPTER I

INTRODUCTION

The outer boundary of a cell can be defined by the plasma membrane which surrounds and separates individual cells from the environment and other cells. In addition to delineating cells, a plasma membrane also contains proteins that can perform a variety of functions. These functions include regulating the movement of substances across the membrane, detecting extracellular signals, and generating appropriate responses to the signals. Membrane bound glycoproteins are also involved in cellular recognition and adherence to other cells and non-living substrates. The adhesive interactions between cells and their substrates are mediated by cell adhesion molecules in a region outside the plasma membrane. This region can be divided into two segments, the cell coat and the extracellular matrix. Proteins and their carbohydrate side chains extending out from the membrane make up the cell coat. The extracellular matrix is an organized meshwork of proteins and carbohydrates secreted and assembled locally. Macromolecules in both these regions play important roles in the adhesive properties of cells.

1.1 Cellular Adhesion

1.1.1 Cell:Cell Adhesion

The mechanism for the adhesion relies on complementary interactions between the binding sites on the receptor and its ligand pair (Hood et al,1984). A stereochemical fit between complementary molecules provides the molecular basis for these interactions.

Hydrophobic and ionic interactions, hydrogen bonding, and van der Waals forces are either directly involved or act to strengthen the primary forces to give an overall attractive force between the macromolecules (Freifelder, 1982). Self-self interactions can occur between identical receptors on different cells. One such example of homophilic interactions is that between nerve cells mediated by neural cell adhesion molecule (NCAM) (Hoffman and Edelman, 1983). Complementary interactions can also occur between a receptor protein on one cell type and a different macromolecule on another cell type. Examples of these heterophilic interactions include the T cell receptor on a lymphocyte recognizing and binding the foreign antigen in the context of the major histocompatibility complex (MHC) on antigen presenting cells (APC) (Marrack and Kappler, 1986), and CD2 (CD = cluster of differentiation) on T lymphocytes interacting with the broadly distributed lymphocyte function-associated antigen 3 (LFA-3) (Selvaraj et al, 1987; Dustin et al, 1987a). The third possible mechanism of recognition and adhesion involves receptors on different cells recognizing the same multivalent linker macromolecule. Fibronectin is a large extracellular glycoprotein that can act as a bridge between two cells (Hynes, 1986).

1.1.2 Cell:Matrix Adhesion

The extracellular matrix is an insoluble meshwork of proteins and polysaccharides that fill most of the intercellular spaces. Macromolecules within this complex architecture include collagen, laminin, proteoglycans, elastin, hyaluronic acid, fibronectin, and vitronectin (McDonald, 1988). In addition to providing structural integrity, these macromolecules have also been shown to play a critical role in many biological processes including cell migration. In the case of embryonic development, the linkage between a cell and the extracellular component is under strict regulation and disrupting the interactions leads to embryonic defects (Menko and Boettiger, 1987). When a tissue is damaged, the extracellular scaffold usually remains intact and provides an adhesive substrate for guiding repair. The binding of platelets, activated as a result of exposure to collagen at sites of trauma, occurs via inducible

receptors for extracellular matrix proteins and is one of the first steps toward the repair of damaged blood vessels (Gardner and Hynes, 1985; Hynes, 1987). Cellular fibronectin is also expressed during wound healing (Vartio et al, 1987) and in lesions characterized by mesenchymal cell proliferation and connective tissue deposition (McDonald, 1988). The effect of adhesion on cell migration may also play an important role in tumor cell invasion of surrounding tissue. Most malignant cells lack their own extracellular matrix (Hayman et al, 1982; Hedman et al, 1982). However, when purified fibronectin is added to these cells, they adhere more tenaciously to extracellular matrix substrates (Hynes, 1982). Thus, the extracellular matrix may also function to inhibit cell migration. A greater insight into the role that the extracellular matrix plays in these varied biological processes can be gained by examining the receptors for the different extracellular macromolecules.

The interaction of eukaryotic cells with other cells and with the extracellular matrix is mediated by membrane-spanning surface receptors. The receptors for the extracellular macromolecules were found to be related to several independently identified groups of proteins. All these proteins have been grouped together into the integrin supergene family based on structural, functional, and sequence homology (Hynes, 1987; Ruoslahti and Pierschbacher, 1987) and summarized in Table I. Integrins are heterodimers composed of noncovalently linked α and β chains. Each subunit contains a large extracellular domain, a transmembrane region, and a short cytoplasmic domain (Ruoslahti and Pierschbacher, 1987). Ligand binding requires both subunits (Buck et al, 1986; Santoro et al, 1987) as well as a divalent cation such as Ca^{2+} or Mg^{2+} (Pytela et al, 1987). Most of the integrins that bind extracellular substrates do so through the sequence Arg-Gly-Asp (RGD) as evidenced by the ability of RGD-containing oligopeptides to inhibit cellular binding to these substrates (Ruoslahti and Pierschbacher, 1986; Pierschbacher and Ruoslahti, 1984).

TABLE I

INTEGRIN SUPERGENE FAMILY OF CELL ADHESION RECEPTORS

Integrin	Subunits	Distribution	Ligands*	Functions
VLA-1	$\alpha_1\beta_1$	widespread	(COLL, LM)?	wound healing, cell migration
VLA-2	$\alpha_2\beta_1$	widespread	COLL, (LM)?	wound healing, cell migration
VLA-3	$\alpha_3\beta_1$	widespread	COLL, LM, FN	wound healing, cell migration
VLA-4	$\alpha_4\beta_1$	lymphocyte, monocyte	VCAM-1	cell-cell adhesion
VLA-5	$\alpha_5\beta_1$	widespread	FN	wound healing, cell migration
VLA-6	$\alpha_6\beta_1$	widespread	LM	wound healing, cell migration
LFA-1	$\alpha_L\beta_2$	lymphoid, myeloid	ICAM-1, ICAM-2	immune cell adhesion
Mac-1	$\alpha_M\beta_2$	lymphoid, myeloid	FB, C3bi, Factor X	leukocyte adhesion, C3bi receptor
p150/95	$\alpha_X\beta_2$	lymphoid, myeloid	C3bi, other?	leukocyte adhesion, C3bi receptor
IIb/IIIa	$\alpha_{IIb}\beta_3$	platelets	FN, FB, VN, vWF	wound healing
VNR*	$\alpha_V\beta_3$	widespread	VN	cell migration

* **C3bi**, C3bi fragment of complement; **COLL**, collagen; **FB**, fibrinogen; **FN**, fibronectin; **ICAM**, intercellular adhesion molecule; **LM**, laminin; **VCAM**, vascular adhesion molecule; **VN**, vitronectin; **VNR**, vitronectin receptor; **vWF**, von Willebrand factor.

1.2 Supergene Families Invovled in Adhesion

1.2.1 Integrin supergene family

The integrin supergene family consists of cell surface receptors, each mediating specific interactions with the extracellular matrix or other cells. Within the integrin superfamily, there are at least 11 distinct receptors divided into three subfamilies based on one of the three commonly shared β subunits, β_1, β_2, and β_3 (Hynes, 1987; Ruoslahti and Pierschbacher, 1987). The three subfamilies all contain multiple heterodimers and each heterodimer is composed of a unique α subunit associated with a β subunit, which is shared by all members of that subfamily. The β_1 subunit defines one subfamily and is the common subunit of the fibronectin receptor (Pytela et al, 1985), the laminin receptor (Gehlsen et al, 1988), and the VLA proteins (very late activation proteins) (Takada et al, 1987; Hemler et al, 1988). Six distinct VLA proteins have been identified on a wide variety of cell types and most appear to mediate cellular adhesion to the extracellular matrix (Sonnenberg et al, 1988; Wayner et al, 1988). Only VLA-4 appears to be involved in cell-cell interactions, possibly directing lymphocyte homing (Holzmann et al, 1989; Holzmann and Weissman, 1989a). The second subfamily is defined by the β_2 subunit which is shared by the leukocyte integrins: LFA-1, Mac-1, p150/95 (Hogg, 1989; Anderson and Springer, 1987). These molecules appear to be involved in the function of essentially all leukocyte cell types. Finally, the β_3 subunit is shared by the vitronectin receptor and the platelet glycoprotein IIb/IIIa complex which recognizes fibronectin, vitronectin, fibrinogen, and von Willebrand factor (Pytela et al, 1986; Ginsberg et al, 1987). Recently, two β subunits have been identified that are immunologically and biochemically distinct from the three previously identified β subunits (Kajiji et al, 1989; Cheresh et al, 1989). Preliminary studies seem to indicate that these two subunits are not identical and may represent further additions to the integrin supergene family.

1.2.2 The immune system and the Immunoglobulin supergene family

The immune system consists of a network of cells that maintain basic defences against disease-causing microorganisms, parasites, and cancer cells. In order to function properly, the immune system must be able to distinguish between self and non-self and eliminate the latter. Members of two major protein families, the integrin supergene family and the immunoglobulin supergene family, assist in the removal of pathogens. Members of the immunoglobulin (Ig) supergene family have sequence homology with the variable and/or constant domains of Ig. They also have at least one region of conserved Ig-like tertiary protein structure, called a homology unit. A homology unit consists of approximately 110 amino acids which fold into two sheets of anti-parallel β-strands (reviewed in Williams and Barclay, 1988).

The majority of molecules in the immunoglobulin supergene family are lymphocyte surface antigens involved in antigen recognition and cell-cell interactions (Williams, 1985). The T cell receptor (TcR) αβ heterodimer, an Ig-like molecule (Kronenberg et al, 1986), recognizes the foreign antigen in conjunction with autologous MHC molecules (Marrack and Kappler, 1986; Reinherz et al, 1984), which are also members of the Ig supergene family (Lew et al, 1986; Kaufman et al, 1984). Although the specificity of the immune response is determined by the TcR:(antigen/MHC) bonds, they provide little if any strength in actual T cell adhesion. Antigen-independent adhesion of cytolytic T lymphocytes (CTL) to target cells was the first experiment to suggest that molecules other than the TcR mediate immune cell adhesion (Zagury et al, 1975; Martz, 1975). Further evidence came from a monoclonal antibody against the TcR. The antibody was able to inhibit CTL-mediated killing of target cells bearing the cognate antigen but unable to block conjugate formation between the two cells (Spits et al, 1986). The non-specificity of conjugate formation between CTL and target cells lacking the cognate antigen strongly suggested that accessory molecules other than the TcR and (antigen/MHC) that contribute to T cell adhesion.

1.2.3 Identification of adhesion molecules with monoclonal antibodies

The identification of cell surface molecules involved in the immune system has been aided greatly by the advent of monoclonal antibody technology. Monoclonal antibodies (MAbs) are produced by hybridomas which are generated by fusing a myeloma cell line with spleen cells from an immunized animal (Kohler and Milstein, 1975). The antibody secreted by each hybridoma is monoclonal in origin and thus monospecific in recognizing an epitope on the immunizing agent. Hybridoma supernatants are then screened for their ability to interfere with or enhance the immune response. In the case of cell adhesion molecules, the antibodies are selected for their blocking of conjugate formation between CTL and target cells.

1.3 Lymphocyte function-associated antigen 1 (LFA-1)

1.3.1 Identification and characterization of LFA-1

The first lymphocyte adhesion molecule to be discovered was lymphocyte function-associated antigen 1 (LFA-1). It was initially identified on both murine and human lymphocytes by MAbs capable of blocking CTL-mediated killing of target cells (Davignon et al, 1981; Pierres et al, 1982; Dialynas et al, 1982). In addition to CTL-mediated killing, these MAbs were also able to inhibit natural killer (NK) cell-mediated cytotoxicity and antibody-dependent cytotoxicity mediated by granulocytes and peripheral blood mononuclear cells (Kohl et al, 1984; Miedema et al, 1984; Schmidt et al, 1985). In fact, the anti-LFA-1 MAbs have been observed to inhibit virtually every immune response tested that requires T cell adhesion (Martz, 1987; Springer et al, 1987). These MAbs are also able to block lymphokine-activated killer (LAK) cell-mediated cytolysis (Nishimura et al, 1985) and ingestion of Staphylococcus by neutrophils (Ross et al, 1985). In addition to these functions, LFA-1 also appears to be involved in helper-T-functions. The anti-LFA-1 MAbs inhibit T cell proliferation in response to viruses, alloantigens, xenoantigens, and mitogens (Davignon et al, 1981;

Pierres et al, 1982; Krensky et al, 1983; Hildreth and August, 1985; Dougherty and Hogg, 1987). Even T-cell-dependent antibody responses by B cells are inhibited by anti-LFA-1 MAbs, by affecting interactions with T cells or APC, whereas the T-cell-independent responses are unaffected (Davignon et al, 1981; Howard et al, 1986; Fischer et al, 1986). Inhibition of interleukin 2 (IL-2) production by T cell hybrids when stimulated by APC allogeneic cells was also observed in the presence of anti-LFA-1 MAbs, but not with anti-T-cell receptor antibody coupled to Sepharose (Kaufman and Berke, 1983; Golde et al, 1985).

All the inhibition experiments are consistent with the notion that LFA-1 is involved in strengthening adhesive interactions between cells. In the case of lymphocyte proliferation or cytolysis, the anti-LFA-1 MAbs can actually cause the dissociation of preformed conjugates as long as the antibodies are added no later than 2 hrs after the initial contacts are made (Krensky et al, 1984; Spits et al, 1986). After such a time, the antibodies cannot inhibit these functions, suggesting that LFA-1 is involved in the induction of proliferation and cytotoxicity rather than the processes themselves. Further support for an adhesion strengthening role for LFA-1 comes from studies of activated macrophages binding to tumor cells (Strassman et al, 1986). Adhesion is initially weak, but becomes much stronger over a period of 1.5 hr at 37^{o}C. Anti-LFA-1 F(ab')$_2$ does not prevent the initial weak binding, but does block the development of weak to strong adhesion.

In addition to the immune responses already mentioned, LFA-1 has also been implicated in lymphocyte recirculation (Holzmann and Weissman, 1989b; Wawryk et al, 1989), HIV-induced syncytium formation (Hildreth and Orentas, 1989), and adherence of leukocytes to numerous cell types of non-hematopoietic origin which have been stimulated by various cytokines (Mentzer et al, 1986; Dustin et al, 1986; Dustin et al, 1988a).

1.3.2 Increased LFA-1-dependent adhesion

It seems that LFA-1 is not just playing a passive role in adhesion, but rather an active role in mediating and regulating immune responses . Activation of T cells with PMA, or

triggering through CD2, CD3, or TcR crosslinking results in a strongly increased LFA-1-dependent adhesiveness (Keizer et al, 1988; Dustin and Springer, 1989; van Kooyk et al, 1989). The increased adhesiveness due to TcR crosslinking or anti-CD3 IgG antibody appears almost immediately after activation and returns back to normal 30 min after stimulation. A unique antibody against LFA-1 has been identified that is directed against an epitope near the transmembrane segment (Keizer et al, 1988). Addition of this antibody strongly stimulated the adhesive capacity of spontaneously aggregating lymphocytes. This induced aggregation is inhibited by all other anti-LFA-1 MAbs. Another anti-LFA-1 MAb has been identified that seems to be directed against an activation epitope (Pircher et al, 1986). It induces proliferation and interferon-γ (IFN-γ) release by T cell clones and inhibits CTL-mediated killing by the same cells. It seems clear that LFA-1 plays a major role in adhesion-dependent immunological responses.

1.3.3 Tissue Distribution

The distribution of LFA-1 is confined to cells of hematopoietic origin. LFA-1 is expressed by virtually all leukocytes (Kurzinger et al, 1981; Krensky et al, 1983), with the exception of some tissue macrophages (Strassman et al, 1985). It is present on 1/3 (human) to 2/3 (mouse) of bone marrow cells, the majority of thymocytes, neutrophils, monocytes, and peripheral lymphocytes (Kurzinger et al, 1981; Krensky et al, 1983). LFA-1 expression is first detected in the pre-B cell and the late myeloblast stages of their respective lineages. However, LFA-1 may be lost from B cells during terminal differentiation to plasma cells (Miedema et al, 1985). LFA-1 also appears on the neoplastic counterparts of mature immunocompetent lymphocytes (Miedema et al, 1985).

1.3.4 Structure and biosynthesis

LFA-1 is a member of the leukocyte integrin family in which the α_L subunit is noncovalently associated with the β_2 subunit (Sanchez-Madrid et al, 1983). The 180 kd α and

95 kd β subunits are synthesized as separate precursors of 170 kd and 87 kd, respectively. Both precursors contain high mannose N-glycoside carbohydrate groups linked to polypeptide backbones of 130 kd (α_L) and 72 kd (β_2) (Sastre et al, 1986). Heterogeneity in glycosylation has also been reported. The N-linked carbohydrates are sulfated only on T cells (Dahms and Hart, 1985). As well, sialyation patterns differ between B and T cells (Takeda, 1987). In order for further processing to the complex type N-linked carbohydrate structures, the α and β precursors must first associate intracellularly (Ho and Springer, 1983; Springer et al, 1984). This oligo-saccharide modification occurs in the Golgi apparatus from which the mature proteins are then transported to the cell surface.

1.3.5 cDNA cloning of LFA-1 subunits

The cDNAs encoding both the β_2 subunit and the α_L subunit have been isolated and characterized (Kishimoto et al, 1987a; Law et al, 1987; Larson et al, 1989). The amino acid sequences of these subunits indicate that they have the classical features of integral membrane proteins. Both have large extracellular domains with several potential N-glycosylation sites, 6 on the β subunit and 12 on the α subunit. Both subunits have short transmembrane and cytoplasmic segments. The β subunit has an unusually high cysteine content (7.4%) with a strong concentration over a fourfold repeat region (20%). This high cysteine content is believed to give the β subunit a rigid tertiary structure. A significant feature of the α subunit is the three homologous repeats that have strong similarity to the Ca^{2+}-binding "EF-hand loop" sequences of calmodulin, troponin C, and parvalbumin. These may be the putative metal-binding sites responsible for the Mg^{2+}-dependency of leukocyte adhesion. An α subunit epitope has recently been found to be exposed only in the presence of Mg^{2+} and correlates with functional activity (Dransfield and Hogg, 1989). This epitope is absent or expressed at low levels at low temperatures or in the presence of metabolic inhibitors or Mg^{2+}-chelating agents such as EDTA. Activation of T cells results in an

increased LFA-1-dependent adhesiveness (Dustin and Springer, 1989; van Kooyk et al, 1989) which may be due to a Mg^{2+}-induced conformational change exposing a binding site.

1.4 Leukocyte Integrins

1.4.1 Nomenclature

The leukocyte integrins share both structural and functional similarities. The common β_2 subunit has been assigned the cluster of differentiation CD18 and the α chains of LFA-1, Mac-1, and p150/95 have been assigned the designations of CD11a, CD11b, and CD11c, respectively (Hogg, 1989). The structure, biosynthesis, and glycosylation of Mac-1 and p150/95 are similar to those already described for the LFA-1 α subunit. Homology at the amino acid level also exists among these subunits indicating that they are evolutionarily related (Larson et al, 1989). The Mac-1 and p150/95 α subunits have 63% amino acid identity with each other, but only 35% homology with the LFA-1 α subunit. Mac-1 and p150/95 differ from LFA-1 in terms of storage pattern. While all LFA-1 protein is transported directly to the cell surface after synthesis, Mac-1 and p150/95 can also be stored in intracellular vesicles and granules (Bainton et al, 1987). Thus, surface expression can be regulated by both de novo synthesis and upregulation of preformed material. The intracellular pool provides a means of rapidly responding to a sudden change in the environment, such as an infection.

1.4.2 Mac-1

The distribution of Mac-1 is more restricted than that of LFA-1. Mac-1 expression is detected on monocytes, macrophages, granulocytes, large granular lymphocytes, and immature and $CD5^+$ B cells (de la Hera et al, 1988). However, the structural similarity between LFA-1 and Mac-1 suggests that Mac-1 can also function as an adhesion molecule.

The first functional data came from the ability of the anti-Mac-1 Ab to block the binding of monocytes and granulocytes to erythrocytes coated with C3bi, a component of complement (Beller et al, 1982). Mac-1 has been shown to bind any cell type coated with C3bi (Rothlein and Springer, 1985; Ramos et al, 1988). Thus, Mac-1 is equivalent to the complement receptor type 3. This does not appear to be the only function of Mac-1; it seems to mediate non-specific interactions of myeloid cells. Activated neutrophils form homotypic aggregates which can be blocked by the anti-Mac-1 Ab but not by the anti-LFA-1 Abs (Anderson et al, 1986). Anti-Mac-1 Abs can also inhibit granulocyte migration and neutrophil and monocyte chemotaxis and adherence (Anderson et al, 1986; Dana et al,1986; Wallis et al, 1986). Differential blocking by the different antibodies suggest that Mac-1 is a multifunctional receptor with separate domains for each function.

1.4.3 p150/95

The p150/95 protein has a similar distribution to Mac-1, but is also found on some activated lymphocytes. Like Mac-1, p150/95 can also act as a C3bi receptor (Micklem and Sim, 1985; Malhorta et al, 1986). It too probably functions as general adhesion protein. Anti-p150/95 Ab is able to block neutrophil binding to substrates (Anderson et al, 1986). p150/95 has also been shown to be a major contributor to peripheral blood monocyte chemotaxis as well as adhesion to substrates and endothelial cells (Keizer et al, 1987a). In one case, the anti-p150/95 Ab was able to inhibit conjugate formation between CTL and APC (Keizer et al, 1987b).

1.4.4 Phosphorylation and interaction with cytoskeleton

The fibronectin receptor, a member of the integrin supergene family, has been shown to directly interact with the cytoskeleton (Horwitz et al, 1986). It localizes to sites of adhesion when cells come in contact with each other (Hynes, 1986). LFA-1 is similar in that activated T cells exhibit a redistribution of LFA-1 and talin, a cytoskeletal protein, to sites of adhesion

with the APC (Burns et al, 1988; Kupfer and Singer, 1989). The redistribution and increased adhesiveness associated with T cell activation is dependent on a functional cytoskeleton and metabolic energy production since cytochalasin B, a microfilament inhibitor, and sodium azide each block LFA-1 redistribution and adhesion (Rothlein and Springer, 1986; Marlin and Springer, 1987). The increased adhesion coincides with phosphorylation of the β_2 subunit and detachment coincides with dephosphorylation (Buyon et al, 1990). When neutrophils are treated with thiophosphate, which renders phosphorylated proteins insensitive to phosphotases, they exhibit enhanced C3bi binding but do not display the characteristic decrease in binding with time (Wright and Meyer, 1986). Phosphorylation and dephosphorylation may provide a mechanism for regulating leukocyte adhesion and de-adhesion.

1.5 Genetic deficiency in the leukocyte integrin subfamily

1.5.1 Leukocyte adhesion deficiency disease (LAD)

An inherited immunodeficiency has been identified in which the patients suffer from recurrent, life-threatening bacterial and fungal infections (reviewed in Anderson and Springer, 1987). They consistently exhibit a deficiency in adherence-dependent leukocyte functions (Fischer et al, 1988). Symptoms associated with this disease include defective neutrophil mobility and phagocytosis, lack of pus formation, and absence of lymphocytes and granulocytes in infected lesions despite chronic leukocytosis. The designation of leukocyte adhesion deficiency (LAD) for this disease has now been accepted.

Early work on LAD cells suggested that the defects in leukocyte function were secondary to an abnormality in adhesion (Crowley et al, 1980). The use of MAbs revealed that these cells lacked leukocyte integrin expression (Springer et al, 1984; Arnaout et al, 1984; Beatty et al, 1984). The deficiency in LFA-1, Mac-1, and p150/95 has been traced to an

abnormality in the expression of the common β_2 subunit (Marlin et al, 1986; Lisowska-Grospierre et al, 1986). Normal amounts of the three α chain precursors have been detected in patient cell lines (Lisowska-Grospierre et al, 1986). However, the absence of a functional β_2 subunit prevents intracellular association of the heterodimers, which is required for the proper processing and transport to the surface. In mouse x human hybrids formed with LAD cells, the murine β_2 subunit coassociates with the α subunits of both species resulting in interspecies complexes on the cell surface (Marlin et al, 1986). It has recently been shown that transfection of a functional β_2 cDNA into LAD cells restores LFA-1 expression and function (Hibbs et al, 1990). This further indicates the importance of the β_2 subunit in leukocyte integrin expression.

1.5.2 Mutations in the common β_2 subunit

A defective β_2 subunit represents the fundamental molecular basis of LAD. However, the disease is actually the result of any one of several heterogeneous defects in the β_2 gene (Kishimoto et al, 1987b). Based on the severity of the symptoms, the patients are either characterized as severely deficient (completely lacking $\alpha\beta$ dimer expression) or moderately deficient (low but detectable levels of $\alpha\beta$ expression) (Springer et al, 1984; Anderson et al, 1985). The mutations responsible for these symptoms have been grouped into five classes. In one class, no β_2 mRNA or protein precursor is detected and results in a severely deficient phenotype. The second class of mutation is characterized by low levels of both β_2 mRNA and protein precursor resulting in a moderate phenotype. The third class of mutation is a defect in splicing which results in the deletion of a 90 base exon from the final transcript (Kishimoto et al, 1987b; Kishimoto et al, 1989). The aberrantly small protein is degraded (Dimanche et al, 1987). The fourth class of mutation is a point mutation which causes an amino acid change and creates a new N-glycosylation site (Asn-X-Ser/Thr) (Kishimoto et al, 1990). The extra oligosaccharide moiety appears to disrupt the tertiary structure of the β_2 subunit and its ability to associate properly with an α subunit. The fifth class of mutation has normal

amounts of β_2 mRNA and protein precursor. However, the β and α subunits are not processed. The mutation in the β_2 subunit affects α chain linkage and thus processing and transport to the surface.

1.5.3 Chromosomal abnormalities in regions encoding leukocyte integrin genes

The chromosomal localization of the genes encoding the leukocyte integrin subunits have provided some interesting results. The common β_2 subunit gene has been mapped to the 21q22 band of chromosome 21 (Corbi et al, 1988). Aneuploidy of this region, such as that observed in trisomy 21 Down syndrome patients, results in an overexpression of LFA-1 (reviewed in Taylor, 1987). Down syndrome patients have abnormal thymic development, a 20-fold increase in the frequency of acute leukemia, reduced numbers of hematopoietic stem cells, and low immunization responses to vaccines. These abnormalities are thought to be a reflection of the disturbance in LFA-1 gene dosage. The 21q22 band has been identified as a translocation breakpoint (t(3;21)(q25;q21)) associated with the blast phase of chronic myelogenous leukemia (CML) (Rubin et al, 1987). The three α subunit genes have also been localized and found to be clustered together on chromosome 16 (Corbi et al, 1988). Inversions and translocations in this region have also been observed in patients with acute myelomonocytic leukemia (Le Beau et al, 1985).

1.6 Ligands for LFA-1

1.6.1 Intercellular adhesion molecule 1 (ICAM-1)

1.6.1 A) Identification and characterization of ICAM-1

Homotypic aggregation of phorbol ester stimulated lymphoid cells is LFA-1-dependent (Rothlein and Springer, 1986). Lymphoid cells from LAD patients (LFA-1$^-$) do not form these

aggregates. However, when LFA-1^+ cells are mixed with LFA-1^- cells, they coaggregate suggesting the presence of a ligand for LFA-1. A MAb against LFA-1^- cells was identified for its ability to block LFA-1-dependent aggregation (Rothlein et al, 1986). The MAb recognizes an 86-114 kd heavily glycosylated molecule called intercellular adhesion molecule 1 (ICAM-1). ICAM-1 is widely distributed on cells of both hematopoietic and nonhematopoietic origin (Dustin et al, 1986). Expression of ICAM-1 is low on nonhematopoietic cells and resting peripheral blood lymphocytes, but upregulated by activation or by cytokines such as IL-1, tumor necrosis factor (TNF), and interferon-γ (IFN-γ) (Dustin et al, 1986; Dustin et al, 1988a). This increased expression of ICAM-1 correlates with increased LFA-1-dependent adhesion of lymphocytes to induced cells.

The formal proof for LFA-1:ICAM-1 receptor-ligand interactions came from studies with artificial membranes (Marlin and Springer, 1987). Purified ICAM-1 incorporated into planar lipid membranes can bind LFA-1^+ cells. The binding can be specifically inhibited by treating the membranes with anti-ICAM-1 MAb or anti-LFA-1 treatment of cells. This adhesion requires divalent cations such as Mg^{2+} and Ca^{2+}. Energy and functional microfilaments are also required for binding. Sodium azide together with 2-deoxy-D-glucose inhibits energy production and T lymphoblastoid binding to ICAM-1-bearing membranes. The binding is also inhibited by cytochalasin B, a microfilament inhibitor. LFA-1-dependent adhesion is also temperature dependent. Cell binding is maximal at 37^oC, reduced at 14^oC, and completely inhibited at 4^oC. These results could be the effects of metabolic energy production or changes in membrane fluidity. Reciprocal studies do not show the same binding requirements indicating that the active, dynamic process is occuring on the LFA-1-bearing cell side of the interaction (Kishimoto et al, 1990).

Several integrins which act as matrix receptors bind their ligands through the RGD sequence (Hynes, 1987; Ruoslahti and Pierschbacher, 1986). Oligopeptides containing the RGD sequence can block the interactions between these integrins and their ligands. Human ICAM-1 does not contain an RGD sequence, but does contain several RGD-like sequences

(Staunton et al, 1988). However, RGD and RGD-like oligopeptides could not inhibit binding of LFA-1^+ cells to ICAM-1 membranes (Marlin and Springer, 1987) suggesting that integrin binding specificity has diverged from that of other integrins.

1.6.1 B) Inducibility of ICAM-1

Interaction of leukocytes with endothelial cells is mediated by cell adhesion molecules. Although LFA-1 expression is relatively constant, the expression of ICAM-1 is highly regulated. The ability of various cytokines to upregulate ICAM-1 expression on fibroblasts and endothelial cells (Dustin et al,1986; Dustin and Springer, 1988) suggested that LFA-1:ICAM-1 interactions play a major role in leukocyte migration along endothelial cells to sites of inflammation. This model is supported by the observation that anti-ICAM-1 and anti-LFA-1 MAbs block leukocyte binding to cytokine-treated endothelial cells (Dustin and Springer, 1988a) and by the inability of LAD patients to mount an immune response (Anderson and Springer, 1987).

1.6.1 C) Gene Cloning

The gene coding for ICAM-1 has been cloned and sequenced (Simmons et al, 1988; Staunton et al, 1988). It is a member of the Ig supergene family with five Ig-like domains and short transmembrane and cytoplasmic domains. ICAM-1 is most closely related to two adhesion molecules in the adult nervous system, neural cell adhesion molecule (NCAM) and myelin-associated glycoprotein (MAG), both of which have five Ig-like domains. ICAM-1 is 24% homologous with MAG and 20% homologous with NCAM over the five domains. ICAM-1 is also about 20% homologous with several retroviral envelope proteins (Dustin et al, 1988b). This includes gp110 of the human immunodeficiency virus (HIV) which is involved in viral interaction with the T lymphocyte molecule CD4 (Sattentau and Weiss, 1988).

1.6.2 Identification and cloning of ICAM-2

ICAM-1 is not the only ligand for LFA-1. Initial evidence for this came from the finding that LFA-1-dependent adherence of T cells to endothelial cells contains both an ICAM-1-dependent and an ICAM-1-independent component (Dustin and Springer, 1988). The ICAM-1-dependent pathway is inducible whereas the ICAM-1-independent pathway is constitutive. Purified LFA-1 incorporated into membranes strongly binds an ICAM-1$^-$ T cell line (Dustin and Springer, 1989). This new LFA-1 ligand, designated ICAM-2, has been cloned (Staunton et al, 1989). A cDNA expression library was transfected into COS cells and screened for the ability to mediate COS cell adherence to LFA-1-coated Petri dishes. This screening was done in the presence of anti-ICAM-1 MAb. ICAM-2 is an integral membrane protein with two Ig-like domains. These two domains have the highest homology with the two most N-terminal Ig-like domains of ICAM-1 suggesting that the crucial interactions for LFA-1 binding are mediated by these domains. Significantly, the LFA-1 binding region of ICAM-1 has been mapped to domains 1 and 2 indicating that the homology is both structural and functional. The three extra domains of ICAM-1 project the LFA-1-binding site further from the cell surface than ICAM-2. This suggests that LFA-1:ICAM-2 interactions require closer cell-cell contacts and might be weaker than LFA-1:ICAM-1 interactions. Support for this hypothesis comes from several binding and detachment studies. ICAM-2 transfected COS cells are more easily washed off LFA-1-coated plastic than ICAM-1 transfected cells. Anti-ICAM-1 MAbs are able to inhibit binding of various cell lines to LFA-1-coated plastic when the purified LFA-1 is plated at low density. However, when LFA-1 is plated at high density, the anti-ICAM-1 MAbs are not able to completely block binding. This implies that when a limited number of LFA-1 molecules are available, the higher affinity ligand, ICAM-1, will outcompete the lower affinity ligand, ICAM-2.

1.7 LFA-1-Independent adhesion pathways

1.7.1 Identification and characterization of CD2 and LFA-3

In certain cases, anti-LFA-1 MAbs were ineffective in blocking conjugate formation (Shimonkevitz et al, 1985; Clayberger et al, 1985). This suggested the existence of an LFA-1-independent adhesion pathway in mediating lymphocyte-target cell contacts. From the same panel of antibodies that gave rise to the anti-LFA-1 MAb, two other unique MAbs were identified (Krensky et al, 1983). Like the anti-LFA-1 MAb, these MAbs were also selected for their ability to interfere with T-lymphocyte-mediated cytolysis. One of these antibodies recognizes a surface molecule known as CD2 (Springer et al, 1987; Bierer et al, 1989). It has also been called LFA-2, Leu5, and the sheep erythrocyte receptor. CD2 is a single chain glycoprotein of 45-50 kd expressed on thymocytes, peripheral T cells, and most NK cells. The gene has been cloned and the predicted amino acid sequence reveals a transmembrane glycoprotein with Ig-like domains (Sewell et al, 1986; Seed and Aruffo, 1987). The cytoplasmic domain is large (116 amino acids) and unusually rich in prolines and basic residues. The MAbs to CD2 inhibit a variety of T-lymphocyte functions, including T lymphocyte cytolysis and proliferation, NK cell activity, and rosetting of sheep erythrocytes with human T lymphocytes (Krensky et al, 1983; Shaw et al, 1986; Verbi et al, 1982). These anti-CD2 MAbs inhibit antigen-nonspecific T lymphocyte-target cell conjugate formation (Krensky et al, 1984; Shaw et al, 1986).

The other antibody recognizes a surface molecule known as LFA-3 (Sanchez-Madrid et al, 1982; Krensky et al, 1983). It is a single chain glycoprotein with a molecular weight of 55-70 kd. Two different forms of LFA-3 arise by alternate splicing (Dustin et al, 1987b; Makgoba et al, 1989). These isoforms differ in membrane anchorage. One form has a conventional hydrophobic transmembrane stretch of amino acids and the other is linked via a phosphatidyl-inositol glycan moiety. LFA-3 is expressed on essentially all tissues that have been tested, including most blood cells, endothelial, epithelial, smooth muscle, and connective

tissue cells of most organs (Krensky et al, 1983; Selvaraj et al, 1987). Like CD2, LFA-3 is also a member of the Ig supergene family with significant homology to CD2 (Seed, 1987). The anti-LFA-1 MAb also inhibits a number of T lymphocyte functions and conjugate formation between CTL and target cells (Krensky et al, 1984; Shaw et al, 1986).

A ligand for CD2 has been identified. CD2 mediates T lymphocyte adhesion by binding directly to LFA-3 (Selvaraj et al, 1987; Dustin et al, 1987a). Purified CD2 is able to inhibit rosette formation and binding of iodinated anti-LFA-3 Ab to erythrocytes (Plunkett and Springer, 1986; Plunkett et al, 1987; Selvaraj et al, 1987). Reciprocal results have been observed with purified LFA-3. Purified LFA-3 is able to bind $CD2^+$ cells just as pure CD2 binds $LFA\text{-}3^+$ cells. All these interactions can be blocked by anti-CD2 or anti-LFA-3 MAbs.

1.7.2 Role of CD2:LFA-3 interactions in T cell activation

The CD2:LFA-3 pathway clearly functions to promote adhesion by contributing to the avidity with which a T cell binds to its target cell. However, these two molecules also appear to be involved in T cell activation. Two distinct antibodies against CD2 have been shown to recognize epitopes in the LFA-3-binding site (Peterson and Seed, 1987). Incubation of T cells with these two antibodies results in IL-2-dependent proliferation (Meuer et al, 1984; Brottier et al, 1985). The exact physiological significance of activation via CD2 is unclear. However, it has been shown that LFA-3 binding to CD2 provides a partial activation signal (Hunig et al, 1987; Bierer et al, 1988). This activation signal appears to be transmitted in the other direction as well. An antibody binding LFA-3 can induce augmented IL-1 secretion from accessory cells (Makgoba et al, 1989). It is suspected that activation through this pathway may act in conjunction with the antigen-specific response from the TcR/CD3 complex (Yang et al, 1986; Breitmeyer et al, 1987). Mutant T cell lines expressing CD2 but not the TcR/CD3 complex, cannot be activated by the CD2 pathway (Alcover et al, 1988). This may be the result of a physical association between CD2 and the TcR/CD3 complex. Approximately half the surface CD2 molecules can be specifically co-precipitated with the TcR/CD3 complex

(Brown et al, 1989). Adhesion molecules at the cell surface are excellent candidates for mediating signals since the cells are ideally situated to provide regulatory information between themselves.

1.7.3 Role of CD2:LFA-3 interactions in thymic ontogeny

Immature T cell precursors leave the bone marrow and migrate to the thymus (Ezine et al, 1984; Spangrude et al, 1988). They remain in the thymus until they fully develop their T lymphocyte characteristics (Stuntman, 1978; Haynes, 1984). The differentiation process is marked by sequential thymocyte migration from one region to another. Interactions between CD2 and LFA-3 are thought to play important roles in thymocyte migration (Reinherz, 1985). Immature thymocytes express CD2 very early, prior to TcR/CD3 complex expression. These thymocytes are in close association with thymic epithelial cells (LFA-3^+) of the cortex (Singer et al, 1985; Vollger et al, 1987). The anti-CD2 and anti-LFA-3 antibodies block binding of thymocytes to thymic epithelial cells (Vollger et al, 1987; Springer et al, 1987). These interactions may also be sending proliferation signals to the thymocytes. Purified thymic epithelial cells induce proliferation of the most immature thymocytes (Denning et al, 1987). This proliferation may also be induced by purified LFA-3 in the presence of an anti-CD2 antibody (Denning et al, 1988). These results suggest that LFA-3 and CD2 interact to maintain and activate thymocytes in the thymus.

1.7.4 Other lymphocyte adhesion molecules

The LFA-1:ICAMs and CD2:LFA-3 interactions appear to be the major contributors in lymphocyte adhesion. However, other molecules have been described. CD4 and CD8, members of the Ig supergene family, have been shown to interact with MHC Class II and Class I molecules, respectively (Doyle and Strominger, 1988; Rosenstein et al, 1989). These interactions can mediate adhesion when overexpressed, but appear to be minor contributors under normal physiological conditions (Makgoba et al, 1989). E2 is a recently identified

molecule that also appears to be involved in lymphocyte adhesion processes (Gelin et al, 1989). The recently identified VCAM-1 seems to mediate endothelial cell adhesion to lymphocytes by binding to VLA-4, making this the second known interaction between the integrin and immunoglobulin supergene families (Osborn et al, 1989; Elice et al, 1990).

1.8 Summary of LFA-1:ICAM-1 and CD2:LFA-3 interactions

The LFA-1:ICAM-1 and CD2:LFA-3 molecules play important roles in establishing antigen-independent contacts between T cells and various other cells (Shaw et al, 1986; Spits et al, 1986). The LFA-1:ICAM-1 pathway seems to be more elaborate. It is more temperature-, energy-, and Mg^{2+}-dependent (Marlin and Springer,1987). Activation appears to lead to a conformational change in LFA-1 resulting in increased avidity for ICAM-1 (Dustin and Springer, 1989; van Kooyk et al, 1989; Dransfield and Hogg, 1989). The CD2:LFA-3 pathway does not have these requirements but can show increased avidity by the formation of multimeric-LFA-3 complexes (Springer et al, 1987; Dustin et al, 1989). Without these adhesion molecules, only high affinity TcR-bearing cells could mount responses (Martz, 1987). Low affinity cells could not overcome the mutual repulsion between themselves and opposing cells. The consequences of not having adhesion molecules can be lethal. LAD patients usually die before they are two years old (Anderson and Springer, 1987). Burkitt's lymphoma cells downregulate LFA-1, LFA-3, and ICAM-1 and escape immune surveillance (Gregory et al, 1988). After several passages in tissue culture, they acquire high levels of expression of these molecules and show increased sensitivity to lysis by Epstein-Barr virus-specific cytotoxic T lymphocytes.

1.9 Other functions of adhesion molecules

The ability of adhesion molecules to mediate cellular contacts means that they have receptive qualities. Because of their adhesive nature, they can mediate cellular adhesive interactions other than those already mentioned. They include processes such as lymphocyte recirculation and metastasis.

1.9.1 Lymphocyte homing and recirculation

Lymphocytes constantly traffic back and forth between the bloodstream and the lymphatic network (reviewed in Yednock and Rosen, 1989). This recirculation allows the full repertoire of antigenic specificities to be continuously represented throughout the body. The lymphocytes exit the bloodstream and gain access to the secondary lymphoid organs by migrating through the post-capillary high endothelial venules (HEV) (Woodruff et al, 1987). Lymphocytes can distinguish between HEV leading to lymph nodes (HEV_{LN}) and HEV leading Peyer's patch (HEV_{PP}) (Hill et al, 1977) and use the same port of exit out of the bloodstream each time (Hamann and Thiele, 1989). A given lymphocyte is able to consistently recirculate through the same pathway by its ability to recognize structural differences between HEV_{LN} and HEV_{PP}. Cell surface molecules are employed by both lymphocytes and HEV for this discriminating function. The molecules are called "homing receptors" on lymphocytes and "vascular addressins" on HEV (Streeter et al, 1988a).

A number of molecules appear to be involved in lymphocyte interactions with HEV. MEL-14 is a ubiquinated glycoprotein found on lymphocytes preferentially homing to HEV_{LN} (St. John et al, 1986; Siegelman et al, 1986; Lasky et al, 1989; Siegelman et al, 1989). The antibody against murine MEL-14 is able to block lymphocyte binding to HEV_{LN} (Gallatin et al, 1983). The human homologue of MEL-14 is found in two forms, a transmembrane form and a phospholipid linked form (Camerini et al, 1989). This lipid-linked form may be relevant for shedding which would be required following homing to ensure lymphocyte migration in the

lymph. It appears that MEL-14 binds HEV_{LN} by acting as a carbohydrate receptor (Stoolman and Rosen, 1983; Stoolman et al, 1984). Mannose-6-phosphate and fructose-1-phosphate produce an 80-90% inhibition of lymphocyte binding to HEV_{LN}. Sialidase treatment of HEV_{LN} destroys lymphocyte binding (Rosen et al, 1985). The cDNA encoding MEL-14 has a region with high homology to carbohydrate-binding (lectin) domains (Lasky et al, 1989; Siegelman et al, 1989). The vascular addressin which appears to bind MEL-14 is identified by the antibody MECA-79. The antibody selectively blocks binding of lymphocytes to HEV_{LN} (Streeter et al, 1988b). The purified protein is heavily glycosylated and binds lentil lectin (Berg et al, 1989) further supporting the model of MEL-14 binding the HEV_{LN} vascular addressin through a carbohydrate moiety.

Monoclonal antibodies against CD44, also known as Hermes, recognize a homing receptor on lymphocytes specific for HEV_{PP} (Jalkanen et al, 1986; Jalkanen et al, 1987). The antibodies specifically block lymphocyte binding to HEV_{PP}. Although the protein is extensively glycosylated (Stamenkovic et al, 1989; Goldstein et al, 1989), carbohydrates do not appear to be involved in binding. Lymphocyte binding to HEV_{PP} is glycanase resistant and unaffected by carbohydrates (Berg et al, 1989). Sequence analysis reveals that the N-terminus globular domain of CD44 has striking homology to tandemly repeated domains of the cartilege link and proteoglycan core proteins. This region is suspected of binding hyaluronic acid (Jalkanen et al, 1988; Haynes et al, 1989), an extracellular matrix component. The CD44 receptor seems to be defined by the MECA-367 Ab (Streeter et al, 1988a; Streeter et al, 1988b). This addressin (mucosal addressin - MAd) is found on HEV_{PP} and purified MAd is able to bind mucosal HEV-binding lymphocytes (Berg et al, 1989). Energy-transfer experiments between Texas Red-labelled CD44 and fluorescein isothiocyanate-conjugated MAd indicate a very close physical association between the two proteins.

Members of the integrin supergene family also appear to mediate lymphocyte-HEV binding (Holzmann and Weissman, 1989b). A murine lymphocyte cell surface receptor,

LPAM-1, is an αβ heterodimer similar to the human VLA-4 protein (Holzmann et al, 1989). LPAM-1 has been shown to direct Peyer's patch-specific lymphocyte homing. The anti-LPAM-1 MAb can block lymphocyte binding to both HEV_{PP} and appendix HEV (Holzmann and Weissman, 1989b; Berg et al, 1989). LFA-1 is also involved in lymphocyte-HEV binding (Hamann and Thiele, 1989). Inhibition of lymphocyte binding by anti-LFA-1 Ab is incomplete and not tissue specific, unlike antibodies to MEL-14, CD44, and LPAM-1 (Hamann et al, 1988). It appears that LFA-1 is not critical in lymphocyte recirculation, but may play a role in adhesion strengthening. LAD cells do not appear to be defective in HEV binding.

1.9.2 Metastatic capacity of tumor cells

The neoplastic transformation of cells is thought to be primarily caused by somatic mutations (Weinberg, 1983). These cells grow autonomously by circumventing growth factor requirements and regulatory signals and form tumors. The tumor cells can break away, or metastasize, and invade healthy tissues. In order for a cell to break away from the primary tumor, the adhesive bonds that normally hold cells together must be disrupted. In order to invade healthy tissue, the cell must also express the original or a new set of adhesive bonds at the site of migration. Adhesion molecules are the mediators of these bonds.

Membrane expression of ICAM-1 is highly regulated on all cells of hematopoietic origin. Expression is significant on a small subset of bone marrow progenitors (Arkin et al, 1989; Boyd et al, 1989). Maturation of these cells is associated with loss of ICAM-1 expression. It is suspected that the disruption of LFA-1:ICAM-1 interactions may facilitate the entry into circulation of these mature cells. An interesting observation was made when ICAM-1 expression was examined on B-lymphoid tumors (Boyd et al, 1989; Wawryk et al, 1989). Large tumors which form bulky, solitary masses consistently show intermediate to strong ICAM-1 expression. Conversely, lymphoma cells which show a diffuse, infiltrative, non-cohesive pattern of spread exhibit weak or no expression. This is highly suggestive that ICAM-1 expression may be a determinant in whether a cell can metastasize.

ICAM-1 appears to be involved in the metastatic progression of melanoma cells (Johnson et al, 1988; Johnson et al, 1989). ICAM-1 is not normally found on quiescent melanocyte or benign melanocytic lesions. An increase in melanoma tumor size, which is accompanied by ICAM-1 upregulation, results in an increase in the probability of metastasis. It is suspected that this increased expression may be a direct cause of increased metastatic capacity. The melanoma cells may interact with LFA-1 molecules on tumor-infiltrating leukocytes. This may lead to a reduction in homotypic aggregation between melanoma cells, while the increased interactions between melanoma cells and leukocytes may enable melanoma cells to dissociate from the primary tumor.

1.10 Thesis Objectives

Adhesion molecules are of fundamental importance in virtually all developmental processes. Their significance has been demonstrated in the immune system which has several pairs of molecules interacting in order to fine tune the immune response. One such pair is LFA-1:ICAM-1. Their interactions have been studied extensively in the human system. However, in vivo studies of LFA-1:ICAM-1 interactions in humans that are both useful and ethical are very limited; thus an animal model is highly desirable.

The objective of this thesis was to isolate a functional cDNA clone encoding the murine ICAM-1. The structure, function, and distribution of the clone was then to be compared to that of a previously isolated clone. Potential expression and localization of the protein was also examined. The functional cDNA clone was then manipulated to determine whether a soluble ICAM-1 protein could be isolated. A truncated protein would be useful in examining LFA-1 interactions in greater detail. The purpose of these studies was to provide a better basis for the construction of an animal model in the future.

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CHAPTER II

MATERIALS AND METHODS

2.1 ANIMALS

BALB/c mice were purchased from Charles River Canada, Quebec, Canada and maintained in the Animal Facility of the B.C. Cancer Research Centre.

2.2 CELL CULTURE

2.2.1 Established Cell Lines

COS1 cells (Gluzman, 1981), SV40-transformed monkey kidney fibroblasts, were grown in Dulbecco's modified minimum essential media (DMEM) supplemented with 5% fetal calf serum (FCS) and 50 U/ml penicillin and 50 µg/ml streptomycin (antibiotics). NS-1 cells (Kohler et al, 1976), BALB/c plasmacytoma, were maintained in DMEM containing 5% FCS and antibiotics. BW5147 cells (Ralph and Nakoinz, 1973), AKR thymic leukemia, were maintained in DMEM containing 5% FCS and antibiotics.

2.2.2 Mitogen Activation of Mouse Spleen Cells

Single cell suspensions were made from BALB/c spleen. The red blood cells were lysed in 0.17 M Tris·NH_4Cl pH 7.2 (Hunt, 1979) and washed 2x in phosphate buffered saline (PBS). Approximately 10^7 spleen cells were stimulated in 10 ml of RPMI 1640 medium containing 5% FCS, antibiotics, and either 2 µg/ml concanavalin A (ConA) and $5x10^{-5}$ M 2-mercaptoethanol for 48 hrs or 20 µg/ml lipopolysaccharide (LPS) for 24 hrs (Takei, 1985).

2.2.3 Monoclonal Antibodies

The YN1/1.7 monoclonal antibody was originally generated in the Terry Fox Laboratory from a fusion between Y3 cells and Fischer 344 rat spleen cells primed with NS-1 cells (Takei, 1985). YN1/1.7 recognizes the murine surface antigen MALA-2. The YE1/48.10 monoclonal antibody was also generated in this laboratory and recognizes a murine lymphocyte antigen YE1/48 (Takei, 1983). The rat hybridoma line FD441.8 produces anti-mouse LFA-1 antibody (Dialynas et al, 1982) and was obtained from the American Type Culture Collection (Rockville, MD). Antibodies were purified from ascites fluid by ammonium sulfate precipitation (50% saturation), followed by DEAE Affigel Blue (Bio-Rad, Richmond, CA) column chromatography. Fractions containing pure IgG were pooled and used in subsequent experiments.

2.3 DNA ISOLATION AND ANALYSIS

2.3.1 Isolation of High Molecular Weight Genomic DNA

In order to isolate genomic DNA (Gross-Bellard et al, 1977), cells were washed 3x in PBS. Approximately $5x10^7$ cells were suspended in 2 ml of TNE buffer (10 mM Tris pH 8.0, 150 mM NaCl, 10mM EDTA) and gently mixed. To this suspension, 20 µL of 20% SDS and 10 µL proteinase K (stock 10 mg/ml; Sigma Chemical Company; St. Louis, MO) were added and incubated at 37°C for 16 hrs. The lysate was then extracted 3x with TNE-saturated-phenol, 3x with TNE-saturated-(phenol:chloroform) (1:1), and finally 3x with chloroform:isoamyl alcohol (24:1). The aqueous phase was dialyzed against two changes of 4L of TE buffer (10 mM Tris pH 8.0, 1mM EDTA) each for 24 hrs at 4°C. The DNA was quantitated spectrophotometrically (A_{260}=1.0 for 50 µg/ml) and purity was determined (A_{260}/A_{280} ~ 2.0) on an LKB ULTROSPEC 4050 (Cambridge, England).

2.3.2 Isolation of Plasmid DNA

Plasmid mini-preps were prepared by alkaline lysis (Brinboim and Dolby, 1979). Bacterial cultures were grown in 5 ml of LB broth overnight at 37°C and pelleted. The pellet was suspended in 200 µL of GTE buffer (50 mM glucose, 25 mM Tris pH 8.0, 10 mM EDTA) containing 4 mg/ml of lysozyme (Sigma Chemical Co.) and incubated for 5 min at 20°C. To this was added 400 µL of 1% SDS, 0.2 M NaOH and the mixture then incubated for 5 min on ice. Finally, 300 µL of 3 M potassium acetate, 2 M acetic acid was added and the mixture spun in a microfuge (15,000 g) for 10 min at 4°C. The clear supernatant was recovered, extracted with 600 µL of phenol:chloroform:isoamyl alcohol solution (25:24:1), and mixed with 1 ml of isopropanol. The nucleic acids were allowed to precipitate by incubating on ice for 10 min and spinning in a microfuge for 15 min at 4°C. The pellet was dissolved in 100 µL of TE buffer containing 200 µg/ml of RNase A (Sigma Chemical Co.) and incubated at 37°C for 40 min. To the aqueous phase, 60 µL of 7.5 M ammonium acetate pH 7.0 was added. The solution was then incubated on ice for 5 min and spun in a microfuge for 5 min at 4°C. The supernatant was recovered and mixed with 160 µL of ethanol. This was spun for 5 min at 20°C. The DNA pellet was washed with 0.5 ml of 80% ethanol and dissolved in 100 µL of TE buffer yielding a final DNA concentration of 100-200 ng/µL.

Large scale plasmid preps were done in a similar fashion (Maniatis et al, 1982). Overnight bacterial cultures grown in 1L of LB broth were pelleted in 500 ml plastic bottles with sealed lids at 7000 RPM for 10 min. The pellets were suspended in 20 ml of 25% sucrose, 50 mM Tris pH 8.0. To this was added 2 ml of lysozyme solution (10 mg/ml in 0.25 M Tris pH 8.0) and the mixture then incubated for 5 min at 20°C. Following this, 1.5 ml of 0.25 M EDTA pH 8.0 was added and the mixture incubated another 5 min at 20°C. With gentle constant swirling, 15 ml of Triton-X solution (1% Triton-X, 62.5 mM EDTA, 50 mM Tris pH 8.0) was added dropwise over a period of 2 min. The resulting lysate was then spun in Oakridge tubes at 19,000 RPM (JA-20 Rotor; Beckman, Palo Alto, CA) for 35 min at 4°C. The clear lysate was carefully decanted and caesium chloride (BDH; Toronto, Ontario) was added

(0.9 mg/ml lysate), followed by 1/20 lysate volume of ethidium bromide (10 mg/ml stock; Sigma Chemical Co.). The solution was transfered to a 16x76 mm heat sealable Sorvall centrifuge tube (Beckman, Palo Alto, CA) and spun in a VTi50 Rotor in an L8-80M Beckman Ultracentrifuge at 46,000 RPM for 18 hr. The lower band was removed with a hypodermic needle and transfered to a 13x51 mm heat sealable Sorvall tube. The tube was then spun in a VTi80 Rotor at 62,000 RPM for 6 hr. The intense lower band was removed, mixed with 4 changes of caesium chloride-saturated-isopropanol and finally dialyzed against 2 changes of 4L of TE buffer, each time for 24 hr at 4^{o}C.

2.3.3 Restriction Enzyme Digestion

Most of the restriction enzymes used were purchased from Bethesda Research Laboratories (BRL; Gaitersburg, MD). Other suppliers included Pharmacia (Uppsala,Sweden) and Boehringer Mannheim (Penzberg, West Germany). The conditions for the digestions were those recommended by the manufacturer. Approximately 1 µg of plasmid DNA was digested with 2 units of enzyme for 2 hr. Genomic DNA (12 µg) was digested with a large excess of enzyme (10 units per µg of genomic DNA) for 6 hr. Both types of samples were then precipitated by adding 1/10 volume of 2.5 M sodium acetate pH 4.5 and 2 volumes of ice cold ethanol, and incubated on dry ice for 20 min. The solution was centrifuged at 15,000g for 20 min at 4^{o}C and the pellet washed with 0.5 ml 80% ethanol. After discarding the supernatant, the pellet was dried in a vacuum centrifuge and redissolved in 10-30 µL of TAE buffer (40 mM Tris·acetate pH 7.2, 20 mM sodium acetate, 1 mM EDTA). To this, 1/10 volume loading buffer (0.25% xylene cyanol, 0.25% bromophenol blue, 50% glycerol) was added and loaded onto an agarose gel.

2.3.4 Agarose Gel Electrophoresis

Digests of plasmid DNA were loaded onto 1% agarose (Pharmacia) mini-gels (Hoeffer) and electrophoresed in TBE buffer (89 mM Tris, 89 mM borate, 25 mM EDTA, pH 8.3) at 50 volts

for 3-4 hr (Maniatis et al, 1982). Ethidium bromide was present in both the gel and running buffer at a concentration of 500 ng/ml. Digested genomic DNA was loaded onto a 0.8% agarose gel (20.5 x 14.5 x 7 mm) with TAE as running buffer and 500 ng/ml ethidium bromide in both the gel and the buffer. These gels were electrophoresed for 16-18 hr at 35 volts. Molecular weights were determined from molecular weight standards such as λ HindIII fragments and λ(HindIII and EcoRI) fragments (Pharmacia). The gels were photographed under UV light and the markers were easily seen in the Polaroid photograph.

2.3.5 Southern Blotting

The gels containing digested genomic DNA were first treated in 0.1 M HCl for 15 min (Maniatis et al, 1982). Then they were transfered to 0.5 M NaOH, 1.5 M NaCl and incubated for 30 min and finally neutralized in 1.0 M Tris, 2.3 M NaCl for an additional 30 min. The gel was then layed on top of a 3MM Whatman filter paper soaked in 20x SSC buffer (3 M NaCl, 0.15 M sodium citrate pH 7.0). The ends of the filter paper were soaked in a pool of 20x SSC buffer. A piece of Zeta Probe (Bio-Rad Laboratories; Mississauga, Ontario) was cut to the size of the gel and soaked in double-distilled water for 10 min. The Zeta Probe filter was then carefully layered on top of the gel. Four wetted pieces of Whatman paper were then layered on top of the membrane, followed by ~10 cm of paper towels. A glass plate and weight were placed on top of the stack. The dry paper towels draw the 20x SSC buffer upward by capillary action and provide unidirectional transfer of the DNA onto the membrane (Southern, 1975). The transfer was allowed to proceed for 16 hr, after which the Zeta Probe was rinsed in 20x SSC. The DNA was fixed on the membrane by UV cross-linking in a UV Stratalinker 1800 (Stratagene; La Jolla, CA).

2.3.6 Prehybridization

The membrane was incubated in a prehybridization solution of 6x SSC, 10% deionized formamide (BRL), 1% SDS, 1% Blotto (Carnation instant milk), 2 mM EDTA, 0.5 mg/ml denatured salmon sperm DNA (Pharmacia) at 60°C for 4 hr (Turhan et al, 1988).

2.3.7 Oligolabelled DNA Probes

The gel purified fragments were radiolabelled by the oligolabelling procedure (Feinberg and Vogelstein, 1983). Briefly, 20 ng of a DNA fragment was added to 100 ng of randomly generated hexanucleotides in a final volume of 14 µL. This solution was boiled for 3 min and cooled on ice. To this was added 2 µL of 10x HLB buffer (0.5 M HEPES pH 6.9, 0.1 M $MgCl_2$, 0.06 M 2-mercaptoethanol), 2 µL nucleotide mix (dGTP, dATP, dTTP, 2.5 mM each), 2 µL [α-^{32}P]-dCTP (3000 Ci/mmol), and 2 units of Klenow polymerase (Pharmacia). The reaction was allowed to proceed for 1.5 hr at 20°C and then stopped by the addition of 2 µL 10 M NaOH. This was added directly to the hybridization mix.

2.3.8 Hybridization and Washing

The membrane was removed from the prehybridization solution and transfered to the hybridization solution containing 6x SSC, 10% deionized formamide, 1% SDS, 1% Blotto, 2 mM EDTA, 10% dextran sulfate (Sigma Chemical Co.), oligolabelled-probe, and 0.5 mg/ml denatured salmon sperm DNA (Turhan et al, 1988). The filter was incubated at 60°C for 16hr and then washed 3x for 30 min each at 65°C in 0.3x SSC, 0.1% SDS, 0.1% sodium pyrophosphate. The filters were exposed to Kodak XAR films at -70°C for 16-36 hr.

2.3.9 Stripping Blots

In order to strip blots, the filters were boiled in 3 changes of 500 ml each of 0.1x SSC, 1% SDS for 20 min.

2.4 RNA ISOLATION AND ANALYSIS

2.4.1 RNA Isolation

Total ribonucleic acid (RNA) was isolated from various cells using the acid-phenol extraction method (Chomczynski and Sacchi, 1987). Cells were washed 2x in PBS and the pellet was dissolved in 0.5 ml of Solution D (4 M guanidinium thiocyanate, 25 mM sodium citrate pH 7.0, 0.5% sarkosyl, 0.1 M 2-mercaptoethanol). Following this, 50 µL of 2 M sodium acetate pH 4.0, 0.5 ml water-saturated-phenol, and 0.1 ml chloroform:isoamyl alcohol (49:1) were added. This solution was spun at 14,000g for 15 min at 4°C. The aqueous layer, which was free of genomic DNA, was mixed with 0.5 ml isopropanol and put at -70°C for 1 hr. The solution was spun at 14,000g for 20 min at 4°C and the pellet suspended in 300 µL Solution D followed by 300 µL of ethanol. The solution was put at -70°C for another hour and then spun for 20 min at 4°C. The pellet was redissolved in 20 µL of loading buffer (50% deionized formamide, 1x MOPS pH 6-7, 7% formaldehyde, 6% glycerol, 1% bromophenol blue) and heated at 95°C for 2 min before loading onto a gel (Davis et al, 1986).

2.4.2 Agarose Gel Electrophoresis

The samples were loaded into the wells of a 1% agarose gel in 1x MOPS buffer with 2% formaldehyde and 1x MOPS buffer as the running buffer (Davis et al, 1986). The gel was run at 150 volts for 3-4 hr. Molecular weights were determined from the RNA ladder (BRL). After electrophoresis, the lane with the RNA ladder was washed in 500 ml 10x SSC for 30 min. The gel slab was then stained in 200 ml 1x SSC containing 1 µg/ml ethidium bromide and destained in 2 changes of 500 ml of 1x SSC for 1 hr each. The slab was then photographed under UV light.

2.4.3 Northern Blotting

The gel was rinsed for 20 min each in two changes of 500 ml of 10x SSC in order to remove the formaldehyde. The gel was then placed on a piece of Whatman 3MM filter soaked in 10x SSC. The ends of the filter were soaked in a pool of 10x SSC. A piece of water-soaked Zeta Probe was then placed on the gel followed by 4 pieces of wet Whatman filters and ~6 cm of paper towels. A glass plate with a weight was placed on top of the stack and the blotting process allowed to proceed for 16 hr. The membrane was then removed and rinsed in 20x SSC and UV fixed with a UV Stratalinker 1800.

2.4.4 Prehybridization

The membrane was incubated in a prehybridization solution of 1.5x SSPE buffer (20x SSPE: 3.6 M NaCl, 0.2 M NaH_2PO_4, 20 mM EDTA pH 7.4), 1% SDS, 0.5% Blotto, 0.5 mg/ml denatured salmon sperm DNA for 2 hr at 60°C.

2.4.5 Hybridization and Washings

The membrane was transferred to a hybridization solution containing 1.5x SSPE, 1% SDS, 0.5% Blotto, 0.5 mg/ml denatured salmon sperm DNA, and oligolabelled-probe for 18 hr at 60°C. The filter was then washed at 20°C in the following solutions for 15 minutes per wash: a) 2x SSC,0.1% SDS; b) 0.5x SSC, 0.1% SDS; c) 0.1x SSC, 0.1% SDS. The final wash was performed at 65°C for 1 hr. The filter was then exposed to Kodak XAR film.

2.5 cDNA CLONING AND SEQUENCING

2.5.1 Isolation of cDNA Clones

A λgt10 cDNA library was previously screened with oligonucleotides complementary to the tryptic peptide sequences of MALA-2 (Takei, 1985; Horley et al, 1989) and several clones were

isolated. One clone, K3-1.1, had already been isolated and sequenced (Horley et al, 1989). A second clone, K4-1.1, is described in this thesis. The bacteriophage carrying the K4-1.1 clone was propagated in E. coli C600 Hfl and isolated as previously described (Davis et al, 1986). PEG-8000 (Eastman Kodak; Rochester NY) was used in place of PEG-6000. The insert was excised from the phage DNA by EcoRI digestion and purified on a 1% agarose gel in TBE buffer. The gel purified fragment was subcloned into the EcoRI site of pUC19 (United States Biochemical Co.; Cleveland, OH) by standard methods (Maniatis et al, 1982). Important features of pUC19 are shown in Figure 1. The plasmid was grown in E. coli DH5α cells (BRL).

2.5.2 Sequencing

The pUC19 plasmid contains a priming site on either side of the polylinker allowing both strands to be sequenced. DNA sequencing was done by the dideoxynucleotide chain termination method (Sanger et al, 1977) using the T7 sequencing kit (Pharmacia) and [α-^{32}P]-dATP (800 Ci/mmol).

2.6 EXPRESSIONAL ANALYSIS OF cDNA CLONES

2.6.1 Expression Vector pAX82

The pAX82 expression vector (Figure 2), constructed by Dr. R. Kay (Terry Fox Laboratory, Vancouver, B.C.) (manuscript in preparation), is similar to the CDM8 vector previously described (Aruffo and Seed, 1987). The plasmid is propagated in E. coli MC1061/p3 (Yamasaki et al, 1988). The p3 plasmid in these cells contains the ampicillin resistance gene (AMP^r) and the tetracycline resistance (TET^r), both of which contain a premature stop codon making the genes nonfunctional (Seed and Aruffo, 1987). However, when pAX82 enters a cell, it replicates via a bacterial repilcation origin and confers drug resistance through a tRNA gene (Sup f) which is able to place a serine residue at the premature TAG stop codons of AMP^r and

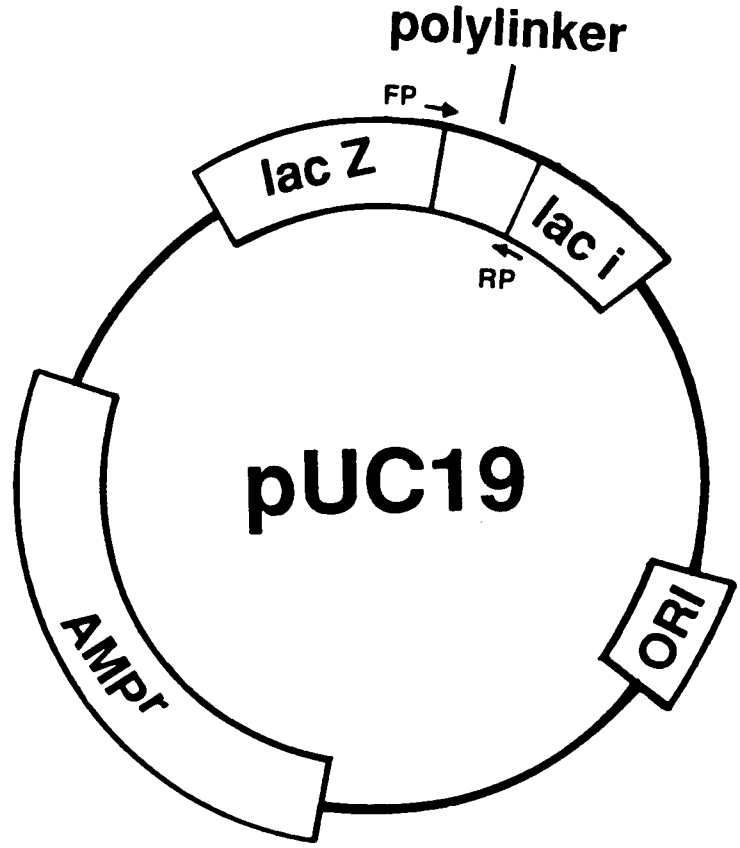
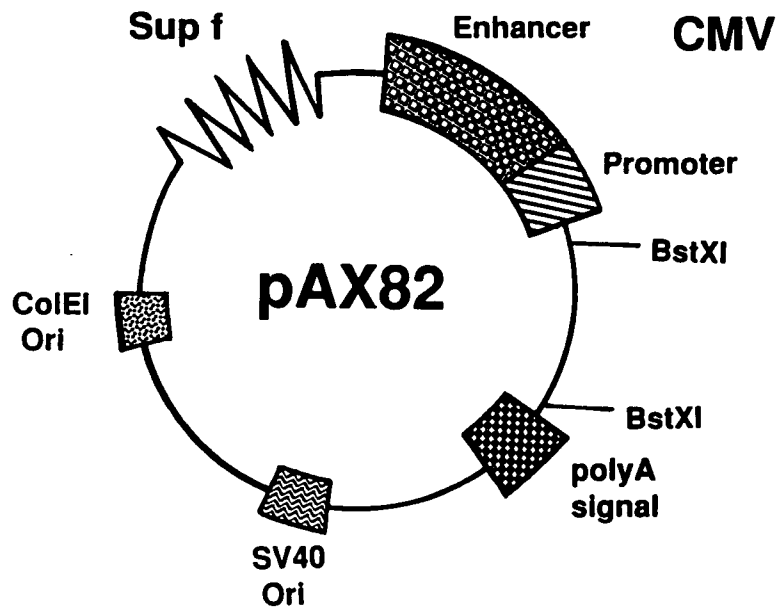


Figure 1 A schematic representation of the pUC19 vector. The vector is used for subcloning and sequencing fragments. The basic features include the ampicillin resistance gene (AMP^r), the pBR322 derived origin of replication (ORI), the β-galactosidase gene (lacZ, laci),and the polylinker in the β-galactosidase gene. Insertion of DNA in the polylinker inactivates the β-galactosidase gene and allows the selection of recombinant plasmid transformed bacteria (white colonies) over the parental plasmid transformed bacteria (blue colonies) when plated on LB agar containing 40 μg/ml X-gal, 150 μg/ml IPTG, 40 μg/ml ampicillin. There are also two priming sites (FP, RP) on either side of the polylinker used for sequencing.

a)



b)

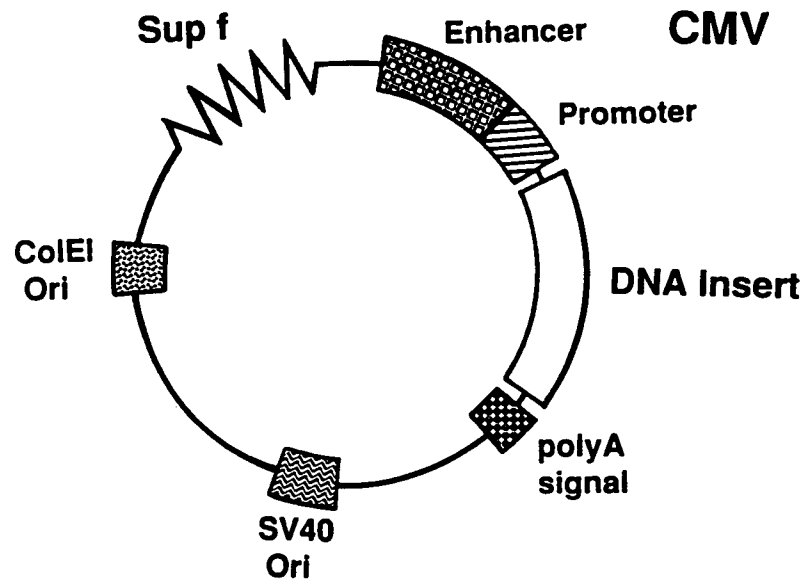


Figure 2 Expression vector pAX82. a) The expression vector is shown with all its features. It is digested with BstXI and doubly cut plasmid is isolated. A fragment with linkers containing 3' TGTG overhang is ligated into the region between the promoter and the polyA signal. b) Once plasmid DNA is transfected into COS1 cell, it replicates via the SV40 origin and transcription is driven by the CMV enhancer/promoter region.

TET^r. The pAX82 vector also has a cytomegalovirus immediate-early promoter/enhancer region to drive transcription, a β-globin polyadenylation (polyA) signal, and an SV40 replication origin to direct plasmid replication in the eukaryotic cells (Kaufman, 1985). There is a unique cloning site adjacent to the transcriptional regulatory elements. Between the promoter and the polyA signal are two identical BstXI sites placed in inverted orientation with respect to each other and separated by a short segment of DNA. The BstXI cleavage site (5'$CCAN_5$'NTGG3') in pAX82 is 5'CCATTGTG'GTGG3' and a four base 3' extension (TGTG) is created. Approximately 10 μg of pAX82 DNA was digested with 125 units of BstXI enzyme at 50°C for 10 hr. The doubly cut plasmid was gel purified from the stuffer DNA and singly cut plasmid on a 0.7% TBE agarose gel.

2.6.2 Ligation of Insert into pAX82

Oligonucleotides having the sequence 5'TAGTTAGTTAGCACA3' and 5'CTAACTAACTA3' were synthesized in an Applied Biosystems DNA synthesizer (provided by Dr. M. Smith's Laboratory, University of British Columbia, Vancouver, B.C.) and purified as described by Atkinson and Smith (1984). They were each kinased separately with T4 polynucleotide kinase (Pharmacia) (Maniatis et al, 1982) and then annealed resulting in a four base 3' extension (CACA) complementary to that of the vector. The linkers (~100 ng) were then ligated to a blunt insert (~400 ng) and the insert then purified on a 1.5% TBE agarose gel. It was then ligated into the doubly cut pAX82 vector (Davis et al, 1986) and competent MC1061/p3 bacteria were transformed and plated on LB agar (40 μg/ml ampicillin, 10 μg/ml tetracycline). The next day, ten isolated colonies were selected and the orientation of the insert (sense vs. antisense) determined by restriction enzyme mapping. Four inserts were used: K3-1.1, K3-1.1Δ5, K4-1.1, and K4-1.1ΔT. Cutting the K3-1.1 insert with Asp700I generated K3-1.1Δ5 and cutting K4-1.1 with ScaI generated K4-1.1ΔT.

2.6.3 Transfection of COS1 Cells

COS1 cells were plated in Falcon 3003 tissue culture plates (Becton-Dickinson; Lincoln Park, NJ) at ~25% confluency. Twelve hours later, they were transfected with pAX82/insert and DEAE-dextran (Hammarskjold et al, 1986). Mock-transfected COS cells contained only DEAE-dextran. Plasmid DNA and plasma membranes are negatively charged. The DEAE-dextran is positively charged and can act as an ionic bridge between the DNA and cells. Glycerol shock treatment of the cells causes uptake of the membrane-bound DNA/DEAE-dextran complexes. The cells were then fed DMEM/5%FCS containing 100 μM chloroquine diphosphate for 5 hrs, which inhibits lysozomal enzymes from degrading the DNA. The media was then replaced with fresh DMEM/5%FCS and cells allowed to grow for 60-70 hr.

2.6.4 Harvesting Transfected Cells

Since binding of cells to Falcon 3003 plates is dependent on divalent cations, they can be removed by chelating agents such as EDTA. The bound cells were first washed with PBS and then incubated in PBS, 0.1% sodium azide, 5 mM EDTA at 37°C for 10 min (Dougherty et al, 1989a). The solution was then used to rinse the plate to loosen any remaining cells and the cells spun down.

2.6.5 FACS Analysis

The harvested cells were divided into 2 batches, one for antibody staining and the other for use as a negative control. Approximately 10^6 cells were dispensed into each tube and centrifuged. The cells in one set of tubes were suspended in 100 μL of YN1/1.7 hybridoma supernatant containing 0.1% sodium azide (NaN_3). The cells in the other set of tubes were suspended in 100 μL Hanks' Balanced Salt Solution, 1% FCS, 0.1% NaN_3 (HBSS/FCS/NaN_3). Both sets of tubes were incubated on ice for 30 min and then washed 3x with cold HBSS/FCS/NaN_3. The cells were then suspended in 50 μL of 1/20 dilution goat anti-rat Ig conjugated to fluorescein isothiocyanate (FITC) (Cooper Biomedical; West Chester, PA) and

incubated on ice for 30 min (Dougherty et al, 1989a). They were then washed 3x with cold HBSS/FCS/NaN_3. The final pellet was suspended in 250 µL of cold HBSS/FCS/NaN_3 containing 2 µg/ml propidium iodide (stains dead cells) and analyzed on a FACStar Plus (Becton-Dickinson).

2.6.6 Lysis of Transfected Cells

Approximately $2x10^7$ cells were dissolved in 1 ml of PBS containing 1% nonidet P-40 (NP-40), 5 mM phenylmethylsulfonyl fluoride and incubated on ice for 1 hr (Dougherty et al, 1989b). The lysates were vigorously vortexed every 10 min. The solution was then spun in a centrifuge at 14,000g for 10 min to pellet the nuclei and other debris. The resulting supernatant was mixed with an equal volume of sample buffer (125 mM Tris pH 6.8, 20% glycerol, 4.6% SDS, 0.004% bromophenol blue). The samples were boiled for 5 min before being applied to a polyacrylamide gel.

2.6.7 SDS-PAGE Analysis

Sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) analysis was carried out by standard methods (Laemmli, 1970) using a Mini-Slab apparatus (8cm x 10cm x 1mm) (Idea Scientific; Corvallis, OR). The buffer in the separating gel consisted of 375 mM Tris pH 8.6, 0.1% SDS, while the running buffer was 192 mM glycine, 250 mM Tris, 1% SDS pH 8.8. The molecular weights were determined from standard markers. The SDS-Blue kit (Sigma Chemical Co.) contains pre-stained proteins of α_2-macroglobulin (180 kd), β-galactosidase (116 kd), fructose-6-phosphate kinase (84 kd), pyruvate kinase (58 kd), fumarase (48.5 kd), lactic dehydrogenase (36.5 kd), and triosephosphate isomerase (26.6 kd). The SDS-200 kit (Sigma Chemical Co.) contains unstained proteins of myosin (205 kd), β-galactosidase (116 kd), phosphorylase b (97.4 kd), bovine albumin (66 kd), egg albumin (45 kd), and carbonic anhydrase (29 kd).

2.6.8 Iodination of YN1/1.7 Antibody

Purified YN1/1.7 antibody (20 μg) was labelled with 0.5 mCi ^{125}I-Na by the chloramine T method (Hunter, 1979). Free iodine was removed by gel filtration on a Pharmacia Nick Translation Column. The specific activity of the iodinated antibody was ~3x10^7 cpm/μg.

2.6.9 Western Blot Analysis

Cell lysates were fractionated by SDS-PAGE and transfered to 0.45 μm nitrocellulose (Schleicher and Schuell; Keene, NJ) as described (Hakomori and Kannagi, 1986). Nonspecific binding sites were then blocked by incubating the filter in NET buffer (50 mM Tris pH 7.5, 5 mM EDTA, 150 mM NaCl, 0.1% NP-40, 1% bovine serum albumin, 0.25% gelatin) at 20^{o}C for 2 hr. The filter was then washed 2x in 100 ml PBS containing 0.05% TWEEN-20 each for 20 min at 20^{o}C. The filter was then incubated overnight at 4^{o}C in 30 ml of HBSS/1%FCS containing 2x10^7 cpm of ^{125}I-labelled YN1/1.7 antibody. Finally, the filter was washed 5x in 20 ml NET buffer for 20 min each at 20^{o}C and exposed to XAR Kodak film.

2.6.10 Indirect Immunoperoxidase Staining

Transfected COS cells were fixed onto glass slides and stained as previously described (Dougherty et al, 1989b). Brielfy, cells were incubated for 30 min at 20^{o}C with 75 μL of hybridoma supernatant. After washing with HBSS, 50 μL of a 1/2000 dilution of horseradish peroxidase-conjugated goat anti-rat IgG (Sigma Chemical Co.) was added to the fixed cells. The reaction was developed by incubating for 5 min at 20^{o}C in PBS containing 0.06% of 3-3'-diaminobenzidine (Sigma Chemical Co.) and 0.012% H_2O_2. The H_2O_2 is converted to oxide radicals which then turn the nearby diaminobenzidine to a brown colour.

2.6.11 Inhibition Studies

300 μL of HBSS/1%FCS/0.1%NaN_3 containing ^{125}I-YN1/1.7 (1x10^7 cpm/ml) were incubated with 150 μL of supernatant for 15 min at 20^{o}C. This solution was first split 3 ways and each

fraction was incubated with $2x10^6$ NS-1 cells at 20°C for 30 min. The cells were then washed 3x with 200 µL of HBSS/1%FCS/0.1%NaN_3 and bound radioactivity counted on a γ-counter (Beckman 5500).

2.7 SOLUBLE ICAM-1 (sICAM-1)

2.7.1 Generation and Purification of Soluble ICAM-1

The K4-1.1 insert was digested with ScaI and ligated to linkers with TAG stop codons in all three reading frames to ensure that pAX82 sequence was not translated (Figure 3). COS1 cells were transfected in Falcon 3025 plates (~2 X 10^6 cells/plate) and the supernatant was harvested 70 hr later. It was incubated with YN1/1.7 antibody coupled to Affi-gel 10 (Bio-Rad) on ice for 4 hr with constant agitation. Beads were washed thoroughly with 1L of 10 mM Tris pH 7.5 overnight. The adsorbed antigen was eluted from the column by washing with 15 ml 50 mM glycine pH 2.9, 0.15 M NaCl and collecting 1 ml fractions. Each fraction was neutralized with 1 M Tris pH 8.0 and assayed for its ability to block binding of ^{125}I-YN1/1.7 antibody to NS-1 cells. Fractions with soluble ICAM-1 (sICAM-1) were pooled and incubated with YE1/48 antibody coupled to Affi-gel 10 for 4 hr on ice. The flow through was collected and the column was washed with 5 ml 10 mM Tris pH 7.5 which was pooled with the first fraction. This was incubated with the YN1/1.7 antibody coupled beads for 4 hr on ice and washed with 1L of 10 mM Tris pH 7.5. The purified sICAM-1 was eluted as previously described. Fractions were neutralized, pooled, and concentrated in a Centricon C-30 (Amicon; Danvers, MA). The amount and purity were assessed by SDS-PAGE. The gel was silver-stained (Ohsawa and Ebata, 1983) and the protein was quantitated by comparing the intensity of the band to that of BSA (Boehringer Mannheim).

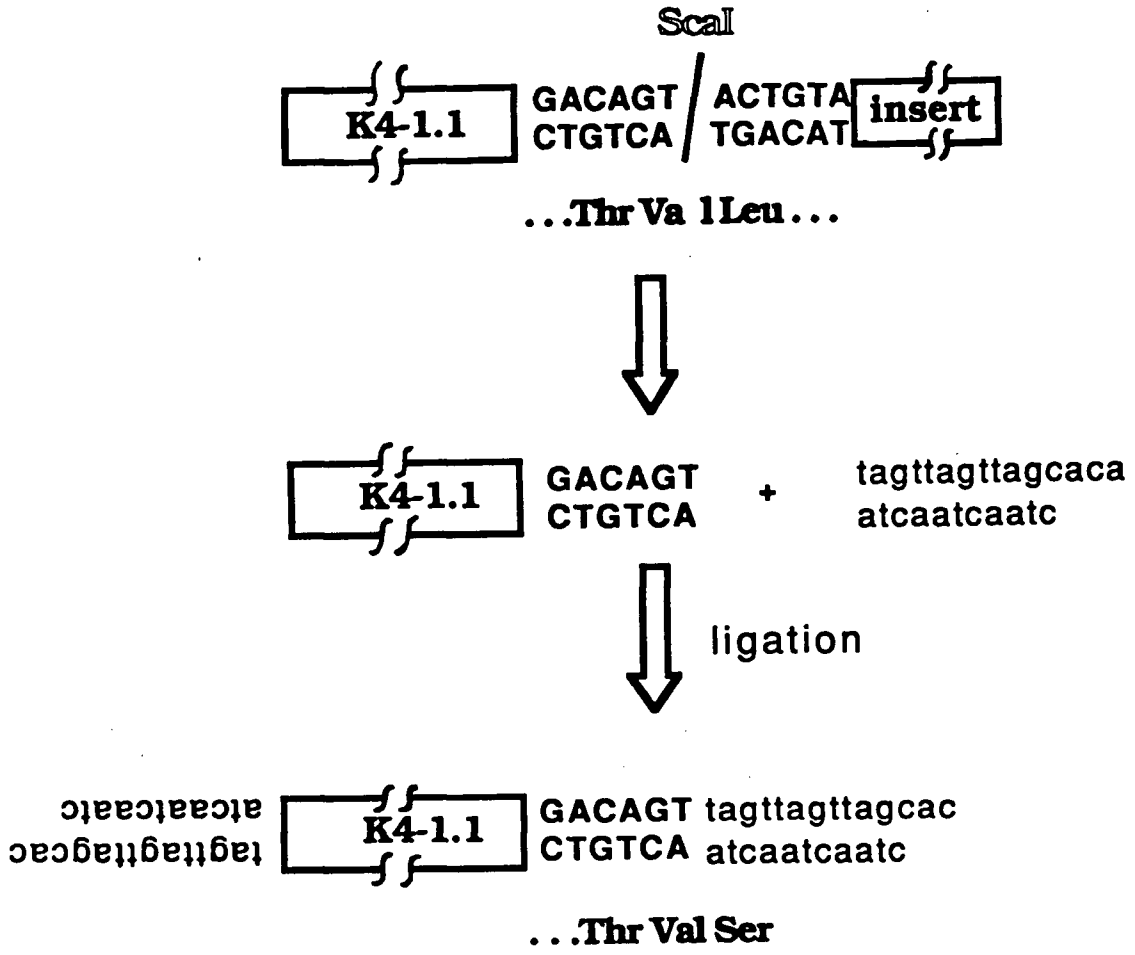


Figure 3 Generation of soluble ICAM-1. The K4-1.1 insert (blunt ended) was cut with ScaI. The larger fragment, which lacked the portion coding for the transmembrane and cytoplasmic regions, was isolated and ligated to linkers. The linkers have TAG stop codons in all three reading frames which ensures that translation will stop at or near the ScaI site. Without a transmebrane region, the protein is secreted by the transfected cells into the medium.

2.7.2 Coupling of sICAM-1 to Microtitre Plates

Purified sICAM-1 was adsorbed to 96-well flat-bottomed microtitre plates (Falcon) (25 ng/well). This was done in 10mM Tris pH 8.0 at 20^{o}C for 1 hr. BSA (0.5%) in 10 mM Tris pH 8.0 was then added and the solution incubated for 1 hr at 20^{o}C. The wells were then washed 3x with PBS.

2.7.3 Binding Assay

BW5147 cells in HBSS/1%FCS were dispensed into sICAM-1-coupled wells (2x10^{5} cells/well). The plate was centrifuged at 300g for 1 min and incubated at 37^{o}C for 30 min. The plate was inverted for 10 min at 20^{o}C for 10 min and then flicked to remove the unbound cells. The extent of cell binding was determined by using a colourimetric assay that measures viable cell number (Mossmann, 1983). This rapid colourimetric assay is based on the tetrazolium salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide). MTT is a colorless substrate for various mitochondrial dehydrogenase enzymes. When the tetrazolium ring is cleaved by the dehydrogenases, the resulting product is colored. The solutions were then read on a Microplate Autoreader EL309 (Bio-Tek Instruments) using a test wavelength of 570nm and a reference wavelength of 630 nm.

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CHAPTER III

RESULTS

The rat monoclonal antibody YN1/1.7 was initially identified by its ability to inhibit the murine mixed lymphocyte response (MLR) (Takei, 1985). It recognizes a 95-100 kd antigen, designated MALA-2, on the surface of proliferating murine lymphocytes. This antigen was purified and tryptic peptide sequences were determined (Horley et al, 1989). From these sequences, oligonucleotides were constructed and used to screen a λgt10 cDNA library from NS-1 cells. Several clones, including K3-1.1, were previously isolated and sequenced (Horley et al, 1989). The K3-1.1 clone has significant homology to the human ICAM-1 gene. This homology together with the ability of purified MALA-2 to bind LFA-1^+ cells in a specific manner suggested that MALA-2 is the murine homologue of ICAM-1. However, the amino acid sequence deduced from the K3-1.1 cDNA has an unusual N-terminal sequence atypical of type I transmembrane proteins. Therefore, an additional cDNA clone, K4-1.1, was isolated and characterized. The sequence of K4-1.1 and the function, expression, and distribution of the two clones, K3-1.1 and K4-1.1, are described in this thesis.

3.1 NUCLEOTIDE AND AMINO ACID SEQUENCE ANALYSIS

The nucleotide sequence of the K3-1.1 clone has been previously determined and found to be 3031 bp long (Horley et al, 1989). It has a 552 bp 5' untranslated region, a 1593 bp open reading frame , and a 3' untranslated region of 851 bp. Using the K3-1.1 clone as a probe, the cDNA library was screened and additional clones were isolated. One of these

clones, K4-1.1, was further characterized. The nucleotide sequence of the K4-1.1 clone is shown in Figure 4a. The K4-1.1 clone has a 29 bp 5' untranslated region, a 1611 bp open reading frame, and a 3' untranslated region of 851 bp. It is identical to that of the K3-1.1 clone, with the exception of the 5' end. The difference in nucleotide sequence covers the entire 5' untranslated region and approximately the first 100 bp of the open reading frame (Figure 4b). The 3' untranslated region of the two clones, which are identical, contain a polyA signal (AATAAA) followed by a polyA tail. The regions also contain four ATTTA sequences which contribute to mRNA instability (Shaw and Kamen, 1986; Schuler and Cole, 1988). This sequence also appears three other times in the K3-1.1 clone, twice in the 5' untranslated region and once in the open reading frame. The K4-1.1 clone has an ATG translation start site at nucleotide 30. A purine (G at position 27) three nucleotides upstream of the ATG and another purine (G at position 33) just downstream of the ATG conforms to the optimal translation initiation sequence of CC(A/G)CCATGG (Kozak, 1986,1987). The K3-1.1 clone has 10 potential translation start sites in the 5' untranslated region. However, they all have short open reading frames encoding proteins no bigger than 97 amino acids (Table II). The ATG at position 553 can initiate translation of a 507 amino acid protein. But the sequence around the ATG does not conform very well to the consensus sequence. Only the ATG at position 14 agrees with the accepted sequence.

The ATG at postion 30 in the K4-1.1 clone initiates translation of a typical type I transmembrane protein. The ATG at position 553 of the K3-1.1 clone also initiates translation of a transmembrane protein. Both proteins are virtually identical in sequence, differing only in the amino terminal (Figure 5). The amino terminal peptide for K3-1.1 corresponding to the leader sequence in K4-1.1 is unusually hydrophilic with six of the first 11 residues being charged. In contrast, only two of the first 25 residues in the K4-1.1 clone are charged giving it the typical features of a leader sequence. The encoded proteins have a large extracellular domain (461 residues, K4-1.1; 456 residues, K3-1.1), a typical 23 residue transmembrane region, followed by a cluster of charged residues in the cytoplasmic domain

a)

(figure legend on next page)

b)

Figure 4 Sequence of the two MALA-2 cDNA clones. a) (previous page) Complete nucleotide sequence of the K4-1.1 cDNA clone and predicted amino acid sequence are shown. The potential glycosylation sites are marked by ---CHO--- and the transmembrane region is underlined with a bold line. Cysteine residues are in bold letters. The polyadenylation signal (AATAAA) is underlined and the ATTTA sequences are boxed. The amino acid sequence is numbered from the predicted cleavage site of the signal peptide. b) The nucleotide and predicted amino acid sequence of the K3-1.1 cDNA clone is shown. Only the 5' end of the sequence is shown. The ATTTA sequences are boxed and the ATG codons in the 5' untranslated region are underlined. The AGG trimer at position 654 is overlined and the sequence identical to K4-1.1 is in bold and underlined.

TABLE II

AMINO ACID SEQUENCES OF HYPOTHETICAL PROTEINS TRANSLATED FROM ATG CODONS IN 5' UNTRANSLATED REGION OF K3-1.1 cDNA CLONE

Nucleotide position of ATG	Amino acid sequence
14	MANIRNLLKPLSPTVQKLNIKASYTLILTYAAKRIILNKS
102	MQQRELS
148	MLF
205	MHASVPGICMCVQTGVCTCACLLRPEIYVLHLPLLLST
209	MQVYLVFVCVCLRVYAHVLAC
232	MCVQTGVCTCACLLRPEIYVLHLPLLLST
252	MHMCLPVEARDLCSTSSSIALYLNQVLSDSARLSGQQAGESSCLCLPSSASQV MPRFLCGCWGSELRTSGLHSKPLLTEPLLRPLTFLLIFFYYSHN
258	MCLPVEARDLCSTSSSIALYLNQVLSDSARLSGQQAGESSCLCLPSSASQVMP RFLCGCWGSELRTSGLHSKPLLTEPLLRPLTFLLIFFYYSHN
411	MPRFLCGCWGSELRTTSGLHSKPLLTEPLLRPLTFLLIFFYYSHN
425	MWLLGI

K3-1.1: Met·Ile·Thr·**His·Arg·His**·Pro·Val·**Arg·Glu·Lys**·Ser·Ile·**Asn**·Ser·Tyr·**Gln**·Phe·Ile·Lys·Glu·Lys·Gln·Phe·Pro·Ala·Glu·Asn . . .

K4-1.1: Met·Ala·Ser·Thr·**Arg**·Ala·**Lys**·Pro·Thr·Leu·Pro·Leu·Leu·Leu·Ala·Leu·Val·Thr·Val·Val·Ile·Pro·Gly·Pro·Gly·Asp·Ala·Gln·Val·Ser·Ile·His·Pro·Arg . . .

Figure 5 Differences in amino acid sequence between the K3-1.1 and K4-1.1 clones. Translation from position 553 in clone K3-1.1 and position 30 in clone K4-1.1 would yield two slightly different proteins. The apparent leader peptides encoded by the two cDNA clones are in large print and underlined with the hydrophilic or charged residues in bold print. The suspected point of cleavage is marked by an arrow resulting in two processed proteins that converge in sequence after only nine amino acids.

(28 residues). The extracellular region contains five Ig-like domains with nine potential glycosylation sites (Asn-X-Ser/Thr), and an RGD and an RGE sequence. Comparison of the sequence has revealed that it has significant homology to the human ICAM-1 at both the nucleotide level (Horley et al, 1989) and at the protein level (Figure 6). The overall identity of the K4-1.1 encoded protein with human ICAM-1 is 54% at the amino acid level. The figure also shows that the two most N-terminal domains of MALA-2 share significant homology with ICAM-2 (31%).

3.2 EXPRESSION OF THE K3-1.1 AND K4-1.1 CLONES

In order for a molecule to function in cellular adhesion, it must be expressed at the cell surface. The two clones, K3-1.1 and K4-1.1, were therefore examined for their ability to be expressed at the surface of transfected cells. The K3-1.1 and K4-1.1 cDNAs were subcloned into the transient expression vector pAX82. Since the presence of multiple ATG start codons in the 5' untranslated region was thought to interfere with expression of the K3-1.1 clone, an additional cDNA insert (K3-1.1Δ5) lacking the 5' untranslated region was also generated. The cDNA clones were introduced into COS cells by DEAE-dextran and the transfected cells were analyzed for the expression of the genes and their protein products.

3.2.1 FACS Analysis

Cell surface expression of the various clones was initially examined by FACS analysis. Three days following transfection, COS cells were harvested and stained with YN1/1.7 antibody and FITC-conjugated goat anti-rat IgG. Expression was detected only with the K4-1.1 clone, but not with the K3-1.1 or K3-1.1Δ5 clones (Figure 7a).

a)

```
                                  1
MALA-2  MASTRAKPTLPLLLALVTVVIPGPG-DAQVSIHPREAFLPQGGSVQVNCSSSCKEDLSLGL  35
ICAM-1  MAPSSPRPALPALLVLLGALFPGPGGNAQTSVSPSKVILPRGGSVLVTCSTSCDQPKLLGI  35
         A     P LP LL L     PGPG  AQ S  P    LP GGSV V CS SC     LG

MALA-2  ETQWLKDE-LESGPNWKLFELSEIGEDSSPLCFENCGTVQSSASATITVYSFPESVELRPL  95
ICAM-1  ETPLPKKELLLPGNNRKVYELSNVQEDSQPMCYSNCPDGQSTAKTFLTVYWTPERVELAPL  96
        ET   K E L  G N K  ELS   EDS P C  NC   QS A    TVY  PE VEL PL

MALA-2  PAWQQVGKDLTLRCHVDGGAPRTQLSAVLLRGEEILSRQPVGGHPKDPKEITFTVLASRGD  156
ICAM-1  PSWQPVGKNLTLRCQVEGGAPRANLTVVLLRGEKELKREPAVG---EPAEVTTTVLV-RRD  153
        P WQ VGK LTLRC V GGAPR  L  VLLRGE  L   P  G    P E T TVL  R D

MALA-2  HGANFSCRTELDLRPQGLALFSNVSEARSLRTFDLPATIPKLDTPDLLEVGTQQKLFCSLE  217
ICAM-1  HGANFSCRTELDLRPQGLELFENTSAPYQLQTFVLPATPPQLVSPRVLEVDTQGTVVCSLD  214
        HGANFSCRTELDLRPQGL LF N S    L TF LPAT P L  P  LEV TQ    CSL

MALA-2  GLFPASEARIYLELGGQMPTQESTNSSDSVSATALVEVTEEFDRTLPLRCVLELADQILET  278
ICAM-1  GLFPVSEAQVHLALGDQRLNPTVTYGNDSFSAKASVSVTAEDEGTQRLTCAVILGNQSQET  275
        GLFP SEA   L LG Q      T   DS SA A V VT E   T  L C   L  Q  ET

MALA-2  QRTLTVYNFSAPVLTLSQLEVSEGSQVTVKCEAHSGSKVVLLSGVEPRPPTPQVQFTLNAS  339
ICAM-1  LQTVTIYSFPAPNVILTKPEVSEGTEVTVKCEAHPRAKVT-LNGVPAQPLGPRAQLLLKAT  335
          T   Y F AP   L   EVSEG  VTVKCEAH   KV  L GV   P  P  Q  L A

MALA-2  SEDHKPSFFCSAALEVAGKFLFKNQTLELHVLYGPRLDETDCLGNWTWQEGSQQTLKCQAW  400
ICAM-1  PEDNGRSFSCSATLEVAGQLIHKNQTRELRVLYGPRLDERDCPGNWTWPENSQQTPMCQAW  396
         ED   SF CSA LEVAG    KNQT EL  LYGPRLDE DC GNWTW E SQQT  CQAW

MALA-2  GNPSPKMTCRRKADGALLPIGVVKSVKQEMNGTYVCHAFSSHGNVTRNVYLTVLYHSQNNW  461
ICAM-1  GNPLPELKC-LKDGTFPLPIGESVTVTRDLEGTYLCRARSTQGEVTREVTVNVL--SPRYE  454
        GNP P   C  K     LPIG    V     GTY C A S  G VTR V   VL  S

MALA-2  TIIILVPVLLVIVGLVMAASYVYNRQRKIRIYKLQKAQEEAIKLKGT-APPP          512
ICAM-1  IVIITVVAAAVIMGTAGLSTYLYNRQRKIKKYRLQQAQKGTPMKPNTQATPP          506
          II V    VI G      Y YNRQRKI  Y LQ AQ        T A PP
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Figure 6 Comparison of MALA-2 sequence with the two human ICAM sequences. a) The amino acid sequence of MALA-2 (K4-1.1 sequence) is aligned with the human ICAM-1 sequence. The third line shows the amino acids shared by the two sequences. b) (next page) The sequences of the first two domains of MALA-2 and ICAM-1 are compared to the ICAM-2 sequence. Residues conserved in all three proteins are shown on the fourth line.

b)

```
MALA-2      DAQVSIHPREAFLPQGGSVQVNCSSSCKEDLSLGLETQWLKDE-LESGPNWKLF   53
ICAM-1        QTSVSPSKVILPRGGSVLVTCSTSCDQPKLLGIETPLPKKELLLPGNNRKVY   52
ICAM-2    SDEKVFEVHVRPKKLAVEPKGSLEVNCSTTCNQPEVGGLETSL-NKILLDEQAQWKHY   57
                    P         GS  V CS  C       G ET        L       K

MALA-2    ELSEIGEDSSPLCFENCGTVQSSASATITVYSFPESVELRPLPAWQQVGKDLTLRCHV   111
ICAM-1    ELSNVQEDSQPMCYSNCPDGQSTAKTFLTVYWTPERVELAPLPSWQPVGKNLTLRCQV   110
ICAM-2    LVSNISHDTVLQCHFTCSGKQESMNSNVSVYQPPRQVILTLQPTLVAVGKSFTIECRV   115
            S    D     C    C    Q          VY  P  V L   P     VGK  T  C V

MALA-2    DGGAPRTOLSAVLLRGEEILSRQPVGGHPKDPKEITFTVLASRGDH--G-ANFSCRTE   166
ICAM-1    EGGAPRANLTVVLLRGEKELKREPAVG---EPAEVTTTVLV-RRDHH-G-ANFSCRTE   162
ICAM-2    PTVEPLDSLTLFLFRGNETLHYETFGKAAPAPQEATATFNS-TADREDGHRNFSCLAV   172
             P    L    L RG    L            P E T T      D    G  NFSC

MALA-2    LDLRPQGLALFSNVSEARSLRTFDLPATIP                           196
ICAM-1    LDLRPQGLELFENTSAPXQLQTFVLPATPP                           192
ICAM-2    LDLMSRGGNIFHKHSAPKMLEIYEPVSDSQ                           202
          LDL    G    F    S     L
```

a)

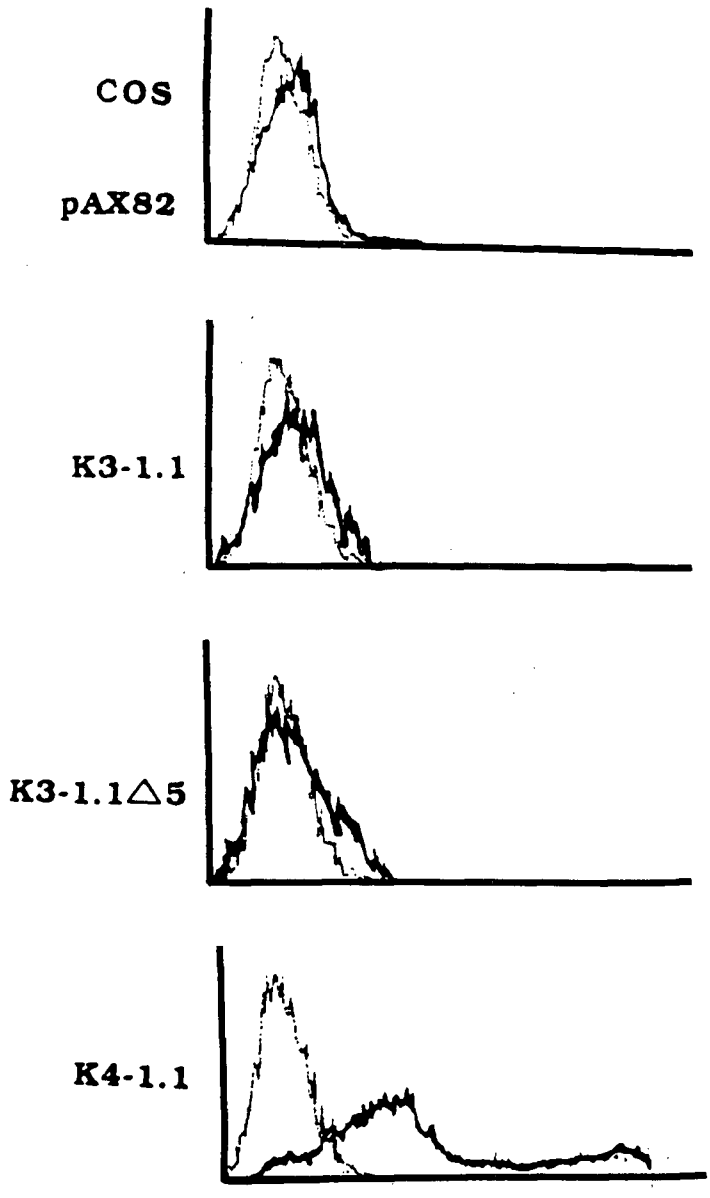
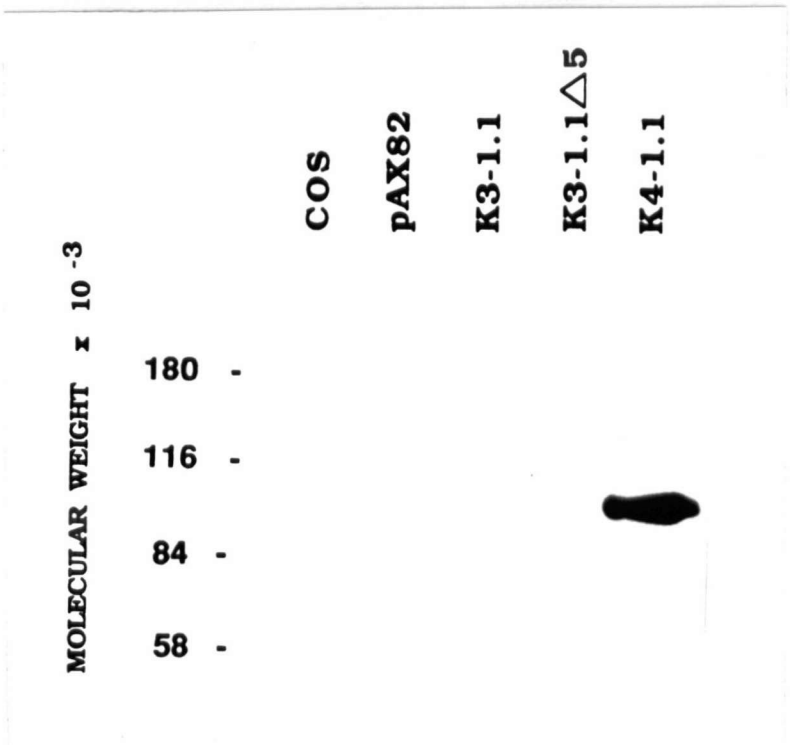
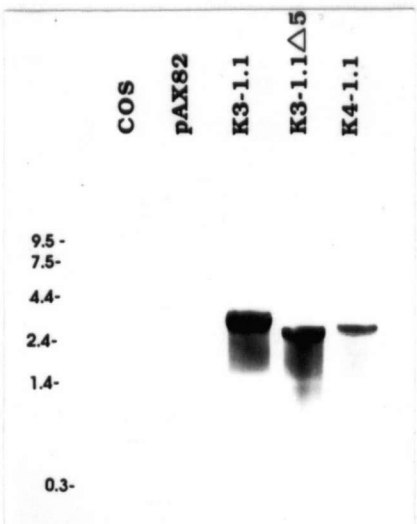


Figure 7 Analysis of transfected COS1 cells. a) Cells were indirectly stained with the YN1/1.7 hybridoma supernatant (dark line) or with HBSS/1%FCS (thin line), followed by FITC-conjugated goat anti-rat IgG. Dead cells were stained with propidium iodide and gated out based on red fluorescence. Data are plotted with cell number on the vertical axis versus fluorescence intensity (log) on the horizontal axis. b) (next page) Lysate from approximately 2 X 10^6 transfected cells was applied to a 7.5% polyacrylamide gel. After electrophoresis and blotting, the filter was probed with ^{125}I-YN1/1.7 antibody and washed. The markers were from the SDS-Blue kit. c) (next page) Total RNA from ~ 2 X 10^6 transfected cells (~10μg) was electrophoresed and blotted onto Zeta Probe. The filter was probed with the K4-1.1 insert.

b)


MOLECULAR WEIGHT x 10 -3
180 -
116 -
84 -
58 -
COS
pAX82
K3-1.1
K3-1.1△5
K4-1.1

c)


COS
pAX82
K3-1.1
K3-1.1△5
K4-1.1
9.5 -
7.5-
4.4-
2.4-
1.4-
0.3-

3.2.2 Western Blot Analysis

Total cell lysate from transfected cells were fractionated on SDS-PAGE and transfered to nitrocellulose. The filter was probed with ^{125}I-YN1/1.7 antibody providing a more sensitive assay for intracellular protein than immunoperoxidase staining. A ~95 kd band appeared only in the K4-1.1 lysate (Figure 7b) indicating that neither intracellular nor cell surface MALA-2 protein could be detected in the K3-1.1 or K3-1.1Δ5 transfected COS cell lysates.

3.2.3 Northern Blot Analysis

Total RNA from transfected cells was blotted onto Zeta Probe and probed with the K4-1.1 insert. Bands corresponding to the size of each message were detected for the K3-1.1, K3-1.1Δ5, and K4-1.1 transfected cells (Figure 7c). This reveals that the lack of expression of K3-1.1 and K3-1.1Δ5 was not due to poor transcription.

3.2.4 Immunoperoxidase Staining

Transfected cells were fixed and examined for intercellular expression and localization of the K3-1.1 and the K4-1.1 proteins by indirect immunoperoxidase staining. Intercellular protein was not detected in the K3-1.1 or the K3-1.1Δ5 transfected cells. However, the K4-1.1 transfected cells demonstrated not only positive staining but also punctated staining (Figure 8).

3.3 NORTHERN BLOT ANALYSIS OF VARIOUS BALB/c TISSUES

Total RNA from various lymphoid cells was electrophoresed and blotted. When the filter was probed with the K4-1.1 insert, two bands of ~3.2 kb and ~2.6 kb were detected in NS-1 cells and LPS-stimulated spleen cells (Figure 9). However, only the 2.6 kb band was detected in ConA-stimulated spleen cells. A very faint 3.2 kb band was detected in the thymus and a

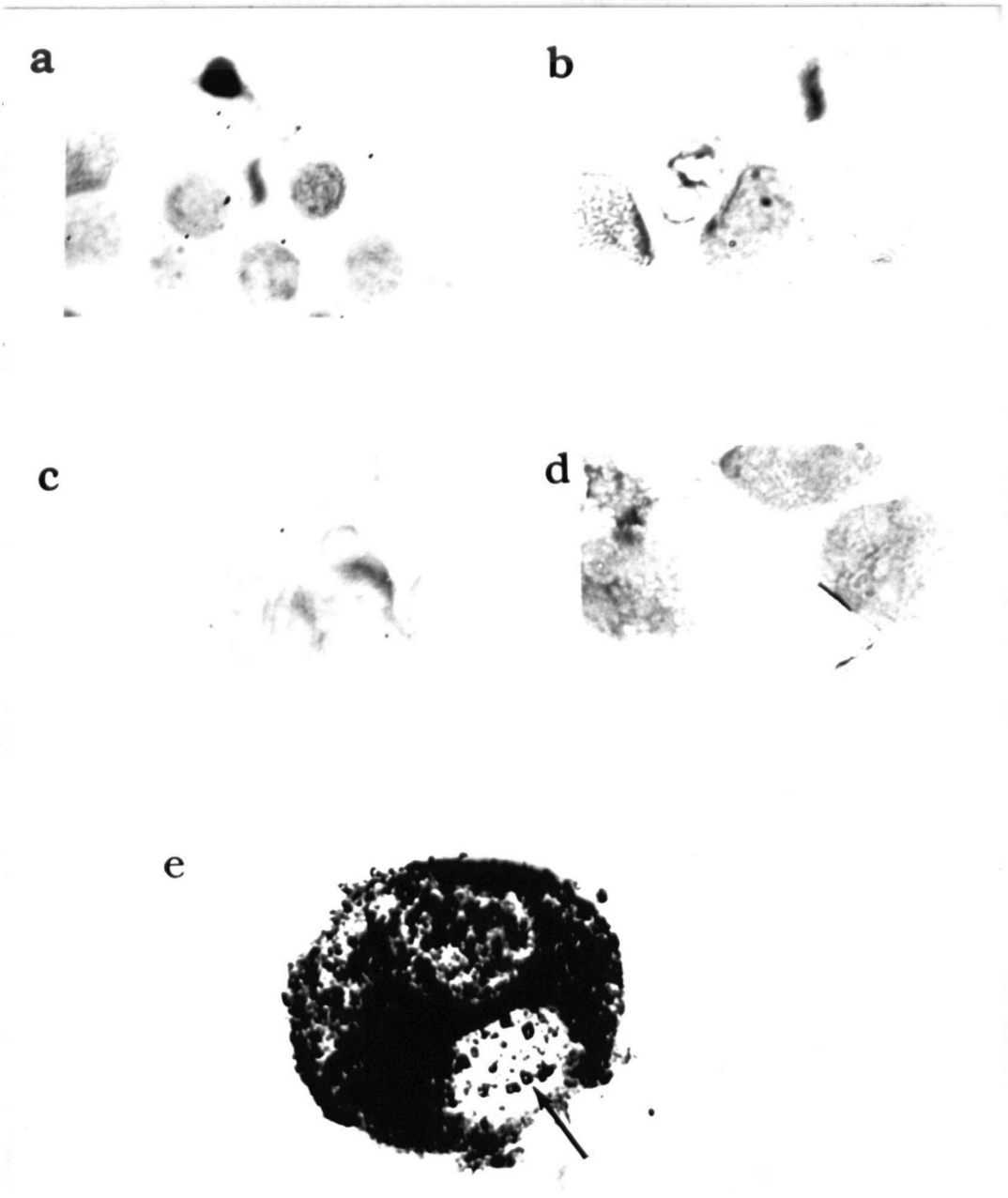


Figure 8 Indirect immunoperoxidase staining of transfected cells. Cytospin preparations of transfected COS cells were fixed and stained for MALA-2 expression using the YN1/1.7 antibody and an indirect immunoperoxidase assay. The cells stained were a) COS1, b) pAX82, c) K3-1.1, d) K3-1.1Δ5, and e) K4-1.1. The arrow in figure 8e indicates a site of punctated staining.

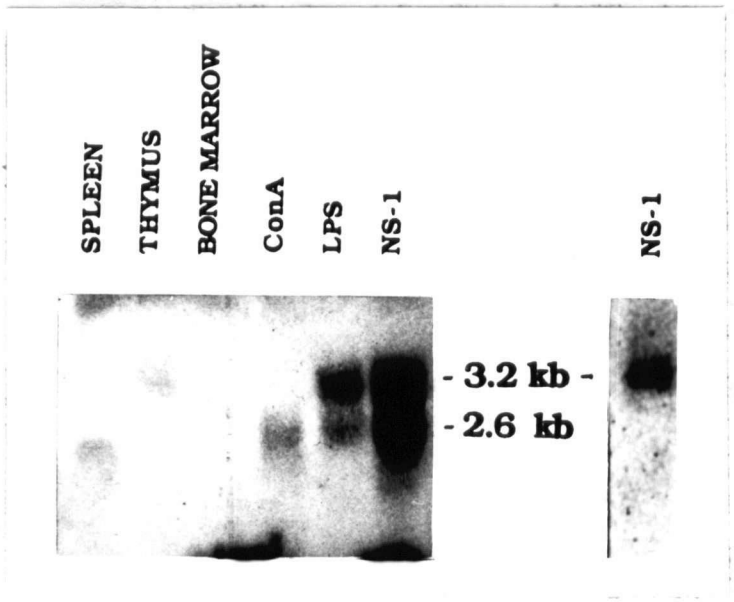


Figure 9 Northern blot analyis of MALA-2 in various lymphoid cell populations. All cells were of BALB/c origin. Total RNA (~10μg) from the various cells was run on a formaldehyde gel and the filter was probed with the K4-1.1 insert (six lanes on the left). Total RNA from NS-1 cells was probed with 5' Asp700I fragment of the K3-1.1 clone (far right lane).

faint 2.6 kb band in the spleen. When the filter was probed with the 5' Asp700I fragment of K3-1.1 (5' untranslated region), which is unique to K3-1.1, only the 3.2 kb band was detected in NS-1 cells. These results strongly suggest that the 3.2 kb transcript is represented by the K3-1.1 clone (3031 bp) and the 2.6 kb message is represented by the K4-1.1 clone (2525 bp). These two transcripts may be the result of alternate splicing. This is supported by the appearance of an AGG (exon-exon joining) trimer in the region where both sequences converge (Figure 4).

3.4 SOUTHERN BLOT ANALYSIS

BALB/c spleen genomic DNA was digested with two sets of restriction enzymes and transfered to Zeta Probe. One filter contained digests with enzymes that do not cut the K4-1.1 cDNA. When this filter was probed with the K4-1.1 insert, bands no smaller than 4.4 kb were detected (Figure 10a). All lanes appear to have single bands, indicating that most of the genomic structure is contained within these bands and that it is a single copy gene. When the filter was probed with the 5' end of K3-1.1, single band lanes were also observed (Figure 10b). They were different form those generated with the K4-1.1 insert, with the exception of PvuI which gave a band of ~23 kb with both probes. Fragments in this size range cannot be resolved efficiently and the possibility that the two probes hybridize to two distinct but similar size fragments cannot be excluded. Therefore, no fragment was clearly identified to contain both the K4-1.1 coding region and the 5' untranslated region of K3-1.1. A second filter contained digests with enzymes that cut the K4-1.1 insert. A ~9.8 kb HindIII fragment was detected with both the K4-1.1 probe and the 5' end of K3-1.1 (Figure 11). This may be the result of close genomic linkage between the two fragments further supporting the notion of alternate splicing in generating the K3-1.1 and K4-1.1 clones. Probing the filters with the 5' end of K3-1.1 also demonstrated that the sequence is endogenous to the mouse genome.

a)

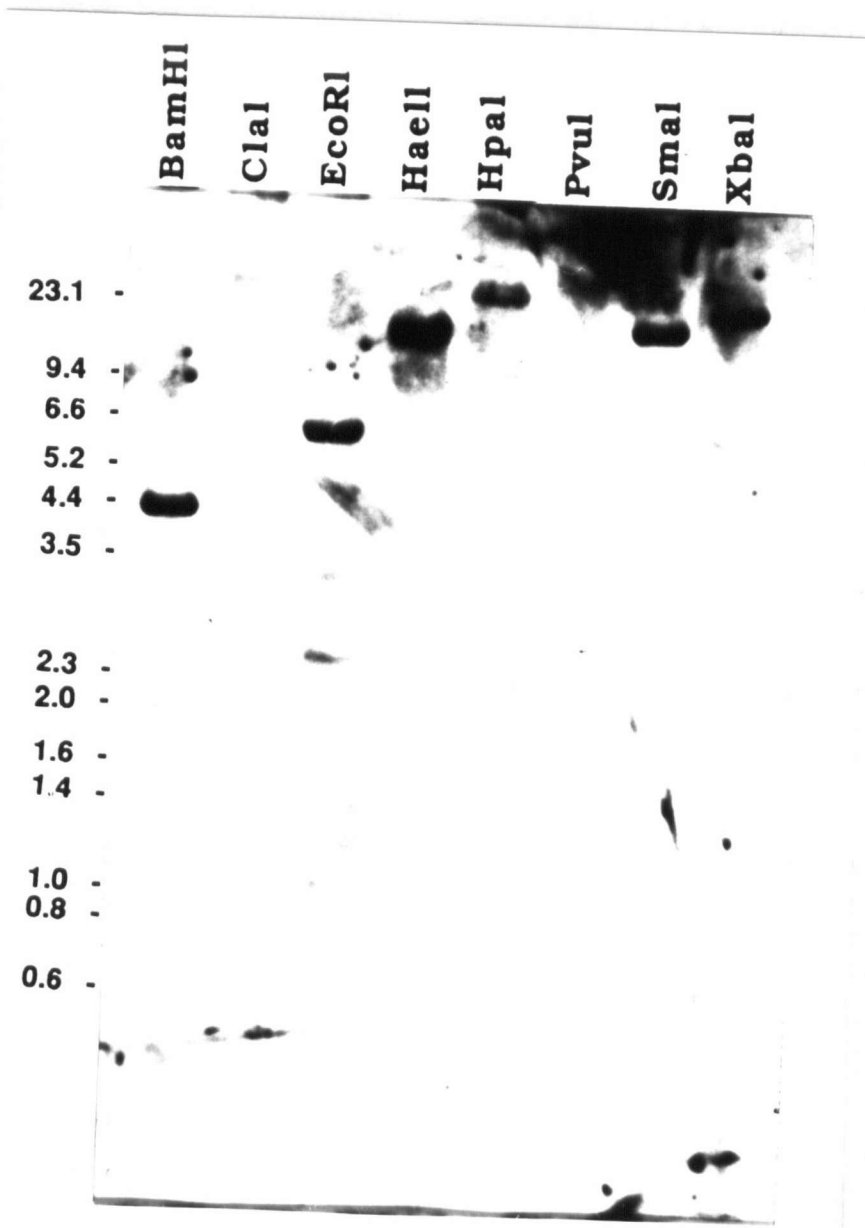
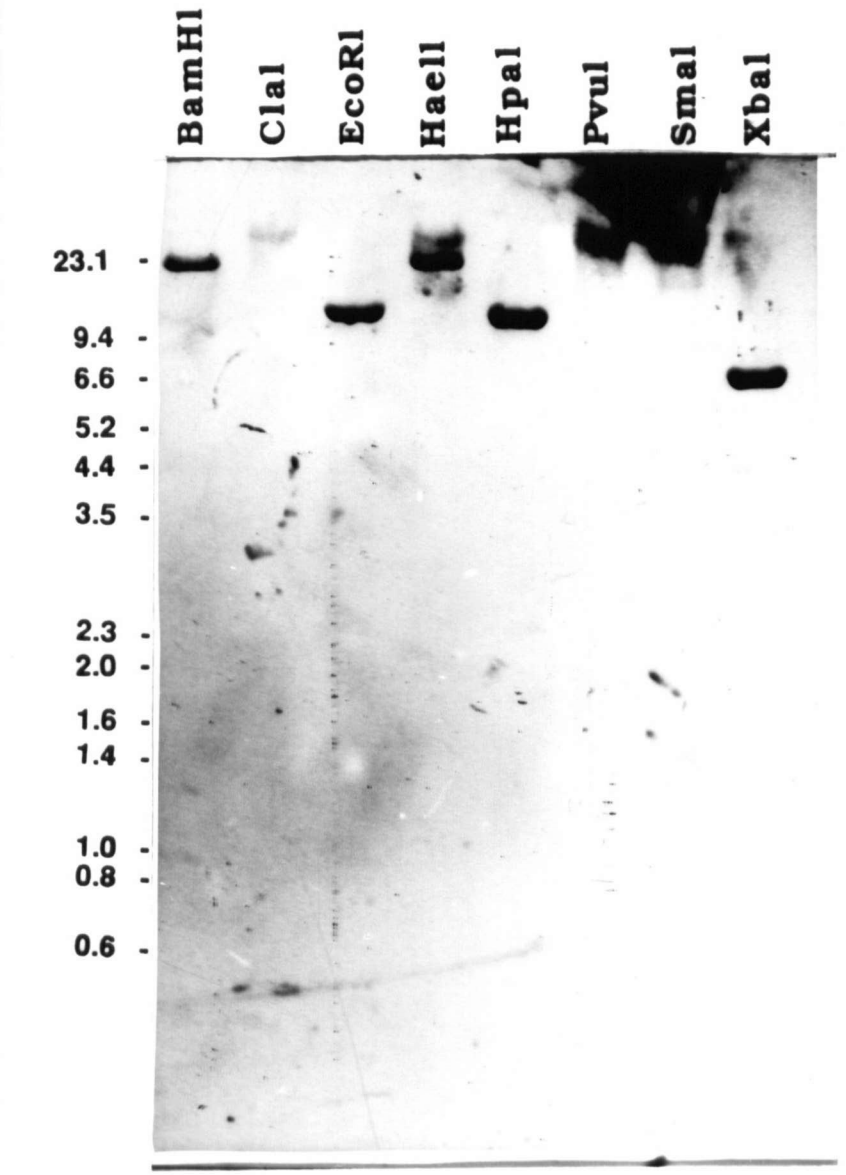


Figure 10 Genomic Southern analysis using restriction enzymes that do not cut the K4-1.1 insert. BALB/c spleen DNA was digested with various enzymes that do not cut the K4-1.1 insert, electrophoresed on a 0.8% agarose gel, and blotted. The filter was probed with either the K4-1.1 insert (a) or the 5' Asp700I fragment of the K3-1.1 insert (b) (next page).

b)


BamHI
ClaI
EcoRI
HaeII
HpaI
PvuI
SmaI
XbaI
23.1
9.4
6.6
5.2
4.4
3.5
2.3
2.0
1.6
1.4
1.0
0.8
0.6

a)

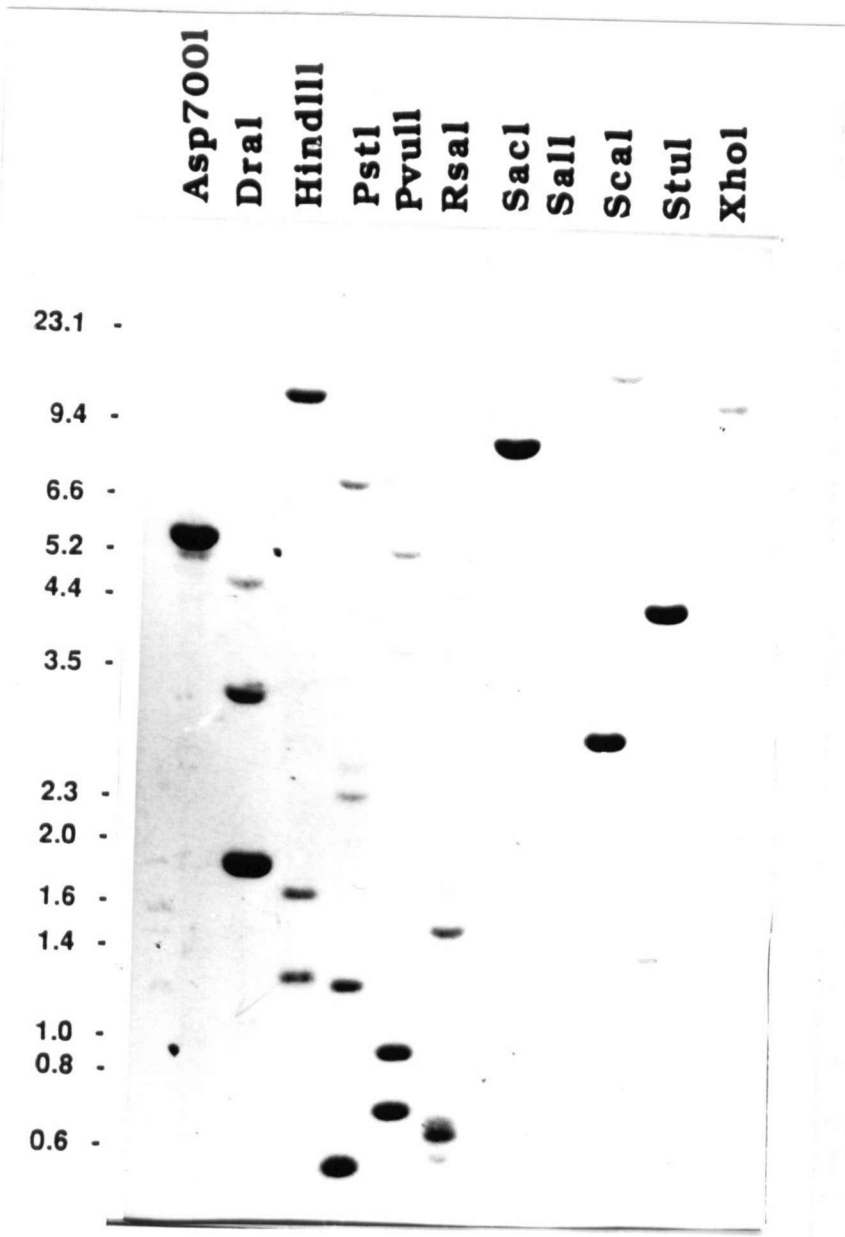
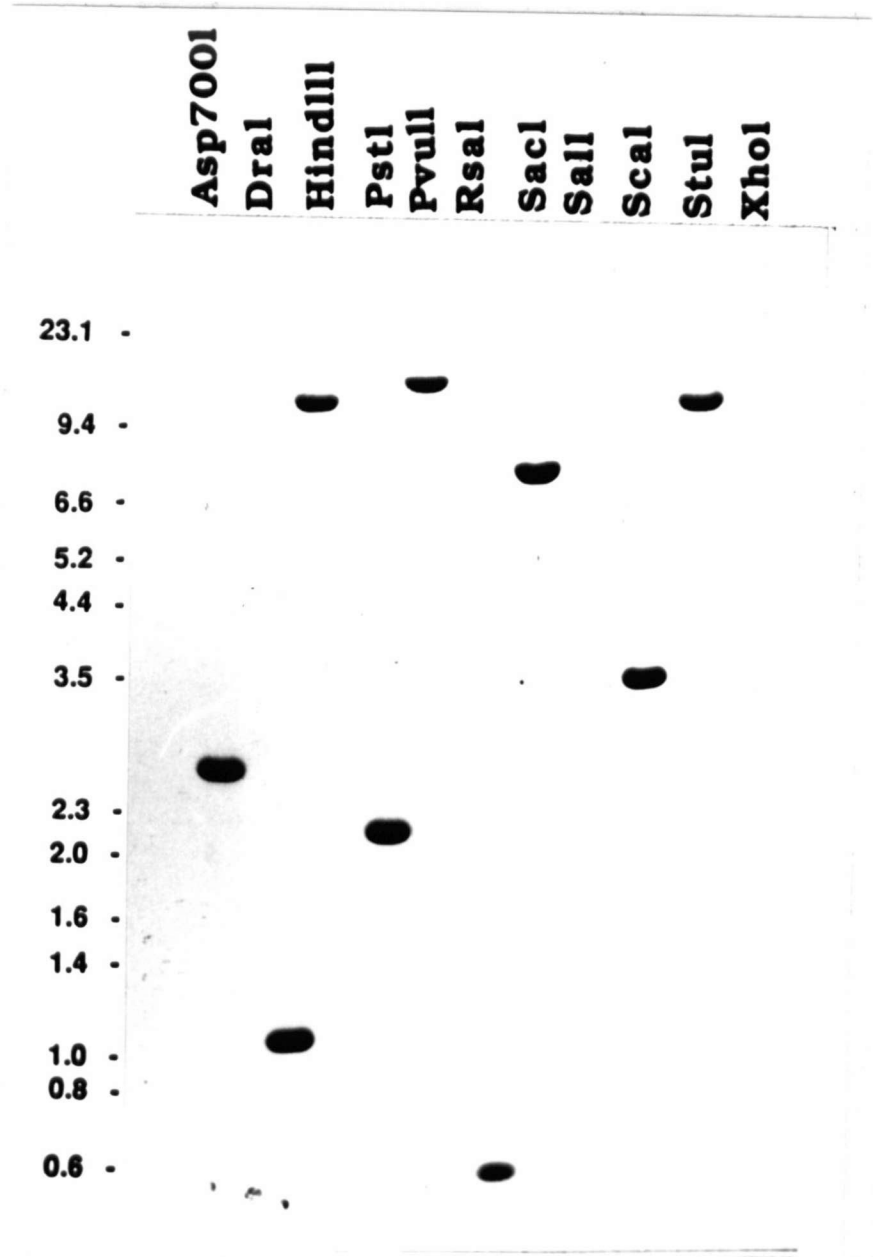


Figure 11 Genomic Southern analysis using restriction enzymes that cut the K4-1.1 insert. BALB/c spleen DNA was digested with various enzymes that cut the K4-1.1 insert, electrophoresed on a 0.8% agarose gel, and blotted. The filter was probed with either the K4-1.1 insert (a) or the 5' Asp700I fragment of the K3-1.1 insert (b) (next page).

b)


Asp700I
DraI
HindIII
PstI
PvuII
RsaI
SacI
SalI
ScaI
StuI
XhoI
23.1
9.4
6.6
5.2
4.4
3.5
2.3
2.0
1.6
1.4
1.0
0.8
0.6

3.5 SOLUBLE ICAM-1 (sICAM-1)

Since both ICAM-1 and LFA-1 are membrane bound molecules, their interaction with each other is difficult to study. As a first step to further investigate this molecular interaction, I attempted to produce soluble ICAM-1 (sICAM-1) by deleting the transmembrane region of the K4-1.1 insert. This was achieved by digesting the K4-1.1 insert with ScaI. In order to avoid the addition of vector-encoded peptide(due to the lack of a termination codon), BstXI linkers with TAG stop codons in all three reading frames were ligated to the ScaI digested cDNA. The translated protein lacks a hydrophobic anchor and should thus be secreted into the medium.

3.5.1 Initial Testing of Supernates

COS cells were transfected and supernates were harvested 70 hrs later. They were then concentrated five fold and assayed for the ability to block the binding of ^{125}I-YN1/1.7 antibody to NS-1 cells. The results in Table III show that only the K4-1.1ΔT supernatant was able to block binding. There is no detectable protein in the control supernates.

3.5.2 Purification of sICAM-1

COS cells were transfected with K4-1.1ΔT/pA82 and the secreted protein was purified from the supernatants by antibody affinity chromatography. Purity and quantity were assessed by SDS-PAGE and silver-staining (Figure 12). The truncated protein, which lacks a transmembrane and cytoplasmic domain, is ~6 kd smaller than the intact, membrane-bound ICAM-1. This is expected since the truncated protein is 56 amino acids shorter than the membrane-bound form. The total yield was approximately 6 μg of protein from 2 X 10^7 cells (10 plates).

TABLE III

BLOCKING OF ^{125}I-YN1/1.7 ANTIBODY TO NS-1 BY SUPERNATES OF TRANSFECTED COS1 CELLS

Transfected COS cell supernatant	Radioactivity bound to NS-1 cells (cpm)
COS	2.3×10^5
pAX82	2.2×10^5
K3-1.1	2.4×10^5
K3-1.1Δ5	2.3×10^5
K4-1.1	2.1×10^5
K4-1.1ΔT	1.5×10^5

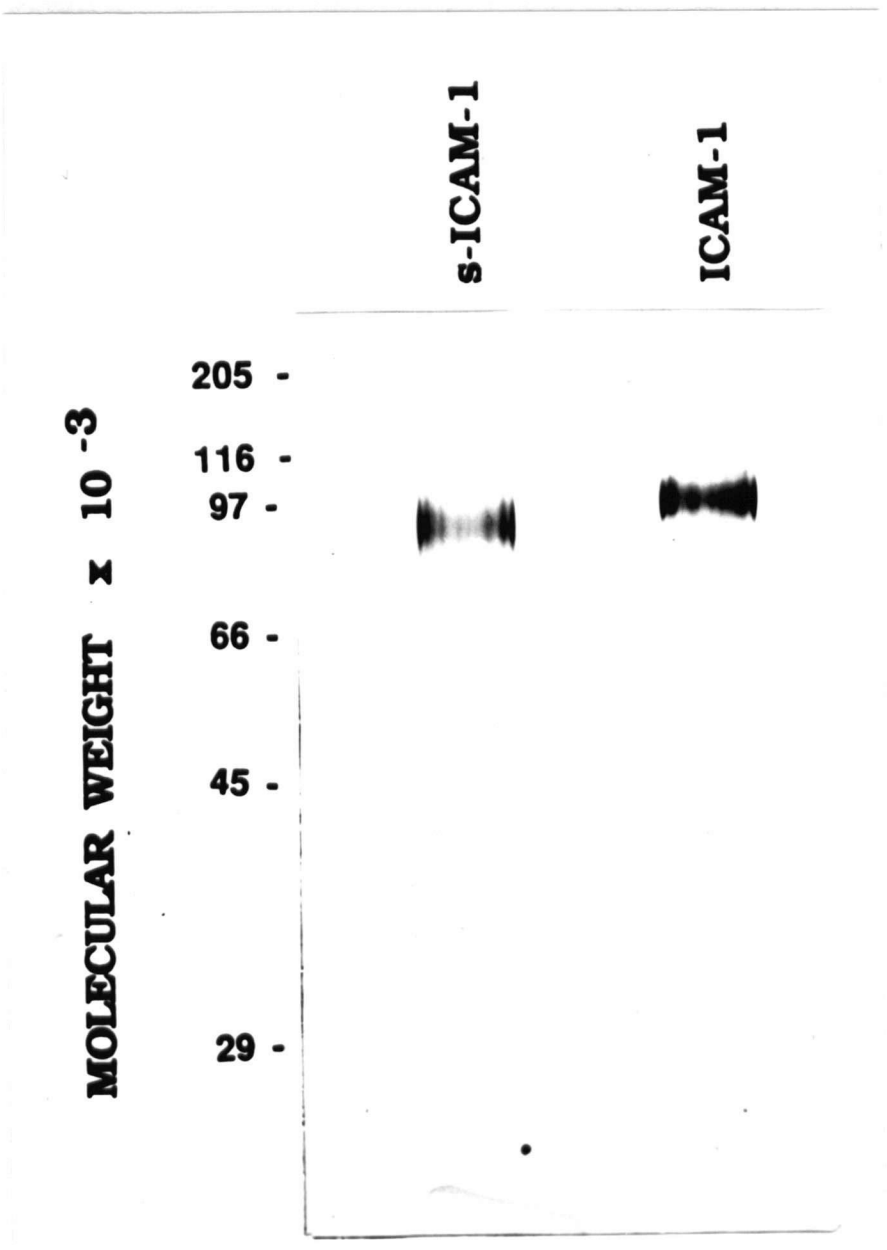


Figure 12 Assessment of the purity and yield of the purified sICAM-1. The purified sICAM-1 and the intact ICAM-1 were run on a 7.5% SDS-PAGE and silver stained. The markers used were from the SDS-200 kit.

3.5.3 Binding of BW5147 cells to sICAM-1

In order to determine whether the removal of the transmembrane and cytoplasmic domains might disrupt the ability of sICAM-1 to bind LFA-1^+ cells, a binding assay was performed. The affinity purified sICAM-1 was immobilized in the wells of microtitre plates. The extent of binding was determined by a colourimetric assay for mitochondrial enzyme activity (MTT assay). The results of the assay are shown in Table IV and show that sICAM-1 can still bind cells in a specific manner.

TABLE IV

BINDING OF BW5147 CELLS TO sICAM-1 IMMOBILIZED TO MICROTITRE WELLS

Blocking antibody	$A_{(570-630)}$
none	0.142 ± 0.010
YN1/1.7	0.020 ± 0.002
anti-LFA-1	0.010 ± 0.004
YN1/1.7 + anti-LFA-1	0.003 ± 0.001

3.6 REFERENCES

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Kozak, M. (1987) An analysis of 5'-noncoding sequences from 699 vertebrate messenger RNAs. Nucleic Acid Res. 15:8125.

Schuler, G.D. and Cole, M.D. (1988) GM-CSF and oncogene mRNA stabilities are independently regulated in trans in a mouse monocytic tumor. Cell 55:1115.

Shaw, G. and Kamen, R. (1986) A conserved AU sequence from the 3' untranslated region of GM-CSF mRNA mediates selective mRNA degradation. Cell 46:659.

Takei, F. (1985) Inhibition of mixed lymphocyte response by a rat monoclonal antibody to a novel murine lymphocyte activation antigen (MALA-2). J. Immunol. 134:1403.

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CHAPTER IV

CONCLUSIONS AND DISCUSSION

The role of adhesion molecules in the immune system has been demonstrated to be one of great importance. Antibodies against both human and murine adhesion molecules have been shown to disrupt virtually every immune response tested. The YN1/1.7 monoclonal antibody was generated by immunizing a rat with NS-1 cells. The antibody reacted strongly with activated and proliferating murine lymphocytes but only weakly with resting lymphocytes. A notable feature of the antibody was its ability to inhibit the mixed lymphocyte response. This suggested that the antigen it recognizes, termed MALA-2, plays a direct role in lymphocyte activation. Subsequent studies to purify and sequence the molecule as well as to isolate a cDNA clone (K3-1.1) encoding it, suggested that MALA-2 is the murine homologue of ICAM-1. Purified MALA-2 was able to bind LFA-1^+ cells in a specific manner further supporting the notion that it is the murine ICAM-1.

The studies presented in this thesis have focussed on the sequence, distribution, and function of a second cDNA clone (K4-1.1) which also has significant homology to human ICAM-1. Comparison of the K4-1.1 clone with K3-1.1, the previously isolated clone, in conjunction with the YN1/1.7 antibody studies has allowed molecular characterization of MALA-2 at both the nucleic acid level and the protein level.

The sequence homology between the protein encoded by the K4-1.1 clone and the human ICAM-1 is significant and extends over the entire protein. There are stretches of 19 residues beginning at residue 153 in the second Ig-like domains of both proteins which are

identical. This may be a critical region for binding to LFA-1 in both species. The positions of the cysteine residues, which maintain the Ig-like structure (Williams and Barclay, 1988), are conserved. Each of the five domains contains ~100 amino acids and exhibits homology with members of the Ig supergene family (Horley et al, 1989). There is both an RGD and an RGE sequence in the second domain. The RGE sequence is conserved; however, the RGD corresponds to an RRD in the human sequence. Although it has been shown that RGD and RGE peptides do not inhibit cellular binding to human ICAM-1 (Marlin and Springer, 1987), this has not yet been tested in the mouse system. It is of particular interest to examine whether the murine LFA-1, a member of a supergene family that can recognize ligands through RGD sequences (Hynes, 1987), can interact with ICAM-1 through RGD and RGD-like sequences. This can be achieved by deleting or modifying the sequences and quantitatively examining the ability of the mutated proteins to bind LFA-1.

The MALA-2 sequence also has homology to the human ICAM-2 sequence. It has been shown that the two Ig-like domains of ICAM-2 have the highest degree of homology with the two most N-terminal domains of the human ICAM-1 (Staunton et al, 1989). The homology between ICAM-2 and the first two domains of MALA-2 and human ICAM-1 are also striking. The cysteine residues are conserved as are the arginine-glycine of RGE and the aspartic acid of RGD. There are no long stretches of conserved residues as there are in the MALA-2/ICAM-1 comparison. The conserved residues are more or less evenly spaced. It is also interesting that most of the residues conserved between MALA-2 and ICAM-2 are also present in ICAM-1. This suggests that ICAM-1, ICAM-2, and MALA-2 bind LFA-1 by similar mechanisms via the first two N-terminal domains. It will be interesting to see if MALA-2 can bind human LFA-1 and if human ICAM-1 and ICAM-2 can bind murine LFA-1.

The sequence homology of MALA-2 with human ICAM-1 shows that the two molecules are structurally similar. The similarity is also functional; purified MALA-2 immobilized on microtitre wells is able to bind ConA-stimulated spleen cells (Horley et al, 1989). The binding is specifically inhibited by the YN1/1.7 antibody or the anti-murine LFA-1 antibody. MALA-2

and human ICAM-1 are also virtually identical in size and cellular distribution (Takei, 1985; Prieto et al, 1989). The summation of the structural, functional, and distributional evidence strongly supports the conclusion that MALA-2 is the murine homologue of ICAM-1.

Aside from the above mentioned similarities, MALA-2 and human ICAM-1 also have similar cytokine response profiles. Activation of lymphocytes results in a 100-fold increase in cell surface expression of ICAM-1 (Dustin and Springer, 1988). This has also been observed for MALA-2 (Takei, 1985). Expression can be induced dramatically by cytokines such as IFN-γ, IL-1, or TNF (Dustin et al ,1986). Initial experiments with fibroblasts and HL-60 cells suggest that upregulation of ICAM-1 occurs at the level of transcription (Dustin et al, 1986; Simmons et al, 1988). A possible mechanism for controlling the downregulation of ICAM-1 may reside in the AUUUA destabilizing sequence in the 3' untranslated region. When these sequences are present in a transcript, the message is rapidly degraded. The K4-1.1 MALA-2 insert has four of these sequences and the human ICAM-1 has two AUUUAs (Staunton et al, 1988). Thus, upregulation can be controlled at the level of transcription and degradation may be controlled by decreasing mRNA stability.

The bulk of this work covers the isolation of a functional cDNA clone encoding MALA-2 and comparison to a previously isolated clone (Horley et al, 1989). The sequence of the newly isolated K4-1.1 clone is virtually identical to that of the previously isolated K3-1.1. The only difference lies in the 5' end. The K3-1.1 clone has a very long 5' untranslated region containing 10 ATG start sites and two ATTTA destabilizing sequences. The K4-1.1 clone has a small 5' untranslated region. Cell surface expression is detected on K4-1.1 transfected COS cells as determined by FACS analysis. Expression is not detected on K3-1.1 transfected cells. It was suspected that the large 5' untranslated region may be disrupting translation from the ATG at position 553. The 10 ATGs may be interfering with translation from the ATG at position 553 and the two mRNA destabilizing sequences may be causing degradation of the message (Shaw and Kamen, 1986; Schuler and Cole, 1988). However, the removal of this potentially disruptive region from the K3-1.1 clone, which generated K3-1.1Δ5, did not result

in cell surface expression. The N-terminal sequence of the K3-1.1 protein appears to be very hydrophilic with six of the first 11 residues being charged. Therefore, the K3-1.1 protein lacks a typical leader peptide, which is needed for cross-membrane transport in the endoplasmic reticulum. I therefore tested the possibility that the protein was accumulating in the cytoplasm. However, no intracellular protein was detected in K3-1.1 or K3-1.1Δ5 transfected cells as revealed by immunoperoxidase staining and western blot analysis. On the other hand, northern blot analysis of transfected cells revealed that lack of expression for K3-1.1 and K3-1.1Δ5 was not due to poor transcription of these cDNAs. The problem could thus be due to a very poor translation initiation sequence around the ATG at position 553 (Kozak, 1986, 1987). The C(A/G)CC upstream of the ATG is normally thought to pair with a noncontiguous complementary sequence in the 18S ribosomal subunit (Sargan et al, 1982) in a fashion similar to that of the Shine-Dalgarno sequence in E.coli (Kozak, 1983). The (A/G) three nucleotides upstream of the ATG appears to be most important for the alignment of the mRNA with the ribosome. The G just downstream of ATG appears to enhance mRNA-tRNA interactions. It is thought that AUGG in mRNA may form a four base pair interaction with the CCAU in the anticodon loop of initiator Met-tRNA. The sequence around the ATG at position 553 of K3-1.1 (ATTCATGA) does not make either of these two apparently important interactions. Another possibility is that the K3-1.1 protein is translated, but the hydrophobic transmembrane segments aggregate intracellularly and cause the protein to be degraded. Thus, it is not known whether the lack of K3-1.1 protein is due to a deficiency in translation of mRNA or a degradation of translated protein.

Expression of the K4-1.1 encoded protein was readily detected by FACS analysis, immunoperoxidase staining, and western blot analysis of transfected cells. Immunoperoxidase staining revealed that some of the staining was punctated. This could reflect patchy expression on the surface, or intracellular storage pools such as storage granules or Golgi apparatus. Both have been previously observed in human ICAM-1. Uropod localization of ICAM-1 has been observed in a T cell clone (Dougherty et al, 1988). Some cells

also have ICAM-1 in storage granules. COS cells transfected with human ICAM-1 also show punctuated staining (Kishimoto et al, 1990). This type of localized distribution on the cell surface may facilitate interactions with LFA-1 on other cells. Activation has been shown to lead to increased surface expression of intracellularly stored ICAM-1. The four ATTTA sequences in the 3' untranslated region may also provide a mechanism for downregulating expression. An AUUUA-specific mRNA binding protein has been detected which binds very tightly to the sequence and causes degradation of the mRNA (Malter, 1989). In K4-1.1 transfected COS cells, it was not determined whether the punctuated staining was on the surface or intracellularly localized.

The expression of MALA-2 mRNA in different lymphoid tissues closely parallels previous studies on cell surface expression by FACS analysis and tissue staining (Takei, 1985; Prieto et al, 1989). Northern blot analysis of various BALB/c lymphocyte populations revealed that messages of two different sizes may be found. In NS-1 cells, two very intense bands of 3.2 kb and 2.6 kb were detected. It is likely that the two messages are representatives of the two clones, since the 3.2 kb message correlates well with the size of the K3-1.1 clone and the 2.6 kb message correlates with the size of the K4-1.1 clone. When NS-1 total RNA lane was probed with the 5' end of K3-1.1, only the 3.2 kb band was detected. This ruled out the possibility that the 5' end of K3-1.1 was a cloning artifact and strengthened the likelihood that the 2.6 kb and 3.2 kb messages are represented by the K4-1.1 and K3-1.1 clones, respectively. This correlates well with the high level of expression on the cell surface. Freshly isolated spleen cells and ConA-stimulated spleen cells express the 2.6 kb message while the thymus expresses the 3.2 kb message at very low levels. Neither message is detected in the bone marrow. Both messages are expressed in LPS-stimulated spleen cells. Two messages have also been detected for both the human ICAM-1 and ICAM-2 (Staunton et al, 1988; Staunton et al, 1989). It is interesting to note that the functional clone of MALA-2, ICAM-1, and ICAM-2 is always the smaller of the two messages. The function of the K4-1.1 clone appears to be in mediating adhesion at the cell surface. The function of the K3-1.1 clone is

not known. No protein was detected intracellularly, at the cell surface, or in the supernates of transfected cells. It was thought that one of the 10 ATGs in the 5' untranslated region might start translation of a protein with homology to a protein with a known function. This might have given some insight into the possible function of K3-1.1. However, no protein with significant homology was found.

Since MALA-2 has been shown to be a single copy gene, it does not appear that the identical coding regions of both clones are encoded by separate portions of the genome. This has lead to the proposal that the two messages are generated by alternate splicing. Evidence in favor of this hypothesis includes the results form the northern blot of NS-1 RNA probed with the K4-1.1 clone and the 5' end of the K3-1.1 clone. There is also an AGG trimer in the area where both clones converge in sequence. AGG is the usual sequence formed at an exon-exon boundary when an intron is removed (Mount, 1982). A 9.8 kb HindIII fragment that hybridizes to both K4-1.1 and the 5' end of K3-1.1 was identified indicating that the two sequences are physically linked on a region of genomic DNA. This is consistent with the alternate splicing hypothesis. Moreover, this is not an unusual process. Alternate splicing in the 5' ends of transcripts has been observed in c-abl (Ben-Neriah et al, 1986). Two distinct 5' exons, which are transcribed form separate promoters, are spliced to a common set of exons. This may be the case for MALA-2, however the significance of alternate splicing in MALA-2 and the mechanism for controlling differential splicing in different lymphoid tissues are not known.

By removing the region that codes for the transmembrane and cytoplasmic portions of the K4-1.1 insert, a soluble form of MALA-2 can be generated. The truncated protein lacks a hydrophobic anchor that normally would maintain it in the plasma membrane and is thus secreted. Truncation does not affect the glycosylation of the protein since it is only 6 kd smaller than the membrane bound protein. This decrease in molecular weight is consistent with the absence of 56 amino acids from the C-terminus. The protein has been shown in preliminary binding studies to be functionally active. This was not unexpected since the

protein consists of almost the entire extracellular region. The binding is specific since it can be inhibited by the YN1/1.7 antibody, or the anti-murine LFA-1 antibody, or both.

The sICAM-1 protein may provide some valuable insight into the binding properties of ICAM-1. The ^{125}I-labelled soluble protein can be used in measuring binding constants for different lymphoid cells; this can be done with and without RGD- and RGE-containing oligopeptides in order to determine the extent of their contribution to LFA-1-binding. It can also be used to alter immune responses by binding LFA-1 and block cellular contacts. This may be very important since sICAM-1 (human) has been shown to block rhinovirus infection in vitro (Marlin et al, 1990) and may soon be used as a remedy for "preventing infection of cells by the common cold virus" (Bangham and McMichael, 1990).

Thus the findings presented in this thesis may prove to be useful in future studies of the regulation of immune responses in vivo, since the antibody together with the functional clone, K4-1.1, can provide reagents for such studies. Most of the work on ICAM-1 has been done with the human system in vitro. The mouse ICAM-1 antibody and cDNA will allow an animal model to be constructed and used in the examination of the in vivo role of ICAM-1 in immune responses. As for the K3-1.1 clone, its function remains unknown.

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