ICAM-1 INTERACTIONS IN IMMUNE RESPONSE

by

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ABSTRACT

Intercellular adhesion molecule-1 (ICAM-1, CD54) and its interactions with LFA-1 and Mac-1 are critical for the proper functioning of our immune system. In addition to these associations, other molecules such as α-actinin of the actin cytoskeleton, and a sialoglycoprotein, CD43, have been suggested to bind human ICAM-1 but the precise nature of these interactions and their physiological significance are not known. This thesis has examined the possible associations of ICAM-1 with these later two molecules, in the murine system, to further elucidate the mechanisms responsible for the upregulation of ICAM-1 adhesion during inflammatory and immune responses.

Punctate distribution of ICAM-1 on the surface of fibroblasts suggested the association of ICAM-1 with cytoskeletal components. However, detergent extraction of cell lines expressing endogenous ICAM-1, as well as those transfected with truncated ICAM-1 lacking the cytoplasmic domain or recombinant ICAM-1 anchored by glycosyl-phosphatidyl inositol, suggested there is very little or no association of ICAM-1 with the cytoskeleton. In all cases, ICAM-1 was completely soluble. To determine if ICAM-1 associated with another molecule which in turn associated with the cytoskeleton, chemical cross-linking studies were performed. In Western blot analysis, a band of approximately 200 KD was observed which suggests ICAM-1 may self associate in a fibroblast and T cell hybridoma cell line. It was concluded that murine ICAM-1 does not associate with the cytoskeleton in the cell types tested and that there must be some other underlying mechanism responsible for the punctate distribution of ICAM-1 on fibroblasts. Whether this affects cell adhesion remains to be determined.

The report demonstrating the binding of human ICAM-1 to a leukocyte sialoglycoprotein, CD43, lead to further investigation of this interaction in the murine system in this thesis. Fibroblasts transfected with murine CD43 were unable to

adhere to soluble immobilized ICAM-1 in an adhesion assay. As well, soluble ICAM-1 coated microspheres were unable to bind to $CD43^+$ fibroblasts. The results of these studies conclude murine CD43 interaction with ICAM-1 can not be detected by these conventional methods and thus bring into question the original reports on CD43/ICAM-1 interaction.

A new model system was established in an attempt to detect a more sensitive interaction that may not withstand an adhesion assay. This system utilized cell death via the cross-linking of an ICAM-1/Fas chimeric molecule as a read out for an association. Although this system was unable to determine whether CD43 and ICAM-1 interact, it did suggest that LFA-1/ICAM-1 interaction may be one of monovalency. This however, requires further investigation.

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List of Abbreviations

Abbreviation	Meaning
Ab	antibody
APC	antigen presenting cell
ATP	adenosine triphosphate
BSA	bovine serum albumin
BS^3	bis(sulfosuccinimidyl) suberate
CAM	cell adhesion molecule
CD	cluster of differentiation
cDNA	complementary deoxyribonucleic acid
CPM	counts per minute
DABCO	1,4-diaminobenzidine tetrahydrochloride
DMEM	Dulbeco's modified minimal essential medium
DMSO	dimethyl sulfoxide
DSS	disuccinimidyl suberate
ECL	enhanced chemiluminescence
EDTA	ethylene diamine tetraacetic acid
FACS	fluorescence activated cell sorter
FAK	focal adhesion kinase
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
GαRIgG	goat anti rat immunoglobulin G
GPI	glycosyl-phosphatidyl inositol

HAT	hypoxanthine aminopterin thymidine
HBSS	Hank's balanced salt solution
HRP	horse radish peroxidase
ICAM	intercellular adhesion molecule
IFN	interferon
Ig	immunoglobulin
IL	interleukin
kD	kilodaltons
kb	kilobases
LAD	leukocyte adhesion deficiency
LFA	lymphocyte function associated antigen
mAb	monoclonal antibody
MHC	major histocompatibility complex
NGF	nerve growth factor
NGFR	nerve growth factor receptor
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
pBST	pBluescript
PCR	polymerase chain reaction
PI	propitium iodide
PI-PLC	phosphatidylinositol-specific phospholipase C
PMA	phorbol myristate acetate
PMSF	phenylmethylsulfonyl fluoride

PVDF	polyvinylidene difluoride
RPM	revolution per minute
SDS	sodium dodecyl sulphate
sICAM-1	soluble ICAM-1
SVEC	small vessel endothelial cells
TBS	tris buffered saline
TCR	T cell receptor
TNF	tumour necrosis factor
Tris	tris(hydroxymethyl)aminomethane
Tween 20	polyoxyethylenesorbitan monolaurate

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INTRODUCTION

1.0 Cell Adhesion Molecules in the Immune System

Cell adhesion molecules (CAM's) were first identified in embryonic development where they maintain cells in specialized locations, creating an organized structure. Later, it was discovered that these CAM's are also required for the proper functioning of cells (Edelman, 1983). For example, to communicate, two cells must come in contact with one another to allow transmission of signals across their membranes. However, cells normally have a negative charge and therefore a natural repulsion occurs between them. CAM's can overcome this natural force to allow contact between cells (Dustin and Springer, 1991).

A large number of cell adhesion molecules have been identified, many of which are critical for immune function and inflammatory response. However, unlike the permanent adhesion required in solid tissue, the cell adhesion molecules involved in the immune system mediate transient adhesion and are therefore precisely regulated. For example, leukocytes are never adhesive under resting conditions. This allows them to circulate through the blood and lymph in surveillance of foreign material. The presence of foreign matter and cytokines and chemoattractants induced from it, then triggers adhesive events which allow the interaction of lymphocytes with other cells to initiate a response. It is clear that the rapid transition between adherent and non adherent states is extremely important for surveillance and response mechanisms. Hence, cell adhesion and its fine tuned regulation are fundamental to the proper functioning of our sophisticated immune system (Springer, 1990).

Most cell/cell, cell/matrix interactions critical for immune function involve multiple combinations of interacting CAM's. Many of these molecules belong to one of three main supergene families, the selectins, integrins and immunoglobulins (Pardi et al., 1992). One example is the multistep process which enables leukocytes

to transmigrate from the circulation into tissue where they perform their function. Briefly, leukocytes in circulation utilize selectin molecules for their initial weak attachment to the vessel wall under normal blood flow. This results in leukocyte rolling. If a signaling event occurs, integrin molecules on the leukocyte surface become activated to a high avidity state and they can now bind their ligands, immunoglobulin family molecules, which are induced on the endothelium. The rolling phenomenon is terminated and the cell comes to a stop by the firm attachment resulting from the integrin/immunoglobulin interaction. Finally the cell alters its shape and migrates between the endothelium, again, with the aid of different CAM's (Mackay et al., 1993). From this example, it is evident, the immune system is very complex and relies on many different cell adhesion molecules for its successful routine function.

2.0 ICAM-1

Intercellular adhesion molecule-1 (ICAM-1, CD54) is one cell adhesion molecule which plays a significant role in immune and inflammatory response. It was originally defined by a monoclonal antibody that inhibited phorbol ester-stimulated homotypic aggregation of lymphocytes (Rothlein et al., 1986). The corresponding murine molecule (MALA-2, at that time) was also identified by a monoclonal antibody which inhibited mixed lymphocyte response (Takei, 1985). This molecule, ICAM-1, is an 80-115 kD heavily glycosylated cell surface protein. Both human (Staunton et al., 1988) and murine (Siu et al.,1989, Horley et al., 1989) ICAM-1 have been cloned and have 50% protein and 65% DNA homology between them (Siu et al., 1989). It is a member of the immunoglobulin supergene family by its five tandomly linked extracellular immunoglobulin-like domains (Staunton et al., 1988).

ICAM-1 is expressed in low levels on both lymphocytic and myeloid lineages as well as epithelial and endothelial cells. However, expression on the cell surface

can be upregulated by inflammatory mediators such as IFNγ, TNFα, and IL-1. Thus ICAM-1 expression is highly elevated in inflammatory tissue which is suggestive of an important role in immune response. Resting lymphocytes lack ICAM-1, but expression is induced over a period of days during T and B cell activation (Dustin et al., 1986). The time course of this induction indicates transcription and translation are required for this event. The regulation of ICAM-1 on non-leukocytes is even more dramatic. Although ICAM-1 is absent from most cells in normal non lymphoid tissues, except for endothelial cells which have low level expression, local immune response can result in a rapid increase in ICAM-1 expression on endothelial cells and induction within hours on epithelial and mesenchymal cells. In vitro, 4 to 48 hours are required to reach maximal levels of ICAM-1 after which expression remains elevated in the presence of these cytokines (Dustin et al., 1986).

The implication of ICAM-1 function in immune response from its inducible expression has been confirmed by studies both in vitro and in vivo. Its role in antigen presentation was established by a B cell line with defective expression of ICAM-1, which was unable to present antigen. This function was restored by the introduction of functional ICAM-1 into this cell line (Dang et al., 1990). ICAM-1 deficient mice have also been generated. The mutant cells from these mice lacking ICAM-1 were unable to function as stimulator cells in a mixed lymphocyte response. Transfection of ICAM-1 into these mutant cells was sufficient to correct the defect in antigen presentation (Sligh et al., 1993). Therefore, ICAM-1 is required on antigen presenting cells to elicit a full T cell activation response. ICAM-1 deficient mice also exhibit prominent abnormalities in inflammatory responses. Neutrophil emigration in response to chemical peritonitis is impaired and contact hypersensitivity to 2,4-dinitrofluorobenzene is suppressed by 74% in these mice (Sligh et al., 1993). In another study, resistance to septic shock was observed in ICAM-1 deficient mice. This indicated ICAM-1 is critically involved in leukocyte/endothelial interactions

which are essential to lethal shock induced by high dose of gram negative endotoxin and also in the massive T cell activation that occurs in sepsis by gram-positive exotoxins (Xu et al., 1994).

From the examples above, ICAM-1 appears to be a multi-function molecule which plays a vital role in immune response. Its versatility is also reflected in its ability to interact with various different molecules. The association of ICAM-1 with two different β_2 integrins, LFA-1 (Rothlein et al., 1986, Staunton et al., 1988) and MAC-1 (Smith et al., 1989, Diamond et al., 1991), is well established. More recently, reports have also suggested ICAM-1 can bind CD43 (Rosenstein et al., 1991), a cytoskeletal protein, α-actinin (Carpen et al., 1992), fibrinogen (Languino et al., 1993), and hyaluronan (McCourt et al., 1994). In addition, ICAM-1 is the receptor for human rhinoviruses (Staunton et al., 1989, Greve et al., 1989), and Plasmodium falciparum infected erythrocytes (Berendt et al., 1989).

2.1.0 Leukocyte Function Associated Antigen

Leukocyte function associated antigen-1, LFA-1, was one of the first cell adhesion molecules discovered to play a major role in immune function. It was identified by a monoclonal antibody capable of inhibiting T cell killing (Davignon et al., 1981). Later, it was found to account for the antigen independent adhesion that is induced by prolonged antigenic stimulation of T cells in vitro (Springer, 1990). LFA-1 is a heterodimeric cell surface glycoprotein belonging to the integrin family. It consists of a unique α chain (180 KD) non-covalently associated with a β chain (95 KD) which it shares with two other leukocyte integrins, MAC-1 and p150,95 (Kurzinger and Springer, 1982, Sanchez-Madrid et al., 1983). LFA-1 is constitutively expressed on cells of hematopoietic lineage however it can be altered qualitatively to affect its adhesive function. This was first recognized by the ability of phorbol esters to rapidly increase lymphocyte aggregation mediated by LFA-1 without altering the cell surface density of this molecule (Rothlein et al., 1986).

Later, a conformational change in the molecule which was identified by specific monoclonal antibodies, was found to be responsible for this phenomenon (Keizer et al., 1988). Thus, LFA-1, like many integrin molecules, has various activation states which affect its ability to bind ligand. From initial studies, it was hypothesized that inside-out signaling is responsible for the regulation of this high avidity state (Dustin and Springer, 1991). This was later verified by a study which deleted the cytoplasmic domain of the β subunit of LFA-1. This truncation eliminated LFA-1 binding to ICAM-1 and also its sensitivity to phorbol esters. Therefore, the signals for activation appear to be initiated from within the cell (Hibbs et al., 1991). Regulation of integrin avidity is also cell-type dependent. For example, LFA-1 on lymphocytes is controlled by activation of the cells, whereas LFA-1 on COS cells is constitutively active (Larson et al., 1990). The precise mechanisms for regulation of integrin avidity remain unknown.

Drugs that inhibit metabolic ATP production or that disrupt the cytoskeleton abrogate the ability of LFA-1 positive cells to bind purified ICAM-1. Therefore, LFA-1 interaction with its ligand also requires a functional cytoskeleton and metabolic energy. In addition, divalent cations and temperature are critical factors for this interaction (Marlin and Springer, 1987).

2.1.1 LFA-1 interaction with ICAM-1

The interaction of ICAM-1 with LFA-1 is well established. This was the first example of an immunoglobulin supergene family molecule binding an integrin (Staunton et al., 1988). In fact, this interaction was unexpected since ICAM-1 is unlike other integrin ligands and does not contain the putative integrin recognition sequence, RGD. The LFA-1 binding site is located in domain 1 of the extracellular portion of ICAM-1 (Staunton et al., 1988).

These two molecules represent a central component of cell/cell contact mediated immune response. For example, in vitro, critical events such as T cell

mediated B cell activation and antibody production, and T cell mediated cytotoxicity are blocked by antibodies to these molecules (Boyd et al., 1988). In vivo, ICAM-1 has been reported to play a critical role in graft rejection. Anti-ICAM-1 antibodies can inhibit T cell mediated injury in a non-human primate with renal allografts (Cosimi et al., 1990). Also, combined administration of anti-LFA-1 and ICAM-1 antibodies can prevent cardiac allograft rejection in a murine model (Isobe et al., 1992).

In addition, these molecules can mediate adhesion between T cells and endothelium. Adhesion of activated T lymphocytes to cultured endothelial cells is mostly inhibited by monoclonal antibody to LFA-1 (Haskard et al., 1986). ICAM-1 antibodies only partially block this interaction suggesting there is another ligand for LFA-1, but this will be discussed later. Also, transendothelial migration of lymphocytes and granulocytes in vitro is inhibited by monoclonal antibodies to LFA-1 (Smith et al., 1989). Therefore it is clear this interaction is involved in the transmigration process from circulation into tissue.

As eluded to above, in addition to a role in adhesion between cells, ICAM-1/LFA-1 interaction can synergize with other molecules to provide costimulatory signals for T cell activation. In vitro, ICAM-1, when coimmobilized with anti-CD3, which alone is unable to stimulate cells, can induce T cell proliferation (Van Seventer et al., 1990). In another study, when ICAM-1 is present on the surface of MHC class II+ antigen presenting cells, a lower concentration of specific antigen can be used to stimulate a T cell response (Kuhlman et al., 1991). ICAM-1 on these cells is also able to enhance the antigen-specific T cell response (Siu et al., 1989). Therefore, it is well established both in vitro and in vivo, that ICAM-1 and its association with LFA-1 is a significant player in immune function.

2.2.0 MAC-1 and interaction with ICAM-1

In transendothelial migration induced by activation of endothelial cells by cytokines, LFA-1/ICAM-1 interaction is important for adherence of unstimulated neutrophils to endothelial cells. However, stimulated neutrophils utilize a different receptor for ICAM-1. MAC-1 (CD11b, CD18) another leukocyte integrin, binds domain 3 of ICAM-1 extracellular region (Diamond et al., 1991, Smith et al., 1989). The importance of this interaction is exemplified in Leukocyte Adhesion Deficiency (LAD) patients where CD18 (β_2) expression is defective. These individuals have defects in neutrophil adhesion and extravasation and therefore are unable to mount an effective immune response against infectious microorganisms. This leads to severe recurrent infections (Dustin and Springer, 1991). The ICAM-1 deficient mice previously mentioned also display leukocytosis due to the decreased emigration of neutrophils out of circulation (Xu et al., 1994). Monoclonal antibodies against ICAM-1, CD11b, CD18 but not CD11a can block neutrophil migration in an in vivo rabbit lung inflammation model (Barton et al., 1989). Thus the interaction of ICAM-1 with this other β_2 integrin, Mac-1, is also critical for immune function.

2.3.0 Other ligands for ICAM-1

The major serogroup of human Rhinoviruses, causative agents in 50% of common colds, utilize ICAM-1 as their cellular receptor (Staunton et al., 1989). Although it binds domain 1 and 2 of ICAM-1 extracellular region, it is distinct from the site of LFA-1 binding (Staunton et al., 1990).

Erythrocytes infected with Plasmodium falciparum, the causative agent of malaria, also bind to a specific region of domain 1 and perhaps domain 2 of ICAM-1 (Berendt et al., 1992). These infected erythrocytes do not circulate in peripheral blood as normal, but instead adhere to the endothelium post capillary venules via ICAM-1. This allows for parasite development and eventually the erythrocyte ruptures releasing the next generation of blood stage parasites. A soluble ICAM-1

immunoglobulin G1 immunoadhesin can inhibit these infected erythrocytes from binding ICAM-1 bearing surfaces and also promote the phagocytocis and destruction of these infected cells (Staunton et al., 1992). Therefore, in addition to the positive role it plays in initiating an immune response through its interaction with the two β_2 integrin family members, ICAM-1 also participates in the infection process itself by binding directly to the infecting organism.

ICAM-1 has been reported to bind the extracellular matrix protein, fibrinogen. This interaction is thought to provide a bridging mechanism between leukocytes and endothelium either through two ICAM-1 molecules on opposing cells or through Mac-1 and ICAM-1 (Languino et al., 1993). More recently, ICAM-1 is suggested to be the receptor for a polysaccharide, hyaluronan, found in connective tissue. This interaction may have important implications in many pathological conditions, although further investigation is required (McCourt et al., 1994).

2.4.0 CD43

CD43 (also known as leukosialin, sialophorin, or gp 115) is a heavily sialylated glycoprotein composed of approximately 60 % carbohydrate (Remold-O'Donnell and Rosen, 1990). This molecule was first identified in the rat where it was referred to as W3/13 (Brown et al., 1981). It is expressed on the surface of T lymphocytes, monocytes, neutrophils, platelets, and some B cells (Remold-O'Donnell et al., 1987). The extracellular region resembles acidic mucin molecules with expanded structure and dense negative charge. It is subject to alternative glycosylation pathways that are cell type specific. For example, human leukosialin has been characterized into two forms: a 115 kD thymocyte/lymphocyte/monocyte form and a 135 kD neutrophil/platelet form (Remold-O'Donnell and Rosen, 1990). Unlike other cell surface molecules which have distinct structural domains, CD43 lacks introns in the coding sequence (Shelley et al., 1990, Cyster et al., 1990). The murine leukosialin (Ly48) has also been cloned (Baecher et al., 1990). The

cytoplasmic domain of rat W3/13, murine and human CD43 is the most conserved region suggesting this portion has an important functional role (Cyster et al., 1990). It is also interesting to note that both human and murine CD43 map to the same region of chromosome as the alpha chain of LFA-1. This linkage has therefore been maintained in evolution, although the significance of this is not known (Baecher et al., 1990).

Defective expression, that is, decreased quantity and abnormal molecular weight of CD43 on circulating T lymphocytes, is associated with the X-linked immunodeficiency disorder, Wiscott Aldrich Syndrome. This disorder has a clinical course which involves progressive T cell depletion, susceptibility to opportunistic and pyogenic infections, inability to produce antibodies against polysaccharide antigens, thrombocytopenia, and severe eczema (Ardman et al., 1990). Although CD43, an autosomal gene, is not the primary defect, it does however suggest that CD43 may play an important role in immune function (Arnaiz-Villena et al., 1992). In fact, in subsequent studies involving the transfection of CD43 into a murine T cell hybridoma, CD43 enhanced the antigen-specific activation of T cells (Park et al., 1991). As well, antibodies to CD43 have a modest costimulatory effect on other cells such as monocytes (Nong et al., 1989) and can provide regulatory signals for homo-heterotypic T cell adhesion. There is also evidence that autocrine production of IL-2 may be transduced through CD43 (Axelsson et al., 1988). A significant number of individuals with HIV infection have circulating anti-CD43 auto-antibodies and these may contribute to the immunodeficiency of AIDS patients (Ardman et al., 1990). A glycosylation defect resulting in hyposialylation of CD43 on HIV infected leukemic T cells is associated with impaired CD43 mediated homotypic aggregation. This may also explain the large amount of auto-antibodies in these patients as non-sialylated CD43 may not be recognized as self (Lefebvre et al., 1994). Therefore,

much interest has been generated in this molecule and its role in lymphoid cell function.

2.4.1 CD43 interaction with ICAM-1

In further studies, a monoclonal antibody, which blocked binding of Daudi cells to plate bound CD43, identified a possible ligand, ICAM-1 (Rosenstein et al., 1991). Further investigation of this interaction assessed the formation of conjugates between beads which had CD43 incorporated on the surface and those with ICAM-1 on the surface. Results suggest that this is a receptor/ ligand pair and therefore ICAM-1 may participate in immune function through its association with this molecule. However, no further studies have established whether this interaction is cis (on the same cell surface) or trans (on two different cells) between membrane bound molecules. It is not known in which orientation this apparent binding occurs, if at all. There is also evidence to suggest that ICAM-1 may co-cap with CD43 in the plane of the membrane (G.J. Dougherty, unpublished) however this remains to be investigated further.

2.4.2 CD43 Inhibitory Role in Adhesion

CD43 has a negative effect on cell/cell interactions as well. The LFA-1/ICAM-1 interaction which is necessary for T lymphocyte adhesion to HeLa cells was disrupted when these HeLa cells were transfected with CD43 (Ardman et al., 1992). This may be due, in part, to the large degree of CD43 sialylation which varies with cell type and degree of differentiation and activation, imparting a highly negative charge. This repellent nature of CD43 positive cells compared to untransfected cells, is also supported by the proposed extended structure of the CD43 molecule making adhesion sterically unfavourable (Ardman et al., 1992). To test CD43 function in its native environment on leukocytes, a CD43 negative human T cell line was generated by targeted disruption of the CD43 gene. In this model, T lymphocyte homotypic adhesion and binding of these cells to two distinct ligands

were enhanced with these CD43 negative cells (Manjunath et al., 1993). Thus, in these studies, CD43 opposes cell adhesion.

It is also interesting to note that neutrophils aggregate upon CD43 crosslinking in both a CD18 dependent and independent manner, whereas lymphocyte and monocyte aggregation is dependent only on CD18 (Kiujpers et al., 1992). Thus, the role of CD43 in adhesion remains poorly understood.

3.0 ICAM-2,-3

Since adhesion of leukocytes to some cell types such as resting endothelium was only partially blocked by antibody to ICAM-1 while it was completely blocked by antibody to LFA-1, an additional ligand for LFA-1 was indicated (Rothlein et al., 1986). This second ligand, ICAM-2 was cloned by transfection of COS cells with an endothelial cDNA library and selection of clones which bound to LFA-1 coated plates in the presence of blocking ICAM-1 monoclonal antibody (Staunton et al., 1989). Murine ICAM-2 was also isolated from a lymphoma library utilizing human ICAM-2 cDNA as a probe. Human and murine ICAM-2 have 60% amino acid identity (Xu et al., 1992).

ICAM-2 was identified as another member of the ICAM family by its two Ig-like domains which have 35% amino acid homology with the two N-terminal domains of ICAM-1 (Staunton et al., 1989). ICAM-2 differs from ICAM-1 in both distribution and inducibility which implies functional differences. ICAM-2 is largely restricted to endothelium and certain interstitial cells. Although its expression on resting lymphocytes is several fold higher than ICAM-1, ICAM-2 is not inducible (de Fougerolles et al., 1991). ICAM-2 transfected COS cells are more readily detached than ICAM-1 transfected cells from LFA-1 coated plastic . ICAM-1 therefore has a greater affinity for LFA-1 (Staunton et al., 1989).

Because there was still an LFA-1-dependent, ICAM-1-independent phenomenon which was not accounted for by ICAM-2, a third ligand for LFA-1 was

suggested (de Fougerolles et al., 1991). An antibody that completely inhibited adhesion of a variety of cell lines to purified LFA-1 in the presence of anti-ICAM-1 and -2 antibodies, identified the third ligand, ICAM-3 (de Fougerolles et al., 1992). It has 5 Ig-like domains which are highly homologous to those of human ICAM-1 (32% identity) and human ICAM-2 (37% identity) (de Fougerolles et al., 1993). The LFA-1 binding site on ICAM-3 is localized to domains I and II (Fawcett et al., 1992). In contrast to ICAM-1 and -2, ICAM-3 is highly expressed on all leukocytes but absent from endothelial cells. In conventional adhesion assays, it is the most functionally important ICAM on resting lymphocytes (de Fougerolles et al., 1992). In fact, ICAM-3 can regulate LFA-1/ICAM-1 dependent cell/cell interaction of T lymphoblasts (Arroyo et al., 1994).

Although distribution patterns and expression levels of these molecules are quite different from ICAM-1, it has been difficult to delineate the exact roles of ICAM-2 and -3 from those of ICAM-1. However, ICAM-1 remains the major ligand for LFA-1 on activated T lymphocytes. A report also suggests that different conformations of LFA-1 are required to support adhesion to ICAM-1,-2, and -3 and that these ligands may all bind on different sites of the LFA-1 molecule (Binnerts et al., 1994).

4.0 Fas Molecule

Fas is a 35kD cell surface, single spanning transmembrane protein which is expressed in both lymphoid and non-lymphoid tissues, although activated lymphocytes have a higher expression level than resting cells. It belongs to a family of proteins that includes TNF receptor, NGF receptor, B cell CD40, and T cell OX40 (Itoh et al., 1991). The extracellular sequence contains cysteine residues that are well conserved with other members of the family, especially NGFR. This region also contains two potential N-linked glycosylation sites. The cytoplasmic domain is abundant in charged amino acids and has 51% homology to TNF receptor cytoplasmic region. This region of Fas is required for the transmission of the

transmembrane signal for apoptosis. It is also the location of the lpr mutation in mice, which invokes a lymphoproliferative disorder similar to systemic lupus-erythematosus in humans (Watanabe-Fukunaga et al., 1992). The binding of anti-Fas antibody to Fas positive cells induces apoptosis, a process of programmed cell death (Itoh et al., 1991). The cytolytic activity of Fas is indistinguishable from that of TNF, including its enhancement by treatment with IFNγ. As well, Fas antigen and TNF receptor co-down regulate when incubated with anti-Fas antibodies or TNF. Therefore, the cytolytic activity of TNF may be mediated by Fas antigen associated with TNF-R (Yonehara et al., 1989). Fas is expressed at high levels in the thymus, suggesting that it may also be involved in negative selection. This may explain the presence of auto reactive T cells in lpr mice (Watanabe-Fukunaga et al., 1992).

It has been suggested that the gld defect in mice (inducing a disease similar to that of lpr mutation) may represent a lack of functional ligand for Fas (Cohen and Eisenberg, 1992). More recently, using a soluble Fas-IgG Fc fusion protein, the Fas ligand was identified on the cell surface of a cytotoxic T cell hybridoma. The ligand is a type II transmembrane protein which belongs to the TNF family. It is expressed on activated splenocytes and thymocytes which suggests a role in T cell mediated cytotoxicity (Suda et al., 1993). It has also been confirmed that this Fas-ligand gene is mutated in gld mice (Lynch et al., 1994).

5.0 Regulation of Cell Adhesion

Cell adhesion can be regulated through various mechanisms. The level of expression of a molecule on the cell surface can have a great effect on the adhesive properties of a cell. Many molecules are upregulated on the cell surface by the presence of various cytokines. Such is the case for ICAM-1 which is normally found in very low levels on the cell surface. Upon activation of the cell and release of inflammatory cytokines such as IL-1 and TNFα, its expression is highly induced and it can now mediate many more adhesive interactions (Dustin et al., 1986).

Another means of regulating cell adhesion is through a qualitative change in a molecule which can affect the affinity of that molecule for another. For example, LFA-1 is normally in a low avidity state on the surface of resting T cells and therefore binds very weakly to ICAM-1. However, upon stimulation of these cells in vitro with phorbol ester, a conformational change is induced such that LFA-1 can now participate in firm adhesion with ICAM-1 (Dustin and Springer, 1989).

Cell adhesion can also be regulated on a long time scale by changes in cell surface charge as occurs with the CD2/LFA-3 interaction. Negative surface charge, mostly on sialic acid residues of glycans, combined with the bulk of surface glycoproteins and proteoglycans, make cell interaction energetically unfavourable. By decreasing the surface charge glycocalyx density on T lymphocytes, activation of the cell allows the CD2/LFA-3 mechanism to mediate adhesion (Dustin and Springer, 1991).

5.1 Distribution

Mobility of integral proteins within their fluid membrane plays a central role in the mechanism of intercellular adhesion and communication as well. Thus the resulting distribution of a molecule on the cell surface can also affect cell adhesion. It is hypothesized that a greater number of interacting molecules at the contact area will create a stronger more stable adhesion since the multivalency will establish a high avidity interaction (Singer, 1992). For example, monovalent soluble ICAM-1 is unable to bind LFA-1. However, when sICAM-1 is immobilized on microspheres it is able to bind and competitively inhibit binding of LFA-1 to immobilized purified ICAM-1 (Welder et al., 1993). This is also illustrated in a glass-supported planar membrane bilayer system. In this system, mobile GPI anchored form of LFA-3 is 8 fold more potent than the immobile transmembrane form of LFA-3 for adhesion of Jurkat T lymphoma line under gentle wash conditions. The difference is even more dramatic under high shear conditions (Dustin and Springer, 1991). This model

demonstrates again, the importance of lateral mobility and hence distribution, in the regulation of cell adhesion.

However, the cell membrane is a fluid component whereby molecules diffuse freely and randomly on non-polarized cells. Thus for a distinct distribution pattern to occur, there must be some mechanism which can regulate this. Several mechanisms have been proposed which could explain a more localized, non random distribution pattern.

5.1.1 Capping

Mutual capping, co-capping, and syn-capping are all thought to create a localized distribution of molecules on the cell surface. The mutual capping theory states the binding of one molecule to its receptor on another cell will result in a massive redistribution of molecules within the two contacting membranes. That is, once a small number of receptor/ligand pairs bind one another, it allows cell/cell contact long enough for the diffusion of many more receptor/ligand pairs, since this decreases the energy of formation for later bonds. This is very similar to what occurs with antibody binding and hence the name, capping. The result is an increase of receptor/ligand pair at the site of cell/cell contact. It is suggested, if this clustering does not occur, the cells will come apart again. Co-capping is similar but involves two molecules which are ordinarily independent of one another. The initial binding of the first receptor/ligand pair also decreases the free energy of formation for the second type of receptor/ligand pair (which otherwise would not form) (Singer, 1992). In syn-capping, the first molecule develops an affinity for another molecule during cellular activation, which ordinarily is independent. Thus when the first molecule is induced to cluster the second molecule co-clusters with it. For example, the TCR and CD4 are normally independent of one another. Upon TCR binding to ligand, a conformational change is induced such that it associates with the CD4 molecule. Thus when the TCR clusters, CD4 also co-clusters with it at the cell contact region.

It is proposed that these capping events always occur during the direct molecular interaction of one cell surface with another, unless molecular mobility in the membrane is inhibited. This capping phenomenon may be important not only for adhesion but also for signaling since many cytokines activate cells by mechanisms that involve dimerization or higher aggregation of the receptors (Singer, 1992).

5.1.2 Association with the Cytoskeleton

Cytoskeletal association may control the distribution of cell surface molecules in the membrane. Previously, cell adhesion molecules have been reported to interact with cytoskeletal proteins. It is this linkage which is often crucial to maintain structural integrity, and membrane stability. For example, adherens junctions and focal adhesions are two well defined junctions between cells and cell and substratum, respectively. They are created by the clustering of cadherins, in the case of adherens junctions, and integrins, in focal adhesions, which are associated with the underlying cytoskeleton in these areas. These interactions utilize the cell adhesion protein to link the extracellular matrix or other cell surface molecule to the structural framework of the cell, the cytoskeleton. In fact, it is this property from which the name 'integrin' was derived (Dustin and Springer, 1991). Most studies of integrin/cytoskeletal interactions have been based on a fibronectin receptor, $\alpha_5\beta_1$ integrin. It can directly bind to cytoskeletal proteins such as talin and α-actinin through the β1 cytoplasmic domain. Binding of fibronectin to this receptor can induce redistribution of this molecule, from a diffuse pattern to clusters at the focal adhesions (Luna and Hitt, 1992). In addition, the cytoskeletal protein, talin, can redistribute to the contact area as well in the specific interaction of T helper cell with B-APC (Singer, 1992). Thus focal adhesion structure is controlled from both sides of the plasma membrane. Cytoskeletal association can also influence the distribution of MAC-1 on the cell surface. This results again in greater local concentration of this molecule at the site of cell contact (Detmers et al., 1987). The

result of this redistribution is a multivalency or greater avidity of the molecule on the cell surface which is critical for adhesive function.

5.2 Cytoskeletal Association and Other Roles

Cytoskeletal linkage is also critical for cell motility. CAM's are thought to provide the 'foot hold' for cellular interactions during migration (Dustin and Springer, 1991, Dustin et al., 1992). Movement of most cells involves cell surface protrusions at the leading edge. The mechanism of these protrusions is not well understood but membrane skeleton interactions and actin accessory molecules are involved. Proteins such as talin and ponticulin, which nucleate actin filament assembly, often localize to this area. Actin filament crosslinking may also stabilize the protrusions and proteins in the fluid membrane, which are both required for motility (Luna and Hitt, 1992).

Focal adhesion kinase, (FAK), is a protein tyrosine kinase which colocalizes with components of cellular focal adhesions such as tensin and talin. The formation of focal adhesions is important for activation of FAK. Also changes in protein phosphorylation, possibly mediated via this kinase, in turn, regulate structure and function of focal adhesions in vivo. The precise function of FAK in interactions between integrins and cytoskeleton is not clear at present. However, the cytoskeleton and this associated kinase may be critical for transduction of signals necessary for regulation of cellular interactions and ultimately cell shape and motility (Zachary et al., 1992).

5.3 ICAM-1 and Association with the Cytoskeleton

Although the integrins are clearly involved in interactions with components of the cytoskeleton, relatively little is known about the interactions of the immunoglobulin family members with the cytoskeleton. Recently published results suggest that ICAM-1 associates directly with α-actinin of the actin containing cytoskeleton (Carpen et al., 1992). In this study by Carpen et al., ICAM-1, both full-

length and a GPI-linked form, were expressed in COS cells. ICAM-1 displayed very localized distribution whereas when GPI-anchored, a more uniform distribution of ICAM-1 was observed. When the microfilament cytoskeleton was disrupted by the drug, cytochalasin B, the distribution of full-length ICAM-1 was altered to a more uniform pattern similar to the GPI-linked form which remained unchanged. From this, they concluded the cytoplasmic domain of ICAM-1 is associated with the cytoskeleton and that this interaction is responsible for the distinct distribution pattern of ICAM-1 observed on the cell surface (Carpen et al., 1992). Utilizing an affinity matrix column generated from synthetic 28-residue peptides representing the entire predicted cytoplasmic domain of ICAM-1, they identified α-actinin as an ICAM-1 binding protein. This protein bound purified ICAM-1 and colocalized with it in these cells. The area close to the membrane spanning region of ICAM-1 was identified as the binding site. However, the interaction was dependent on the positively charged residues and not the order of the residues themselves (Carpen et al., 1992).

There is conflicting evidence from previous studies in this laboratory concerning the role of the cytoplasmic domain in control of the distribution of ICAM-1 on the cell surface. Although a distinct punctate distribution pattern was observed on the surface of A20 cells, when both a full length and cytoplasmic truncation of ICAM-1 were transfected into either T28 or L cells, both forms had a similar distribution pattern. This suggested that the cytoplasmic domain is not involved in distribution of this molecule (Welder, M.Sc. thesis). Therefore, due to these conflicting results, it is not fully clear if or how ICAM-1 may associate with the cytoskeleton.

Thesis Objective and Hypothesis

The long term goal of this research is to define the interactions of ICAM-1 with other molecules to further elucidate the complete role of ICAM-1 in immune

response. Previous research has established the interactions of ICAM-1 with the integrins, LFA-1 and MAC-1, and the important role these play in adhesion and signaling in a variety of cell/cell interactions of the immune system. However, the two other ICAM's identified, -2 and -3, which also bind LFA-1 have complicated our understanding of ICAM-1's role in immune response. The association of human ICAM-1 with other molecules such as CD43, and the cytoskeleton, have also been eluded to but not clearly defined. Nor have these later interactions been studied in the murine system.

Since ICAM-1 mediates a relatively weak interaction, as demonstrated in this laboratory previously by comparing monovalent soluble ICAM-1 with multivalent soluble ICAM-1 coated microspheres in adhesion to LFA-1, the multivalency of ICAM-1 is clearly important for its function. For this reason, the distribution and stability of ICAM-1 on the cell surface may have a significant influence on cellular adhesion mediated via ICAM-1. Cytoskeletal association can affect distribution of other CAM's such as MAC-1 and β1 integrins. Therefore, the investigation of ICAM-1 association with the cytoskeleton may lead to an understanding of how distribution of this molecule is regulated and further, how adhesive events including those required during extravasation and migration of leukocytes into tissue are controlled. From the literature and previous studies completed in this laboratory the following hypothesis was generated. ICAM-1 can associate with the cytoskeleton but does not require the cytoplasmic domain for this interaction. Instead, this may be mediated through the transmembrane domain or extracellular domain via association with another protein. The cytoskeleton may be involved and necessary for ICAM-1 function, not only to increase avidity of interaction with LFA-1 but also to provide an anchoring mechanism for ICAM-1 which LFA-1 and MAC-1 can utilize in transendothelial migration.

In the previous investigation of CD43/ICAM-1 interaction, these molecules were not simultaneously membrane bound and thus the results of these studies did not establish a physiological interaction between these two molecules mediating cell/cell adhesion. Therefore, the reported interaction between CD43 and ICAM-1 may represent either an interaction of these molecules on opposing cells (trans interaction) or those on the same plane of membrane (cis interaction). Nonetheless, this interaction is important to study since CD43 plays a significant role in immune response as demonstrated by the clinical immunodeficiency syndrome associated with its defective expression. Therefore, this research is proposed to define a biological association between these molecules utilizing murine ICAM-1 and CD43. We hypothesize that CD43 can associate with ICAM-1 in a cis interaction, although this association may be weaker than with LFA-1. Therefore, an alternative method to the conventional adhesion assay may be required for detecting this interaction. Clarification of this interaction may impart a novel role for ICAM-1 in signal transduction since CD43 has been shown to provide such signaling properties. This, in turn, could lead to a novel means of immune modulation.

MATERIALS AND METHODS

1.0 Cell Culture

The murine fibroblastoid cell line L, the B cell lymphoma A20, the T cell hybridoma T28, the myeloma NS-1, the monocytic cell line P388, the endothelial cell line SVEC, were all maintained in DMEM 5% FBS (Hyclone Laboratories, Inc., Logan, Utah) in a 37 °C, 5% CO_2 incubator.

2.0 DNA Constructs and Transfected Cell Lines

2.1 ICAM-1

Two constructs of ICAM-1 had been previously generated in our laboratory, a full length and a cytoplasmic truncation. The full length, EH, is a cDNA encoding the complete, functional murine ICAM-1. PCR amplification of a portion of the EH clone was used to generate the cytoplasmic truncation. An oligonucleotide (TTCAGCTCCGGTCCTGACCC) complementary to the sequence upstream of the HindIII site in the extracellular domain 4, and an oligonucleotide (TCTAGATCTGGCGGTTATAAACAT) which incorporated a BglII site, were used as 5' and 3' primers, respectively. The resulting product which was digested with HindI II and BglII, encoded part of the extracellular domain 4, extracellular domain 5, and the first three amino acids of the cytoplasmic domain. A fragment encoding the first 3 extracellular domains and part of domain 4, obtained by HindIII digestion of the cDNA was ligated to the PCR fragment and the result was a cDNA encoding ICAM-1 without the majority of the cytoplasmic domain. This was sequenced and further subcloned into the expression vector, pBCMGS (Karasuyama et al., 1989) for transfection into fibroblast cell line, L, and T cell hybridoma cell line, T28.

2.2 GPI-linked ICAM-1

To generate a GPI-linked form of ICAM-1, the extracellular domain was ligated with the GPI anchor sequence from the Heat Stable Antigen, M1/69. The GPI anchor was generated by PCR (using a BioOven II, BioTherm Corp., Fairfax, Virginia) from a full length M1/69 cDNA clone obtained from Dr. Rob Kay (Terry Fox Laboratory). Oligonucleotides (CAGGATCCGGGGTGGCAGCTCCCTGCAG) and (TTCTAGAGAGATATGGTAACCACCAATT) with BamHI and XbaI sites incorporated, respectively, were used as 5' and 3' primers to generate a 200 base pair product containing the GPI linkage sequence. The 50 µl reaction mixture contained 150 ng of cDNA template, 0.2 mM dNTP, and 2.5 U Pfu (Stratagene Cloning Systems, La Jolla, California). The PCR conditions were 93 °C for 30 seconds, 50 °C for 30 seconds, and 72 °C for 1.5 minutes, for 20 cycles. A portion of the ICAM-1 extracellular region was generated by PCR using the primers (GGAATTCCGAAGGCCTTTTTCCTGCCTC) and (TTGGATCCGGTACAGTACTGTCAGGTAC) and EH3K, a full length ICAM-1 cDNA clone in pBluescript, as a template. The PCR conditions were similar to above. This product was then digested with PstI (internal site) and BamHI (incorporated into 3' primer) and inserted into EH3K clone in which PstI/BamHI fragment (containing the 3' end of the extracellular domain, transmembrane and the cytoplasmic domain) had been cut and removed. The result was the full extracellular domain of ICAM-1 in pBluescript. This clone was then digested with BamHI and XbaI to allow ligation of the GPI anchor PCR product. Finally, the construct was subcloned into XhoI/NotI restriction sites of the expression vector pBCMGS. $4X10^6$ T28 cells were electroporated with 10µg DNA using a BioRad Gene Pulser at a voltage of 0.4 kV, and capacitance of 125 µFD. Cells were selected in 0.5 mg/ml G418 (Life Technologies Inc., Grand Island, N.Y.) and confirmed by FACS analysis for ICAM-1 expression. GPI linkage was confirmed by

treatment of cells with 10 U/ml PI-PLC (purified from Bacillus thuringiensis by Dr. Rob Kay) in DMEM (no serum) for one hour at 37 °C, followed by staining with YN1/1 antibody conjugated to FITC, and observation by FACS analysis.

2.3 CD43

Because the CD43 gene has no introns, it was cloned from a BALB/c mouse embryo genomic library (CLONTECH Laboratories, Inc., Palo Alto, California). Four CD43 positive plaques had been previously identified through primary screening. These plaques were further purified by secondary and tertiary screening, using a partial CD43 PCR fragment previously generated from a cDNA clone prepared from the murine T cell line EL4, missing the 5' end, as a probe. Phage DNA was isolated using λ Quick Kit (Bio/Can Scientific Inc., Mississauga, Ont.) and digested with BamHI which generated a 2.3 Kb fragment containing the full CD43 sequence. This fragment was then cloned into the plasmid, pBluescript. Since there were no convenient restriction sites available flanking the CD43 coding sequence and due to the possible intron hypothesized from human CD43 at -35 to -432, the murine CD43 gene was obtained by PCR using the above as a template. A 5' primer was generated (GGTCCGAATTCCTGCTCCTGCCT) which incorporated an EcoRI site and the 3' primer (TAGAACTAGTGGATCCGGGG) was immediately downstream of the BamHI site belonging to pBST. The PCR conditions of 30 seconds at 94 °C, 30 seconds at 55 °C and 1 minute at 72 °C, for 25 cycles generated a product containing the full CD43 coding sequence with the putative ribosomal binding site. This was cloned into EcoRI and BamHI sites of pBST and subsequently, the XhoI /NotI restriction sites of the vector, pBCMGS for expression in a murine fibroblast cell line, L. After $CaPO_4$ mediated transfection (Ausubel et al., 1989), clones were selected by G418 resistance. Clones were plucked and tested for CD43 expression by FACS analysis utilizing anti-mouse leukosialin antibody conjugated to FITC (PharMingen, San Diego, CA).

2.4 ICAM-1/Fas Chimeric Molecule

To generate an ICAM-1/Fas chimeric molecule, a full length Fas PCR clone previously generated in our lab by methods similar to Watanabe-Fukunaga 1992, was used as a template to PCR amplify the cytoplasmic and transmembrane region of the Fas molecule. Restriction sites, SmaI and BamHI were incorporated in the 5' (AGTCCCGGGAAATCGCCTAT) and 3' (AAGGGATCCAAAGCTGGGTA) oligonucleotide primers, respectively. The PCR conditions were 94°C for 15 seconds, 50°C for 30 seconds and 72°C for 15 seconds, for 20 cycles. The PCR product was digested with SmaI and BamHI and subcloned into pBluescript to generate the Fas pBST clone. The extracellular domain of ICAM-1 was obtained by restriction digest with EcoRI and ScaI of a murine ICAM-1 cDNA clone, EH3K, previously established in our laboratory. This fragment was then cloned into the EcoRI, SmaI sites of the Fas pBST clone. Finally, this clone was sequenced by the Sanger method (Ausubel 1989) at the ICAM-1-extracellular/Fas-transmembrane junction site to confirm the construct sequence was in frame. It was then further subcloned into XhoI/NotI restriction sites of the eukaryotic episomal expression vector, pBCMGS. A murine fibroblast L cell line stably expressing this molecule was generated by $CaPO_4$ mediated transfection, selection of clones by G418 resistance and FACS analysis. These cells were further transfected with thymidine kinase, generously donated by Dr. Rob Kay (Terry Fox Laboratory), and selected in HAT medium (DMEM supplemented with 100 μM hypoxanthine, 0.4 μM aminopterin, 16 μM thymidine and 3 μM glycine). ICAM-1/Fas Chimeric$^+$ L(TK^+) cells were used for ^{3}H-thymidine incorporation experiments.

3.0 Fluorescent Microscopy

Suspension cells were harvested and chilled on ice. Cells were aliquoted into microfuge tubes and washed in ice-cold PBS before fixing in freshly prepared 1% paraformaldehyde/PBS for 20 minutes on ice with gentle agitation. Fixed cells were

then washed once with PBS and 2 times with HBSS + 5% FBS before staining with primary antibody undiluted supernatant for one hour on ice. Once again, cells were washed 3 times in HBSS + 5% FBS followed by secondary staining with GαRIgG conjugated to FITC (Cappel, Cooper Biomedical, Malvern, PA) for 45 minutes on ice. After a final washing step, cells were resuspended in HBSS with 2% DABCO (Aldrich, Milwaukee, WIS), an anti-photobleaching agent, and the cell suspension was mounted on microscope slides. Cells were observed under a Zeiss microscope equipped with an objective for epifluorescence. Photos were taken with Kodak Tri-X pan 400 film.

Adherent cells, fibroblasts (L), were seeded at 10^4 cells per well in 8 well Lab-Tek Chamber Slide Tissue Culture chambers (Nunc, Inc., Naperville, IL) which had been previously coated with 0.5% gelatin. Cells were grown overnight and then fixed in 1% paraformaldehyde/PBS for 20 minutes at room temperature. Staining procedure was similar to above, only cells were stained and washed on the slide. Finally the wells were removed, a drop of HBSS was added and the slide was covered with a coverslip. Cells were observed in a similar manner to above.

4.0 Detergent Extraction

Single cell suspensions were prepared from adherent cell cultures (L cells) by incubation with 8 mM EDTA at 37°C for 5 minutes. 10^6 suspension cells were then extracted in lysis buffer (20 mM pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 0.2 mM PMSF, 0.5 mg/ml leupeptin and aprotinin) for 10 minutes on ice. Lysate was centrifuged at 10,000 rpm in an eppendorf centrifuge 5414 (Brinkman Instruments Ltd., Rexdale, Ontario) for 10 minutes at 4° C. Supernatant fraction was transferred to a new tube and the remaining pellet was washed in lysis buffer. Both the pellet and the supernatant fraction were suspended in non reducing SDS sample buffer (0.2M Tris pH 6.8, 4% SDS, 10% glycerol, 0.04% Bromophenol Blue).

Samples were centrifuged by a Beckman Airfuge for 20 minutes and boiled 5 minutes before loading on SDS PAGE.

5.0 Chemical Cross-linking and Detergent Extraction

10^6 cells were suspended in 500 µl HBSS with or without 0.1 mM DSS (dissolved fresh in DMSO) (Pierce, Rockford, Illinois) and rotated at 4 °C for one hour. Cells were then centrifuged at 1500 rpm for 5 minutes and the pellet was washed 2 times before extracting as above. In the case of cross-linking alone, the pellet was simply resuspended in SDS sample buffer, as above.

6.0 Western blot analysis

Samples were boiled 5 minutes prior to loading onto a 7.5% polyacrylamide discontinuous gel. A high molecular weight prestained protein standard (Life Technologies Inc., Gaithersburg, MD) was simultaneously run to estimate size of proteins. The samples were separated by electrophoresis in a Mini V 8.10 Vertical Gel Electrophoresis Apparatus (BRL Life Technologies Inc., Gaithersburg, MD) at 180 volts. The proteins were then transferred to Immobilon P Transfer Membrane (Millipore Corp., Bedford, MA) using a TE Series Transphor Electrophoresis Unit (Hoefer Scientific Instruments, San Francisco) for one hour at 60 volts. The membrane was then preincubated in 5% blotto (Carnation Skim Milk Powder), TBS (10mM TrisCl pH 7.4, 0.15M NaCl)/.08% Tween 20 for 1 hour at room temperature. The filters were then incubated with primary antibody (either hybridoma supernatant (1:4) or purified antibody (1 µg/ml) in 5% blotto TBS/Tween for 45 minutes. The filter was washed once for 15 minutes, two times for 5 minutes and incubated further with secondary antibody, GαRIgG conjugated to horseradish peroxidase (Cappel Cooper Biomedical Inc., Malvern, PA) diluted 1 in 10,000 in 5% blotto TBS/Tween. After the last wash, filters were developed by an ECL Detection Kit (Amersham Life Sciences, Oakville, Ontario) and exposed to X-ray film (Kodac XAR) for 15 seconds to 30 minutes.

7.0 Adhesion Assay

Wells of a F96 Polysorp Nunc-Immuno Plate were coated with soluble ICAM-1 (50 µl of 25 µg/ml in 0.1M $NaHCO_3$) or BSA (50 µl of 200 µg/ml in 0.1M $NaHCO_3$) for 1 hour at room temperature. Wells were then washed with HBSS and blocked with BSA for 45 minutes at room temperature. Meanwhile, cells were harvested with 8 mM EDTA/PBS, washed 3 times with HBSS (without serum) and labeled with 1 mg/ml Calcein AM (Molecular Probes Inc., Eugene, Oregon) for 40 minutes at 37°C. Labeled cells were then washed 3 times in prewarmed HBSS and resuspended in HBSS + 5% FBS to give a final concentration of 4 X 10^6 cells/ml. 50 µl of cell preparation were added per well and plates were spun at low speed for a few seconds to allow cell contact with the bottom of the wells before incubating at 37 °C for 15 minutes. Unbound cells were removed by repeatedly adding prewarmed HBSS and flicking the plate 6 times. Fluorescence was determined by CytoFluor 2300 Fluorescence Measurement System. The percentages of adherent cells were estimated from a standard curve drawn from the fluorescent readings of the known concentrations of labeled cells added to wells after the final wash.
Falcon 96 well tissue culture plates were seeded with 2X10^4 ICAM-1/Fas chimeric$^+$ cells and incubated overnight to establish a monolayer. PMA activated and calcein labeled splenic T cells were then added to the wells and the adhesion assay proceeded similar to above except splenic T cells were incubated in wells for 10 minutes at 37°C and wells were washed 10 times.

8.0 Isolation of Splenic T cells

BALB/c mice, approximately 3 months old, were sacrificed and the spleen removed. Cells were extracted from the spleen and suspended in RPMI 1640 medium + 5% FBS. After centrifugation at 1100 rpm for 5 minutes, cells were resuspended in 1-1.5 ml prewarmed media as above. They were then loaded onto a prewashed nylon wool column which had been incubated at 37 °C, 5% CO_2

overnight. After incubation in the column for 1 hour at 37 °C, cells were eluted with 10-15 ml of prewarmed RPMI 1640 medium + 5% FBS. In order to lyse the red blood cells, 17mM Tris,144mM NH_4Cl was added to the cells for 4 minutes at room temperature. Again cells were washed in this solution and finally with RPMI 1640 medium after which the cells were counted. The isolated splenic T cells were activated with 50 ng/ml PMA (Sigma, St Louis, MO) for 20 minutes at 37 °C. Cells were then washed 3 times in HBSS to remove the FBS before labeling the cells with Calcein AM in a similar manner to above.

9.0 Bead Binding Assay

Soluble ICAM-1 was coated to 2.138 μm Polybead Polystyrene Microspheres (Polysciences, Inc., Warrington, PA). Beads were mixed with 0.1 M $NaHCO_3$ and 100 μg/ml soluble ICAM-1 for 2 hours at room temperature. Beads were then centrifuged at 3500 rpm for 2 minutes, and suspended in 5% blotto/PBS. Beads were again inverted for 30-60 minutes at room temperature before centrifugation, followed by final resuspension in the original volume with 5% blotto/PBS. 0.1% NaN_3 was added to the bead suspension for storage at 4 °C. Control beads were coated with 5% blotto in a similar manner. Beads were prewashed in 5% blotto/PBS before use, to remove NaN_3.

For the binding assay, cells were seeded at 10^4 cells/well in duplicate in 96 well tissue culture plate (Falcon Microtest III , Becton Dickinson, Lincoln Park, New Jersey) and grown overnight at 37 °C, 5% CO_2. 10 μl of beads (sICAM-1 or control) were added with either 50 μl media or YN1/1 supernatant and incubated for 25 minutes at 37 °C. Wells were washed extensively 10 times with prewarmed media. Final wash was in PBS before fixing with 0.5 % glutaraldehyde/PBS for 5 minutes at room temperature. Cells were stained with Giemsa stain 5-7 minutes and then rinsed with water. PBS was added and bead binding was observed and photographed using a Nikkon Diaphot microscope.

10.0 Cell Death Assay

Fibroblasts transfected with different forms of ICAM-1 were seeded in triplicate at 2.5 X 10^4 cells/well in 96 well Falcon tissue culture plate and grown overnight at 37 °C, 5% $C0_2$. Antibody, either purified YN1/1 (2 μg/ml) or YE1/30 (2 μg/ml) was added to appropriate wells and incubated overnight under similar conditions. Cell death was observed and photographed with a Nikkon Diaphot microscope.

11.0 ^{3}H-Thymidine Incorporation

Fibroblasts expressing ICAM-1/Fas chimeric molecule or full length ICAM-1 were seeded at different cell densities in triplicate in 96 well Falcon tissue culture plate and grown overnight at 37 °C, 5% CO_2. Antibody, YN1/1 (2 μg/ml), or cells expressing high-avidity LFA-1 or CD43 which had been irradiated at 10 000 rads, were added to appropriate wells and incubated overnight. 1.0 μCi of ^{3}H-Thymidine (Dupont, NEN Products, Boston, MA) was added per well and incubated for 4 hours at 37 °C, 5% CO_2. 20 μl of 0.5 M EDTA (100 mM final) was added per well for 5 minutes before cells were harvested with a 1295-001 Cell Harvester (LKB Wallac, Skatron, Norway). Radioactivity was determined by a 1205 Betaplate Liquid Scintillation Counter (LKB Wallac).

RESULTS

1.0 ICAM-1 Association with the cytoskeleton

1.1 Generation of ICAM-1 Constructs and Transfected Cell Lines

To study the participation of different domains of ICAM-1 in the regulation of surface distribution and interaction with the cytoskeleton, several different constructs of ICAM-1 were generated. A full length, and cytoplasmic truncation were previously constructed in our laboratory. Briefly, the full length construct, EH, is a cDNA, encoding the complete, functional murine ICAM-1. The cytoplasmic truncation construct, PCR, encodes all of the extracellular and transmembrane regions, the first three amino acids of the cytoplasmic region and one irrelevant amino acid (Figure 1). Fibroblast (L) cells and the T cell hybridoma, T28, expressing these constructs, either full length or cytoplasmic truncation of ICAM-1, had previously been generated and were analyzed by FACS (Figure 2).

In addition to these, ICAM-1 that lacked the transmembrane and cytoplasmic domains but anchored into the plasma membrane by a glycosyl-phosphatidyl inositol (GPI) linkage was also generated. The GPI anchor linkage sequence was generated by PCR amplification from heat stable antigen, M1/69 cDNA, generously donated by Dr. Rob Kay (Kay et al., 1990). The resulting construct encoded ICAM-1 extracellular domain missing the last five amino acids of domain 5, followed by three irrelevant amino acids and a GPI linkage sequence (Figure 1). To confirm the proper, in frame sequence of the ICAM-1/GPI construct, the clone was sequenced. This construct was then subcloned into the expression vector, pBCMGS and transfected into T28 cells. To confirm that ICAM-1 was truly GPI linked when expressed on the surface, cells were treated with PI-PLC, stained with antibody against ICAM-1 and analyzed by FACS. GPI-anchored proteins are cleaved from the cell surface by PI-PLC (Low and Saltiel, 1988, Ferguson et al., 1988). As expected, the FACS profile showed a significant decrease in YN1/1 staining when

A) ...RNVYLTVLYHSQNNWTIIILVPVLLVIVGLVMAASYVYNRQRKIR...

B) ...RNVYLTVLYHSQNNWTIIILVPVLLVIVGLVMAASYVYNRQI

C) ...RNVYLTVLY-----RIR**GGSSLQST**...

D) ...RNVYLTV-------G**NRLWLLTI**...

Figure 1

Partial amino acid sequences of ICAM-1 constructs

A) The carboxy-terminal end of the extracellular domain, transmembrane domain (underlined) and the partial cytoplasmic domain of full-length ICAM-1 (EH)

B) Cytoplasmic truncation of ICAM-1 (PCR)

C) Junction sequence of GPI-linked ICAM-1 (GPI-anchoring signal sequence derived from M1/69 is in bold)

D) Junction sequence of ICAM-1/Fas chimeric molecule (Fas sequence is shown in bold)

(transmembrane region is underlined; ---missing amino acids; irrelevant amino acids are double underlined)

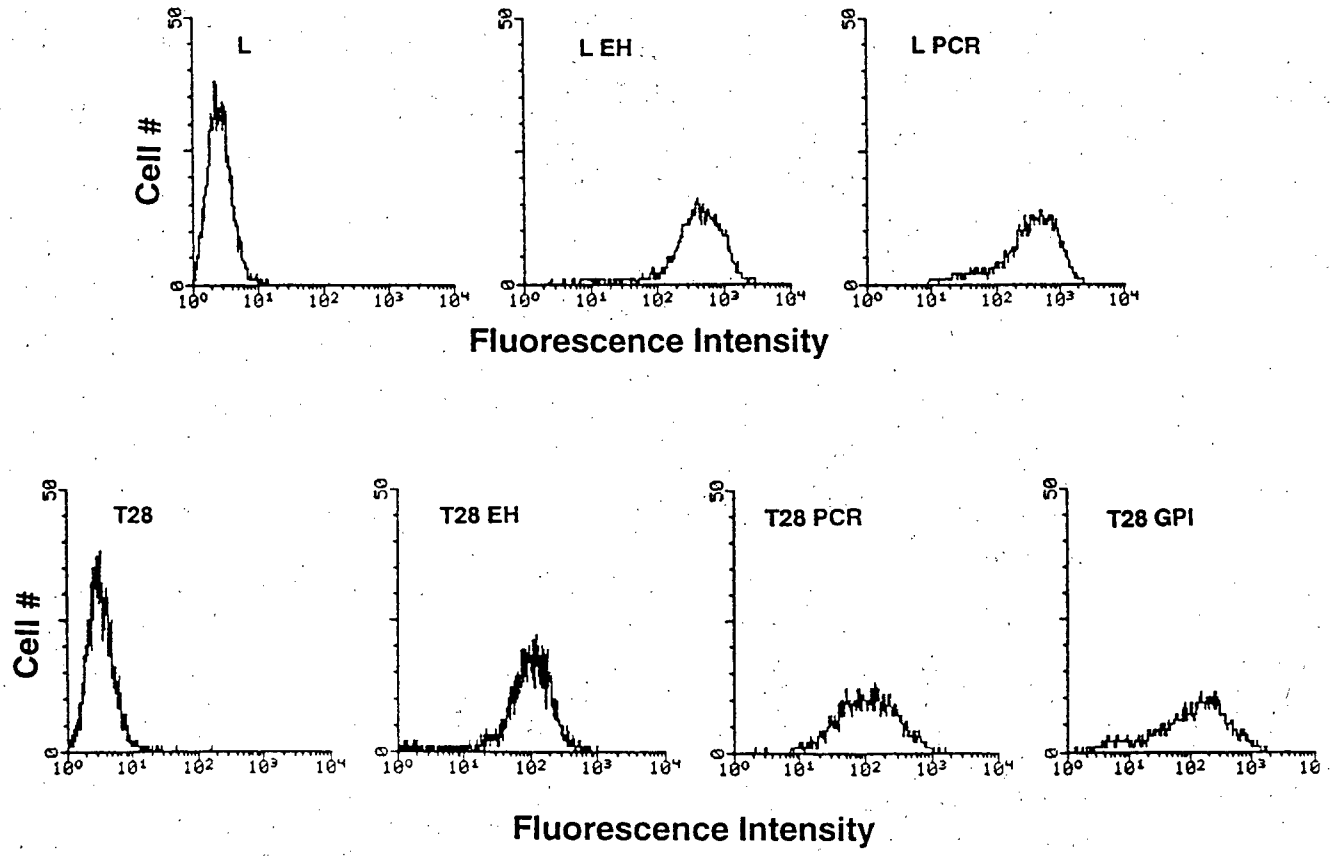


Figure 2

Flow cytometric analysis of L cells and T28 cells transfected with various forms of ICAM-1

Untransfected (L or T28) and transfected (LEH, LPCR, T28EH, T28PCR, T28GPI) cells were stained with mAb YN1/1, against ICAM-1, followed by GαRIgG conjugated to FITC. Dead cells were excluded by PI staining.

cells were treated with PI-PLC (Figure 3). This reduction in ICAM-1 staining by PI-PLC treatment was comparable to that of Thy-1, a known GPI-linked protein, as detected by YE1/30 antibody. In contrast, LFA-1, stained with TIB213, was not affected by PI-PLC treatment. This was used as a negative control since LFA-1 does not contain a PI-PLC cleavage site.

1.2 Distribution Studies

To determine the role of the cytoplasmic and transmembrane domains in cell surface distribution of ICAM-1, various cell lines expressing different forms of ICAM-1 were analyzed by fluorescent microscopy. Cells were fixed in 1% paraformaldehyde before staining to prevent capping of ICAM-1 by antibody binding. ICAM-1 was distributed in a patchy pattern on the surface of fibroblasts, regardless of the presence or absence of the cytoplasmic domain (Figure 4). The microspikes had highly concentrated ICAM-1 at their tips. In contrast, the three different forms of ICAM-1, full-length, cytoplasmic truncation, and GPI-linked, all had a more uniform distribution pattern when expressed in the T cell line (Figure 5). ICAM-1 appeared to be more concentrated at the point of cell contact. However, such localization was not prominent, and was observed with all the different forms of ICAM-1. YE1/21 staining (antibody against CD45) was used as a control, which showed uniform distribution.

1.3 Detergent Extraction

The difference in distribution of ICAM-1 on the surface of fibroblasts and T cells, along with previous observations of punctate distribution of ICAM-1 on the B cell lymphoma A20 (Welder, M.Sc. Thesis, 1992), suggested there is a cell type specific mechanism that regulates the distribution ICAM-1, in a fluid membrane. ICAM-1 may associate directly or indirectly (via another molecule) with a cytoskeletal component which may result in the variable distribution pattern observed. To test this hypothesis, cytoskeletal association was determined by

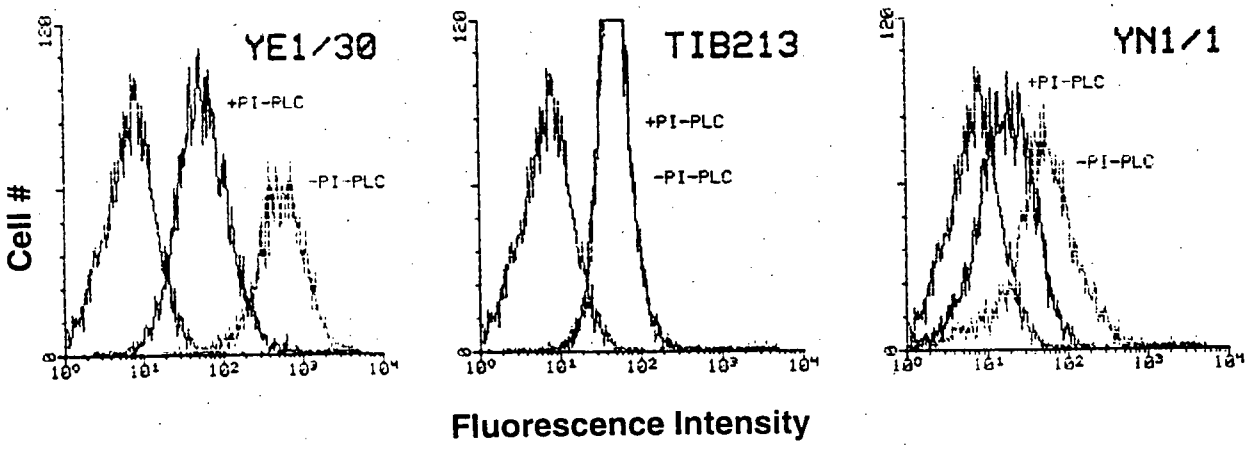


Figure 3

Flow cytometric analysis of T28 cells treated with PI-PLC

Cells were first treated with or without 10U/ml PI-PLC in serum free DMEM for 1 hour at 37 °C. The cells were then labeled with either anti-Thy-I, YE1/30, anti-LFA-1, TIB213, or anti-ICAM-1, YN1/1 followed by GαRIgG conjugated to FITC. The background histogram represents T28 cells stained with secondary antibody only. Dead cells were excluded by PI staining.

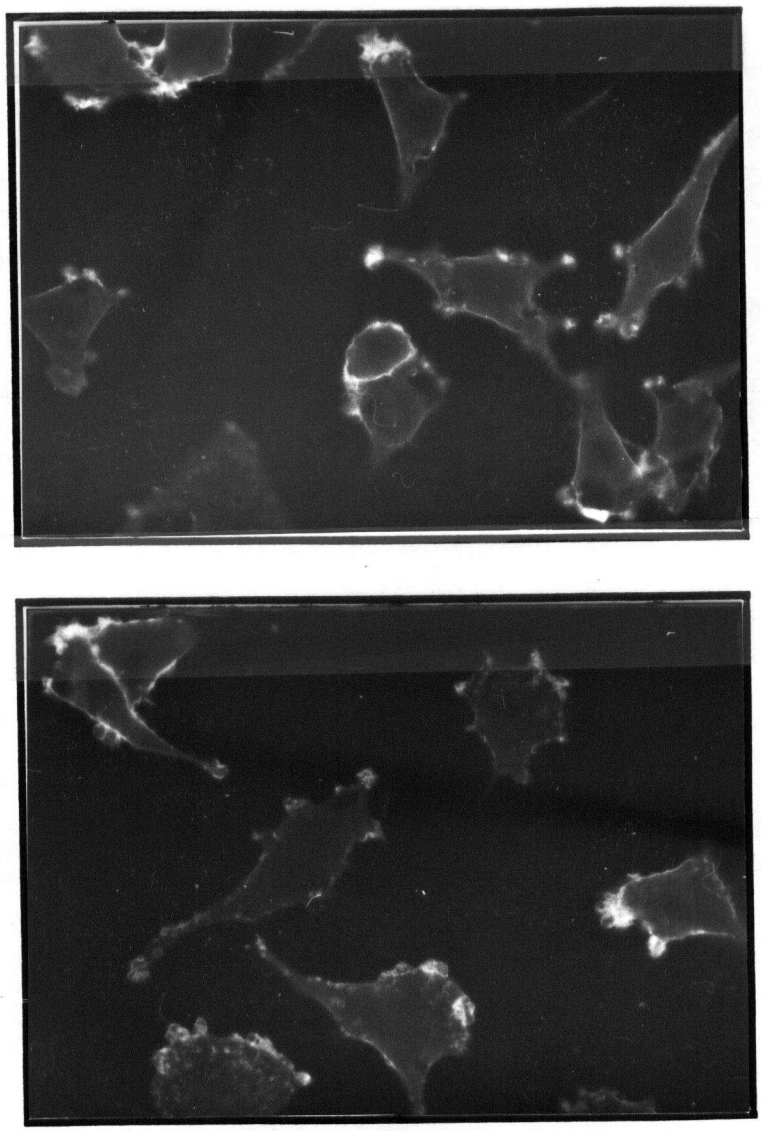


Figure 4

ICAM-1 distribution on L cells

Fibroblast (L) cells transfected with either full-length ICAM-1 (LEH) or cytoplasmic truncation (LPCR) were seeded on a chamber slide and fixed with 1% paraformaldehyde before staining with mAb YN1/1 (anti-ICAM-1) followed by Gα RIgG conjugated to FITC. Cells were photographed under a Zeiss microscope equipped with epifluorescence using a Leitz 40X10.75 objective.

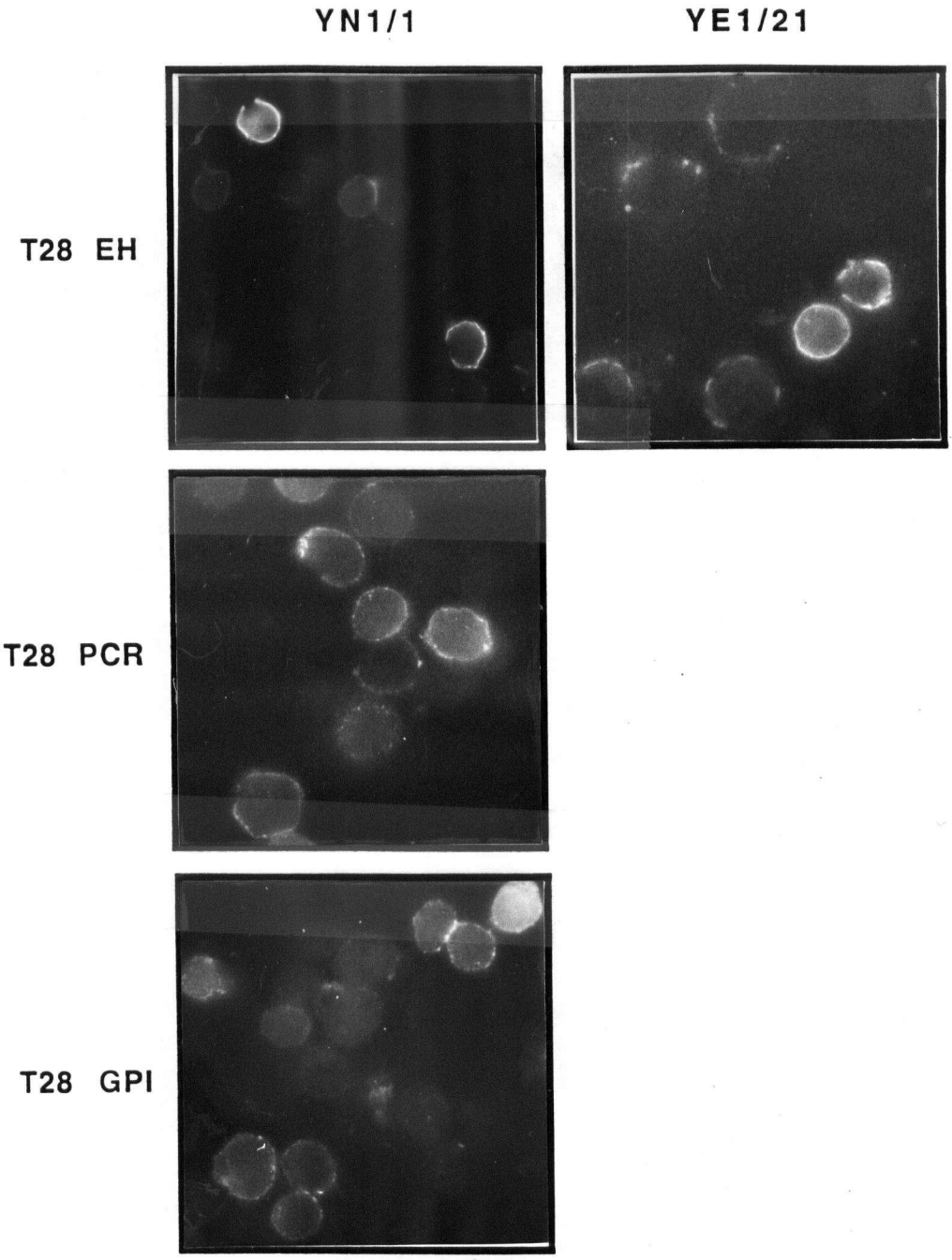


Figure 5

ICAM-1 distribution on T28 cells

The T cell hybridoma (T28) transfected with various constructs of ICAM-1, full-length (T28EH), cytoplasmic truncation (T28PCR), or GPI-linked (T28GPI), were fixed in 1% paraformaldehyde before staining with mAb YN1/1 (anti-ICAM-1) or control mAb YE1/21 (anti-CD45) followed by GαRIgG conjugated to FITC. The cell suspension was mounted on a slide and the photo was taken under a Zeiss microscope equipped with epifluorescence using a Leitz 40X10.75 objective.

detergent extraction. Previous studies have shown that non-ionic detergent insolubility is a reliable indication of cytoskeletal association. The molecules associated with the cytoskeleton remain in the detergent insoluble cytoskeletal fraction (Geppert 1991, Albrecht 1988). ICAM-1 on L cells was partially insoluble when extracted with 1% Triton X-100 at various temperatures (Figure 6). To quantitate the amount which was insoluble, the detergent soluble fraction was titrated against the insoluble fraction. Approximately 2% of ICAM-1 on fibroblasts was insoluble (Figure 7). T28 cells transfected with the different forms of ICAM-1 as well as two clones of A20, one expressing high avidity LFA-1(A8), the other only low avidity LFA-1 (CL7), were also tested. Since A20 cells express endogenous ICAM-1, it was of interest to establish if LFA-1/ICAM-1 interaction altered cytoskeletal association. ICAM-1 was completely soluble in 1% Triton X-100 in all cases (Figure 8). Another molecule, Thy-1, which is GPI-anchored and has been reported to have variable solubility, was tested as a control to determine whether insoluble proteins could be detected in the pellet fraction of these cells. Figure 8 shows a significant portion of Thy-1 was insoluble, confirming that some proteins are insoluble in 1% Triton X-100.

1.4 Chemical Cross-linking and Detergent Solubility

Because all forms of ICAM-1 were almost completely soluble in all cell lines tested, despite the variable distribution patterns observed, we tested whether the detergent extraction conditions disrupted a potential association of ICAM-1 with the cytoskeleton. Cells were first treated with a permeable chemical cross-linker, DSS, which covalently links two molecules in close association, before detergent extraction was initiated. In addition, two more cell lines, P388, a monocyte cell line and NS-1, a myeloma, both expressing endogenous ICAM-1, were also tested since the previous cell lines examined, with the exception of A20, do not express endogenous ICAM-1. The chemical cross-linker had no effect. That is, ICAM-1

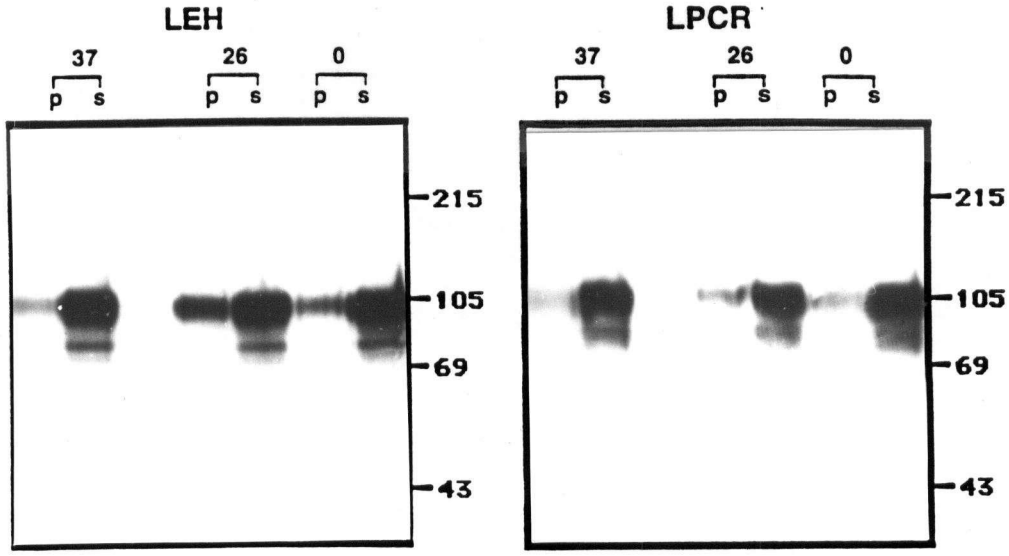


Figure 6

Detergent extraction of L cells at various temperatures

Fibroblast cells (L) transfected with either full-length ICAM-1 (LEH) or cytoplasmic truncation (LPCR) were extracted with 1% Triton X-100 extraction buffer at 37 °C, 26 °C, or on ice. The insoluble pellet (p) and the detergent soluble extract (s) were resolved on 7.5% SDS-PAGE, blotted onto PVDF (Immobilon) and probed with mAb YN1/1 followed by GαRIgG conjugated to HRP. Blots were visualized using an ECL Detection kit and exposed to X-Ray film. The numbers show the sizes (KD) of the prestained protein standards.

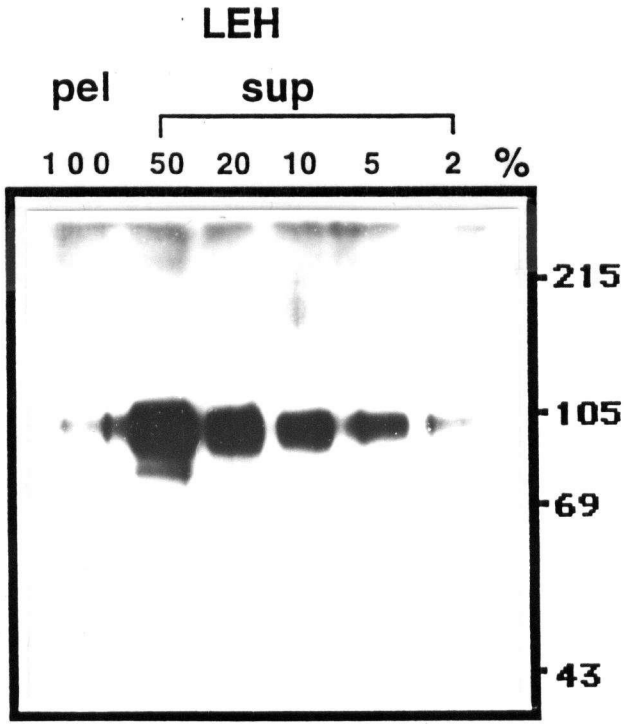


Figure 7

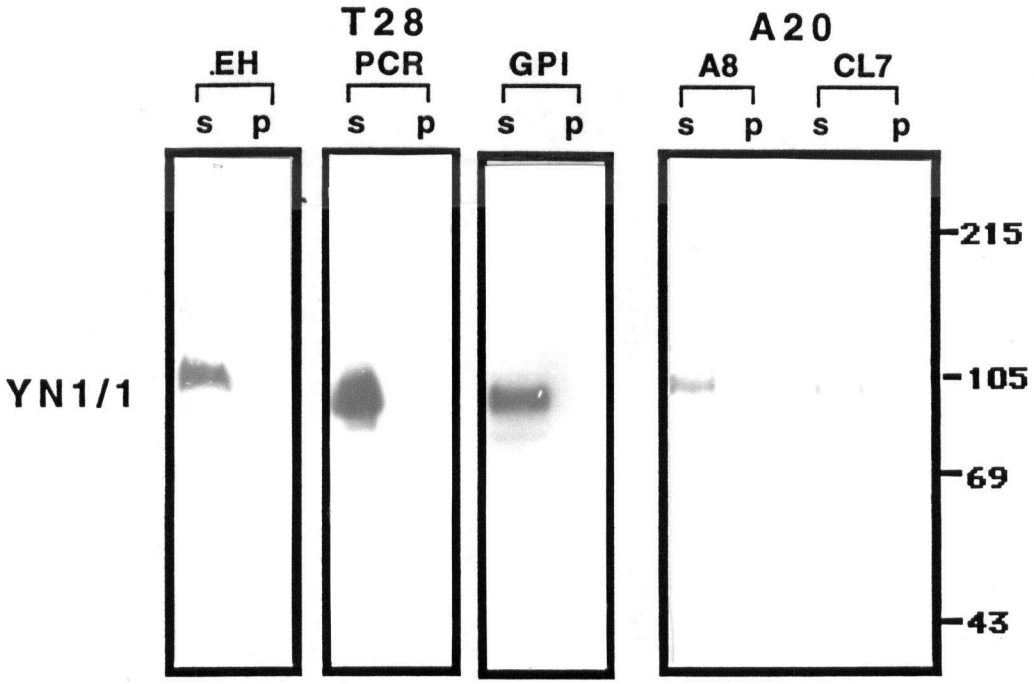
Quantitation of insoluble ICAM-1 after detergent extraction of L cells

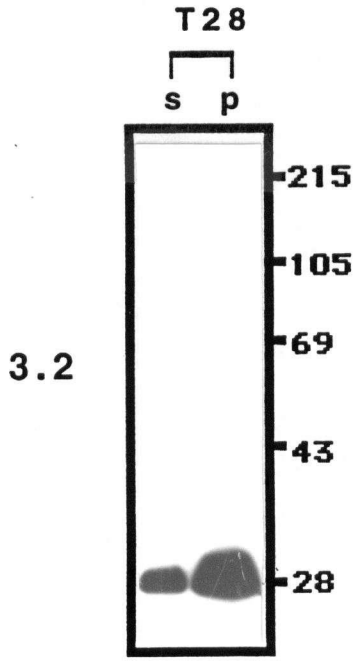
Fibroblasts transfected with full-length ICAM-1 (LEH) were extracted with 1% Triton X-100 extraction buffer at 4 °C. Varying amounts (50,20,5,2%) of the total detergent soluble extract (sup) were titrated against the insoluble fraction (pel). Fractions were then resolved on 7.5% SDS-PAGE, blotted onto PVDF (Immobilon) and probed with mAb YN1/1 followed by GαRIgG conjugated to HRP. The blot was visualized using an ECL Detection Kit and exposed to X-Ray film. The numbers show the sizes (KD) of the prestained protein standards.

Figure 8

Detergent extraction of T28 and A20 cells

A T cell hybridoma T28, transfected with various forms of ICAM-1, full-length (T28EH), cytoplasmic truncated (T28PCR) or GPI-linked (T28GPI) and two different clones of an ICAM-1^{+}B cell hybridoma A20, adherent (A8), or non adherent (CL7), were extracted with 1% Triton X-100 extraction buffer at 4 °C. The insoluble pellet (p), and the detergent soluble extract (s) were resolved on 7.5% SDS-PAGE, blotted onto PVDF (Immobilon) and probed with mAb YN1/1 (anti-ICAM-1) or control mAb 3.2 (anti-Thy-1) followed by GαRIg conjugated to HRP. Blots were visualized by using an ECL Detection kit and exposed to X-Ray film. The numbers show the sizes (KD) of the prestained protein standards.


T28
A20
EH
PCR
GPI
A8
CL7
s p
s p
s p
s p
s p
YN1/1
215
105
69
43


T28
s p
3.2
215
105
69
43
28

remained completely in the detergent soluble fraction (Figure 9,10). Although some GPI-linked ICAM-1 on T28 cells was detergent insoluble, the results were highly variable in three separate experiments (Figure 11).

1.5 Chemical Cross-Linking

Because some high molecular weight bands were detected on the Western blots when cells were cross-linked and detergent extracted in the above experiments, chemical cross-linking studies were utilized to determine if ICAM-1 associates with another molecule which in turn can bind the cytoskeleton. Following our hypothesis, this could also explain how ICAM-1 without the cytoplasmic domain on L PCR cells, could result in the punctate distribution pattern. When fibroblasts transfected with full-length ICAM-1 were treated with various concentrations of the permeable chemical cross-linker DSS, a band of approximately 200 KD was observed (Figure 12). A similar band was detected for all constructs of ICAM-1 in different cell types when the same cross-linker was used, although it was much less pronounced in T28 cells (Figure 13). Also, with increasing DSS concentration, a lower sensitivity of both bands was observed. This could be due to chemical modification of the epitope which the antibody recognizes. Another explanation could be that aggregates are formed which do not run into the gel. An endothelial cell line, SVEC, which does not express LFA-1, was transfected with ICAM-1, and similarly tested. Again, a faint but distinct band of approximately 200 KD was detected (Figure 13). Another chemical cross-linker BS3, an impermeable analogue of DSS, was used to determine if cytoplasmic cross-linking was responsible for what appeared to be self association or dimer formation. Similar results were observed with this cross-linker. A prominent 200 KD band was detected with fibroblasts, but only a very faint 200 KD band was observed for T28 cells. No additional band was

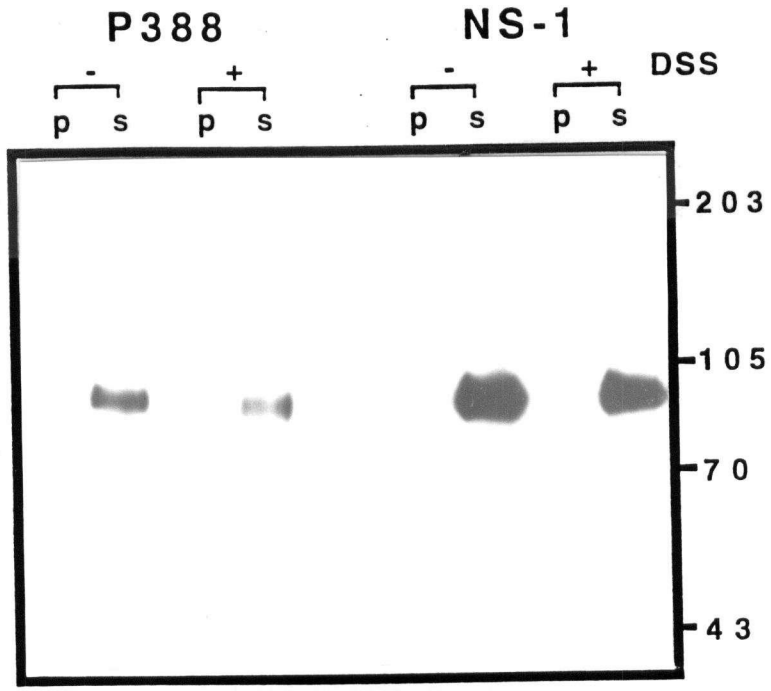


Figure 9

Chemical cross-linking and detergent extraction of P388 and NS-1 cells

The monocyte cell line, P388, and the myeloma cell line, NS-1, both expressing endogenous ICAM-1, were treated with or without (+/-) 0.1 mM DSS for 1 hour at 4 °C followed by extraction in 1% Triton X-100 extraction buffer. The insoluble pellet (p) and the detergent soluble extract (s) were resolved on 7.5% SDS-PAGE, blotted onto PVDF (Immobilon) and probed with mAb YN1/1 followed by GαRIgG conjugated to HRP. Blots were visualized using an ECL detection kit and exposed to X-Ray film. The numbers show the sizes (KD) of the prestained protein standards.

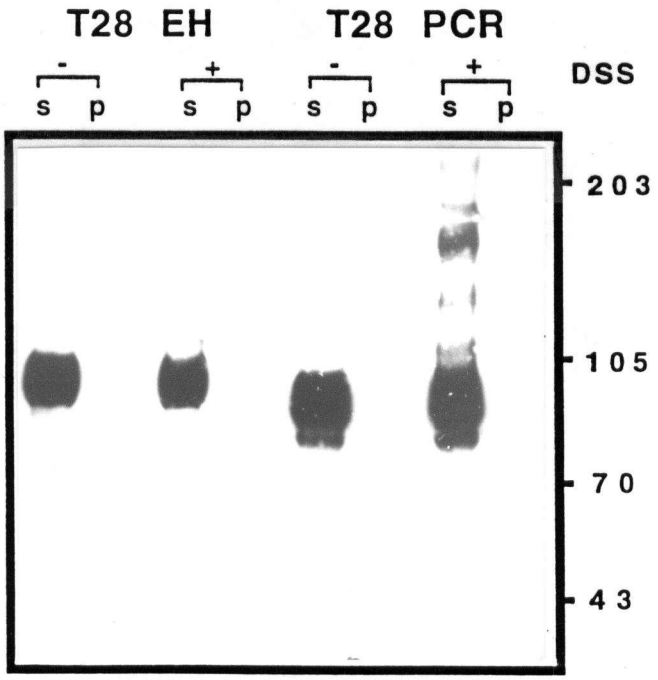


Figure 10

Chemical cross-linking and detergent extraction of T28 cells

T cell hybridoma T28, transfected with various forms of ICAM-1, full-length (EH), or cytoplasmic truncation (PCR), were treated with or without (+/-) 0.1 mM DSS followed by extraction in 1% Triton X-100 extraction buffer. The detergent insoluble pellet (p) and the soluble extract (s) were resolved on 7.5% SDS-PAGE blotted onto PVDF (Immobilon) and probed with mAb YN1/1 followed by GαRIgG conjugated to HRP. The blots were visualized using an ECL Detection Kit and exposed to X-Ray film. The numbers show the sizes (KD) of the prestained protein standards.

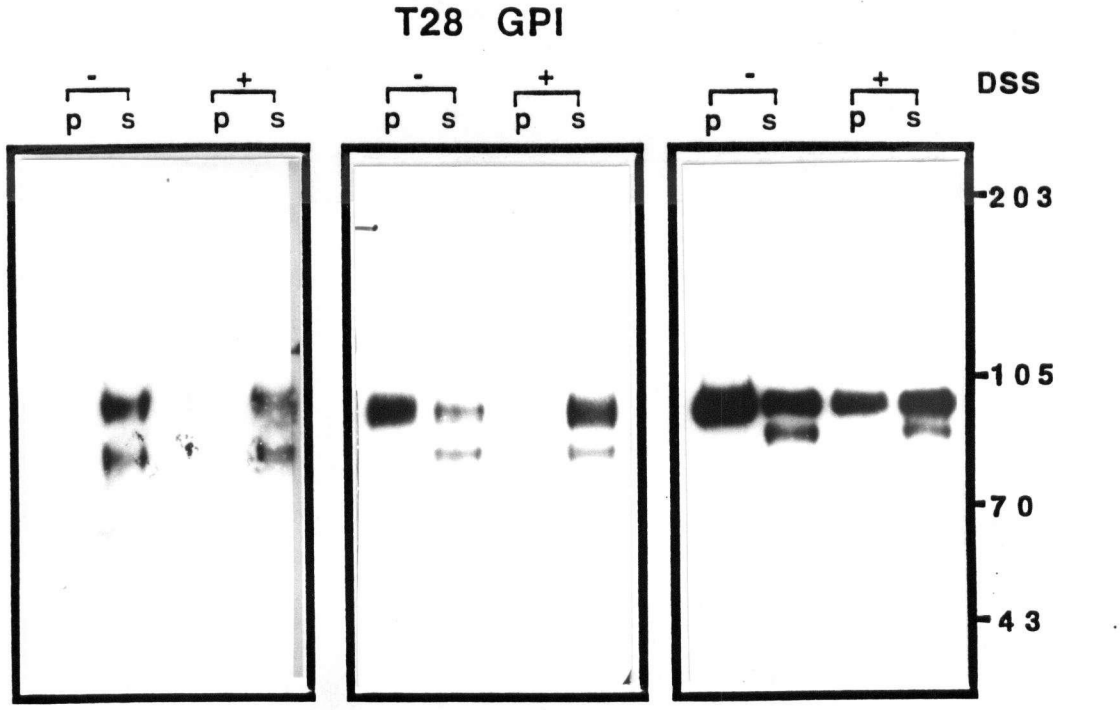


Figure 11

Detergent solubility of GPI-linked ICAM-1 after cross-linking

A T cell hybridoma T28, transfected with ICAM-1/GPI construct was treated with or without (+/-) 0.1 mM DSS followed by extraction in 1% Triton X-100 extraction buffer. The detergent insoluble pellet (p) and the soluble extract (s) were resolved on 7.5% SDS-PAGE blotted onto PVRD (Immobilon) and probed with mAb YN1/1 followed by GαRIgG conjugated to HRP. The blots were visualized using an ECL Detection Kit and exposed to X-Ray film. The numbers show the sizes (KD) of the prestained protein standards. The results of three separate experiments are shown.

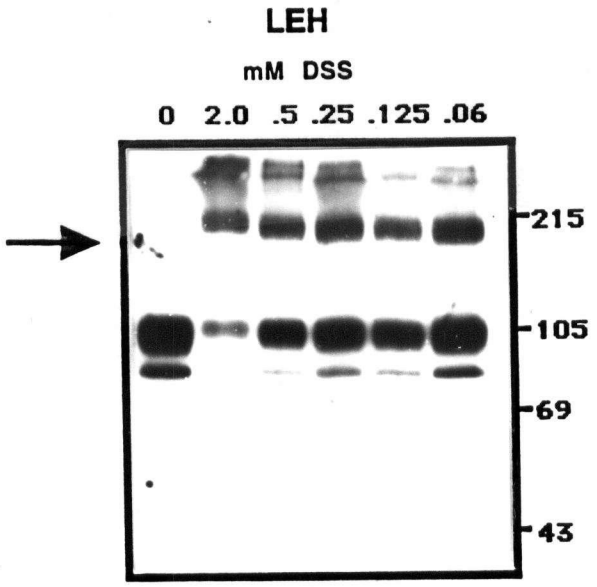


Figure 12

Chemical cross-linking of L cells by DSS

A fibroblast cell line L, expressing ICAM-1 was treated with various concentrations (0, .06, .125, .25, .5, 2 mM) of chemical cross-linker, DSS for 1 hour at 4 °C. Cells were lysed in 4 % SDS loading buffer, resolved on 7.5% SDS-PAGE, blotted onto PVDF (Immobilon) and probed with mAb YN1/1 followed by GαRIgG conjugated to HRP. Blots were visualized using ECL Detection Kit and exposed to X-Ray film. The numbers represent the sizes (KD) of the prestained protein standards.

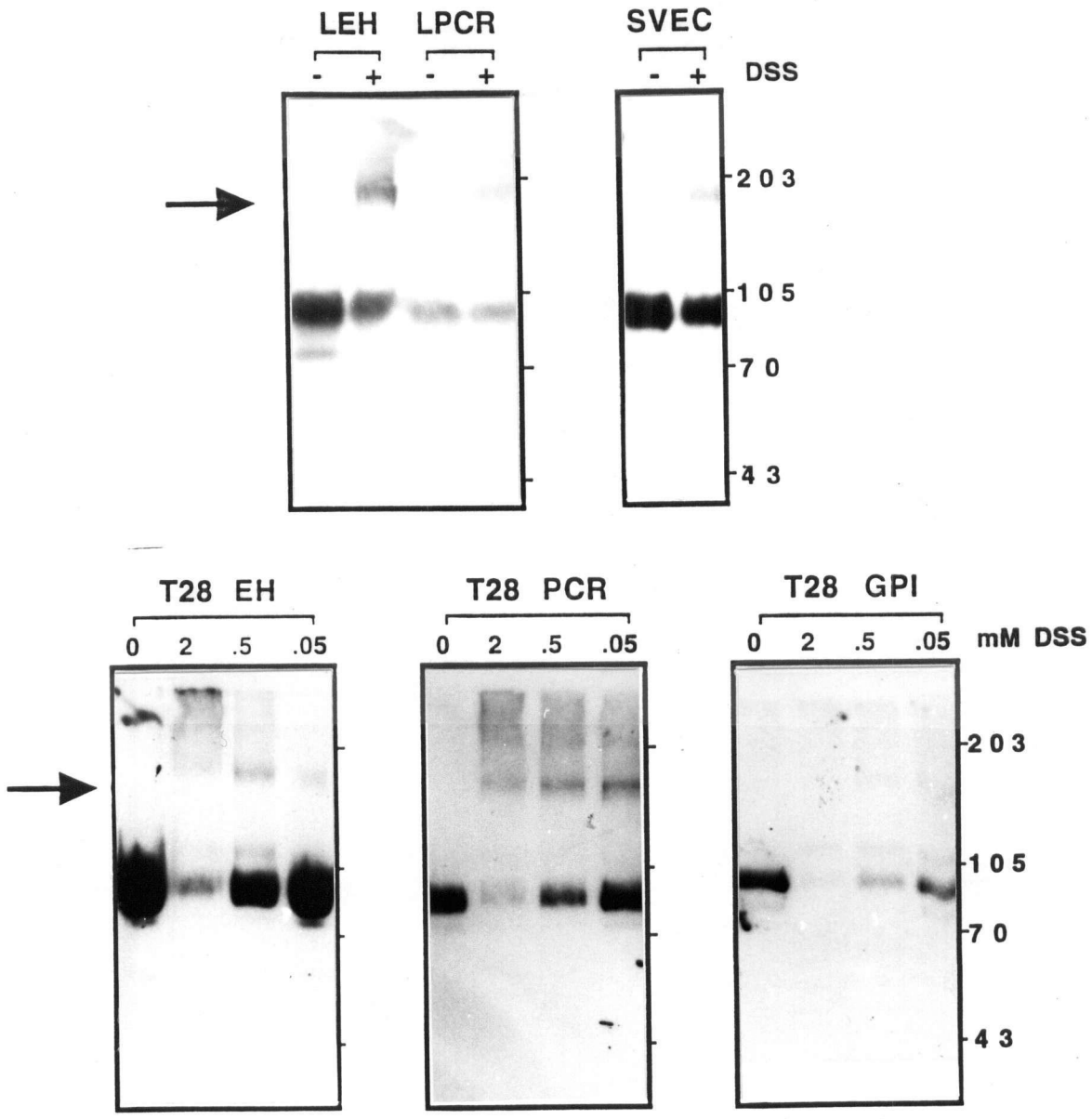


Figure 13

Chemical cross-linking of various cells lines by DSS

Various cells lines expressing different ICAM-1 forms (EH, full-length; PCR, cytoplasmic truncation; GPI, GPI-linked) were treated with 2 mM chemical cross-linker, DSS for 1 hour at 4 °C. Cells were lysed in 4 % SDS loading buffer and resolved on 7.5% SDS-PAGE blotted onto PVDF (Immobilon) and probed with mAb YN1/1 followed by GαRIgG conjugated to HRP. Blots were visualized using ECL Detection Kit and exposed to X-Ray film. The numbers show the sizes (KD) of the prestained protein standards.

detected with A20 cells (Figure 14). This suggested cytoplasmic cross-linking is not involved. Thus a small percentage of ICAM-1 molecules appear to be present in dimers on the cell surface of various cell types.

Table 1 summarizes the results obtained from the studies of distribution and cytoskeletal association along with observation of homotypic aggregation. In the cell lines tested expressing various forms of ICAM-1, no correlation could be made between these parameters. The cells expressing ICAM-1 did not require patchy distribution of this molecule on the cell surface to spontaneously aggregate, as hypothesized. Also, distribution of ICAM-1 did not correlate with cytoskeletal association as determined by detergent solubility.

2.0 ICAM-1 Association with CD43

2.1 Generation of CD43$^+$ Cells

To study ICAM-1 association with CD43, a cell line expressing CD43, but not other known ligands for ICAM-1, was established. A murine CD43 construct was generated by PCR from a genomic clone (Figure 15). This was possible since there are no introns in the coding sequence of the CD43 gene (Shelley et al., 1990). The resulting construct encoded the complete murine CD43 with the putative ribosomal binding site. The clone was sequenced from both the 5' and 3' end to confirm the entire sequence was present. By $CaPO_4$ mediated transfection and subsequent selection in G418, an L cell clone expressing a reasonably high level of CD43 on the surface was established (Figure 16).

2.2 Adhesion Assay

An adhesion assay was the first step taken to examine a possible interaction between ICAM-1 and CD43 in the murine system. Calcein AM labeled CD43$^+$ L cells or control L cells (LFA-1 transfected or untransfected) were incubated in wells

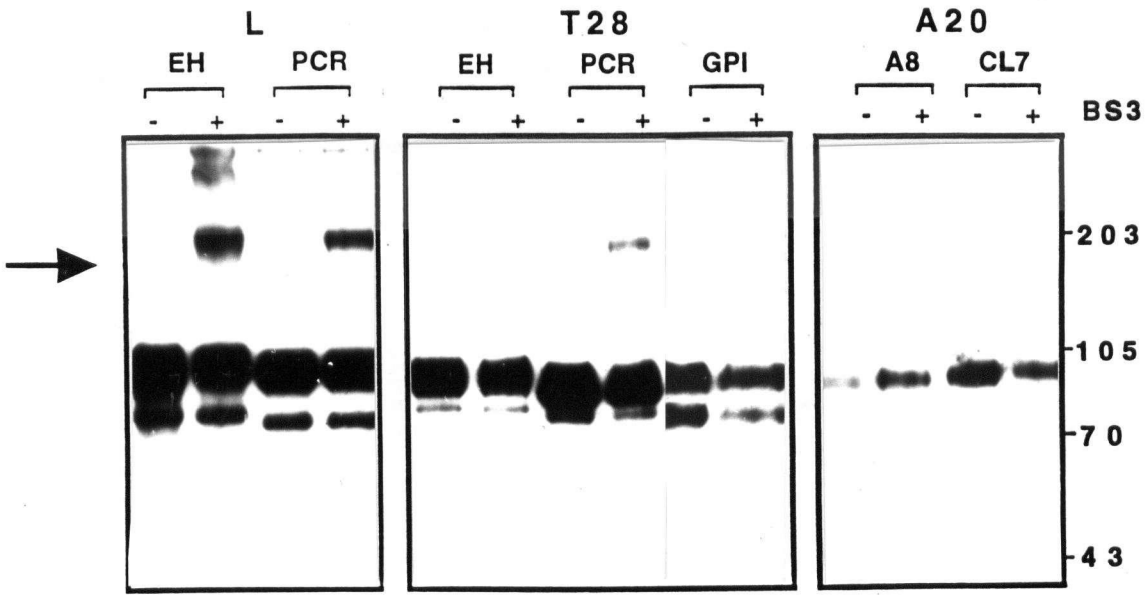


Figure 14

Chemical cross-linking of various cell lines, by BS3

Various cells lines expressing different ICAM-1 forms (EH, full-length; PCR, cytoplasmic truncation; GPI, GPI linked) were treated with 2 mM of chemical cross-linker, BS3, for 1 hour at 4 °C. Cells were lysed in 4 % SDS loading buffer and resolved on 7.5% SDS-PAGE blotted onto PVDF (Immobilon) and probed with mAb YN1/1 followed by GαRIgG conjugated to HRP. Blots were visualized using ECL Detection Kit and exposed to X-Ray film. The numbers show the sizes (KD) of the prestained protein standards.

Table 1

Summary of ICAM-1 characteristics on different cell types

The cell lines studied were L (fibroblast), T28 (T cell hybridoma), A20 (B cell lymphoma), P388 (monocyte), and NS-1 (myeloma). The various ICAM-1 constructs transfected into the first two cell lines were full-length (EH), cytoplasmic truncation (PCR) and GPI-linked (GPI). + (yes), +++ (very much), +/- (variable), - (no), N/A (not available)

Cell	Ability to Aggregate	Distribution	Soluble	X-link & Soluble	Hi mol wt band
L EH	adherent	patchy	98%	N/A	+
L PCR	adherent	patchy	98%	N/A	+
T28 EH	+	uniform	+	+	+/-
T28 PCR	+	uniform	+	+	+/-
T28 GPI	+++	uniform	+	+/-	+/-
A20 A8	+	punctate	+	N/A	-
A20 CL7	+++	punctate	+	N/A	-
P388	+	N/A	+	+	N/A
NS-1	-	N/A	+	+	N/A

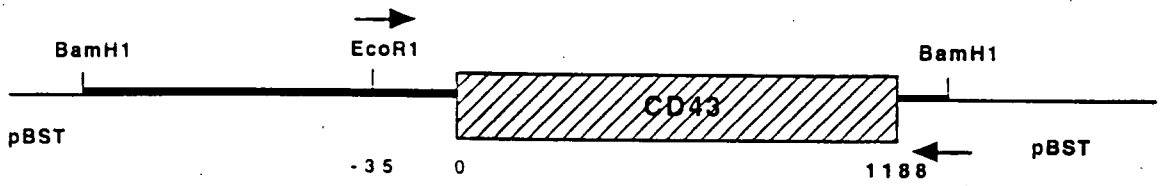


Figure 15

Generation of CD43 gene by PCR

Phage DNA was isolated from a purified plaque which contained the CD43 gene. This was digested with BamH1 and the resulting 2.3 Kb fragment containing CD43 gene, was cloned into the BamH1 site of the vector, pBluescript. Oligonucleotide primers (arrows) were generated to PCR amplify the CD43 gene and flanking sequences using the above genomic clone as a template.

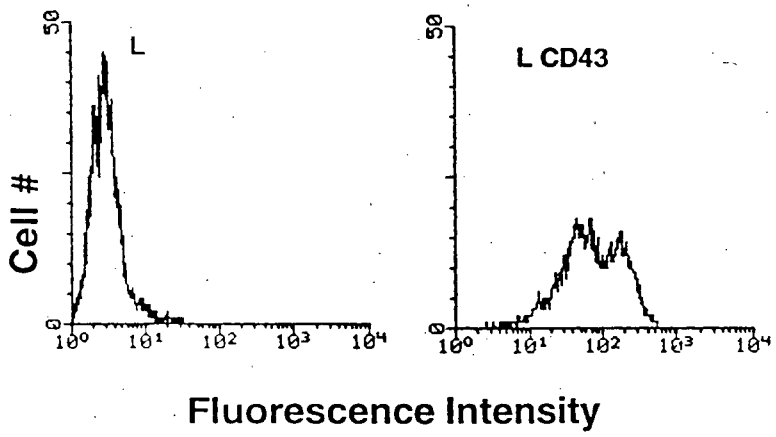


Figure 16
Flow cytometric analysis of L cells transfected with murine CD43
Untransfected (L) and transfected (LCD43) clones were labeled with biotinylated anti-CD43 antibody followed by strepavidin conjugated to FITC. Dead cells were excluded by PI staining.

coated with soluble ICAM-1. Nonadherent cells were removed by several washing steps. CD43$^+$ L cells did not bind significantly more than the control untransfected L cells to immobilized ICAM-1 (Figure 17). The positive control, LFA-1 transfected fibroblasts expressing high-avidity LFA-1 (Pyszniak 1994) had a significant degree of binding, which was inhibited by the mAb YN1/1 (Figure 17).

2.3 Bead Binding Assay

In addition to the adhesion assay above, the binding of microspheres coated with soluble ICAM-1 to CD43 transfected L cells was tested. These beads were incubated in wells with a monolayer of CD43$^+$ fibroblasts or control cells (untransfected or LFA-1 transfected L cells). After extensive washing to remove nonadherent beads, wells were observed by microscope. The beads appear as small black dots in the photos (Figure 18). The small degree of ICAM-1 coated beads bound to CD43$^+$ L cells was not significantly higher than the negative control coated beads bound to similar cells or the soluble ICAM-1 coated beads to untransfected fibroblasts. It was clearly insignificant when compared to the positive control of soluble ICAM-1 coated beads bound to LFA-1$^+$ L cells (Figure 18). Therefore, no interaction between ICAM-1 and CD43 was detected by these methods.

3.0 Fas chimeric Model

3.1 Generation of ICAM-1/Fas Chimeric Cells

The interaction between murine CD43 and ICAM-1 may be of very low affinity and thus may be very difficult to detect in a standard adhesion or bead binding assay. Therefore, an alternative approach was taken to study this potentially weak interaction. To utilize the ability of the Fas molecule to mediate apoptosis as a read out for an association with ICAM-1, an ICAM-1/Fas chimeric construct was made.

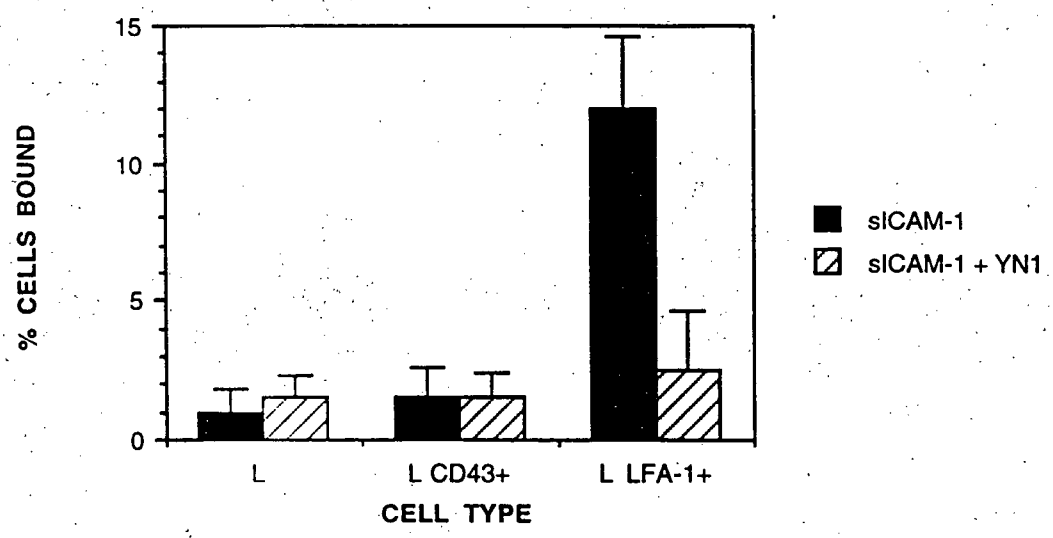


Figure 17

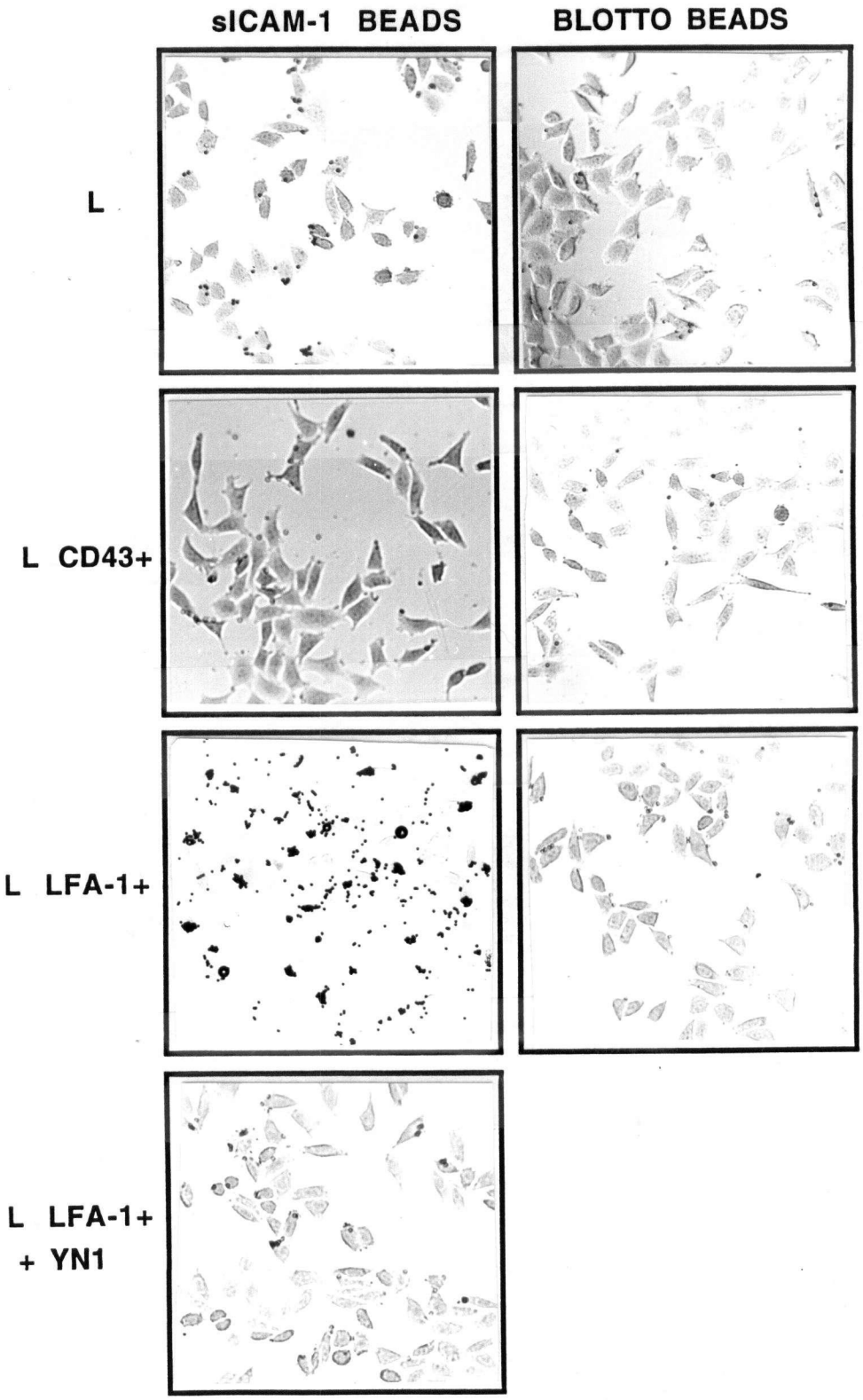
Adhesion Assay for CD43+ L cells to immobilized ICAM-1

Soluble ICAM-1 was adsorbed to wells of a 96 well plate. Fibroblasts (L) transfected with and expressing either CD43 (LCD43) or LFA-1 (L LFA-1) were labeled with calcein and added to wells in the presence or absence of mAb YN1/1 (anti-ICAM-1). Following 15 minute incubation and extensive washing, fluorescence was detected by CytoFluor 2300 Fluorescence Measurement System. The percentage of cells bound was determined from a standard curve.

Figure 18

Bead Binding Assay

Untransfected or transfected L cells expressing either CD43 (LCD43+) or LFA-1 (L LFA-1+) were seeded in 96 well plate. sICAM-1 or blotto coated beads were added to wells in the presence or absence of mAb YN1/1 (anti-ICAM-1), incubated at 37 ° C for 25 minutes, and washed extensively. The cells were fixed and stained before photo was taken using a Nikon Diaphot microscope.


sICAM-1 BEADS
BLOTTO BEADS
L
L CD43+
L LFA-1+
L LFA-1+
+ YN1

A PCR product which encoded the 5 extracellular domains of ICAM-1 (missing the last 7 amino acids of domain 5 and containing one irrelevant amino acid) was ligated with the complete transmembrane and cytoplasmic domain of the Fas molecule (with 2 amino acids from the extracellular domain) (Figure 1). Fibroblasts were transfected with this construct and clones were selected by G418 resistance. Expression of the chimeric molecule was confirmed by FACS analysis (Figure 19).

3.2 Cell Death Assay

Preliminary experiments were performed to establish if binding of anti-ICAM-1 antibody (YN1/1) to ICAM-1/Fas chimeric molecules on the transfected L cells induces their death. This was expected since anti-Fas antibody can induce apoptosis of cells expressing the Fas molecule (Itoh et al., 1991). When antibodies were incubated with L cells expressing the chimeric molecule, approximately 50% of the cells died upon treatment with YN1/1 antibody but not with control antibody, YE1/30 (anti-Thy-1), nor when L cells expressing full-length ICAM-1 were treated with the same antibodies (Figure 20). These results demonstrated that the Fas portion of the chimeric molecule is functional and transduces signals leading to cell death upon antibody binding. The next step was to determine if ligand binding could also generate the same.

3.3 Ligand binding and Cell Death

To quantitate the degree of cell death induced by the binding of ligand to ICAM-1/Fas chimeric molecule, a ^{3}H-thymidine incorporation assay was utilized. The transfected L(TK^{-}) cells were further transfected with thymidine kinase to enable dividing L cells to incorporate ^{3}H-thymidine. Since dying or dead cells should no longer take up thymidine, the degree of cell death can be determined by a reduced incorporation of ^{3}H-thymidine. As expected, YN1/1 treatment of the chimeric^{+} L cells reduced incorporation of ^{3}H-thymidine by approximately 50% (Figure 21).

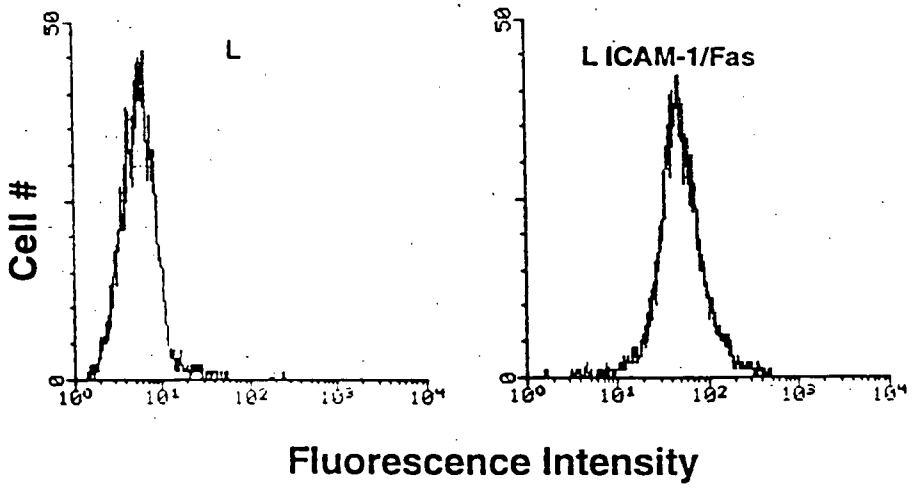


Figure 19

Flow cytometric analysis of L cells transfected with ICAM-1/Fas chimeric molecule

Untransfected (L) and transfected (LICAM-1/Fas Chimeric) clones were labeled with anti-ICAM-1 mAb, YN1/1 followed by GαRIgG conjugated to FITC. Dead cells were excluded by PI staining.

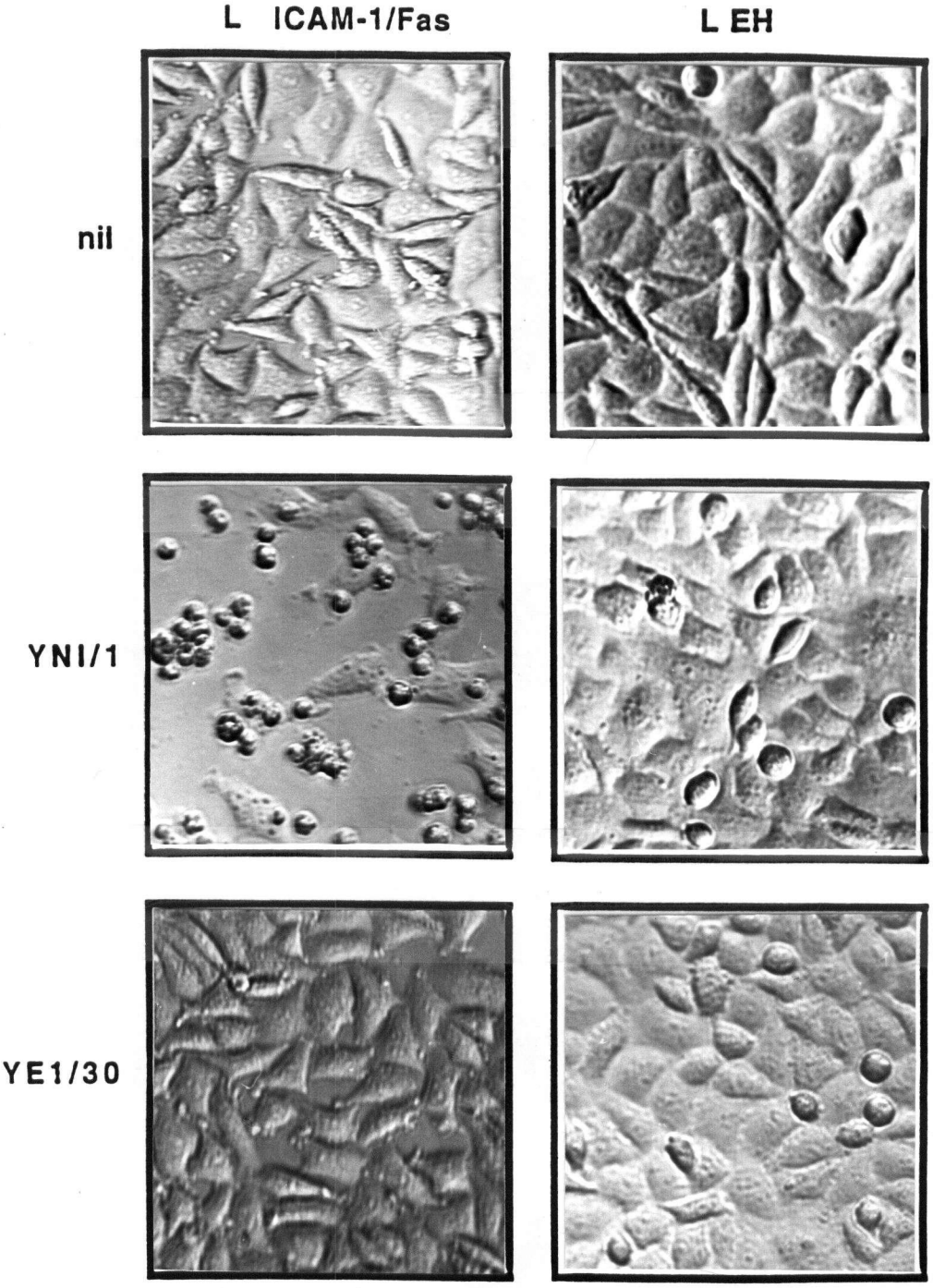


Figure 20

Cell Death Assay

Fibroblasts (L), transfected with either full-length ICAM-1 (L EH) or a chimeric ICAM-1/Fas (L ICAM-1/Fas) were grown overnight in 96 well plates. mAb, YN1/1 (anti-ICAM-1) (2μg/ml) or control antibody, YE1/30 (anti-Thy-1) (2μg/ml) was added and cells were further grown overnight before cell death was observed and photographed with a Nikkon Diaphot microscope.

However, when the chimeric$^+$ cells were incubated with either irradiated LFA-1$^+$ or CD43$^+$ L cells, incorporation of ^{3}H-thymidine was similar to the controls, indicating that cell death was not induced (Figure 21).

3.4 Cell Adhesion Assay: Activated Splenic T cells to ICAM-1/Fas Chimeric Cells

To confirm that the ICAM-1 portion of the chimeric molecule is functional, the binding of LFA-1$^+$ cells to ICAM-1/Fas chimeric transfected L cells was tested. PMA activated splenic T cells were used as a source of LFA-1$^+$ cells since LFA-1 transfected fibroblasts gave too high a background due to the adherent nature of fibroblasts themselves. Approximately 50% of activated splenic T cells bound to both of the chimeric clones #7 and #11 (Figure 22), whereas less than 30% bound to untransfected L cells. Almost all activated splenic T cells bound to ICAM-1 transfected L cells. The results indicate that the chimeric molecule is able to bind LFA-1. The low degree of binding of splenic T cells to the chimeric molecule is probably due to the relatively low level of expression of the chimeric molecule on transfected L cells as compared to that of the full length ICAM-1 (Figure 23). Whether the level of the binding of LFA-1 to the chimeric molecule is too low to induce detectable cell death remains to be determined.

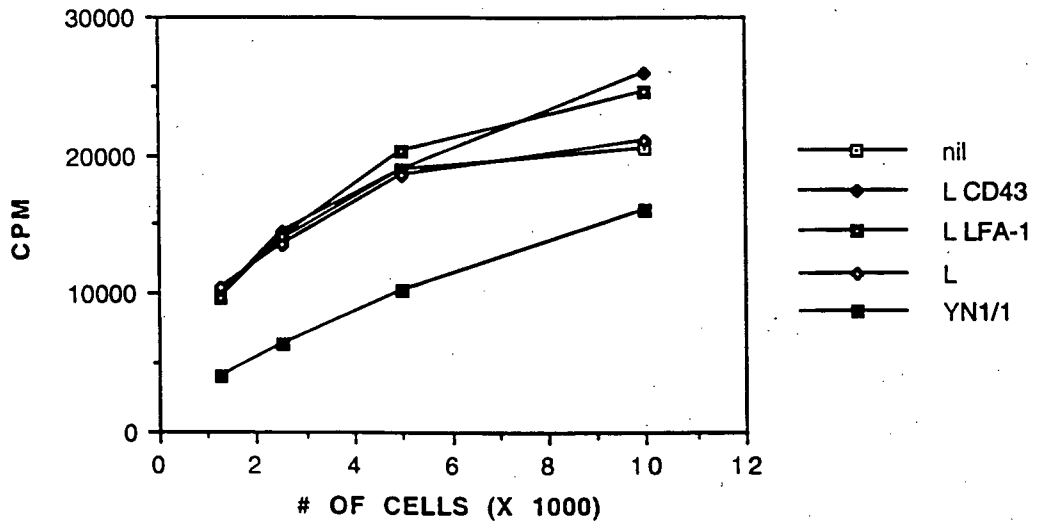


Figure 21

^{3}H-thymidine incorporation assay for cell death

Fibroblasts transfected with ICAM-1/Fas were grown overnight in 96 well plate at different cell densities. Untransfected fibroblasts(L) or fibroblasts expressing hi avidity LFA-1 (L LFA-1) or mCD43 (L CD43) were irradiated and added to wells for 24 hours. mAb, YN1/1 was also added at this time. Cells were then incubated with 1.0 μCi/well ^{3}H-thymidine for 4 hours before harvesting.

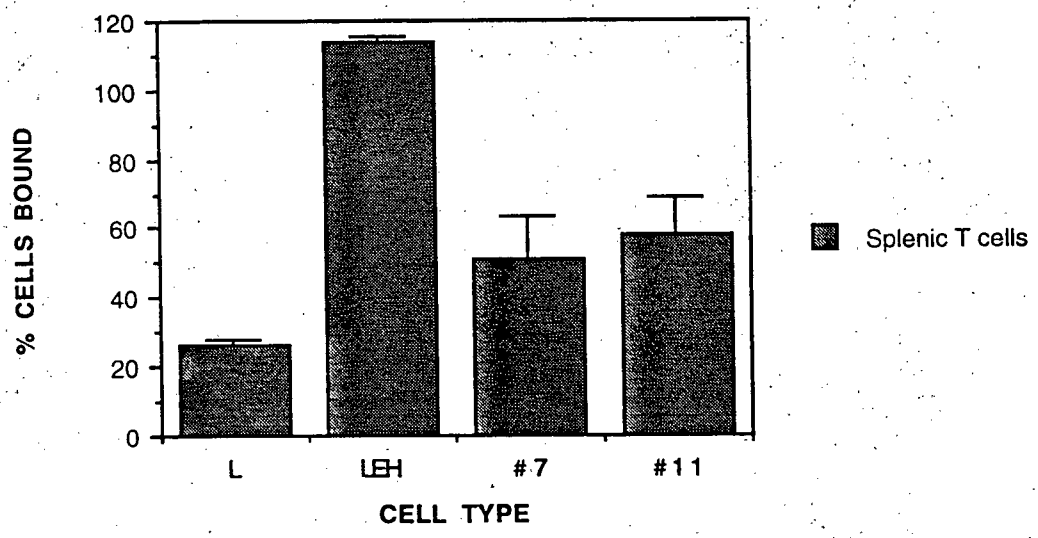


Figure 22

Adhesion Assay of PMA activated splenic T cells to ICAM-1/Fas chimeric+ L cells

2 X 10^4 chimeric$^+$ L cells (#7, #11) or control cells were seeded in wells of a 96 well plate. PMA activated splenic T cells labeled with calcein were added to wells and incubated for 10 minutes at 37 °C before extensive washing. Fluoresence was detected by CytoFluor 2300 Fluorescence Measurement System. The percentage of cells bound was determined from a standard curve.

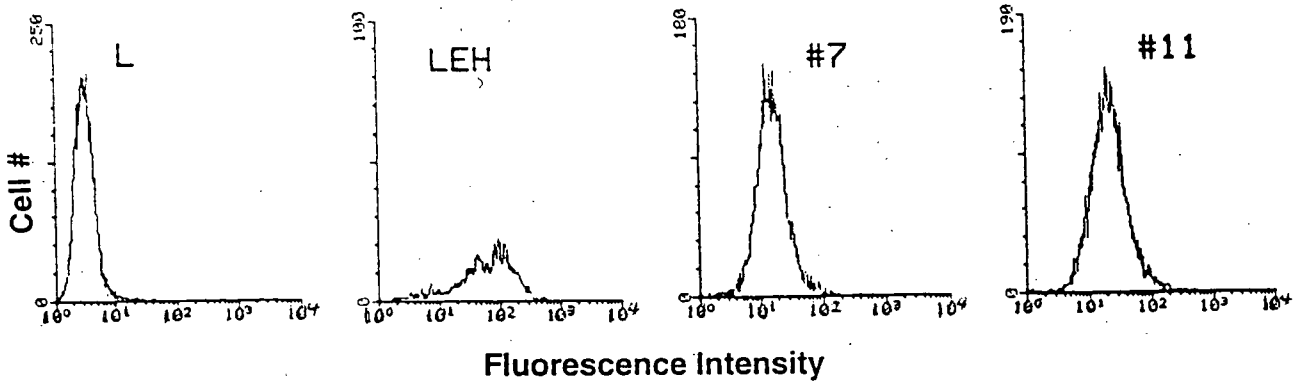


Figure 23

Flow cytometric analysis of L cells transfected with ICAM-1 or ICAM-1/Fas chimeric molecule

Untransfected (L), ICAM-1 transfected (LEH), and two clones of ICAM-1/Fas chimeric transfected (#7, #11) cells were stained with mAb YN1/1, followed by Gα RIgG conjugated to FITC. Dead cells were excluded by PI staining.

DISCUSSION

1.0 ICAM-1 Association with the Cytoskeleton

One of the goals of this thesis was to investigate the association of ICAM-1 with the cytoskeleton. It was hypothesized that the distribution pattern of ICAM-1 on the cell surface is determined by its association with the cytoskeleton, and that the distribution of ICAM-1 may affect its cell adhesion function. A previous study in our laboratory has shown monovalent soluble ICAM-1 is unable to bind LFA-1. However, when it is immobilized on microspheres, these ICAM-1 coated beads can bind and competitively inhibit binding of LFA-1 to immobilized purified ICAM-1 (Welder et al., 1993). Thus, multivalency appears to be important for the binding of ICAM-1 to ligand. Localization of ICAM-1 on the cell surface may establish a similar multivalency to aid in its cell adhesion function.

Fluorescence microscopy has demonstrated that ICAM-1 on L cells shows a unique clustered distribution pattern, which is enriched on microspikes. Carpen et al. has also shown that human ICAM-1 on COS cells is localized to microvillar projections whereas a GPI-linked version of ICAM-1 on similar cells is evenly distributed over the surface. From this observation, the cytoplasmic domain of ICAM-1 was suggested to be responsible for this difference in distribution (Carpen et al., 1992). To further investigate this, in addition to a GPI-linked form, we generated a truncated ICAM-1 lacking the cytoplasmic domain. Several cell lines expressing these different forms of ICAM-1 were established to clarify if indeed, this region of the molecule participates in the regulation of distribution and cytoskeletal interactions. The results from this study however, were inconsistent with the hypothesis. Although different patterns of ICAM-1 distribution were observed among transfected fibroblasts and T cells, the distributions among the various forms of ICAM-1 remained similar. This suggests that the cytoplasmic domain does not participate in determining the distribution of ICAM-1 on the cell surface. However,

from the observation in the fluorescent microscopy study that various distribution patterns do exist between different cells types, it was predicted that ICAM-1 can associate with the cytoskeleton directly or indirectly to allow this cell type specific clustering on the cell surface to occur. This in fact was observed in the previous study by Carpen et al. Disruption of the cytoskeleton by cytochalasin B changed the localization of ICAM-1 to an even pattern but had no effect on the GPI-linked ICAM-1. A 28-residue peptide, representing the cytoplasmic domain of ICAM-1, was synthesized and used to make an affinity matrix column to characterize the cytoskeletal protein which could associate with ICAM-1. α-Actinin was eluted from the column and was shown to bind purified ICAM-1 (Carpen et al., 1992). However, when investigated in this thesis using detergent solubility experiments, the results were unexpected. ICAM-1 was completely solubilized in all cell types suggesting it does not associate with the cytoskeleton. These results therefore contradict the earlier experiments by Carpen et al.

The conflicts may be explained, in part, by the different cell types used in these studies. Fibroblasts and T cells may utilize different mechanisms for regulating distribution than the adherent COS cells and EBV- transformed B cells that were used in the previous study. Also, because the fibroblasts were in suspension when detergent extracted, cytoskeletal associations that occur in the normal adherent state, may have been disrupted. Murine ICAM-1 was also used in this thesis which may account for these different results as well. The methods used to detect cytoskeletal association were also very different.

The method of detergent extraction to determine cytoskeletal association, has been widely used. To identify an mIgM capping correlation with cytoskeletal association, a previous study used a combination of fluorescent staining, detergent extraction and flow cytofluorometric analysis. When a secondary mAb, which recognized the FITC-labeled mIgM, was added to the cells before disruption by

detergent, the cytoskeletons were positive for fluorescence. Fluorescence was lost if cross-linking antibody was not added. This indicated the capping of mIgM induced the association of this molecule with the cytoskeleton (Albrecht and Noelle, 1988). This method was also used to determine the association of various T cell surface molecules with the cytoskeleton and the effect of cross-linking and activation on these interactions (Geppert and Lipsky, 1991). In this later study, CD2, CD4, CD8, CD11a/CD18, CD44 and class I MHC were from 7-50% resistant to detergent solubilization. It was concluded that a fraction of these molecules are constitutively associated with the cytoskeleton. Cross-linking these molecules with secondary antibody further enhanced the detergent insolubility. They also suggested that the association with cytoskeleton is a dynamic process that varies with the state of activation or differentiation of cells, as in the case of CD2, CD3, and CD44 (Geppert and Lipsky, 1991). However, in these initial studies, spleen cells and resting peripheral blood T cells were used, respectively. If a specific state of activation and differentiation of cells is required for cytoskeletal association, then perhaps the fibroblast and T cell lines used in this thesis were not appropriate. This may then explain why no cytoskeletal association was observed with ICAM-1 in this thesis. Also, if cytoskeletal association was sensitive to detergent extraction in these cells, perhaps a cross-linking antibody could be used in future experiments to increase the ability of this method to detect insoluble proteins, similar to the experiment by Geppert and Lipsky.

As eluded to above, association with the cytoskeleton may also be cell-type specific. In an investigation of CD44 association with the cytoskeleton, CD44 was completely detergent soluble in epithelial cells while in fibroblasts a significant portion remained in the insoluble fraction. These results imply that whatever the mechanism in fibroblasts to allow cytoskeletal association, this mechanism does not exist in epithelial cells (Neame and Isacke, 1993). We found that a very small

amount of ICAM-1 was Triton X-100 insoluble in the fibroblast cell line but not in lymphocytes. This suggests that a mechanism that is unique to fibroblasts is involved in coupling ICAM-1 to the cytoskeleton. In addition, all of the cell types used were non-polarized and thus it may have been more difficult to detect an association with the cytoskeleton in these cell types. Cell surface molecules on polarized cells have a much clearer association with the cytoskeleton (Mays et al., 1994). Therefore these cells would be a good model to work with in future experiments.

In addition to the truncated ICAM-1 lacking the cytoplasmic domain, a GPI-linked ICAM-1 was constructed to determine if the absence of the transmembrane domain affected distribution and cytoskeletal association, thereby establishing a role for this region. The transmembrane region, as well as the cytoplasmic domain of MHC class II molecule was recently reported to participate in cytoskeletal association as well as the cytoplasmic domain (Chia et al., 1994). Although this may mediate a weaker interaction than the cytoplasmic domain, an alternative or additional cytoskeletal binding site in the transmembrane domain could maintain an attachment site if the cytoplasmic domain was occupied by signaling proteins thereby making it unavailable (Chia et al., 1994).

GPI-linked form of ICAM-1 on T28 cells had quite uniform distribution, similar to other forms of ICAM-1 in T28 cell line. Therefore, the transmembrane domain does not affect distribution of ICAM-1 in these cells. Carpen et al. also reported similar uniform distribution of GPI-linked ICAM-1 when expressed on COS cells. Unfortunately, this molecule was not appropriate to study cytoskeletal association using the method of detergent extraction. Several studies have shown that GPI anchored proteins are solubilized poorly by nonionic detergents such as Triton X-100 (Low and Saltiel, 1988). Because they are limited to the extracellular side of the cell, they can not bind to the cytoskeleton directly. In another study, GPI-linked

proteins did have variable solubility although this was not due to cytoskeletal association. Rather, the insolubility was related to the environment of the protein at the time of solubilization and not to the intrinsic resistance to solubilization or nonspecific aggregation (Brown and Rose, 1992). Therefore the role of the transmembrane region of ICAM-1 in establishing an interaction with the cytoskeleton could not be investigated.

Unfortunately a positive control for the solubility experiment was not found. Although several molecules remain in the insoluble fraction upon detergent extraction signifying cytoskeletal association, as previously described (Geppert and Lipsky, 1991), an antibody which could detect one of these proteins by Western blot analysis, was unavailable. Thy-1 was used as a control to establish that proteins could be detected in the insoluble fraction. However, because this molecule is GPI-linked, the insolubility was not due to cytoskeletal association as described above and therefore was not the most effective control.

It was unexpected that ICAM-1 did not associate with the cytoskeleton in this thesis. In a recent study, ICAM-3 localized to the heading uropod after treatment of lymphoid cells with a specific activating antibody against ICAM-3 (Campanero et al., 1994). In fluorescent studies, linear arrays of myosin were identified in the uropods. Treatment of these cells with a myosin disrupting drug caused a uniform distribution of ICAM-3 on the cell surface. This suggested a myosin interaction was responsible for this distinct distribution of ICAM-3. A similar cytoskeletal disrupting drug could be used in future experiments with the fibroblasts in this thesis to determine if a cytoskeletal interaction is responsible for the punctate distribution of ICAM-1 observed on these cells.

The results from this thesis suggest ICAM-1 is not significantly associated with the cytoskeleton in the cells examined. They also suggest the cytoplasmic domain is not required for the distinct distribution patterns observed. Thus, there

must be some other mechanism which can regulate the distribution of ICAM-1 on the surface of these cells. The distribution and lack of cytoskeletal association also do not correlate with the ability of these cells to aggregate and therefore it remains unknown if these variables affect cell adhesion function.

The dimer formation of ICAM-1 which is suggested by the chemical cross-linking studies is consistent with a recent report using flow cytometric energy transfer to detect the lateral distribution of several different molecules on either T cell or B cell surface. These findings suggest that ICAM-1 molecules are at least dimers or higher oligomers in the plasma membrane of a T cell line while they are mostly expressed as monomers at the surface of a B cell line (Bene et al., 1994). This does correlate somewhat with our findings. In this thesis, ICAM-1 on T cells yet not on B cells showed this apparent dimer formation. However, it should be noted, ICAM-1 on B cells is endogenous whereas T cells had been transfected and expressed quite high levels of ICAM-1. Whether this is significant remains to be examined. These results also suggest either the extracellular or transmembrane domain is required for this potential self-association since the high molecular weight band was observed with the cytoplasmic truncated ICAM-1 as well.

A previous study determined the oligomeric nature of murine CD23, FcεRII, by a similar chemical cross-linking experiment. Only this formation had sufficient affinity/avidity to bind its ligand, IgE (Dierks et al., 1993). Thus, dimer formation may have important consequences for ICAM-1 function as well and should therefore be further investigated.

The suggested dimer formation of ICAM-1 from cross-linking studies can also be supported by evidence from the Fas chimeric model, which will be discussed in more detail later. If ICAM-1 is a monomer on the cell surface, then simple cross-linking of the ICAM-1/Fas chimeric molecule by antibody should not have induced cell death, since it is now known that more than a divalent interaction is required to

induce the signal for apoptosis as the ligand for the Fas molecule is trimeric in nature (Suda et al., 1993). Thus if ICAM-1 is found on the surface as at least a dimer, this could explain why cell death was observed by cross-linking initiated by a divalent antibody.

2.0 ICAM-1 and CD43 Interaction

To this point, CD43 remains a mystery molecule. It plays a significant role in T cell activation yet, its ligand has not been clearly defined. Its large size and high degree of negative charge suggest that it would not be involved in cell adhesion. However, there is some evidence that CD43 binds to ICAM-1. The experiments in this thesis regarding CD43 were inspired by those previous results of Rosenstein et al. that suggested human ICAM-1 is the ligand for CD43. They demonstrated that anti-ICAM-1 mAb could block Daudi cell binding to plate bound purified CD43 and also a $CD43^+$ T cell hybridoma could bind immobilized recombinant human ICAM-1. In addition, CD43 and ICAM-1 were incorporated onto separate cell sized beads and upon incubation, 42% conjugates were formed. These initial adhesion assays and bead aggregation studies using purified recombinant soluble ICAM-1 and CD43, did not establish an interaction between these two molecules when expressed on the cell surface. Thus, a physiological interaction was not established. Our goal was to determine if this CD43/ICAM-1 interaction can mediate cell/cell adhesion and whether the interaction is cis or trans. However, our results did not detect an interaction between murine ICAM-1 and CD43.

To test cell adhesion mediated by these molecules, L cells expressing CD43 were generated to eliminate the possible interaction of ICAM-1 with its other ligands in adhesion assays. L cells expressing an ICAM-1/Fas chimeric molecule were also generated to detect a potentially weak interaction by a new model system utilizing induction of cell death. To examine a cis interaction, an attempt was made to express both CD43 and the chimeric molecule on the same cell surface. If a cis

interaction exists, it was hypothesized, CD43 cross-linking by antibody binding would initiate death of these co-transfected cells. As well, cells expressing a CD43/Fas chimeric molecule were also generated for the reciprocal experiment. This, however, was unsuccessful. Although the results of this thesis are not conclusive, they do appear to contradict those of Rosenstein. The methods of cell adhesion and bead binding using sICAM-1 were similar to those of Rosenstein et al., however the results of these conventional assays in this thesis were negative. There may be several explanations for this.

This study utilized a murine system whereas the studies of Rosenstein involved human CD43 and ICAM-1. In addition, CD43 in this study was expressed on a fibroblast cell line, unlike the COS cells used in the previous investigation. This cell type difference could alter the binding function of CD43 and/or ICAM-1 as has been shown for the integrin molecule, LFA-1. When expressed on fibroblasts, LFA-1, is constitutively avid and able to bind ICAM-1, unlike on lymphocytes where adhesion between these molecules is dependent on cellular activation (Dustin and Springer, 1991).

The expression level of CD43 on transfected fibroblasts may not have been sufficient to support cell adhesion. However, it is also possible that the interaction between murine ICAM-1 and CD43 in this system, is very weak and as a result, is difficult to detect in vitro utilizing an adhesion or bead binding assay. This is highly plausible as both of these methods require a harsh washing regime that may disrupt a weak interaction. Thus, based on the results from the adhesion and bead binding assays, the interaction of murine ICAM-1 with CD43 can not be completely ruled out. It remains to be shown whether ICAM-1 on a cell surface can associate with CD43 on a cell as well. Also, if this pair do interact, a cis or trans interaction must be determined. Most likely the interaction is cis, since ICAM-1 and CD43 have been observed to co-cap on the surface of certain cells (G. Dougherty, unpublished). The

evidence that suggests CD43 decreases cell adhesion by its large size and highly negative charge also make a cis interaction more favourable. A cis interaction would however be difficult to establish.

To overcome the problem of very weak interactions and high levels of background in cell adhesion assays, the Fas chimeric model system was developed. This system would also be able to detect cis interaction between CD43 and ICAM-1 by co-expression on a cell. The initial results using antibody to induce cell death were promising for the new model system. However, neither LFA-1 positive cells nor CD43 positive cells caused cell death even though a cell adhesion assay showed that LFA-1 positive cells bound to the ICAM-1/Fas chimeric molecule. But, because activated splenic T cells bound to the ICAM-1/Fas chimeric positive L cell monolayer considerably less than the positive control, namely L cells transfected with the full length ICAM-1, it is difficult to conclude whether there was sufficient adhesion between LFA-1 and ICAM-1/Fas to induce cell death. It should be noted, the expression level of ICAM-1 on the positive control cells, LEH, was approximately 5 fold higher than the level of the ICAM-1/Fas chimeric. Therefore, these two cell types can not be compared directly with regards to their binding capabilities. It has been observed in this laboratory, that LFA-1 requires a certain threshold level of expression of ICAM-1 for adhesion to occur (Pyszniak, unpublished). This may explain why the level of binding of splenic T cells to chimeric positive cells was half as much as to full-length ICAM-1 positive cells. Although there was still significant binding of LFA-1 positive splenic T cells to the chimeric molecule, the degree of binding may have been insufficient to induce cell death. It is possible that a certain threshold level of binding is required to induce cell death as well. With antibody binding, the majority of chimeric molecules were likely cross-linked and therefore cell death was readily induced.

Because LFA-1 positive cells did not induce cell death, yet a significant number of LFA-1 positive cells bound to the chimeric molecule, it is also possible that this interaction did not cross-link the chimeric molecule. This would suggest the LFA-1/ICAM-1 interaction is monovalent. In that case, antibody binding may not always be a good representation of ligand binding since, it can result in very different signaling.

The recent identification of the Fas ligand may also explain some of the results obtained above. The ligand is a type II transmembrane protein which belongs to the TNF family. There are some conserved amino acids with TNFα and TNFβ suggesting the Fas ligand has a similar structure to these molecules. Also there are two cysteine residues in the extracellular domain of the Fas ligand which correspond to those in TNFα. From these similarities, it is hypothesized that the Fas ligand is also trimeric in structure which suggests that perhaps more than a divalent interaction is required for transduction of the apoptotic signal (Suda et al., 1993). If that is the case, it is not surprising that LFA-1 was unable to induce cell death. It also suggests, this system may not be a good one to establish new interactions as very few interactions are probably trimeric in nature. Thus, further investigation of ICAM-1 interaction with CD43 is required in the murine system. If an interaction does occur, it is too weak to be detected by typical adhesion assays and therefore a another method must be developed to investigate this.

A cis interaction may exist between ICAM-1 and CD43 molecules. This may explain why L cells expressing both CD43/Fas and ICAM-1 or ICAM-1/Fas and CD43 molecules failed to grow. Cells positive for both of these proteins may not be viable since a cis interaction between these molecules may induce their death. Further investigation with the necessary controls is required to confirm these observations.

The interactions of ICAM-1 with molecules that make up the cytoskeleton and CD43 remain unclear. It seems this molecule and its interactions are quite elaborate, as illustrated by recent observations that ICAM-1 is a receptor for hyaluronan (McCourt et al., 1994). The complete role of ICAM-1 in immune and inflammatory response is still unraveling. Therefore, further investigation of this molecule and its interactions is required.

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