FUNCTIONAL ANALYSIS OF ICAM-1:LFA-1 INTERACTION IN CELL ADHESION

by

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We accept this thesis as conforming to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

March 1992

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Date 31 March 1992
Abstract

This thesis presents the results of a project aimed firstly at exploring the use of a soluble form of ICAM-1 to inhibit cellular immune responses, which in general rely heavily on the interaction between the counter receptors ICAM-1 and LFA-1, and aimed secondly at studying the mechanisms regulating ICAM-1:LFA-1 mediated cell adhesion. Functional characterization of the purified sICAM-1 demonstrated that radio-iodinated sICAM-1 could bind to LFA-1 positive cells, albeit with an apparently low affinity. However, when immobilized on plastic, sICAM-1 was fully functional, efficiently facilitating LFA-1:ICAM-1 mediated cell adhesion. Two mechanisms of inducing LFA-1:ICAM-1 mediated cell adhesion were characterized: stimulation by the phorbol ester PMA and stimulation by the divalent cation Mn++. PMA induced adhesion was dependant on a functional actin cytoskeleton as judged by the inhibitory effect of cytochalasin B. PMA induced adhesion could not be inhibited by sICAM-1, suggesting that this cell adhesion was mediated by low affinity ICAM-1:LFA-1 interaction. In contrast, Mn++ induced adhesion seemed to be mediated by an increase in the affinity of the ICAM-1:LFA-1 interaction; Mn++ induced homotypic aggregation could be specifically inhibited by sICAM-1. While monovalent sICAM-1 could not efficiently inhibit PMA induced adhesion, a multivalent form of sICAM-1 could inhibit PMA induced ICAM-1:LFA-1 mediated cell adhesion, demonstrating the importance of multivalent interaction in LFA-1:ICAM-1 mediated cell adhesion. Fluorescence microscopic studies aimed at determining the cell surface distribution of ICAM-1 and LFA-1 clearly showed that the distribution of ICAM-1 could be differentially regulated. With LFA-1, the regulation of it's cell surface distribution could not be demonstrated using immunofluorescence microscopy. Studies were conducted to define a role for the cytoplasmic domain of ICAM-1 by transfecting ICAM-1 with or without it's cytoplasmic domain into ICAM-1 negative cells. These studies demonstrated that removal of the cytoplasmic domain of ICAM-1 resulted in a decreased but
not abolished aggregative phenotype. However, a precise role for the cytoplasmic domain of ICAM-1 in determining the cell surface distribution of ICAM-1 could not be defined.

These results are discussed in relation to the literature concerning soluble adhesion molecules and the regulatory mechanisms governing cell adhesion. It seems likely that sICAM-1, at least in its monovalent form, has little potential as an inhibitor of *in vivo* immune responses. It is hypothesized that there are three primary mechanisms regulating cell adhesion mediated by ICAM-1 and LFA-1. The first mechanism is simply the amount of LFA-1 or ICAM-1 a given cell expresses on its surface, the second mechanism is the modulation of the affinity of the interaction between ICAM-1 and LFA-1, and the third mechanism is the regulation of the distribution of adhesion molecules on the cell surface. Each of these regulatory mechanisms is discussed with reference to the results presented in this thesis as well as the results in the literature.
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<tr>
<td>Ab</td>
<td>antibody</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
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<tr>
<td>CD</td>
<td>cluster of differentiation</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>CHO</td>
<td>chinese hamster ovary</td>
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<tr>
<td>CPM</td>
<td>counts per minute</td>
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<tr>
<td>cyto. B</td>
<td>cytochalasin B</td>
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<tr>
<td>DAB</td>
<td>3,3'-diaminobenzidine tetrahydrochloride</td>
</tr>
<tr>
<td>DABCO</td>
<td>1,4-diazobicyclo-(2,2,2)-octane</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's modified minimal essential medium</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylene diamine tetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme linked immunosorbent assay</td>
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<tr>
<td>FACS</td>
<td>fluorescence activated cell sorter</td>
</tr>
<tr>
<td>FCS</td>
<td>fetal calf serum</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
</tr>
<tr>
<td>GVHD</td>
<td>graft versus host disease</td>
</tr>
<tr>
<td>fMLP</td>
<td>formyl-methyl-leucyl-phenalanine</td>
</tr>
<tr>
<td>Hanks'</td>
<td>Hanks' balanced salt solution</td>
</tr>
<tr>
<td>Hepes</td>
<td>N-2-hydroxyethylpiperazine</td>
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<tr>
<td>hr.</td>
<td>hour</td>
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<tr>
<td>ICAM</td>
<td>intercellular adhesion molecule</td>
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<tr>
<td>IFN</td>
<td>interferon</td>
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<td>Ig</td>
<td>immunoglobulin</td>
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IL: interleukin
inh.: inhibitor
kd: kilodaltons
LAD: leukocyte adhesion deficiency
LFA: lymphocyte function associated
MHC: major histocompatibility complex
MALA: murine activation lymphocyte antigen
min.: minute
mRNA: messenger RNA
MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
OVA: ovalbumin
PAGE: polyacrylamide gel electrophoresis
PBS: phosphate buffered saline
PCR: polymerase chain reaction
PFHM: protein free hybridoma medium
PKC: protein kinase C
PMA: phorbol myristate acetate
RPM: revolutions per minute
SDS: sodium dodecyl sulphate
SN: supernatant
sICAM-1: soluble ICAM-1
TcR: T cell receptor
TNF: tumour necrosis factor
Tris: tris(hydroxymethyl)aminomethane
T.R.: transferrin receptor
TRITC: tetramethylrhodaminly isothiocyanate
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*Happy is the man who finds wisdom,*  
*and the man who gets understanding,*  
*for the gain from it is better than gain from silver*  
*and its profit better than gold. Proverbs 2: 13,14*
Introduction

Adhesion molecules

Adhesive interactions between cells or between cells and the extracellular matrix are mediated, by definition, by adhesion molecules. Adhesion, and therefore adhesion molecules, are fundamentally important to all multicellular animals. Adhesive interactions mediated by adhesion molecules are responsible to a large extent for the organization and maintenance of the various tissues and organs of the body, as well as being required for a wide range of biological functions. Since a cell is essentially a sphere with a net negative surface charges, electrostatic forces tend to repel cells from one another, and adhesive interactions are required to stabilize cell:cell contact. A stereochemical fit between complimentary molecules, which is stabilized by hydrophobic interactions, ionic interactions, hydrogen bonding and other van der Waals interactions, provides the molecular basis for adhesive interaction (1).

As an example of the general importance of adhesion molecules, consider the cadherins, a group of adhesion molecules responsible for maintaining multicellular structures, especially in the embryonic development of vertebrates (2). Cadherins often function by homotypic interaction, and have a strict requirement for calcium ions. It has been observed that anti-cadherin antibodies disrupt normal embryonic development (3). As I will discuss in more detail later, antibodies directed against adhesion molecules utilized by the immune system effectively inhibit the function of the immune system.

Adhesion molecules in the immune system

The immune system of higher animals is a particularly interesting system in that it is comprised of a network of individual cells circulating throughout the body, yet the system functions in a highly organized and regulated fashion. The organization and regulation of the immune system is, to a great extent, due to the use of a variety of adhesion molecules by the
The general 'job description' of the immune system is to discriminate self from non-self, and eliminate the latter. Such discrimination necessitates cell:cell recognition, and such cell:cell recognition is facilitated and stabilized by adhesion molecules. Adhesion molecules in the immune system facilitate a variety of functions ranging from lymphocyte recirculation and homing to lymphocyte:target or lymphocyte:accessory cell interactions (reviewed in 4). Adhesion molecules of the immune system are involved in cell:cell as well as cell:matrix interactions. Most of the adhesion molecules utilized by the immune system belong to one of two major groups, those groups being the integrins and the Ig superfamily.

Members of the Ig superfamily all have in common the Ig homology unit, a linear stretch of about 110 amino acids which generally folds into a secondary structure consisting of 2 anti-parallel $\beta$ sheets stabilized by one disulfide bond. This domain structure was of course first characterized in immunoglobulin molecules. Members of the Ig superfamily usually have Ig-like homology units tandemly repeated. Of particular interest to this thesis is ICAM-1, a member of the Ig superfamily which has five extracellular Ig-like homology units (5). The Ig superfamily has been well reviewed (6).

Members of the integrin family all have in common a two chain structure. Each integrin molecule is a non-covalently associated $\alpha \beta$ heterodimer. Certain $\beta$ chains are found associated with various $\alpha$ chains, hence the $\beta_1$, $\beta_2$, and $\beta_3$ subfamilies. The structure and function of the integrins has been well reviewed (7). The family of integrins is involved in a variety of functions ranging from immune cell adhesion to wound healing. Of particular interest to this thesis is the $\beta_2$ integrin LFA-1.

Leukocyte adhesion deficiency disease (LAD) is a human hereditary disease in which patients suffer from life-threatening microbial infections (reviewed in 8). The disease is characterized by defective neutrophil mobility and phagocytosis, and the absence of lymphocytes and granulocytes in infected lesions despite chronic leukocytosis. The cause of this disease has been traced to a deficiency in $\beta_2$ integrin expression (LFA-1, Mac-1, and p150/95) resulting from decreased or complete lack of expression of functional $\beta$ chain (9).
The physiological importance of the β2 integrins is underscored by the serious consequences of the lack of their expression in LAD.

The β2 integrin lymphocyte function-associated antigen, LFA-1, was originally discovered by mAb which inhibit T cell mediated killing (10). Since that time a number of Ab to LFA-1 have been characterized, and all of those which inhibit T cell adhesion inhibit nearly every immune response involving T cells. LFA-1 has truly lived up to its name, lymphocyte function-associated antigen-1. In addition, the importance of adhesive contributions to T cell functions is emphasized by the importance of LFA-1:ICAM-1 interaction in T cell function. The interaction between LFA-1 and ICAM-1 is one of a number of interactions involved in the recognition and adhesion of T cells, as depicted in Figure 1.

The counter receptor for LFA-1, ICAM-1, was originally identified using the phorbol ester induced homotypic aggregation assay (11). In this assay, lymphocytes or lymphocytic cell lines aggregate only after stimulation by phorbol esters such as PMA. Aggregation in this assay is completely inhibited by Ab to LFA-1. It was observed that LFA-1 negative cells could co-aggregate with LFA-1 positive cells, suggesting that there was a counter receptor for LFA-1 on the LFA-1 negative cells. ICAM-1 was identified by immunization of mice with these LFA-1 negative lymphocytes and screening for Abs capable of inhibiting phorbol ester induced aggregation (12). Molecular cloning of ICAM-1 showed that it was a member of the Ig superfamily, with five extracellular Ig-like homology units (5). The presence of ICAM-1 confers the potential for ICAM-1:LFA-1 mediated adhesion (13). In addition to ICAM-1, ICAM-2 has been characterized as another counter receptor for LFA-1. ICAM-2 was originally defined by the ability of antibodies to LFA-1, but not by antibodies to ICAM-1, to inhibit the binding of T cells to endothelial cells (14). Expression cloning resulted in the isolation of a cDNA encoding ICAM-2, a molecule which has only 2 Ig-like homology units with 35% identity to the two N-terminal Ig-like homology units of ICAM-1 (15). Indeed there is evidence for a third counter receptor for LFA-1, based on the ability of certain cell lines to aggregate in an LFA-1 dependant but ICAM-1 and 2 independent manner (16).
Figure 1  A schematic representation of the molecules involved in T cell recognition and adhesion.
LFA-1 is expressed constitutively, but its expression is restricted to leukocytes (17). In contrast, ICAM-1 is highly inducible by inflammatory mediators. In the absence of inflammation, ICAM-1 is expressed on only a very few cells; however, ICAM-1 expression is induced by a number of inflammatory mediators including IL-1, TNF, and γIFN (18). ICAM-1 expression is inducible on a variety of cell types including hemopoietic, fibroblastic and endothelial cells. ICAM-2 expression differs from ICAM-1 expression markedly in that it is expressed well basally on endothelial cells and is not upregulated by inflammatory mediators (19).

It is clear that there are multiple ICAM counter receptors for LFA-1. One recent report also suggests that there may indeed be multiple counter receptors for ICAM-1 (20). This report demonstrates that purified, immobilized ICAM-1 can serve as an adhesion receptor for CD43, a molecule found expressed at high levels on T lymphocytes, monocytes, neutrophils, platelets, and activated B lymphocytes. The in vivo, functional significance of ICAM-1:CD43 interaction has yet to be determined.

In addition to the two families of adhesion molecules already discussed, another group is emerging with important contributions to leukocyte adhesion. This group is known as the selectins (reviewed in 4), named for the common N-terminal lectin-like domain shared by all of its members. In addition to the Ca++-dependant lectin-like domain, members of the selectins also have in common an EGF motif next to the lectin-like domain and short consensus repeats with homology to complement regulatory proteins. Included in the group of selectins are CD62, ELAM-1 and LECAM-1, also known as Mel-14/LAM-1. Selectins are thought to be involved in the binding of leukocytes to endothelium at the sites of inflammation as well as in lymphocyte recirculation. One selectin known as CD62, PADGEM, or GMP-140 is stored in the Weible-Palade bodies of endothelial cells, and is rapidly mobilized to the cell surface following stimulation by products of the clotting cascade (21, 22). CD62 has been shown to mediate the rolling of neutrophils on endothelium (23), a process the authors argue is facilitated by the length of the selectin molecule and its potential for rapid association and dissociation.
kinetics. Another report have shown that the ligand for CD62 may be the selectin LECAM-1 on neutrophils (24). At least for neutrophils, the rolling adhesion mediated by selectins appears to be a prerequisite for static adhesion mediated by integrins.

Lymphocytes are constantly patrolling the entire body in search of foreign antigens. Adhesion molecules allow lymphocytes to travel throughout the body and scrutinize the entire body for foreign antigens. The general scheme of this patrol route begins with the lymphocytes traveling through the blood circulatory system, then at various points in the vasculature they emigrate through the endothelium either into LN or directly into the tissues. The processes and molecules involved in lymphocyte recirculation have been well reviewed (25, 26, 27). Lymphocytes emigrating directly into the tissue usually do so at sites of inflammation, from which point they travel to a nearby LN via the afferent lymphatic vessels. From the LN, lymphocytes return to the blood via the efferent lymphatics and the thoracic duct. Lymphocytes enter the LN from the blood stream by binding to specialized post-capillary venules cells called 'high' endothelial venules (HEV). 'Recirculation' receptors on lymphocytes and molecules termed 'addressins' on endothelial cells have been identified by Ab capable of blocking the binding of lymphocytes to endothelium (27), and likely represent counter receptors for lymphocyte recirculation. Candidate molecules implicated in this process include CD44, LFA-1, VLA-4, and LECAM-1 on lymphocytes and ELAM-1, CD62, hyaluronic acid, and poorly defined carbohydrate moieties on endothelium (reviewed in 4). A degree of specificity has been observed in lymphocyte recirculation; a given lymphocyte will continually recirculate either through peripheral LN or through the Peyer's patch. Therefore, site specific receptors on lymphocytes termed homing receptors are likely involved. Such homing receptors on lymphocytes include the selectin LECAM-1 which seems to facilitate the homing of lymphocytes to peripheral LN (28), and the VLA-4 α-subunit associated with either of two integrin β-subunits, which seems to facilitate homing to the Peyer's patch (29). Memory T cells have been observed to emigrate preferentially from the blood directly into
tissue (30), and it is interesting to note that memory T cells show an increased expression of the candidate homing receptors CD44 and VLA-4 (4).

As stated above, the role of the immune system is to distinguish self from non-self and eliminate the latter. The cells that accomplish this task are the B and T lymphocytes. B lymphocytes recognize soluble or cell-bound antigens via membrane bound Ig, and react by secreting Ig directed against the recognized antigen. B lymphocytes are regulated such that they do not respond to self. T lymphocytes recognize only cell-associated antigens; therefore, they require cell:cell contact in order to function. A schematic representation of the molecules involved in the cell:cell recognition and adhesion of T cells is given in Figure 1. The antigen specificity of T cell recognition is mediated by the TcR complex (31, 32), which recognizes antigenic peptides presented on the opposing cell surface in the context of Class I or Class II MHC. CD4 or CD8 on the T cell also bind to Class II or Class I respectively (33). In addition to the TcR/CD4/CD8 recognition of MHC/peptide, two sets of adhesion molecule counter receptors have been demonstrated to play a crucial role in T cell recognition. These interactions are between LFA-1 and ICAM-1, as discussed above, and between CD2 and LFA-3.

The regulation of adhesion in the immune system

One of the interesting aspects of many adhesion systems, including some systems utilized by the immune system, is the ability of these systems to be regulated. Adhesion mediated by many adhesion molecules is not simply a random process facilitated by the binding of counter receptors, but rather adhesion mediated by many molecules can be specifically modulated by the cell. As shown in Figure 1, the major adhesion molecule interactions in T cell recognition are between LFA-1 and ICAM-1 and between CD2 and LFA-3. Both of these two adhesion systems are subject to regulation by the cell.

Both CD2 and LFA-3 are members of the Ig superfamily. Adhesion mediated by CD2:LFA-3 interaction is thought to be strictly dependant on T cell activation; activated T cells but not resting T cells form rosettes with autologous erythrocytes expressing LFA-3 (34).
difference between activated and resting T cells with respect to adhesion mediated by CD2 and LFA-3 is thought to be the surface charge of the T cells (35). Upon T cell activation, T cells begin de novo glycoprotein biosynthesis with altered degrees of glycoprotein sialation. Sialic acid is responsible to a large extent for the net negative charge of the cell surface, and activated T cells have less sialic acid and therefore less negative surface charge (36). It is believed that this reduction in negative surface charge reduces the electrostatic repulsion between cells and allows adhesion mediated by CD2 and LFA-3. The reported dissociation constant for the CD2:LFA-3 interaction is only $10^{-6}$ M (37, 38); therefore, it is easy to see how adhesion mediated by CD2 and LFA-3 could be regulated so delicately as by surface charge. In addition, it is believed that ligation of CD2 on T cells results in the transduction of signals which are capable of synergizing with TcR derived signals (39). Therefore, with the CD2:LFA-3 interaction we see a regulated adhesion system which is also capable of functioning in signal transduction.

Adhesion mediated by LFA-1 and ICAM-1 is particularly interesting for several reasons. One of these reasons is that adhesion mediated by LFA-1 and ICAM-1 is not simply a passive process in which complementary counter receptors recognize each other stereochemically. Rather, adhesion mediated by LFA-1 and ICAM-1 is both finely regulated and metabolically dependant. Treatment of cells with sodium azide, which blocks the generation of ATP in cells, abolishes LFA-1:ICAM-1 mediated adhesion (13). Similarly, when cells are cooled to 4°C LFA-1:ICAM-1 mediated adhesion is completely abolished (13). This is not the case for all adhesion molecule systems, for example CD2:LFA-3 mediated adhesion occurs readily at 4°C (40), as does adhesion mediated by NCAM, another Ig superfamily member (41). Incidentally, adhesion mediated by certain members of the cadherins is also metabolically dependant (2).

As with the CD2:LFA-3 system discussed above, adhesion of T cells mediated by ICAM-1 and LFA-1 is also believed to be strictly dependant on T cell activation. However, an increase in ICAM-1:LFA-1 mediated adhesiveness occurs in a matter of minutes (42) as
compared to the 12-24 hours required for *do novo* glycoprotein biosynthesis. Therefore, the mechanism(s) regulating LFA-1 mediated adhesion is completely different than that regulating CD2 mediated adhesion. The increase in adhesion mediated by LFA-1 and ICAM-1 has been clearly shown to be the result of a change in LFA-1 as opposed to a change in ICAM-1. This distinction was made through the use of adhesion assays in which the binding of cells to either plastic absorbed LFA-1 or ICAM-1 was quantitated (42). The adhesion of cells to immobilized ICAM-1, but not to immobilized LFA-1 was increased substantially by stimulation with the phorbol ester PMA, clearly showing that the change induced by phorbol ester is on the side of LFA-1 and not ICAM-1. The increase in LFA-1 mediated adhesion has carefully been termed an increase in LFA-1 avidity, since for the multivalent binding between cells the distinction between affinity and avidity, a subtle but important distinction, cannot be made. Without a monovalent form of one of the two counter receptors, a clear measurement of affinity cannot be made; therefore, one cannot distinguish between the affinity of monovalent interaction and avidity, which is the sum of the affinity of monovalent interaction and the number of interactions occurring.

Phorbol esters, namely PMA, are diacylglycerol analogs, and therefore stimulate PKC directly. PKC is an enzyme that facilitates the phosphorylation of proteins in the cell on serine or threonine residues. It has been shown that the β chain of LFA-1 is a substrate for PKC, and in fact is phosphorylated on serine following PMA stimulation (43). It has also been shown that while the β chain can be phosphorylated upon PKC upregulation, the α chain of LFA-1 is phosphorylated constitutively (44). Phosphorylation on tyrosine residues, of which the LFA-1 β chain has one, has not to our knowledge been demonstrated for LFA-1.

In addition to PMA stimulation, Dustin and Springer have also studied the effect of stimulating T cells via the TcR on LFA-1:ICAM-1 mediated adhesion (42). The results show that with respect to LFA-1 avidity, stimulation of T cells via the TcR does not mirror the effects of PMA stimulation. Rather, they observed that the kinetics were markedly different; TcR stimulation resulted in a rapid but transient avidity increase while PMA stimulation resulted in a
slower but sustained avidity increase. The kinetics of the increase following stimulation by PMA is in good agreement with the kinetics of LFA-1 β chain phosphorylation by PMA stimulation (44). These results suggest that the normal control of LFA-1 mediated adhesion is relatively complex; with respect to LFA-1 avidity, TcR derived signals do not simply turn on PKC, but PKC upregulation may be part of TcR derived signals.

Adhesion of cells to absorbed ICAM-1 is dependant on activation from the so called low to high avidity forms of LFA-1, as well as a functional actin cytoskeleton and divalent cations (5, 11, 12, 13). It has been assumed that the avidity of LFA-1 is determined by it's affinity for ICAM-1, and that PKC mediated phosphorylation of the LFA-1 β chain, stimulated by phorbol ester or TcR ligation, regulates the affinity of LFA-1 (42). Again, in the absence of an assay for the binding of sICAM-1 in solution, the relationship between the affinity of LFA-1 for ICAM-1 and it's avidity could not be determined.

The major source of confusion in the literature concerning LFA-1 regulation is the distinction between avidity and affinity. Most of the literature has favored conformational change models for LFA-1 avidity increases, which usually imply increases in the affinity of the interaction between LFA-1 and ICAM-1. With respect to LFA-1, two groups have characterized 'activated' forms of LFA-1 through the use of mAb. Figgodor et al. have raised a mAb, NKI-L16, which binds to an epitope of LFA-1 dependant on divalent cations and cellular activation (45, 46, 47). This Ab also facilitates LFA-1:ICAM-1 mediated homotypic aggregation of cell lines, presumably by stabilizing an activated LFA-1 conformation. Based on their data, this group has postulated the existence of three distinct conformational forms of LFA-1 on the cell corresponding to distinct stages of activation (48). Dransfield and Hogg have similarly characterized an LFA-1 epitope dependant on active metabolism and divalent cations through the use of their mAb, Ab24 (49). With respect to LFA-1 regulation, the distinction between affinity and avidity is covered at length in the Discussion of this thesis.

While increasing affinity is one mechanism whereby avidity is increased, it is by no means the only mechanism capable of increasing avidity. Logically, there is at least one
mechanism apart from affinity by avidity can be regulated. By increasing the number of adhesion molecules interacting, the net strength of interaction between cells can be altered. The rapid inducibility of ICAM-1 is an example of how a cell may increase its likelihood of adhering. It has been shown that monocytes mobilize cytoplasmic ICAM-1 to their surfaces relatively quickly following adherence to fibronectin (50). The distribution of adhesion molecules on the surface of cells would also determine the overall strength of interaction between cells. High local concentrations of adhesion molecules at the site of cell:cell contact would increase the avidity of interaction. Indeed, it has been shown that Mac-1 on neutrophils aggregates in the absence of ligand upon PMA stimulation (51). This aggregation was shown to correlate with increased ligand binding ability in the absence of increased Mac-1 expression. In the case of T cells, there have been several reports from a single laboratory on the localization of adhesion molecules to sites of contact between T cell and target or accessory cell (52, 53). Reports from this laboratory have also suggested an association between LFA-1 and talin, a protein associated with the actin cytoskeleton, mediated by serine/threonine phosphorylation events (54, 55). However, this work has failed to address the sequence of events that is occurring: do adhesion molecules aggregate thus facilitating adhesion, or does adhesion result in the mutual capping of counter receptors? There is a report in literature of the localization of ICAM-1 on uropods of cloned T cells (50). Interestingly, this cell line forms large homotypic aggregates, and LFA-1 does not seem to co-localize on the cells' uropods. Staunton et al. have observed that ICAM-1 expressed on transfected COS cells exhibits a punctate staining pattern, even when the entire cytoplasmic domain is deleted (56). The role of adhesion molecule distribution in the regulation of adhesion is, at present, not well characterized.

The association of adhesion molecules with the cytoskeleton

Functionally, many adhesion molecules appear to be associated with the actin cytoskeleton. These observations were first made with adherent cells in culture. The regions
of these cells at which contact is maintained between the cell and the substrate have been termed the focal adhesions, or adhesion plaques (reviewed in 57). The adhesion plaque is the point at which an adherent cell, such as a fibroblast, is adhering to extracellular matrix proteins absorbed to the culture vessel. Extracellular matrix proteins which function in such a manner include fibronectin and vitronectin, both of which have the RGD sequence known to be the ligand for their cellular receptors. On the cell's plasma membrane at the focal adhesion were found high concentrations of integrin molecules such as certain β₁ and certain β₃ integrins, which were later characterized as being receptors for fibronectin and vitronectin. Inside the cell at the focal adhesion were found cytoplasmic proteins such as actin, α-actinin, talin, and vinculin. Actin forms long polymers which make up the insoluble actin cytoskeleton visualized as stress fibres in adherent cells. Vinculin, talin, and α-actinin are proteins which have been shown to associate with the actin cytoskeleton. Talin is associated with the focal adhesions (58), α-actinin has been shown to associate directly with actin (59), and vinculin has been shown to associate with talin (60), with α-actinin (61) and with other yet uncharacterized actin binding proteins (62). Talin has been shown to associate directly with the fibronectin receptor (63). In addition to the integrins, the actin cytoskeleton has also been shown to be associated with other types of adhesion molecules including the cadherins (2). It seems logical that the interaction between the cytoskeleton and adhesion molecules could control the distribution of adhesion molecules on the surface of the cell, as discussed above.

Studies by Marlin and Springer demonstrated that LFA-1 mediated adhesion of lymphocytes was dependant on a functional actin cytoskeleton (13). Studies by Singer et al. have implicated talin in integrin mediated lymphocyte adhesion (54, 55), and also suggested that PKC mediated phosphorylation events mediate the association of talin with LFA-1. It is known that talin and vinculin are substrates for PKC (64, 65), and as previously mentioned so is the β chain of LFA-1. Interestingly, a cytoplasmic protein, 86,000 in molecular weight, has been shown to associate with LFA-1 (66). This is a molecular weight similar to that of several poorly characterized proteins found associated with the fibronectin receptor (67, 68). These
data collectively show that the actin cytoskeleton is involved in LFA-1:ICAM-1 mediated adhesion. More specifically, it seems that PKC mediated phosphorylation events regulate interactions between the actin cytoskeleton and LFA-1, thus regulating LFA-1 avidity.

**Physical aspects of adhesion**

Another aspect of cell adhesion which should be considered is that of the distances involved (reviewed in 4). The term glycocalyx has been used to represent the complex surface of the cell, made up mostly of protruding proteins and carbohydrates (4). Electron microscopy and X-ray crystallography have provided data on the shape and size of several leukocyte surface molecules. As mentioned previously, the glycocalyx has a net negative charge due mostly to sialic acid on glycoproteins. For cells to adhere, their glycocalyces must interact, and adhesive interactions must overcome the electrostatic repulsion. Molecules such as the TcR, MHC, CD2, LFA-3, and ICAM-2 extend less than 10 nm from the plasma membrane; therefore, interactions between these molecules must either be of a high affinity themselves, or the cell:cell association involving these molecules must be stabilized by other interactions. LFA-1 and ICAM-1 extend farther away from the plasma membrane, about 15-20 nm, and therefore could interact with a lesser degree of interdigitation of the two opposing glycocalyces. CD45, a glycoprotein expressed at very high levels on leukocytes, extends about 28 nm from the plasma membrane; therefore, closely associated cells may be required to exclude CD45 at the points of contact. Since the cytoplasmic domain of CD45 has tyrosine phosphatase activity, signalling may be affected. The selectin CD62 which is involved in neutrophil rolling, extends about 40 nm from the plasma membrane of endothelial cells, and one of it's possible ligands, the selectin LECAM-1 (23), extends about 15 nm on neutrophils. CD43, a glycoprotein expressed at very high levels on T cells, neutrophils, and activated B cells, extends about 45 nm from the plasma membrane, and may serve as an alternate counter receptor for ICAM-1 (20). The intermediate distance of the ICAM-1:LFA-1 association, an
interaction between molecules extending 15-20 nm from the plasma membrane, is probably of significance to the general importance of this interaction.

**Soluble versions of adhesion molecules**

A soluble version of an adhesion molecule is a form of the molecule which is no longer anchored to the cell, but is a soluble protein free of the cell. Soluble adhesion molecules could be produced by proteolytic cleavage of a full length, cell associated adhesion molecule; proteolytic cleavage could be done by the cell or in the test tube. Soluble adhesion molecules could also be produced by the cell by alternative splicing of mRNA. Alternatively, soluble adhesion molecules could be produced using recombinant DNA techniques to genetically engineering the coding sequence for the protein such that it is no longer anchored to the cell, but rather completely secreted from the cell. Soluble versions of adhesion molecules may or may not maintain their biological activity as measured by binding to their normal counter receptor.

Soluble versions of adhesion molecules are of interest for several reasons. In general, soluble adhesion molecules are crucial for studying the fine details of cell adhesion mediated by particular adhesion molecules. For example, measurement of the affinity of the interaction between adhesion molecules can only be accomplished with a soluble version of one of the two molecules. It is therefore possible, with a soluble adhesion molecule, to study mechanisms modulating the affinity of a particular adhesion molecule system. Similarly, it is also possible to dissect the mechanisms modulating avidity apart from affinity. As with the CD2:LFA-3 interaction, adhesion molecules may transduce signals in addition to serving as adhesion receptors. Soluble adhesion molecules may allow the dissection of these various accessory functions of adhesion molecules. Soluble adhesion molecules also are hopeful candidates for inhibiting cell adhesion, and therefore inhibiting the biological functions mediated by cell adhesion, such as inflammation and cellular immune responses. With this in mind, it is not surprising that the field of adhesion molecules is a very active field. Indeed, there are several
reports in the literature on the production and functional characterization of soluble versions of adhesion molecules.

Wheelock et al. purified a soluble version of cell-CAM 120/80, a member of the cadherins which is normally involved in the adhesion of epithelial cells (69). This adhesion molecule is found on cells as a 120 kd membrane bound glycoprotein and as a 80 kd proteolysis product in the culture SN of tumor cell lines used for the production of the soluble protein. The purified 80 kd protein was capable of disrupting cell:cell adhesion in cultured epithelial cell lines at relatively low concentrations (0.01 nM, 0.8 ng/ml). Damle and Aruffo produced a recombinant, soluble VCAM-1 fusion protein in COS cells, and found that it produced co-mitogenic signals in CD4 T cells when immobilized on plastic with either anti-TcR or anti-CD3 Ab (70). Lobb et al. expressed recombinant, soluble ELAM-1 in Chinese hamster ovary cells (71). The authors observed that their protein was functional as an adhesion molecule when immobilized on plastic; however, it only weakly inhibited ELAM-1 mediated adhesion. Van Seventer et al. recently reported on the production of recombinant, soluble forms of both VCAM-1 and ELAM-1, and demonstrated that each is functional when absorbed to plastic (72). Soluble Mac-1 has been produced by Dana et al. in COS cells transfected with truncated cDNAs for each of the α and β chains in separate expression vectors (73). Surprisingly, the protein was maintained as a noncovalently associated αβ heterodimer, appeared functional as measured by binding to it's ligand iC3b, and effectively inhibited the binding of polymorphonuclear cells to IL-1 stimulated endothelium.

With respect to ICAM-1, there have been several reports on the production of it's soluble version. Marlin et al. reported originally on the production of soluble human ICAM-1 through recombinant techniques, and it's use as an inhibitor of rhinovirus infection since the rhinovirus receptor is ICAM-1 (74). In addition, during the course of this thesis project a report was published on the production of a recombinant, soluble form of murine ICAM-1 (75). This report concentrated on the accessory function of ICAM-1 as demonstrated by it's effects on antigen presentation by ICAM-1 transfected fibroblasts, and by the activity of
soluble ICAM-1 co-immobilized on plastic with anti-CD3 Ab. However, the authors claim that their soluble protein mediates adhesion of T cells when coated to plastic, and they also claim that their soluble protein blocks ICAM-1:LFA-1 mediated adhesion, although they do not show any data in support of these claims. It is important to note that the soluble murine ICAM-1 reported in this publication is produced in Chinese hamster ovary cells, and it's molecular weight of only 50 kd suggests inappropriate glycosylation. In addition to the production of sICAM-1 through recombinant techniques, several reports on the presence of a circulating, soluble ICAM-1 in serum have been published (76, 77). The concentration of this circulating, soluble ICAM-1 was reported to be in the range of 100 ng/ml (77). One group has shown that upon stimulation with IFN-γ or TNF-α, melanoma cells shed ICAM-1, and that this shed ICAM-1 is capable of inhibiting ICAM-1 mediated adhesion (78).

The report on the shedding of ICAM-1 is one of a number of reports on the shedding of adhesion molecules from cells. MEL-14/Leu-8/LECAM-1 is shed from neutrophils upon activation (79, 80) and it is hypothesized by both groups that this shedding may have some functional significance to adhesion. In addition, a recent report has characterized a soluble form of CD43 present in plasma at concentrations exceeding of 10 μg/ml (81).

The general consensus to date from the published reports on soluble adhesion molecules seems to be that they usually function as adhesion molecules when immobilized on plastic. However, there is some controversy as to the ability of soluble versions of adhesion molecules to inhibit cell:cell adhesion.

**Thesis objectives**

The objectives of this thesis project were twofold: to explore the use of a soluble form of ICAM-1 as an inhibitor of immune responses, and to study in more detail some of the mechanisms regulating cell adhesion mediated by ICAM-1 and LFA-1. Since soluble, murine ICAM-1 was already being produced, the means to do this were readily available, and indeed there were some encouraging reports in the literature along similar lines. The original goal was
first to functionally characterize sICAM-1 and then evaluate its inhibitory potential through the use of MLR as an *in vitro* model as well as GVHD as an *in vivo* animal model system. The long term goal we had in mind was clinical application to graft versus host disease associated with bone marrow transplantation. Through the functional characterization of sICAM-1, the molecule proved inefficient as an inhibitor of LFA-1:ICAM-1 mediated cell adhesion despite the fact that the molecule appeared to be fully functional. Explaining the inability of sICAM-1 to inhibit ICAM-1:LFA-1 mediated adhesion then became an important part of the project. Through out the work we encountered various mechanisms by which ICAM-1:LFA-1 mediated cell adhesion is regulated, and we attempted to characterize these mechanisms in some detail.

The Results section has been divided into five sections; the production and purification of sICAM-1, the functional characterization of sICAM-1, the role of multivalent interaction in LFA-1:ICAM-1 mediated cell adhesion, the cell surface distribution of ICAM-1 and LFA-1, and the role of the cytoplasmic domain of ICAM-1 in cell adhesion. The Discussion section is a synthesis of the information presented in this thesis along with other salient information in the literature, with the goal of clarifying some of the topics that are ambiguous in the literature. One of the main themes that I have attempted to develop is that a variety of mechanisms can potentially regulate cell adhesion, even through a single pair of counter receptors such as LFA-1 and ICAM-1.
Materials and methods

Animals

C57BL/6 and BALB/c mice were purchased from Charles River Canada Ltd. (St. Constant, Quebec, Canada) and were maintained in the Joint Animal Facility of the B.C Cancer Research Centre.

Cell culture

The Moloney murine leukemia virus induced T cell lymphoma MBL-2 (82), the myeloma NS-1 (83), the B cell lymphoma A20 (84), the T cell hybridoma T28 (85), and the fibroblastoid cell line L (86), all murine cell lines used for various analyses, were maintained in DMEM supplemented with 5% FCS and antibiotics in a 37°C, 5%CO2 incubator.

The hybridomas YN1/1.7, FD441.8 (ATCC TIB213), M1/70.15.11.5.HL (ATCC TIB128), KM 81 (ATCC TIB 241), GK1.5, YE1/21.1, and YE1/9.9 were all maintained in DMEM supplemented with 5% FCS and antibiotics in a 37°C, 5% CO2 incubator. All hybridomas secrete rat IgG directed against mouse antigens. YN1/1.7 secretes anti-murine ICAM-1 (87), TIB213 secretes anti-LFA-1 α chain (88), TIB128 secretes anti-Mac-1 α chain (89), TIB241 secretes anti-CD44 (90), GK1.5 secretes anti-CD4 (91), YE1/21.1 secretes anti-CD45 (92), and YE1/9.9 secretes anti-transferrin receptor (92). Cultures of these cells were overgrown, the SN was harvested by centrifugation at 5,000 xg for 45 min., and used as a source of specific Ab. For purification of YN1/1.7 Ab, hybridoma cells were grown up in PFHM-II serum-free medium, and the SN from overgrown cultures was processed as described below.

Resting splenic T cells were purified by passage over a nylon wool column (93) in order to deplete the population of adherent cells. Columns were prepared by packing 1 g of dried nylon wool into a disposable 6 ml disposable syringe, and autoclaving. Nylon wool
columns were equilibrated overnight in a 37°C, 5% CO₂ incubator with RPMI 1640 supplemented with 5% FCS. C57BL/6 or BALB/c mice, 2-10 months old, were then sacrificed and single cell suspensions were made from the spleens in RPMI 1640 5% FCS. Cells from 1-2 spleens in a volume of 1 ml were then loaded on to a nylon wool column, and the column incubated in a 37°C, 5% CO₂ incubator for 1 hr. Non-adherent cells were then eluted with pre-warmed RPMI 1640 5% FCS for about 15 min., depending on the flow rate. Contaminating red blood cells were then lysed with Tris-NH₄Cl (94). One spleen routinely yielded approximately 2x10⁷ nucleated cells. Cells purified by this procedure have been shown by others to represent a population of resting T cells, greater than 90% in purity (93).

Production and purification of sICAM-1

The derivation of the expression system for the production of murine sICAM-1 was previously established in this laboratory by Daniel Lee and Fumio Takei (Lee and Takei, submitted). The overall scheme is that recombinant sICAM-1 is produced by NS-1 cells and then purified from the culture SN by immunoaffinity chromatography.

The starting point was the cloned full length cDNA encoding murine ICAM-1, originally named MALA-2 (95). Briefly, this cDNA was cut with Scal and EcoRI and ligated into pBLUESCRIPT which had previously been cut with EcoRI and XbaI and filled in. This blunt end ligation generated a stop codon immediately after the Scal site, which is conveniently located 21 base pairs 5' of the transmembrane encoding region of MALA-2. From this point on, the murine version of ICAM-1, MALA-2, will simply be referred to as ICAM-1. The cDNA encoding sICAM-1 was then cut out of pBLUESCRIPT using SalI and NotI, and sub-cloned into the XhoI and NotI sites of the eukaryotic episomal expression vector BCMGSNeo (96). The BCMGSNeo vector carries a CMV promoter which efficiently drives the expression of the inserted cDNA in the appropriate eukaryotic host cell. BCMGSNeo-sICAM-1 was then electroporated into NS-1 cells, and transfectants were selected in 1 mg/ml G418 (Gibco Laboratories Life Technologies Inc., Grand Island, NY). Single cell cloning and screening by
immunoblot with YN1/1.7 Ab (see below) yielded the cell line 2706C, which secreted large amounts of sICAM-1 into the culture SN.

A YN1/1.7 Ab immunoaffinity column was prepared for the purification of sICAM-1. Ig was precipitated from overgrown, serum-free YN1/1.7 SN with 50% saturated ammonium sulphate. The precipitate was then dialyzed against PBS, and analyzed by SDS-PAGE under reducing conditions to determine the purity of the Ab preparation. SDS PAGE analysis of material purified by this method demonstrated that the preparation was approximately 80% pure Ig. The immunoaffinity column was made by coupling 3 ml of AFFIGEL 10 beads (Bio-Rad, Richmond, CA) with 6 mg of purified YN1/1.7 Ab in 0.1 M NaHCO₃ overnight at 4°C. The beads were then washed with 100 mM Tris pH 8.0 to block residual coupling sites, and then stored in 10 mM Tris pH 7.5, 0.15 M NaCl, 0.2% azide at 4°C.

2706C cells were selected in 2 mg/ml G418 and then grown up in order to purify sICAM-1. Falcon 3028 flask (Becton Dickinson, Lincon Park, NJ) were seeded with 5 ml of 2 mg/ml G418 selected culture each, along with 45 ml of DMEM 2% FCS + 0.5 mg/ml G418. On day 2, 50 ml DMEM 1% FCS was added to each flask, and on day 4, 100 ml DMEM 1% FCS was added to each flask. On day 7 the culture supernatant was harvested by centrifugation at 5,000 xg for 45 min. at 4°C. 500 ml batches of SN were used to purify sICAM-1, and the entire purification was carried out at 4°C. The SN was loaded onto the YN1/1.7 column at a flow rate of approximately 20 ml/hr. The column was then washed with 200-400 ml of 0.15 M NaCl, 10 mM Tris pH 7.5 at a flow rate of approximately 25 ml/hr. SICAM-1 was then eluted with 0.15 M NaCl, 50 mM diethylamine pH 11.0 at a flow rate of approximately 30 ml/hr. 5 ml fractions were collected into tubes containing 500 µl of 1 M Tris pH 6.0. The fractions were monitored by measuring OD₂₈₀, and fractions containing significant amounts of protein were pooled, concentrated using Centricon-30 microconcentrators (Amicon, Beverly, MA), exchanged 3 times into PBS, and adjusted to a concentration of 1 mg protein (OD₂₈₀) per ml. The purified sICAM-1 was then aliquoted and stored at -20°C. 500 ml batches of SN routinely yielded 1-2 mg of purified sICAM-1.
**Immunoblot**

An immunoblot assay was used to screen cell lines for the production of sICAM-1, to assess levels of sICAM-1 production, and to monitor the purification of sICAM-1. The Minifold II slot blot apparatus (Schleicher and Schuell, Keen, NH) was assembled according to the manufacturer's instructions, and with vacuum applied, samples were loaded. Usually 10 and 100 μl samples of culture SN were loaded into individual slots. Each slot was washed with a 200 μl aliquot of PBS, and the apparatus was disassembled. The nitrocellulose membrane was then washed in 2 changes of PBS and then incubated in PBS 5% blotto + 0.1% NP40 for 10 min. at 20°C. After washing with 3 changes of PBS, the membrane was incubated in YN1/1.7 SN diluted 1/40 with PBS for 1 hr. at 4°C. The membrane was then washed in 2 changes of PBS 5% blotto, 2 changes of PBS, and incubated in peroxidase conjugated goat anti-rat IgG (Bethesda Research Laboratories Life Technologies Inc., Burlington, Ont.) diluted 1/2,000 with PBS for 30 min. at 4°C. After 2 washings in PBS 5% blotto and 2 washings in PBS, PBS containing 0.06% DAB (Bethesda Research Laboratories Life Technologies Inc., Burlington, Ont.) and 0.012% H₂O₂ was added, and the blot was developed for 5-10 min.

**SDS-PAGE analysis**

Preparations of purified sICAM-1 were analyzed by SDS-PAGE in order to confirm molecular weight, purity, and quantitation. This analysis was carried out using a Mini-V 8.10 apparatus (Bethesda Research Laboratories Life Technologies Inc., Burlington, Ont.) according to standard conditions (97). Samples of purified sICAM-1 or known amounts of standard proteins (in order to confirm the quantitation) were diluted 1/2 with 2X non-reducing SDS-PAGE sample buffer, and electrophoresed on 7.5% discontinuous gels. The gels were then removed from the apparatus and stained in a solution of 2% coomassie brilliant blue, 50% methanol, and 10% acetic acid, and then destained in a solution of 5% methanol and 7.5%
acetic acid. Molecular weights were determined from the Bio-Rad high range SDS-PAGE standards (Bio-Rad, Richmond, CA). For radio-iodinated samples, rather than staining, the gel was wrapped in plastic wrap and exposed to X-ray film.

**Radio-iodination of sICAM-1**

Purified sICAM-1 was radio-iodinated by the IODO-GEN method (98). IODO-GEN (Pierce, Rockford IL) was dissolved in chloroform and 100 μl aliquots containing 10 μg/100 μl were dispensed into 12x75 mm borosilicate glass tubes, and the chloroform was then allowed to evaporate overnight. In a fume hood, 10 μg of purified sICAM-1 was mixed with 5 μl of 100 mCi/ml Na\(^{125}\)I (Dupont New England Nuclear, Wilmington DE) in a final volume of 50 μl of PBS. This mixture was then transferred to an IODO-GEN coated tube, and iodination was allowed to proceed for a period of 2 min., after which the reaction was stopped by the addition of 10 μl of 2 mg/ml sodium metabisulfate. The iodinated protein was then separated from unincorporated iodine by gel filtration through a Nick column (Pharmacia, Uppsala, Sweden) which had previously been equilibrated with PBS containing FCS and then PBS. Fractions eluted from the Nick column corresponding to radio-iodinated protein were then pooled, made to 10% FCS, aliquoted, and stored at -20°C. \(^{125}\)I-sICAM-1 concentration was calculated based on an estimated overall recovery of 85%. SICAM-1 was routinely iodinated by this procedure to a specific activity of 2-5x10^4 CPM/ng. Radio-iodinated sICAM-1 was then used either for SDS-PAGE analysis, or for \(^{125}\)I-sICAM-1 binding.

**\(^{125}\)I-sICAM-1 binding assay**

The \(^{125}\)I-sICAM-1 binding assay is a functional assay for sICAM-1 in which the binding of radio-iodinated sICAM-1 to LFA-1^+ cells is quantitated. Parameters for the standard \(^{125}\)I-sICAM-1 binding assay were previously worked out in Fumio Takei's lab by Daniel Lee (Lee and Takei, submitted). LFA-1^+ lymphoid cells (MBL-2, A20, T28) were suspended to a concentration of 2x10^6/ml in binding buffer (either RPMI 1640 supplemented
with 5% FCS and antibiotics or Tris buffered saline with 1 mM Ca\(^{++}\) and Mg\(^{++}\), and the appropriate inhibitors or stimulators were added. All conditions were done in duplicate. \(^{125}\)I-sICAM-1 was then added to a final concentration of between 200 ng/ml (2x10\(^{-9}\) M) and 2 \(\mu\)g/ml (2x10\(^{-8}\) M), and the cells were incubated for 1 hr. at 20\(^\circ\)C. At the end of this 1 hr. incubation, cell bound radioactivity was determined by one of two methods. For high affinity binding, cells were washed 3X binding buffer and then counted in a gamma counter; for low affinity binding cells were spun through oil instead of being washed. To spin through oil, 100 \(\mu\)l aliquots of the cells were layered onto 300 \(\mu\)l aliquots of a mixture of 3 parts dibutyl pthalate (Sigma, St. Louis, MO) and 2 parts dioctyl pthalate (Aldrich, Milwaukee, WIS) and centrifuged at maximum speed in an Eppendorf Microfuge for 2 min. at 20\(^\circ\)C. The tubes were then frozen in dry ice. The bottoms of the frozen tubes containing the pelleted cells were cut from the rest of the tube with a dog toe nail clipper, and the cell pellets containing only bound \(^{125}\)I-sICAM-1 were counted in a gamma counter. Results are expressed as the arithmetic mean of duplicates, and the error bars represent the standard deviation.

Quantitation of the density of sICAM-1 in sICAM-1 coated microtitre wells

In order to better understand the dynamics of the quantitative cell adhesion assay (see below), the density of sICAM-1 on sICAM-1 coated polystyrene microtitre wells was quantitated. This quantitation was accomplished with radio-iodinated YN1/1.7 Ab.

Purified YN1/1.7 Ab was radio-iodinated by the chloramine T method (99). 10 \(\mu\)l of a freshly prepared solution of 0.5 mg chloramine T/ml and 10 \(\mu\)l of 100 mCi/ml Na\(^{125}\)I (Dupont New England Nuclear, Wilmington DE) was added to 50 \(\mu\)g of purified YN1/1.7 Ab in 167 \(\mu\)l of PBS. Iodination was allowed to proceed for a period of 5 min., after which the reaction was stopped by the addition of 50 \(\mu\)l of 2 mg/ml sodium metabisulphate. The iodinated protein was then separated from unincorporated iodine by gel filtration through a Nick column which had previously been equilibrated with PBS containing FCS and then PBS. Fractions eluted from the Nick column corresponding to radio-iodinated protein were then pooled, made to 10%
FCS, aliquoted, and stored at -20°C. $^{125}$I-YN1/1.7 Ab concentration was calculated based on an estimated overall recovery of 85%. YN1/1.7 Ab was radio-iodinated by this procedure to a specific activity of approximately $1 \times 10^5$ CPM/ng.

Serial dilutions of purified sICAM-1 were made in 0.1 M carbonate buffer pH 9.6. Duplicate 30 µl aliquots of these dilutions were then dispensed into the wells of flat bottom 96 well Nunc tissue culture plates (Nunclon Delta, Denmark), and protein was allowed to absorb onto the plastic for 1 hr. at 20°C. The wells were then washed 3X PBS, and residual protein binding sites were blocked by incubation with 200 µl of PBS 5% blotto/well for 30 min. at 20°C. Wells were then washed 3X PBS and incubated with $^{125}$I-YN1/1.7 Ab at 20 µg/ml, a concentration previously determined to be saturating, for 1 hr. at 20°C. Wells were then washed 6X PBS, and bound $^{125}$I-YN/1.7 Ab was dissociated with 2 M NaOH. Aliquots of the dissociated $^{125}$I-YN1/1.7 Ab were then counted in a gamma counter. Again, all conditions were done in duplicate, with less than 10% variation in CPM between duplicates. From the bound CPM/well (arithmetic mean of duplicates) and the specific activity of the $^{125}$I-YN1/1.7 Ab, the number of molecules of YN1/1.7 bound/well was calculated, and from this number and the area of the the microtitre well surface, the density of bound YN1/1.7 Ab molecules and therefore the density of sICAM-1 absorbed to the well was estimated. These calculated sICAM-1 densities are likely to have a relatively large error associated with them, especially at high sICAM-1 densities, since 1 YN1/1.7 Ab molecule can potentially bind to 2 molecules of sICAM-1.

**Quantitative cell adhesion assay**

The quantitative cell adhesion assay is a functional assay for sICAM-1 in which the ability of LFA-1+ cells to bind to plastic absorbed sICAM-1 is quantitated. The protocol for this assay was based on the protocol of Dustin and Springer (41). SICAM-1 was coated to the wells of 96 well flat bottom tissue culture plates as described above for the quantitation of the density of sICAM-1 in sICAM-1 coated wells. All conditions were done in duplicate. After
coating, wells were washed 3X PBS and blocked by the incubation of 100 µl/well of 100 µg/ml BSA or OVA in PBS for 30 min. at 20°C. Wells were then washed 3X PBS, and 100 µl of cells at a concentration of 2x10⁶/ml in RPMI 1640 5% FCS (2x10⁵ cells/well) were dispensed into each well. In some experiments the cells were incubated for 20 min. at 37°C with various inhibitors or stimulators prior to addition to the assay plate. The plate was then centrifuged at 10Xg for 5 min. at 20°C, and then incubated for 5 min. in a 37°C, 5% CO₂ incubator. At the end of this 5 min. incubation, unbound cells were carefully washed from the wells. Wells were washed by flicking the plate of it's contents and gently pipetting 150 µl of medium warmed to 37°C into each well, then repeating this process 5 more times. After the final wash, 100 µl of medium was added to each well. Also at this time 100 µl aliquots of cells diluted to various known concentrations were added in order to generate a standard curve for precise quantitation.

Bound cells were quantitated in the quantitative cell adhesion assay using a convenient colorimetric assay in which the tetrazolium salt MTT is cleaved by mitochondrial enzymes of viable cells, yielding a colored product (100). 10 µl of a solution of 5 mg/ml MTT (Sigma, St. Louis, MO) in PBS was dispensed into each well of the assay plate, and the plate was incubated for 4 hr. in a 37°C, 5% CO₂ incubator. After this incubation, the colored product was solubilized by the addition of 100 µl of 0.04 M HCl in isopropanol to each well and vigorous pipetting. The absorbances of individual wells was then read on a Bio-Tek ELISA plate reader (model EL309) using a test wavelength of 570 nm and a reference wavelength of 630 nm. Nylon wool T cells usually generated a linear standard curve over the range of cell concentrations used, while the cell lines usually generated a standard curve best described by a polynomial expression. The number of cells bound per well in each well of the experiment was then determined by comparison to the standard curve. Again, all conditions were done in duplicate and the results represent the arithmetic mean. Duplicates usually varied by less than 5% in this assay.
In order to study the affects of Mn++ in the quantitative cell adhesion assay, several alterations to the standard protocol had to made. Firstly, since 1 mM Mn++ resulted in the precipitation of MnPO4 from standard RPMI 1640, PO4-free RPMI 1640 had to be used. Secondly, it was found that treatment of primary, resting T cells with mM concentrations of Mn++ resulted in a substantial inhibition of the cells' metabolic activity as measured by the MTT assay. Treatment of cell lines with mM concentrations of Mn++ resulted in only a slight inhibition of metabolic activity as measured by the MTT assay. Therefore, the MTT assay could not be used to quantitate the adhesion of primary T cells to absorbed sICAM-1 in the presence of mM concentrations of Mn++. The Mn++ induced adhesion of primary T cells to absorbed sICAM-1 wells was quantitated by using cells labeled with 51Cr or simply by inspecting the degree of cell binding in the wells of the assay plate. Cells eluted from the nylon wool column were labeled with 51Cr by incubating approximately 2x107 cells for 1 hr. at 37°C in 50 μl FCS with 80 μCi 51Cr (80 μl Na251CrO4 in normal saline (Dupont New England Nuclear, Wilmingdon DE)). Excess 51Cr was then removed by two washes with PO4-free RPMI 1640 supplemented with 10 mM Hepes pH 7.2 and 5% FCS, and then cells were used as in the standard protocol. After spinning the cells onto the plate and incubating at 37°C for 5 min., wells were washed 8X 150 μl of pre-warmed PO4-free RPMI 1640 supplemented with 10 mM Hepes pH 7.2, with or without 1.25 mM Mn++. Instead of washing by flicking, 51Cr labeled cells were washed by aspirating the contents of the wells through a 18 ga. needle. After washing the wells, bound cells were dissociated by incubating the wells with 100 μl aliquots of 5 mM EDTA in PBS for 10 min. at 37°C. The contents of each well was then mixed by pipetting and removed for γ-counting. 51Cr labeled primary T cells were observed to spontaneously release a significant amount of label, especially over the 6 washes in the assay, making quantitation difficult and somewhat un-reproducible from experiment to experiment.

Inhibition of the PMA induced aggregation of MBL-2 cells
Murine cell lines were screened for the ability to form homotypic aggregates upon stimulation by the phorbol ester PMA. Of the cell lines tested MBL-2, a Moloney murine leukemia virus induced LFA-1 and ICAM-1 positive T lymphoma cell line, aggregated reproducibly to the greatest extent. The aggregation of MBL-2 cells was found to be dependant on LFA-1 and ICAM-1 (Figure 7). MBL-2 cells did not, however, aggregate to the extent of certain human cell lines under similar conditions (11).

MBL-2 cells in early logarithmic phase were suspended to a concentration of 4x10^6/ml in RPMI 1640 supplemented with 10% FCS and antibiotics, and 100 µl aliquots were dispensed into the wells of flat bottom microtitre plates (2x10^5 cells/well). To the appropriate wells 5 µl volumes of hybridoma SN were added as inhibitors, as were dilutions of EDTA, cytochalasin B, and volumes of purified sICAM-1. Then 50 µl aliquots of PMA (Sigma, St. Louis, MO), diluted to 200 ng/ml in medium from a stock of 20 µg/ml in DMSO, were dispensed into the appropriate wells. Finally, the volume of each well was made up to 200 µl with medium and the contents of each well was mixed by pipetting. The final concentration of PMA in this assay was 50 ng/ml, the final concentration of EDTA was 5 mM, and the final concentration of cytochalasin B was 20 µM. The plate was then incubated in a 37°C, 5% CO2 incubator for a period of 5 hr., after which the individual wells were photographed on an inverted microscope.

Inhibition of the Mn++ induced aggregation of MBL-2 cells

Cell lines (A20 and MBL-2) were observed to aggregate when suspended in 1 mM Mn++ and used for the 125I-sICAM-1 binding assay. To determine if this phenomenon was mediated by LFA-1 and ICAM-1, a qualitative aggregation assay similar to the PMA induced aggregation assay was carried out. MBL-2 cells were suspended at 4x10^6/ml in 10 mM Hepes pH 7.2, 0.15 M NaCl, 5% FCS. Cells were dispensed in eppendorf tubes, and to the tubes volumes of Ab SNs, EDTA or purified sICAM-1 were added followed by volumes of 1 M MnCl2 and more Hepes/NaCl to give a final concentration of 1.25 mM MnCl2 and 2x10^6
cells/ml. The tubes were then incubated at 37°C for 45 min., at which time the cells were resuspended by vortexing gently, and 100 µl aliquots were dispensed into the wells of flat bottom microtitre plates. The cells were allowed to settle in the wells for 5 min. and then individual wells were then photographed on an inverted microscope.

**Preparation of sICAM-1 coated polystyrene beads**

Uniform polystyrene beads, 2 µm in diameter (Seradyn, Indianapolis IN), were coated with sICAM-1 by physical absorption in a fashion similar to the coating of polystyrene microtitre wells. Absorbed surfactant and low molecular weight contaminants had to be removed from the beads before coating with sICAM-1, and this was accomplished by diafiltration in an Amicon 8010 stirred ultrafiltration cell (Amicon, Beverly, MA). 200 µl of beads (3.7x10⁹ beads) were washed in the ultrafiltration cell, using a 300,000 molecular weight cut off membrane, 2X dH₂O, 2X 0.1 M carbonate buffer pH 9.6, and then suspended in a volume of 1-2 ml 0.1 M carbonate buffer pH 9.6. 10 µl (10 µg) of purified sICAM-1 was added to 100 µl of beads and the tube was gently agitated for 1 hr. at 20°C. Control beads were similarly coated with BSA. After coating, beads were washed once by centrifugation (2,500 RPM, 10 min.), and blocked by a 30 min. incubation at 20°C in 100 µl of PBS containing 50 µg of BSA. Finally the beads were washed by centrifugation twice with RPMI 5% FCS.

**Quantitation of sICAM-1 coated polystyrene beads**

The amount of sICAM-1 coated to the sICAM-1 coated beads was determined using an ELISA in which the detection of sICAM-1, absorbed to microtitre wells, by YN1/1.7 Ab is inhibited by sICAM-1. SICAM-1 was coated to the wells of microtitre wells as described for the quantitation of sICAM-1 in sICAM-1 coated microtitre wells. For the ELISA, wells were coated to approximately 1,600 sites/µm², and blocked by incubation with PBS 5% blotto for 30 min. at 20°C. After washing wells 3X PBS, various volumes of sICAM-1 or BSA coated
beads or known quantities of purified sICAM-1 were added to duplicate wells. Then 100 µl of YN1/1.7 SN diluted 1/80 (a dilution previously determined to be near the end of the titration of this SN by ELISA) was dispensed into each well and incubated for 30 min. at 20°C. Wells were then washed 6X PBS and incubated with peroxidase conjugated goat anti-rat IgG (Bethesda Research Laboratories Life Technologies Inc., Burlington, Ont.) diluted 1/2,000 in PBS 5% blotto for 30 min. at 20°C. After washing the wells 6X PBS, the wells were incubated with 100 µl of substrate consisting of 2 mg/ml OPD (Sigma, St. Louis, MO) in 0.1 M NaPO₄ pH 6.0 with 0.012% H₂O₂, for 15 min. in the dark. The reaction was then stopped by addition of 25 µl of 3 M HCl/well, and the absorbances of individual wells was read on a Bio-Tek ELISA plate reader (model EL309) using a single wavelength of 490 nm. All conditions were done in duplicate, and duplicates usually varied by less than 10%. The % inhibition was calculated by the equation:

\[
\% \text{ inhibition} = \frac{\text{specific } A_{490}(-\text{inhibitor}) - \text{specific } A_{490}(+\text{inhibitor})}{\text{specific } A_{490}(-\text{inhibitor})} \times 100\%
\]

Immobilization of MBL-2 cells to microtitre plates via poly-L-lysine and subsequent binding of sICAM-1 coated beads

In order to initially visualize the binding of sICAM-1 coated beads to LFA-1+ cells, MBL-2 cells were immobilized to plastic via poly-L-lysine (Sigma, St. Louis, MO) and then allowed to bind to coated beads. Flat bottom microtitre wells were coated with 50 µl/well of 100 µg/ml poly-L-lysine in dH₂O for 2.5 hrs. at 37°C, after which time the wells were washed 3X PBS. MBL-2 cells were washed once with PBS, adjusted to 2x10⁶ cells/ml in PBS, and 100 µl was dispensed into each poly-L-lysine coated well. The plate was then centrifuged for 2 min. at 620 RPM and incubated for 30 min. at 20°C. Unbound cells were then washed away by gently washing the wells 3X PBS. 100 µl of RPMI 1640 supplemented with 10% FCS and antibiotics was then dispensed into each well, followed by 10 µl of either sICAM-1 or BSA
coated beads. The plate was incubated for 45 min. in a 37°C 5% CO₂ incubator, and then unbound beads were washed by gently washing the wells 6X with prewarmed, serum free RPMI 1640. Individual wells were photographed on an inverted microscope.

**Fluorescent staining of cells**

Lymphocytic cells were stained in suspension with specific Ab against various cell surface molecules. Prior to staining, cells were fixed in order to 'freeze' the distribution of cell surface molecules, and to prevent Ab induced patching. In some situations, cells were stimulated with PMA or treated with cytochalasin B for periods of time before staining, and in these situations the cultures were treated at 37°C and then chilled on ice before fixation and staining.

Cells were fixed in a solution of 1% paraformaldehyde in PBS. This concentration of paraformaldehyde was previously determined to effectively fix cells by the prevention of lysis in dH₂O. The paraformaldehyde solution was prepared fresh daily by dissolving 1 g of paraformaldehyde in 50 ml of dH₂O with heating and the addition of 1 drop of 1 M NaOH. The dissolved paraformaldehyde was then cooled to 20°C, 50 ml of 2X PBS was added, and the solution was chilled on ice. Lymphocytic cells chilled on ice were disaggregated by gentle pipetting, and aliquoted into eppendorf tubes. Cells were washed once with ice cold PBS, suspended in cold 1% paraformaldehyde in PBS, and incubated on ice for 20 min. with occasional gentle agitation. Fixed cells were then washed once with PBS and twice with Hanks containing 5% FCS.

Fixed lymphocytic cells were stained with primary Abs directed against cell surface molecules by incubation in undiluted tissue culture SN for 1 hr. on ice. After 3 washes with Hanks 5% FCS, cells were then incubated for 45 min. on ice in FITC conjugated goat anti-rat IgG (Cappel, Cooper Biomedical, Malvern, PA) diluted 1/40 with Hanks 5% FCS. In some cases cells were also stained with TRITC-phalloidin (101, Sigma, St. Louis, MO) at the same time as they were stained with 2°Ab. Phalloidin is a protein derived from certain mushrooms
which binds specifically and with a very high affinity to F-actin (102). It has also been shown that this small molecular weight molecule can enter formaldehyde fixed but not viable cells (101). Following the secondary staining cells were washed 3X Hanks 5% FCS and cell pellets were suspended in between 15-50 μl of Hanks containing 2% DABCO (Aldrich, Milwaukee, WIS) as an anti-photobleaching agent.

Adherent mouse L cells were also stained following paraformaldehyde fixation, but the cells were handled differently. Monolayers of L cells were lifted with 5 mM EDTA and plated into dishes containing autoclaved microscope coverslips. When cells had grown to the desired density, the cells attached to the coverslips were fixed and stained. Coverslips were washed in PBS once and then incubated in 1% paraformaldehyde in PBS for 20 min. at 20°C. Coverslips containing fixed cells were then washed 1X PBS, 2X Hanks 5% FCS, and stained with primary Abs directed against cell surface molecules by incubation in tissue culture SN for 1 hr. at 20°C. After 3 washes with Hanks 5% FCS, coverslips were then incubated for 45 min. in FITC conjugated goat anti-rat IgG (Cappel, Cooper Biomedical, Malvern, PA) diluted 1/40 with Hanks 5% FCS. After staining with secondary Ab, coverslips were washed 3X Hanks 5% FCS and then kept in serum free Hanks prior to mounting.

Immunofluorescence microscopy

For lymphocytic cells, slides were made by mounting 10 μl of cell suspension on a microscope slide and sealing the coverslip with melted wax. Coverslips with attached, stained L cells were mounted on microscope slides in 10 μl of Hanks 2% DABCO, and sealed with melted wax. Slides were viewed on a Zeiss microscope, equipped with epifluorescence, using a Leitz 40X/0.75 objective. Photographs were taken with either Kodak Ektachrome ASA 400 or P800/1600 film; using either phase contrast optics, epifluorescence and the green filter for visualizing FITC, or epifluorescence and the red filter for visualizing TRITC. Shutter speeds for exposures under phase contrast were approximately 1/2 sec. Shutter speeds for exposures
under fluorescence varied from 5 sec. with the 400 speed film (for high fluorescence intensity) to 15 sec. with the 800 speed film (for low fluorescence intensity).

**Creation of cell lines expressing ICAM-1 with or without it's cytoplasmic tail**

This work was initiated in Fumio Takei's lab by Mike Butchart, a summer student who put together all of the DNA constructs and did the initial transfections. For transfection of the complete ICAM-1 molecule, the cDNA encoding complete, functional murine ICAM-1(95) was subcloned into BCMGSNeo. For transfection of ICAM-1 without it's cytoplasmic domain, a little more work was involved. Using PCR and the above cDNA as a template, a fragment encoding part of extracellular domain 4, extracellular domain 5, the transmembrane domain, and the first two amino acids of the cytoplasmic domain was generated. The PCR primers were designed such that the 5' end of this fragment had a HindIII restriction site. A fragment encoding the first 3 extracellular domains and part of the fourth, obtained by HindIII digestion of the cDNA, was then ligated to the PCR fragment, and the result was a cDNA encoding ICAM-1 without the majority of it's cytoplasmic domain. This construct was then subcloned into the BCMGSNeo expression vector, where the final construct encodes all of the extracellular domains, the transmembrane region, the first two amino acids of the cytoplasmic domain, and finally two irrelevant amino acids. These two versions of ICAM-1, each in BCMGSNeo, were then electroporated into T28 cells, and also transfected into L cells by calcium phosphate mediated transfection.

T28 transfectants were selected in 1 mg/ml G418, and FACS analysis of initial transfectant populations showed very good expression (data not shown). L transfectants were also selected in 1 mg/ml G418; however, initial expression was poor. High expressers were sorted by FACS and selected again in 1 mg/ml G418. This process yielded populations of L cell transfectants exhibiting strong ICAM-1 expression. In both the transfected T28 and L cells, the cytoplasmic deletion version of ICAM-1 was expressed just as well as the complete ICAM-1 molecule (data not shown).
Analysis of cell lines expressing transfected ICAM-1 with or without its cytoplasmic domain

The transfected T28 cells were compared to the parental T28 line in a PMA induced homotypic aggregation assay similar to that described above for MBL-2 cells. Parental T28 cells and the two ICAM-1 transfectant lines were suspended to $2 \times 10^6$ cells/ml in RPMI 1640 supplemented with 10% FCS and antibiotics, and 1 ml aliquots with or without 50 ng/ml PMA were dispensed into the wells of a 24 well plate. Only 1 hr. of PMA stimulation was required to achieve efficient aggregation of the ICAM-1 transfectants, as compared to the 4-5 hr. required for MBL-2 cells. These 1 ml cultures were then chilled on ice and analyzed by immunofluorescence microscopy after fixation and fluorescent staining of ICAM-1 and other cell surface molecules, as described above.

FACSorted, 1 mg/ml G418 selected L cell transfectants were analyzed by immunofluorescence microscopy after fixation and fluorescent staining of ICAM-1 and other cell surface molecules as described above. This analysis, along with the FACS analysis, demonstrated extremely high levels of ICAM-1 expression, possibly swamping out any subtle distribution effects. The L cell transfectants were therefore grown for approximately 2 weeks in only 100 µg/ml G418 in an attempt to lower the level of ICAM-1 expression, and immunofluorescence analysis was repeated.
Results

Part I: Production and purification of sICAM-1

Previously, the means and the methodology for the production and purification of sICAM-1 had already been worked out in Fumio Takei's lab (Lee and Takei, manuscript in preparation). Briefly, the cDNA encoding a soluble version of murine ICAM-1 (MALA-2) was subcloned into the eukaryotic expression vector BCMGSNeo (96). The cDNA encoding sICAM-1 encodes amino acids 1-453, with the transmembrane sequence starting at amino acid 461 in the complete ICAM-1 protein. The recombinant vector was electroporated into NS-1 cells and transfectants were then selected in 1 mg/ml G418. Culture SNs were screened for the presence of ICAM-1 by immunoblot. Results of such an immunoblot are shown in Figure 2. Detection of sICAM-1 by this method was both rapid and sensitive. Single cell cloning and re-screening yielded the clone 2706C which secreted large quantities of sICAM-1 into the culture SN. This cell line was used for subsequent large scale protein production. 2706C culture SN was loaded onto a YN1/1.7 Ab column, the column was then washed extensively with 0.15 M NaCl, 10 mM Tris pH 7.4. sICAM-1 was eluted with 0.15 M NaCl, 50 mM diethylamine, pH 11.0. 500 ml batches of SN generally yielded approximately 1 mg of protein as estimated by OD$_{280}$. Eluted material from the column was then either concentrated and exchanged into PBS or re-applied to the column.

Molecular weight, purity, and yield of sICAM-1 was then confirmed by SDS-PAGE analysis. Typical SDS-PAGE results are shown in Figure 3. In this experiment approximately 1 μg of each of two independently purified preparations were run on SDS-PAGE, and the gel subsequently coomassie stained. Purified sICAM-1 migrates with a relative molecular mass of approximately 93,000 under nonreducing conditions. This size is in good agreement with the predicted molecular weight for sICAM-1, since murine ICAM-1 was found to migrate with a relative molecular mass of 95,000 to 100,000 under reducing or nonreducing conditions (87).
The cloned murine ICAM-1 contains 512 amino acids (95), and 59 amino acids were deleted from the original molecule in order to obtain the soluble version.

A more stringent assessment of the purity of the purified sICAM-1 preparations was obtained from the analysis of radio-iodinated sICAM-1. Purified sICAM-1 was radio-iodinated, using the IODO-GEN method, to a specific activity of approximately $4 \times 10^4$ CPM/ng. $^{125}$I-sICAM-1 was then run on SDS-PAGE and the gel subsequently exposed to X-ray film. Figure 4 shows the results of such an experiment in which approximately 25 ng of each of two preparations, one purified by one cycle of affinity chromatography and one purified by two cycles of affinity chromatography, were analyzed. The results clearly show that the material purified by one round of affinity chromatography is very pure. Protein purified by one cycle of affinity chromatography was used for all subsequent experiments.

To summarize the results presented in Part I, highly purified, recombinant murine sICAM-1 was produced. With sICAM-1, two things could be accomplished. Firstly, it's use as an inhibitor of LFA-1:ICAM-1 mediated cell adhesion and immune responses dependent on LFA-1:ICAM-1 interaction could be explored. Secondly, the fine details of LFA-1:ICAM-1 mediated cell adhesion could be explored.
Figure 2  Initial detection of sICAM-1 in culture supernatants and following affinity chromatography purification. Samples were blotted onto nitrocellulose and sICAM-1 was detected by the indirect immunoperoxidase method using YN1/1.7 Ab, peroxidase conjugated goat-anti-rat IgG, and DAB/H2O2 substrate. For samples 1 to 4, (a) represents 10 µl of culture supernatant and (b) represents 100 µl of culture supernatant. The samples were from 1) an intermediate producing cell line, 2) a low producing cell line, 3) the high producing cell line used for large scale protein production, 4) control untransfected NS-1 supernatant, and 5) affinity chromatography purified sICAM-1. For 5, (a) represents 5 µg of purified protein and (b) represents 50 µg of purified protein.
Figure 3  
**Confirmation of molecular weight, purity, and yield of purified sICAM-1.** 
Approximately 1 μg of each of two independently purified batches of sICAM-1 were run on a 7.5% SDS-PAGE and coomassie stained. Molecular weight markers were Bio-Rad high range standards, and molecular weights are indicated.
Assessment of the purity of radio-iodinated sICAM-1. SICAM-1 was radio iodinated by the IODO-GEN method and aliquots containing approximately 25 ng (approximately 2x10^6 CPM) of 125I-sICAM-1 each were run on 7.5% SDS-PAGE and the gel exposed to X-ray film. The batches of protein were purified a) by one cycle of affinity chromatography or b) by two cycles of affinity chromatography.
Part II: Functional characterization of sICAM-1

The first functional assay for sICAM-1 was its ability, in solution, to bind to LFA-1 positive cells. Purified sICAM-1 was radio-iodinated using the IODO-GEN method to a specific activity of approximately $4 \times 10^4$ CPM/ng. The results of several $^{125}$I-sICAM-1 binding assays are shown in Figure 5. Here MBL-2, A20 or T28 cells were incubated at room temperature with various concentrations of $^{125}$I-sICAM-1 for 1 hr., in the presence or absence of excess unlabeled sICAM-1 or EDTA as inhibitors. Figure 5 clearly shows that $^{125}$I-sICAM-1 is capable of binding to LFA-1 positive cells, and in addition since this binding is inhibited both by unlabeled sICAM-1 and the divalent cation chelator EDTA, the binding is most likely mediated specifically by LFA-1. In Figure 5 (a) there appears to be relatively high affinity binding, while in Figure 5 (b) and (c) the binding appears to be of relatively low affinity. The high affinity binding shown in Figure 5 (a) is consistent with the binding assay results obtained by Daniel Lee (Daniel Lee, unpublished data). However, in my hands high affinity binding was much more difficult to demonstrate, and most of the experiments yielded results as shown in Figure 5 (b) and (c). In addition, Figure 5 (c) demonstrates that the addition of 1 mM Mn$^{++}$ enhances the binding of $^{125}$I-sICAM-1 to its receptor. The observed effect of Mn$^{++}$ is in agreement with a recent report concerning the effect of Mn$^{++}$ on the affinity of Mac-1 for its ligands (103), and a report on the effects of Mn$^{++}$ on a $\beta_3$ integrin (104). Indeed a recent report has also shown that Mn$^{++}$ increases both the adhesion of T cells to absorbed ICAM-1 as well as the existence of an LFA-1 activation epitope (105).
$^{125}$I-sICAM-1 binds to LFA-1 positive cells. Purified sICAM-1 was radioiodinated by the IODO-GEN method to a specific activity of approximately 4x10^4 CPM/ng, and its binding to the LFA-1 positive cell lines MBL-2 (a), A20 (b), and T28 (c) was determined. In (a) binding was carried out in RPMI 1640 5% FCS at an $^{125}$I-sICAM-1 concentration of 200 ng/ml (2x10^{-9} M) for 1 hr., then the cells were washed 3X and cell associated radioactivity was determined. In (b) binding was carried out in RPMI 1640 5% FCS at an $^{125}$I-sICAM-1 concentration of 2 μg/ml (2x10^{-8} M) for 1 hr., then aliquots of cells were spun through oil and cell associated radioactivity was determined. In (c) binding was carried out in Hepes buffered saline with 1 mM CaCl$_2$, 1 mM MgCl$_2$, with or without 1 mM MnCl$_2$; cell bound radioactivity was determined as for (b). In all cases unlabeled sICAM-1 (cold inh.) was used in a 100 fold excess to inhibit binding, while EDTA was used at a final concentration of 5 mM. All incubations were done at room temperature.
The second functional assay for sICAM-1 was the quantitative cell adhesion assay. In this assay purified sICAM-1 is coated to the wells of polystyrene microtitre plates, and then LFA-1 positive cells are allowed to bind to the absorbed sICAM-1.

In order to estimate what the density of sICAM-1 was on the sICAM-1 coated plastic, and to compare this to the physiological situation, I quantitated the sICAM-1 absorbed to plastic used for the quantitative adhesion assay. This quantitation was done with $^{125}$I-YN1/1.7 Ab. The results of this quantitation are shown in Figure 6. For the sake of comparison, Kurzinger et al. have estimated that the density of LFA-1 on human peripheral blood lymphocytes is approximately 150 molecules/µm$^2$ (106). Therefore the density achievable in the quantitative cell adhesion assay, approximately 1,600 molecules/µm$^2$, is significantly higher than what is likely to exist in the physiological situation.

Figure 7 shows the results of an early quantitative cell adhesion assay. For this experiment, wells were either coated to a high density of sICAM-1 and then subsequently blocked with BSA, or just blocked with BSA. This experiment was done with the the murine B lymphoblastoid cell line A20, which expresses LFA-1 on it’s cell surface. It is important to note the way this assay is done: the cells are added to the wells, the plate is spun to bring the cells into contact with the coated surfaces, and then the plate is incubated at 37°C for only 5 min. before the unbound cells are washed away. $2 \times 10^5$ cells are added to each well; therefore, almost 100% of the input cells bind under these conditions. Cytochalasin B, an agent which inhibits the actin cytoskeleton by binding specifically to actin and preventing the polymerization of actin (107), effectively inhibits the binding of A20 cells in this assay. This experiment shows that sICAM-1 in solution, at a concentration of up to 100 µg/ml, does not inhibit adhesion.
Figure 6

Quantitation of the density of sICAM-1 in sICAM-1 coated microtitre wells. Serial dilutions of purified sICAM-1 were coated to the wells of flat bottom microtitre plates, the wells were then blocked and incubated with a saturating concentration of $^{125}\text{I}$-anti-ICAM-1. The amount of bound $^{125}\text{I}$ anti-ICAM-1 was then determined, and from the quantity of $^{125}\text{I}$-anti-ICAM-1 bound to wells, the density of sICAM-1 was calculated. This standard curve was used to estimate the density of sICAM-1 for all of the subsequent quantitative cell adhesion assays.
Figure 7  
SICAM-1 is functional when absorbed to plastic in the quantitative cell adhesion assay. Microtitre wells were coated with sICAM-1 to a density of about 1,600 sites/µm². 2x10^5 A20 cells per well were then spun down onto the surface of the wells, incubated at 37°C for 5 min., and unbound cells were washed off. Bound cells were then quantitated with MTT. Cytochalasin B was used at a final concentration of 20 µM and sICAM-1 was used at a final concentration of 100 µg/ml to attempt inhibition.
In agreement with the inability of sICAM-1 to inhibit cell adhesion in the quantitative cell adhesion assay, sICAM-1 is unable to inhibit the LFA-1:ICAM-1 mediated homotypic aggregation of MBL-2 cells. The results of such an experiment are shown in Figure 8. In this assay the murine T lymphoma cell line MBL-2 aggregates only after stimulation by PMA. Aggregation is mediated by LFA-1:ICAM-1 interaction as demonstrated by the ability of Ab to either LFA-1 or ICAM-1 to inhibit aggregation. Interestingly, the inhibition by Ab to LFA-1 was consistently more complete than that by Ab to ICAM-1, suggesting the involvement of another counter-receptor for LFA-1. Aggregation in this assay is also effectively inhibited by EDTA and by cytochalasin B. However, aggregation is not inhibited to any extent by sICAM-1 at a concentration of up to 100 µg/ml.

As mentioned above, the addition of 1 mM Mn++ presumably increases the affinity of LFA-1 for ICAM-1. When I first used Mn++ in the 125I-sICAM-1 binding assay, I noticed that cells suspended in medium containing Mn++ aggregated extensively compared to cells suspended in medium containing Ca++ and Mg++ but no Mn++. This phenomenon appeared to be ICAM-1:LFA-1 dependant since ICAM-1 positive, LFA-1 positive MBL-2 and A20 cells aggregated strongly in response to Mn++, while LFA-1 positive, ICAM-1 negative T28 cells aggregated to a much lesser extent in response to Mn++. In order to characterize this phenomenon, a qualitative aggregation assay similar to the PMA induced aggregation assay was developed. Figure 9 shows the results of such an experiment. In this Figure, MBL-2 cells are shown to aggregate strongly in response to 1 mM Mn++, and this aggregation is LFA-1 and ICAM-1 dependant. However, unlike PMA induced aggregation, Mn++ induced aggregation could be efficiently inhibited with sICAM-1 at 100 µg/ml. Analogous results were obtained with A20 cells (data not shown).
Figure 8  SICAM-1 does not inhibit the PMA induced homotypic aggregation of MBL-2 cells. Photomicrographs of MBL-2 cells a) without PMA, b) with PMA, c) with PMA and anti-LFA-1, d) with PMA and anti-ICAM-1, e) with PMA and cytochalasin B, f) with PMA and EDTA, g) with PMA and DMSO as a control for f), and h) with PMA and sICAM-1. PMA was used at a final concentration of 50 ng/ml, Abs were used at a 1/40 dilution of culture SN (predetermined to be saturating), cytochalasin B was used at a final concentration of 20 µM, EDTA was used at a final concentration of 5 mM, and sICAM-1 was used at a final concentration of 100 µg/ml to attempt inhibition. The assay was photographed following 4 hrs. of incubation at 37°C.
Figure 9  SICAM-1 inhibits the Mn$^{++}$ induced homotypic aggregation of MBL-2 cells. Photomicrographs of MBL-2 cells a) without Mn$^{++}$, b) with Mn$^{++}$, c) with Mn$^{++}$ and anti-LFA-1, d) with Mn$^{++}$ and anti-ICAM-1, e) with Mn$^{++}$ and anti-Mac-1, f) with Mn$^{++}$ and 100 µg/ml sICAM-1, g) with Mn$^{++}$ and 33 µg/ml sICAM-1, and h) with Mn$^{++}$ and 11 µg/ml sICAM-1. All conditions contained 1 mM Ca$^{++}$ and Mg$^{++}$. Mn$^{++}$ was used at a final concentration of 1 mM, Abs were used at a 1/40 dilution of culture SN (predetermined to be saturating), and sICAM-1 was used at the final concentrations indicated to attempt inhibition. The assay was photographed following 45 min. of incubation at 37°C.
Early on in the project we hypothesized that since the density of sICAM-1 on the sICAM-1 coated plastic was so high in the quantitative cell adhesion assay, inhibition by sICAM-1 in solution might be difficult, especially since the cells are spun down onto the surface of the well and then allowed to bind. We predicted that sICAM-1 might be better able to inhibit adhesion in the quantitative adhesion assay if the wells were coated to a lower density and if cells were incubated with the sICAM-1 before being added to the plate. Figure 10 shows the results of such an experiment. The results clearly show that adhesion is inhibitable by cytochalasin B and anti-LFA-1, but not by sICAM-1 at a final concentration of 100 µg/ml, even when the cells were preincubated with the sICAM-1 for 20 min. and then assayed for adhesion in wells coated to only 250 sites/µm². This experiment also shows that for A20 cells PMA stimulation increases adhesion, but only at lower densities of absorbed sICAM-1. My observation that cell lines bind efficiently to high densities of absorbed sICAM-1 without PMA stimulation is in contrast to the findings of Springer's group with human ICAM-1 (13, 42). These reports suggest that even at high densities of ICAM-1, lymphoblastoid cells require PMA stimulation in order to adhere. In fact I have observed that the cell line MBL-2 binds efficiently to absorbed ICAM-1 over a range of densities, and binding could not be specifically increased by PMA stimulation. In addition, cytochalasin B does not efficiently inhibit the adhesion of MBL-2 cells in the quantitative adhesion assay, despite the ability of cytochalasin B to effectively inhibit the PMA induced homotypic aggregation of MBL-2 cells. In contrast the cell line A20, which was used in Figures 7 and 10, is effectively inhibited by cytochalasin B from adhering to plastic absorbed sICAM-1.
Figure 10  
Cell adhesion in the quantitative cell adhesion assay is stimulated by PMA and inhibited by anti-LFA-1 and cytochalasin B but not by sICAM-1. Experiment performed as for Figure 7 except that wells were coated to varying sICAM-1 densities, and cells were pretreated with inhibitors/stimulators for 20 min. at 37°C before being added to the plate. PMA was used at a final concentration of 50 ng/ml. TIB 213 is anti-LFA-1, and was used at a 1/40 final dilution of culture SN.
There were a number of problems associated with using cell lines in the quantitative cell adhesion assay. Firstly, all of the cell lines tested often gave high backgrounds, that is they bound to blocked plastic or even uncoated plastic appreciably. Secondly, as noted above there was disagreement as to the requirement for PMA stimulation in cell adhesion. The work of Springer et al. has suggested that stimulation by PMA or TcR cross-linking is an absolute requirement for the adhesion of lymphoid cells to absorbed ICAM-1 (42). The findings of this study with murine cell lines suggested that cells can bind efficiently to high densities of absorbed sICAM-1, and only in some cases at lower densities of sICAM-1 is PMA stimulation required. Therefore, we wanted to obtain a population of cells that would consistently bind to plastic absorbed sICAM-1 with a low background, but only after stimulation by PMA. Resting splenic T cells, purified over a nylon wool column, fulfilled these criteria. Figure 11 shows the results obtained when nylon wool T cells were used in the quantitative cell adhesion assay. It is clear that these cells bind to immobilized sICAM-1 in a sICAM-1 density dependent and PMA dependent fashion. Adhesion is also effectively inhibited by Ab to LFA-1, and cytochalasin B.

Resting splenic T cells were also used to quantitate the Mn++ induced adhesion described above. Since Mn++ treatment precluded the use of the MTT assay for the quantitation of cell adhesion (see Materials and Methods), 51Cr labeled nylon wool T cells were required for these experiments. Mn++ clearly induces LFA-1:ICAM-1 mediated adhesion in the quantitative cell adhesion assay, as shown in Figure 12. However, sICAM-1 in solution at a concentration of 65 μg/ml was incapable of inhibiting Mn++ induced adhesion, in contrast to the ability of sICAM-1 to inhibit the Mn++ induced aggregation of cell lines. Subsequent experiments with sICAM-1 concentrations of up to 110 μg/ml failed to clearly demonstrate inhibition of Mn++ induced adhesion by sICAM-1 in the quantitative cell adhesion assay (data not shown). Surprisingly, cytochalasin B did inhibit the Mn++ induced adhesion of resting T cells to absorbed sICAM-1, as shown in Figure 12.
Figure 11  

Cell adhesion of resting T cells in the quantitative cell adhesion assay is absolutely dependent on PMA stimulation. Resting splenic T cells were purified over a nylon wool column and then used in the quantitative cell adhesion assay. The experiment was performed as for Figure 10.
Figure 12  
**Mn**^{++} induces ICAM-1:LFA-1 mediated adhesion in the quantitative cell adhesion assay. Resting splenic T cells were purified over a nylon wool column, labeled with \textsuperscript{51}Cr, and then used in the quantitative cell adhesion assay. The experiment was performed as for Figure 10 with several exceptions. \textsuperscript{51}Cr was used at 1.25 mM, and all wells containing \textsuperscript{51}Cr were washed with \textsuperscript{51}Cr containing medium. SICAM-1 was used at a final concentration of 65 µg/ml in solution to attempt inhibition. Bound cells were quantitated by γ counting following dissociation with 5 mM EDTA.
In an attempt to get a picture of how cytochalasin B was inhibiting adhesion so efficiently, the cellular morphology of cells treated with cytochalasin B was compared to that of untreated cells. To do this, untreated or cytochalasin B treated MBL-2 cells were used in the quantitative cell adhesion assay, except that their cellular morphology was observed throughout the experiment. As shown in Figure 8, cytochalasin B effectively inhibits the PMA induced aggregation of MBL-2 cells, but as noted above cytochalasin B does not effectively inhibit the binding of these cells to plastic absorbed sICAM-1. The results of this experiment are shown in Figure 13. In this experiment cells were photographed following the 5 min. incubation at 37°C before and after the wells were washed of unbound cells. Cytochalasin B clearly induces a morphological change in the cells observable before unbound cells were washed away. Cytochalasin B treated cells appear more uniformly round than do untreated cells, and the membranes of treated cells were smoother than untreated cells. It was hoped that more effective flattening of untreated cells on the plastic absorbed sICAM-1 as compared to the cytochalasin B treated cells might be observed in this experiment; however, the magnification and resolution of the microscope seemed to be inadequate for this purpose. Following the washing away of unbound cells, the cells treated with cytochalasin B return substantially to their characteristic morphology.

In summary of the functional characterization of sICAM-1, the purified recombinant sICAM-1 binds to LFA-1+ cells and functions just as cellular ICAM-1 when immobilized on solid surfaces. PMA stimulation increases ICAM-1:LFA-1 mediated cell adhesion; however, sICAM-1 does not inhibit PMA stimulated, ICAM-1:LFA-1 mediated cell adhesion. Mn++ enhances the binding of 125I-sICAM-1 to LFA-1+ cells and increases ICAM-1:LFA-1 mediated cell adhesion. In contrast to PMA induced homotypic aggregation, Mn++ induced homotypic aggregation is inhibited by sICAM-1. Also of significance is the importance of the actin cytoskeleton for ICAM-1:LFA-1 mediated adhesion.
Figure 13  

Cytochalasin B induces morphological changes in cells. MBL-2 cells which were either a) untreated or b) treated with cytochalasin B (20 μM) for 20 min. at 37°C, were then centrifuged onto sICAM-1 coated microtitre wells (coated to 1600 sites/μm²), incubated for 5 min. at 37°C, and then photographed. Unbound cells were then washed away and the wells were photographed again.
Part III: The role of multivalent interaction in LFA-1:ICAM-1 mediated cell adhesion

The results presented above demonstrate that sICAM-1 is ineffective in inhibiting PMA induced cell adhesion. It seems that the most reasonable explanation for this observation is that the binding of sICAM-1 to LFA-1 is not of sufficiently high affinity. Cell:cell adhesion mediated by LFA-1 and ICAM-1 is likely mediated by multivalent interaction. Low affinity monovalent binding is unlikely to effectively compete with multivalent binding between membrane LFA-1 and either membrane ICAM-1 or plastic immobilized sICAM-1. If the inability of monovalent sICAM-1 to inhibit PMA stimulated cell adhesion is solely due to it's monovalency then an artificially multivalent form of sICAM-1, with the same affinity for it's receptor, would be expected to effectively inhibit cell adhesion. The approach we took to test this hypothesis was to create a multivalent form of sICAM-1 by coating it to uniform polystyrene beads. Since sICAM-1 coated to polystyrene microtitre plates is functional as determined by it's adhesive capabilities, we expected that sICAM-1 coated to polystyrene beads would also be functional.

Polystyrene beads, 2 μm in diameter, were coated with purified sICAM-1 in a similar fashion to the coating of the polystyrene microtitre wells. These beads were then assayed using an ELISA in order to quantitate the amount of sICAM-1 coated to the beads. The results of this assay are shown in Figure 14. In Figure 14a), sICAM-1 coated beads, control BSA coated beads, or known amounts of purified sICAM-1 were used to inhibit an ELISA in which sICAM-1 coated to microtitre wells was detected with YN1/1.7 Ab. The results clearly show that the sICAM-1 coated beads, but not the BSA coated beads, inhibit the binding of YN1/1.7 Ab to sICAM-1 coated to the ELISA plate. Using the calculated % inhibition values for the known amounts of purified sICAM-1, a standard curve was generated (Figure 14b). This standard curve was then used to estimate the amount of sICAM-1 in the preparation of sICAM-1 coated beads. It was estimated that the final suspension of sICAM-1 coated beads contained approximately 60 μg/ml of sICAM-1, and since the beads were coated at a concentration of 100
\( \mu g/ml \), an approximate coating efficiency of 60% was achieved. When this concentration was used, along with the approximate concentration of beads in the preparation, to calculate the density of sICAM-1 on the beads, a density of approximately 6,000 sites/\( \mu m^2 \) was obtained. This density is extremely high when compared to both the density obtained on the polystyrene microtitre plates and the density of LFA-1 on human neutrophils mentioned above. It is likely that the high surface area to volume ratio of the polystyrene beads explains their ability to absorb protein so efficiently.

These sICAM-1 coated beads bound to MBL-2 cells as shown in Figure 15. The sICAM-1 coated beads also specifically inhibited LFA-1:ICAM-1 mediated cell adhesion in the quantitative cell adhesion assay, as shown in Figure 16. The sICAM-1 coated beads were shown in this experiment to specifically inhibit cell adhesion under conditions where monovalent sICAM-1 was completely incapable of inhibiting cell adhesion. It is important to note that in Figure 16 the amount of inhibitor is given in terms of \( \mu l \) per 100 \( \mu l \) final assay volume/well; therefore, since the sICAM-1 coated beads only contain about 1/20 the sICAM-1 per unit volume as the monovalent sICAM-1, the 10 \( \mu l \) designations correspond to sICAM-1 concentrations of 100 \( \mu g/ml \) for the monovalent sICAM-1 but only 5 \( \mu g/ml \) for the sICAM-1 coated beads. In addition, 10 \( \mu l \) of coated beads corresponds to approximately 150 beads per cell in the assay. The inhibition of adhesion in the quantitative cell adhesion assay by blocking LFA-1 with sICAM-1 coated beads was never complete, and was always plagued by a nonspecific background inhibition problem, especially at high ratios of beads:cells. It would seem that at high densities of beads, when the cells are spun down onto the surface of the plate, so are some of the beads, and the beads simply get between the cells and the plate. However, the inhibition shown in Figure 16 is specific, and even marginal inhibition in this assay is probably significant in light of the inability of monovalent sICAM-1 to inhibit the \( Mn^{++} \) induced adhesion in the quantitative cell adhesion assay (Figure 12). The experiment shown in Figure 16 therefore supports the notion that the reason for the inability of sICAM-1 to inhibit cell adhesion is simply that monovalent sICAM-1 is incapable of competing with the
multivalent sICAM-1 on the coated microtitre wells; since both forms of the molecule have the
same low affinity for their receptor, only multivalent interactions are stable enough to persist.
The same argument holds for the inability of sICAM-1 to inhibit the PMA induced homotypic
aggregation of MBL-2 cells; monovalent sICAM-1 is incapable of competing with the
multivalent ICAM-1 on other cells.

In summary of the results presented in Part III, through the use of a multivalent form of
sICAM-1 the importance of multivalent interaction in LFA-1:ICAM-1 mediated cell adhesion
was demonstrated. The results also support the notion that PMA stimulated, ICAM-1:LFA-1
mediated cell adhesion is the result of low affinity binding.
Quantitation of sICAM-1 coated to polystyrene beads. An ELISA assay, in which sICAM-1 coated to microtitre wells was detected with YN1/1.7 Ab and indirect immunoperoxidase development, was used to quantitate the amount of sICAM-1 coated to the polystyrene beads. In (a) the binding of YN1/1.7 Ab was inhibited with known quantities of purified sICAM-1, sICAM-1 coated polystyrene beads, or BSA coated beads. The volumes of beads indicated in (a) represents volumes of the final suspension of coated beads per 100 μl in the ELISA assay. The data from the inhibition of the ELISA with known quantities of sICAM-1 was then used to generate a standard curve (b) from which the amount of sICAM-1 in the sICAM-1 coated beads could be estimated. From the standard curve it was estimated that the final suspension of sICAM-1 coated beads contained approximately 60 μg sICAM-1/ml.
Figure 15  
SICAM-1 coated polystyrene beads bind to MBL-2 cells. Photomicrographs of MBL-2 cells which were immobilized on plastic via poly-L-lysine, incubated for 45 min. at 37°C with polystyrene beads previously coated with a) sICAM-1 or b) BSA, and washed of unbound beads. Microtitre wells were incubated with 10 µl of bead suspensions in final volumes of 100 µl, corresponding to approximately 6 µg/ml sICAM-1 and 3x10^8 beads/ml.
Figure 16  Multivalent sICAM-1 coated polystyrene beads inhibit cell adhesion in the quantitative cell adhesion assay while monovalent sICAM-1 does not. PMA treated resting T cells were incubated for 20 min. at 37°C with either sICAM-1, sICAM-1 coated beads, BSA coated beads, or nothing. The cells were then used in the quantitative cell adhesion assay in wells coated to approximately 940 sites/µm². Experiment was performed as described for Figure 10. For monovalent sICAM-1, 10 µl corresponds to a final concentration of 100 µg sICAM-1/ml, whereas for the sICAM-1 coated beads 10 µl corresponds to only 5 µg sICAM-1/ml and approximately 150 beads/cell.
Part IV: Analysis of the distribution of LFA-1 and ICAM-1 on the cell surface

The functional characterization of sICAM-1 presented above provides somewhat confusing information. sICAM-1 appears functional as measured by the \(^{125}\text{I}-\text{sICAM-1}\) binding assay, as well as in the quantitative cell adhesion assay. In addition, sICAM-1 is capable of inhibiting the Mn\(^{2+}\) induced aggregation of cell lines, aggregation which seems to be facilitated by an increase in the normally low affinity of the interaction between ICAM-1 and LFA-1. However, sICAM-1 is very inefficient at inhibiting LFA-1:ICAM-1 mediated cell adhesion mediated by PMA stimulation. In addition, the actin cytoskeleton is essential for LFA-1:ICAM-1 mediated cell adhesion, especially adhesion stimulated by PMA.

The results with sICAM-1 coated beads explain why the monovalent form of sICAM-1 is incapable of inhibiting ICAM-1:LFA-1 mediated adhesion. These results also stress the importance of multivalent interaction in ICAM-1:LFA-1 mediated cell adhesion. In light of the results with sICAM-1 coated beads, we proposed that since ICAM-1:LFA-1 mediated adhesion was dependant on multivalent interaction, local concentrations of adhesion molecules on the cell surface might be important in determining the adhesive phenotype of LFA-1 and ICAM-1 positive cells. We therefore made several predictions which I set out to test. The first was that high localized concentrations of adhesion molecules on the surface of cells might facilitate adhesion, at the same time as making inhibition by sICAM-1 in solution difficult. We also predicted that PMA stimulation may affect the distribution of adhesion molecules, especially LFA-1, on the cell surface, as is the case for Mac-1 (51). While these predictions may seem somewhat 'hand-wavey', as discussed in the Introduction of this thesis, there is a great potential for the distribution of adhesion molecules to have significant effects on cell adhesion. In addition, relatively little is known about the distribution of adhesion molecules on the cell surface, and what effects this distribution may have on cell adhesion. Therefore the distribution of LFA-1 and ICAM-1 on the cell surface was studied to determine what relationship, if any there is between the distribution of adhesion molecules and cell adhesion.
The first distribution studies were done with MBL-2 cells. Since this cell line aggregates in an LFA-1:ICAM-1 dependant fashion in response to PMA stimulation (Figure 8), we hoped to see some differences in the distribution of adhesion molecules, particularly LFA-1, upon PMA stimulation. The results of one such experiment are given in Figure 17. In order to visualize the distribution of cell surface molecules we used immunofluorescence microscopy because of the relative ease of this technique. As shown in Figure 17, the distribution of LFA-1 and ICAM-1 is relatively uniform over the cell surface, not significantly different from the control molecule CD45, which incidentally was chosen as a control because of it's high level of expression and it's apparent lack of association with the cytoskeleton (108), suggesting uniform distribution. In addition, Figure 17 demonstrates the lack of any change in the distribution of either LFA-1 or ICAM-1 following PMA stimulation, a consistent observation over several independent experiments. The way the MBL-2 cells were treated before fixation and staining was to culture them under the same conditions as for the PMA induced aggregation assay, except in 1 ml cultures with or without PMA, for 4 hours. Aggregated, PMA stimulated cultures as well as non-aggregated, unstimulated cultures were then chilled on ice for about 10 min. in order to 'freeze' the normally fluid plasma membrane, disaggregated by pipetting, and then fixed with paraformaldehyde on ice before staining.
Figure 17  

The distribution of cell surface molecules on unstimulated or PMA stimulated MBL-2 cells. Photomicrographs of MBL-2 cells which were either unstimulated or stimulated with PMA at 37°C for 4 hrs., chilled on ice, disaggregated by pipetting, fixed with 1% paraformaldehyde, stained with rat IgG antibodies to various cell surface molecules, and then stained with FITC goat-ant-rat IgG. Cells were stained and subsequently observed under the microscope in suspension. For each exposure taken under fluorescence (Fl.), an accompanying exposure was taken under phase contrast (P.C.).
2nd Ab control

Fl.

P.C.
LFA-1

F1.

- PMA

P.C.
In addition to MBL-2, initial distribution studies were done with A20 cells. Typical results of the analysis of A20 cells is shown in Figure 18. A20 behave quite differently than MBL-2 cells; A20 cells spontaneously form homotypic aggregates in culture while MBL-2 cells grow mostly as single cells with few aggregates. A20 cells also do not aggregate well in response to PMA. A20 cells were therefore not analyzed after PMA stimulation. Since studies to functionally characterize sICAM-1 demonstrated the importance of the actin cytoskeleton, we decided to fluorescently visualize cytoplasmic actin concurrently with cell surface staining. We hoped to see some association between the actin cytoskeleton and adhesion molecules, especially LFA-1. Figure 18 shows results from A20 cells which were either untreated or treated with cytochalasin B, and then stained for actin or various cell surface molecules. There were two very striking observations with this experiment. The first was that ICAM-1 was very often found localized to single spots on the surface of A20 cells. This observation was consistent over several experiments with A20 cells, and it is not likely the result of Ab induced capping because the cells were fixed prior to staining and this punctate staining pattern was not seen with any other primary Ab used, or with any other cell line analyzed. The other striking observation shown in Figure 18 is the very different actin staining patterns in untreated and cytochalasin B treated cells. Untreated cells exhibited heterogeneous actin staining, between cells and within individual cells. The majority of untreated cells stained relatively weakly for actin, while a minority of cells stained very intensely often with a polarized staining pattern. In sharp contrast, the cytochalasin B treated cells exhibited much less heterogeneous actin staining between cells but consistently patchy staining within individual cells. The actin staining of MBL-2 cells was very similar to that shown for A20 cells in Figure 18 (data not shown).

With respect to the punctate ICAM-1 staining of A20 cells shown in Figure 18, a correlation with actin staining is unclear. In (cytochalasin B) untreated cells the ICAM-1 spots appeared often but not always to correlate with areas of the cell staining intensely for actin. In cytochalasin B treated cells the ICAM-1 spots most often correlated to regions staining intensely for actin, although cytochalasin B treated cells showed far more consistently intense
and punctate actin staining than untreated cells. The LFA-1 staining of A20 cells shown in Figure 18 demonstrated an even distribution, not unlike the distribution of LFA-1 on MBL-2 cells. No dramatic association between LFA-1 and actin was observed.

For Figure 18 cytochalasin B was added to aggregated A20 cells in culture, the cells were incubated for 30 min. at 37°C, chilled on ice, fixed, and then stained. Over the 30 min. incubation with cytochalasin B the cells did not disaggregate, and as shown in Figure 18 the cytochalasin B treatment did not cause the punctate ICAM-1 spots to dissociate. Another experiment was done where aggregated cells were disaggregated by pipetting, treated with cytochalasin B for 30 min. at 37°C, and then analyzed by immunofluorescence microscopy. In this experiment the ICAM-1 spots persisted even after disaggregation of the cells and treatment with cytochalasin B (data not shown).

To summarize the initial studies on LFA-1 and ICAM-1 distribution, while differential distribution of ICAM-1 could clearly be shown on different cell lines, the effect of PKC stimulation on the distribution of LFA-1 could not be shown, at least through the use of fluorescence microscopy.
Figure 18

The distribution of cell surface molecules and intracellular actin on/in cytochalasin B treated or untreated A20 cells. Photomicrographs of A20 cells which were either untreated or treated with cytochalasin B at 37°C for 20 min., and then handled as described for Figure 17 except that the cells were stained with a combination of FITC-goat-ant-rat IgG and TRITC phalloidin. For each exposure taken under fluorescence with the green filter to visualize FITC (Gr. Fl.) or with the red filter to visualize TRITC (Rd. Fl.), an accompanying exposure was taken under phase contrast (P.C.).
ICAM-1 / actin

Gr.Fl.

-cytochalasin B

Rd.Fl.

P.C.
Part V: The role of the cytoplasmic domain of ICAM-1 in cell adhesion

As discussed briefly in the Introduction of this thesis, the distribution of adhesion molecules on the surface of the cell can potentially play an important role in the strength of cell:cell adhesion. However, the role of adhesion molecule distribution in the regulation of adhesion is, at present, poorly characterized. Part IV of the Results section of this thesis addresses the topic of the distribution of adhesion molecules, but presents only partially satisfying results. What effect, if any, does PKC stimulation have on the distribution of adhesion molecules, especially LFA-1? What are the molecular mechanisms controlling the distribution of adhesion molecules? These are two of the questions still to be answered. With respect to ICAM-1, it is clear from my own results with A20 cells and the results of others that ICAM-1 can be highly localized on the cell surface. Staunton et al. have observed that ICAM-1 expressed on transfected COS cells exhibits a punctate staining pattern, even when the entire cytoplasmic domain is deleted (56). It would seem logical that the cytoplasmic domain be involved in the regulation of ICAM-1 cell surface distribution via cytoplasmic links. We therefore wanted to repeat the studies of Staunton et al. in our own system, and attempt to determine what role, if any, the cytoplasmic domain of ICAM-1 has on both the distribution of ICAM-1 and on the function of ICAM-1.

The approach we took was to transflect ICAM-1 with or without it's cytoplasmic domain into ICAM-1 cells and compare both the function and the distribution of both versions of the molecule. The constructs were made and the transfections were done by a summer student in our Lab, Mike Buchart. The entire cDNA encoding murine ICAM-1 or the cDNA deleted of the majority of the coding sequence for the cytoplasmic domain were subcloned into BCMGSNeo. We chose two different cell lines to transflect, a lymphoid cell line in the hope of comparing both the function and distribution of the two molecules, and a fibroblastoid cell line as a back-up for distribution studies.

The lymphoid cell line chosen was the T cell hybridoma T28 (85). Each construct was electroporated into T28 cells, transfectants selected in 1 mg/ml G418, and expression checked
by FACS. In culture, cells with the two different versions of ICAM-1 behave much differently. This difference is shown in Figure 19. Cells with the entire ICAM-1 molecule form large, tight homotypic aggregates while cells with ICAM-1 deleted of its cytoplasmic domain form fewer, loose homotypic aggregates. This difference is not likely due to differences in levels of expression, since FACS analysis showed very similar levels of expression, and even populations of cells expressing clearly lower levels of the entire molecule as compared with the cytoplasmic domain deletion exhibited the same behavior in culture as shown in Figure 19 (data not shown). Both versions of ICAM-1 conferred the ability to form tight homotypic aggregates upon PMA stimulation, as shown in Figure 20. In addition, Figure 20 also demonstrates the importance of the cytoplasmic domain in mediating homotypic aggregation in the absence of stimulation; when resuspended and incubated at a relatively high cell concentration, cells with the entire ICAM-1 molecule aggregate more efficiently than do cells with the molecule deleted of its cytoplasmic tail. The T28 cells with the entire ICAM-1 molecule did aggregate to a greater degree upon PMA stimulation as compared to the cells with ICAM-1 deleted of its cytoplasmic domain. Therefore, the effect of ICAM-1's cytoplasmic domain and the LFA-1 avidity affect of PMA appear to be additive.
Figure 19  T28 cells transfected with ICAM-1 with or without it's cytoplasmic domain behave differently in culture. Photomicrographs of flask cultures of T28 cells transfected with the BCMGSNeo vector carrying a) the complete ICAM-1 cDNA, or b) the cDNA encoding ICAM-1 without it's cytoplasmic domain.
Figure 20  

T28 cells transfected with ICAM-1 with or without its cytoplasmic domain aggregate in response to PMA. Photomicrographs of unstimulated or PMA stimulated a) parental T28 cells, b) T28 cells transfected with the entire ICAM-1, and c) T28 cells transfected with ICAM-1 deleted of its cytoplasmic domain. Cultures were photographed after 1 hr. at 37°C. PMA was used at a final concentration of 50 ng/ml.
The cell surface distribution of both versions of ICAM-1, LFA-1, and the control cell surface molecule CD45 on the transfected T28 cells is shown in Figure 21. Figure 21 in fact shows the very cultures, which were analyzed by immunofluorescence analysis after fixation and antibody staining, as are shown in figure 20. Figure 20 shows 1 ml cultures of T28 cells which were photographed after a 1 hr. incubation with or without PMA. These cultures were then chilled on ice, fixed on ice, and stained with antibodies to various cell surface molecules. Figure 21 shows the relatively high expression level of both of the transfected versions of ICAM-1. Both versions of transfected ICAM-1, with and without the cytoplasmic domain, exhibited somewhat punctate distributions, although clearly not as punctate as the distribution of ICAM-1 on A20 cells. The distribution of ICAM-1 was similar with or without PMA stimulation. The one striking observation of this experiment was the difference in the distribution of LFA-1 with or without PMA stimulation. Figure 21 shows that on PMA stimulated, aggregated T28 cells transfected with complete ICAM-1, LFA-1 exhibited a more patchy distribution than the unstimulated, less tightly aggregated cells. This observation was also made on the PMA stimulated, aggregated T28 cells transfected with ICAM-1 deleted of its cytoplasmic domain (data not shown). The LFA-1 staining was, however, more punctate on the cells with the entire ICAM-1 molecule; therefore, the extent of punctate LFA-1 staining on the aggregated, ICAM-1 transfected T28 cells seems to correlate with the extent of aggregation.
The distribution of cell surface molecules on unstimulated or PMA stimulated T28 cells expressing transfected forms of ICAM-1. Photomicrographs of T28 cells expressing either complete ICAM-1 (ICAM-1 transfected) or ICAM-1 without it's cytoplasmic tail (ICAM-1 del.cyto. transfected) which were unstimulated or stimulated with PMA for 1 hr. at 37°C, and then handled as described for Figure 17. For each exposure taken under fluorescence (Fl.), an accompanying exposure was taken under phase contrast (P.C.).
ICAM-1 transfected / ICAM-1 stained
ICAM-1 transfected / ICAM-1 stained

F1.

+ PMA

P.C.
ICAM-1 del cyto. transfected / ICAM-1 stained

Fl.

PMA

P.C.
ICAM-1 del. cyto. transfected / ICAM-1 stained
ICAM-1 transfected / LFA-1 stained
ICAM-1 transfected / LFA-1 stained

Fl.

+ PMA

P.C.
ICAM-1 del. cyto. transfected / CD45 stained

Fl.

- PMA

P.C.
Figure 22 shows the distribution analysis of the untransfected T28 cells, and serves as a control for Figure 21. The cells shown in Figure 22 were treated exactly the same way as those shown in Figure 21, except of course that as shown in Figure 20 the untransfected T28 cells do not aggregate appreciably in response to PMA. As shown in Figure 22 the untransfected T28 cells are essentially ICAM-1 negative, in agreement with FACS analysis, except for occasional slight staining. The distribution of LFA-1 on unstimulated or PMA stimulated parental T28 cells was indistinguishable, in contrast to the more punctate LFA-1 distribution on PMA stimulated, aggregated ICAM-1 transfected T28 cells. Taken together these observations strongly suggests that the change in LFA-1 distribution on the ICAM-1 transfectants occurred as a result of aggregation, rather than before aggregation; therefore, it cannot be concluded that PMA stimulation results in the alteration of LFA-1 distribution on the cell surface such that increased localized concentrations of LFA-1 promote cell:cell adhesion. The increased patchyness of LFA-1 following PMA induced aggregation of the ICAM-1 transfected T28 cells is possibly a result of the high level of ICAM-1 expression on the ICAM-1 transfected T28 cells, especially since a change in LFA-1 distribution was never observed on MBL-2 cells following PMA induced aggregation. PMA stimulation likely affects the ICAM-1 transfectants by increasing the avidity of LFA-1 for ICAM-1, which facilitates ICAM-1:LFA-1 mediated aggregation. I speculate that the punctate LFA-1 distribution on the PMA stimulated, aggregated transfectants was due to mutual capping with ICAM-1 on opposing cells. Because there is so much ICAM-1 expressed on the surface of the transfected cells, a form of mutual capping likely occurs where since the density of one of the two counter receptors is in such excess the capping of only one counter receptor, the one expressed at a lower density, is observed.
Figure 22  The distribution of cell surface molecules on unstimulated or PMA stimulated, untransfected T28 cells. Photomicrographs of parental, untransfected T28 cells which were handled as described as for Figure 21. For each exposure taken under fluorescence (Fl.), an accompanying exposure was taken under phase contrast (P.C.).
ICAM-1

F1.

-PMA

P.C.
LFA-1

F1.

- PMA

P.C
As described above, the distribution of both forms of transfected ICAM-1, with and without its cytoplasmic domain, appeared similar on transfected T28 cells. The distribution could at best be described as somewhat patchy. However, the two forms of ICAM-1 did have different functional characteristics; the complete ICAM-1 molecule mediated cell adhesion more efficiently in unstimulated cells. Therefore, it still seemed likely that the cytoplasmic domain may mediating subtle distribution effects not easily observed on lymphoid cells. We wanted to express these same ICAM-1 molecules on flat, adherent cells in order to more carefully analyze any distribution effects mediated by the cytoplasmic domain. The same two ICAM-1 constructs, with and without the cytoplasmic domain, each in BCMGSNeo, were transfected by calcium phosphate mediated transfection into the murine fibroblastoid cell line L. The distribution of both versions of ICAM-1 as well as other control cell surface molecules on the transfected L cells is shown in Figure 23. Both versions of ICAM-1 were expressed over the entire surface of the cells, and both versions exhibited a somewhat punctate distribution pattern on the cell surface, as is demonstrated especially well on the full length ICAM-1 transfected, ICAM-1 stained photomicrograph. The actual microscopic observation of the slides did not suggest, however, any significant difference in the distributions of the two versions of ICAM-1. As described in the Materials and Methods section of this thesis, initial expression of both versions of transfected ICAM-1 on FACSorted L cells was very strong, and the transfectants had to be grown in low concentrations of G418 in order to lower the expression to a level where subtle observations concerning distribution could be made. The cells shown in Figure 23 were grown in only 100 µg/ml G418 for approximately 2 weeks before analysis, and as a result there was some heterogeneity in the levels of ICAM-1 expression. In general, the most punctate distributions were observed on cells expressing lower levels of transfected ICAM-1.
Figure 23 The distribution of cell surface molecules on L cells expressing transfected forms of ICAM-1. Photomicrographs of L cells expressing either complete ICAM-1 (ICAM-1 transfected) or ICAM-1 without its cytoplasmic tail (ICAM-1 del.cyto. transfected) which were fixed with 1% paraformaldehyde, stained with rat IgG antibodies to various cell surface molecules, and then stained with FITC-goat-ant-rat IgG. L cells were grown on glass coverslips prior to immunofluorescence analysis; cells were fixed, stained, and observed while attached to the coverslips. For each exposure taken under fluorescence (Fl.), an accompanying exposure was taken under phase contrast (P.C.).
ICAM-1 del.cryo. transfected / 2nd Ab control

F1.

P.C.
ICAM-1 transfected / CD4 stained

Fl.

P.C.
ICAM-1 transfected / ICAM-1 stained

F1.

P.C.
ICAM-1 transfected / CD44 stained

F1.

P.C.
ICAM-1 del. cyto. transfected / ICAM-1 stained

F1.

P.C.
ICAM-1 del.cyto. transfected / CD44 stained

F1.

P.C.
CD44 was chosen as a control for the transfected fibroblasts because it has been proposed that CD44 and ICAM-1 may associate in the plane of the membrane (G.J. Dougherty, manuscript in preparation). Indeed, the distribution of CD44 was similar to that of ICAM-1 on the ICAM-1 transfected L cells. If CD44 and ICAM-1 do associate in the plane of the membrane, then the cytoplasmic domain of ICAM-1 may not be involved; our results are consistent with the CD44:ICAM-1 association hypothesis. The primary Ab directed against CD4 was used as a negative control since it shares the same isotype as YN1/1.7 Ab.

Parental, untransfected L cells were also analyzed by immunofluorescence as a control for the ICAM-1 transfectants shown in Figure 23. The results of this analysis is shown in Figure 24. Figure 24 shows that the untransfected L cells are ICAM-1 negative, as well as showing the distribution of CD44 and transferrin receptor (T.R.). The distribution of CD44 appeared to be the same on untransfected L cells as it was on ICAM-1 transfected L cells. Transferrin receptor was expressed on L cells at a much lower level, and often exhibited a polarized distribution on individual cells.

To summarize the results of the studies on the cytoplasmic domain of ICAM-1, while the cytoplasmic domain of ICAM-1 could be shown to be of functional importance, the mechanism by which the cytoplasmic domain functions could not readily be defined. The results obtained suggest that the cytoplasmic domain of ICAM-1 does not strongly influence the cell surface distribution of ICAM-1, at least as visualized by fluorescence microscopy.
The distribution of cell surface molecules on untransfected L cells. Photomicrographs of parental, untransfected L cells which were handled as described for Figure 23. For each exposure taken under fluorescence (Fl.), an accompanying exposure was taken under phase contrast (P.C.).
Discussion

Soluble versions of adhesion molecules as inhibitors of cell:cell adhesion

The original goal for this project was to explore the use of sICAM-1 as an inhibitor of the cell mediated immune responses, involving LFA-1:ICAM-1 interaction, in GVHD. However, the results suggested from an early stage that sICAM-1 would be a relatively poor inhibitor of ICAM-1:LFA-1 mediated cell adhesion. It is important to stress that in the two functional assays for sICAM-1 used in this study, namely the binding of radio-iodinated sICAM-1 in solution to LFA-1+ cells and the binding of LFA-1+ cells to sICAM-1 coated plastic, sICAM-1 was considered to be functional as determined by it's ability to bind to LFA-1+ cells; however, the affinity between sICAM-1 and LFA-1 seemed low.

In general there is much speculation and uncertainty regarding the potential for soluble forms of adhesion molecules to inhibit cell adhesion \textit{in vivo}. The report on a soluble cadherin (69) demonstrated inhibition of cell:cell adhesion at relatively low concentrations (low ng/ml range), suggesting cell adhesion is mediated by high affinity interaction. The report on a soluble form of Mac-1 (73) demonstrated that the molecule apparently inhibited cell adhesion at a relatively low concentration (50 ng/ml). The report on recombinant sELAM-1 showed that sELAM-1 in solution was capable of inhibiting only about 60% of the ELAM-1 mediated adhesion in an adhesion assay similar to the one described in this thesis (71). The published report on murine sICAM-1 (75) claimed that the molecule mediated adhesion when coated to plastic and also that in solution the molecule blocks ICAM-1:LFA-1 mediated adhesion, although no data was shown to support either claim. The shedding of ICAM-1 from human melanoma cell lines has recently been reported (78), and the authors show that the shed ICAM-1 apparently inhibits both ICAM-1:LFA-1 mediated cell adhesion and cell mediated cytotoxicity. Neutrophils have been observed to shed Leu-8/MEL-14/LECAM-1 upon activation, and it has been proposed that this shedding "may be a mechanism for rapid
alteration of neutrophil adhesion characteristics" (80). A novel isoform of CD44, CD44R1, contains a potential tryptic cleavage site in its extracellular region (109), and a soluble form of CD44 has been detected in serum (110). Soluble hyaluronic acid will inhibit the binding of cells to plastic absorbed hyaluronic acid (G. Dougherty, personal communication), and others have hypothesized that CD44 mediated adhesion may be modulated by soluble molecules (111). In addition a shed, soluble form of CD43 has been characterized at concentrations of up to 10 μg/ml in plasma (81). While there are many reports in the literature concerning inhibition by soluble forms of adhesion molecules, there is currently no consensus on the potential for soluble forms of adhesion molecules to inhibit cell adhesion in vivo.

Cell:cell adhesion is by its very nature a form of multivalent interaction with different characteristics than monovalent interaction. These characteristics have significant implications for the use of soluble adhesion molecules as inhibitors of cell adhesion. With monovalent binding, stable interaction will only persist if the interaction is of relatively high affinity. However, with multivalent binding, stable interaction will persist even if the interaction between individual molecules is of considerably lower affinity. A good analogy is velcro. The "affinity" between one hook and its interacting loop is extremely low; however, the adhesive interaction between the two multivalent velcro materials is relatively strong. In molecular terms, multivalent interaction decreases the apparent dissociation rate as compared to the corresponding monovalent interaction. The effect of valency and apparent dissociation kinetics has been well studied, especially with antibody molecules, for which the decrease from the divalent to the monovalent state has been reported to increase the apparent rate of dissociation by between five and ten fold (112). The term avidity is used in immunology to represent the net strength of the interaction between one multivalent reagent, such as an antibody molecule, and another multivalent reagent, such as a virion. The net avidity of a multivalent interaction is "a complex function of the valences of both reactants and the affinities of the various determinants involved" (113). It is easy to imagine how for cells interacting with other cells,
the sum of many low affinity interactions can result in a relatively stable, adhesive interaction. The measurement of cell adhesion is, by its very nature, a measure of avidity and not affinity.

In contrast the binding of a soluble version of an adhesion molecule, in solution, to its cellular receptor is a monovalent interaction, and does not benefit from the stabilizing or enhancing properties of avidity. This weak monovalent binding will also not efficiently inhibit cell:cell adhesion, because of the kinetics of the binding. Once cell:cell adhesion is initiated, even if the affinity of the interaction is low, cell:cell adhesion will be maintained because of the effects of avidity. At the molecular level individual counter receptors will associate but then dissociate relatively quickly; however, the overall cell:cell adhesion will persist. If a soluble version of one of the two counter receptors is introduced into the system, it will bind to its counter receptor with the same affinity (and kinetics) as its cell associated analogue. However, since the monovalent dissociation kinetics are relatively high and the membranes of the two opposing cells are already in close proximity, a relatively high concentration of the soluble adhesion molecule would be required to inhibit cell:cell adhesion by saturating the cellular adhesion receptors. Of course the higher the affinity of the monovalent binding, the lower the concentration of the soluble adhesion molecule which would inhibit cell:cell adhesion.

The data presented in this thesis suggests that ICAM-1 binds to LFA-1 with a low affinity, thus ICAM-1:LFA-1 mediated cell adhesion is not easily inhibited by sICAM-1. In order to test this hypothesis a multivalent form of sICAM-1 was used to inhibit ICAM-1:LFA-1 mediated cell adhesion in the quantitative cell adhesion assay. The results obtained support the low affinity hypothesis and explain why monovalent sICAM-1 was unable to inhibit cell adhesion. The results also emphasize the importance of multivalent interaction in LFA-1:ICAM-1 mediated cell adhesion. The results with multivalent sICAM-1 suggested that such a form of the molecule may be useful for inhibition of in vivo immune responses.

With LFA-3, a multimeric form has been compared to the monomeric form with respect to its binding to CD2, its stimulatory effects, and its inhibition of CD2:LFA-3 mediated
adhesion (38). The multimeric form of soluble LFA-3 bound to CD2 more avidly and was more stimulatory than was the monomeric soluble form. In addition, the multimeric form of soluble LFA-3 was far more effective at inhibiting CD2:LFA-3 mediated cell adhesion. The results with multivalent sICAM-1 reported here are in agreement with the results concerning multimeric LFA-3. In addition, since the CD2:LFA-3 interaction is of a relatively low affinity (37), the analogy between the CD2:LFA-3 and ICAM-1:LFA-1 systems supports the notion that the ICAM-1:LFA-1 interaction is of relatively low affinity. Thus it would seem that optimal inhibition of cell mediated immune responses with sICAM-1 will require the use of a multivalent form of sICAM-1.

As mentioned above, several other groups have claimed that sICAM-1 can inhibit cell adhesion (75, 78). It should be noted that the method by which sICAM-1 was produced for this study differs somewhat from the two other published reports on the production of sICAM-1. For murine sICAM-1 (75), the truncated cDNA was subcloned into an expression vector in which expression is driven from the SV40 early promoter and then transfected into CHO cells. SICAM-1 was then purified from the SN by anion exchange followed by immunoaffinity chromatography. Human sICAM-1 was produced by a very similar procedure (74). The murine sICAM-1 which contained only four complete domains and a portion of the fifth had a reported molecular weight of only 50 kd, suggesting inappropriate glycosylation. Since we produce sICAM-1 in lymphocytic cells (NS-1), our protein is likely appropriately glycosylated. Appropriate glycosylation is not trivial, especially for ICAM-1, since it has been shown that glycosylation can influence it's biological activity (114). The report on the shed form of sICAM-1 does not include a rigorous characterization of the molecule (78). Again, the results of this thesis suggest that while monovalent sICAM-1 is inefficient at inhibiting cell:cell adhesion, multivalent sICAM-1 will inhibit cell:cell adhesion more effectively. It is possible that the two reports claiming inhibition by sICAM-1 (75, 78) represent the effects of either aggregated sICAM-1 or membrane bound ICAM-1 vesicles in the case of shed sICAM-1, since
I have shown that only multivalent sICAM-1 is capable of effectively inhibiting ICAM-1:LFA-1 mediated cell adhesion.

**Regulation of LFA-1:ICAM-1 mediated cell adhesion**

Cell adhesion mediated by LFA-1 and ICAM-1 is known to be actively regulated. However, the mechanisms that regulate LFA-1:ICAM-1 mediated cell adhesion are potentially very complex and currently not well understood. The studies in this thesis showed that ICAM-1:LFA-1 mediated cell adhesion can be upregulated by PMA or Mn++. These two effects have different sensitivities to inhibition by sICAM-1. In addition, the cell surface distribution of ICAM-1 is shown to be differentially regulated, at least in different cell lines, and these differences in distribution are hypothesized to have significant implications for cell:cell adhesion (discussed below). Taken together, the results presented in this thesis strongly suggest that there are multiple mechanisms regulating cell adhesion mediated by LFA-1 and ICAM-1.

There are many reports in the literature concerning the mechanisms regulating cell adhesion mediated by LFA-1 and ICAM-1. Springer's group has shown that the adhesion of cells to absorbed ICAM-1 is dependant on a functional actin cytoskeleton, active metabolism, divalent cations, and also activation from the so called low to high avidity forms of LFA-1 (5, 11, 12, 13). The avidity of LFA-1 has been shown to be upregulated by treatment of cells with PMA or by TcR crosslinking (42). It has been assumed that the avidity of LFA-1 is determined by it's affinity for ICAM-1, and that PKC mediated phosphorylation of the LFA-1 β chain, stimulated by phorbol ester or TcR ligation, regulates the affinity of LFA-1 (42, 115). However, in the absence of an assay for the binding of sICAM-1 in solution, the relationship between the affinity of LFA-1 for ICAM-1 and it's avidity could not be determined. Figdor's group has also studied LFA-1 regulation (45). This study demonstrated that T cell signalling through CD2 or CD3 increased the avidity of LFA-1. Signalling through CD2 resulted in a
persistent avidity increase, while signalling through CD3 resulted only in a transient avidity increase.

Multiple mechanisms of Mac-1 avidity regulation have also been reported. Since LFA-1 and Mac-1 share a common β chain, it would seem likely that they also share regulatory mechanisms. Detmers et. al have demonstrated that phorbol ester stimulation results in the aggregation of Mac-1 molecules in the plane of the membrane (51), and there are a number of reports suggesting that the affinity of Mac-1 for its ligands can be regulated (116, 117).

I propose that there are three primary mechanisms regulating cell:cell adhesion mediated by LFA-1 and ICAM-1. The first mechanism is simply the amount of LFA-1 or ICAM-1 a given cell expresses on its surface. The second mechanism is the modulation of the affinity of the interaction between ICAM-1 and LFA-1, by any means the cell may have at its disposal. The third mechanism is the regulation of cell adhesion by the regulation of the cell surface distribution of adhesion molecules. The remainder of the Discussion of this thesis will involve consideration of these proposed methods of cell adhesion regulation, with regard to my results and those of others.

1. Regulation of adhesion by modulating expression levels on the cell surface

Cells will not adhere to other cells unless they express adhesion molecules on their surface. Naturally, the higher the level of adhesion molecules expressed on the surface of a cell, the more avidly it will adhere to other cells expressing the proper counter receptor. Adhesion regulation by regulation of the level of adhesion molecule expression was exemplified in the present study from the aggregative phenotype of the ICAM-1 transfected T28 cells. The parental T28 cells, which express very low levels of ICAM-1, grow in single cell suspension whereas the transfected T28 cells expressing high levels of ICAM-1 grow in homotypic aggregates, demonstrating that expression of ICAM-1 on LFA-1+ cells enables cell:cell adhesion. *In vivo* ICAM-1 is expressed on relatively few cells; however, ICAM-1 expression is induced by a number of inflammatory mediators on a number of cell types (18).
A good example of this mechanism at work in vivo is the induction of ICAM-1 expression on endothelium, facilitating the binding and extravasation of neutrophils, monocytes, and lymphocytes into sites of tissue insult. Another example of this method of adhesion regulation is the ability of monocytes to rapidly mobilize cytoplasmic stores of ICAM-1 to their surface following stimulation (50). In general, the cell surface expression level of ICAM-1 is an important regulatory mechanism for LFA-1:ICAM-1 mediated cell adhesion. However, LFA-1 is constitutively expressed on leukocytes and the avidity of LFA-1 seems to be regulated by other mechanisms.

2. ICAM-1:LFA-1 affinity modulation

The adhesive interaction between cells mediated by ICAM-1:LFA-1 interaction may be regulated by the affinity of the interaction between individual ICAM-1 and LFA-1 molecules. It has long been hypothesized that modulation of affinity regulates cell:cell adhesion (2, 4); however, rigorous testing of this hypothesis has been slow because of the lack of affinity assays in which soluble versions of adhesion molecules bind to their cellular counter receptors. The exception to this is the CD2:LFA-3 system. Using soluble forms of both CD2 and LFA-3, the affinity of the interaction between these molecules has been determined. These studies demonstrated that the affinity of the CD2:LFA-3 interaction is surprisingly low, with a Kₐ of 10⁻⁶ M (37, 38).

In my studies, the measurement of the affinity of LFA-1:ICAM-1 binding was not done mainly because of the difficulties in obtaining consistent results. In some experiments, ¹²⁵I-sICAM-1 seemed to bind to LFA-1+ cells efficiently at concentrations in the ng/ml range, whereas in other experiments concentrations in the μg/ml range were required (Figure 5). There are two possible explanations to account for these discrepancies. The first is that in some experiments ¹²⁵I-sICAM-1 exists in multivalent aggregates; therefore, the presumed high affinity binding is actually multivalent high avidity binding with the same affinity. Why ¹²⁵I-sICAM-1 would exist in aggregates in some experiments and in monomeric form in others is
not known, especially since aliquots of the same batches of frozen protein yielded inconsistent results. The other explanation, which we labored over for some time, is that the iodination process may inactivate the biological properties of the sICAM-1. Again, the inconsistencies in the results are difficult to account for by this explanation. For example, some iodinations yielded apparently active $^{125}$I-sICAM-1, while others using the very same iodination protocol yielded inactive $^{125}$I-sICAM-1. Along this line a more 'gentle' iodination protocol was used in which Na$^{125}$I is oxidized in the absence of protein to give free $^{125}$I, then the free $^{125}$I is transferred to the protein solution and labelling is allowed to proceed without exposing the protein to the potentially harsh oxidizing conditions. However, this method did not improve the results of the binding assay. In addition a two phase iodination protocol, which is reportedly very gentle, was tested without any improvement. With respect to affinity, the other salient data from this study to keep in mind is that PMA stimulated ICAM-1:LFA-1 mediated cell adhesion is not inhibited by sICAM-1 in solution at up to 100 µg/ml. From all of our results it would seem that the affinity of LFA-1:ICAM-1 binding is relatively low, at least in comparison to Ab:Ag or growth factor:receptor systems.

The observation that the normally low affinity interaction between LFA-1 and ICAM-1 can be increased by Mn$^{++}$ is in agreement with the the observed effects of Mn$^{++}$ on affinity in other systems. In the human system, Dransfield et al. have observed that Mn$^{++}$ induces LFA-1:ICAM-1 mediated cell adhesion (105). As well in this experimental system Mn$^{++}$ induces the LFA-1 24 epitope, which is presumably an indication of an LFA-1 conformational change accompanying the increase in the affinity of LFA-1 for ICAM-1. The binding of radioiodinated fibrinogen and factor X to Mac-1 has recently been demonstrated (103), and this binding assay was used to show that Mn$^{++}$ increases the affinity of the interaction between Mac-1 and it's ligands. In addition, the affinity of RGD containing peptides for the $\beta_3$ integrin GPIIb/IIIa has been determined (104), and Mn$^{++}$ was also found to increase the affinity of this integrin for the RGD peptides. Therefore, Mn$^{++}$ appears to have a general effect on integrins; the engagement of Mn$^{++}$, as opposed to Ca$^{++}$ or Mg$^{++}$, by integrins apparently induces some
conformational change in the integrins' extracellular region such that the affinity for its ligand is increased. In contrast to Mn++, we have consistently been unable to detect an increase in the binding of {sup}125I-{sup}-sICAM-1 to LFA-1+ cells upon stimulation with PMA. In addition, Mn++ induced homotypic aggregation could be inhibited by sICAM-1 in solution, whereas PMA induced homotypic aggregation could not. These results suggest that PMA most likely increases the avidity of LFA-1:ICAM-1 mediated cell adhesion without increasing the affinity of ICAM-1:LFA-1 binding.

SICAM-1 was unable to consistently inhibit Mn++ induced adhesion in the quantitative cell adhesion assay, even at a concentration of up to 110 μg/ml (results not shown). However, sICAM-1 did inhibit the Mn++ induced aggregation of cell lines (Figure 9). It would seem that in the quantitative cell adhesion assay, inhibiting adhesion by blocking LFA-1 is difficult unless the inhibiting agent binds with relatively high avidity. The observation that Mn++ induced adhesion in the quantitative cell adhesion assay was inhibited significantly by cytochalasin B was unexpected. This adhesion, presumably mediated by an increase in affinity of the ICAM-1:LFA-1 interaction, was expected to be independent of the actin cytoskeleton (see below). However, adhesion mediated by multiple mechanisms appears to be very sensitive to inhibitors of the actin cytoskeleton.

The possible physiological relevance of Mn++ induced cell adhesion is unclear at this point. Circulating concentrations of Mn++ have been reported to reach 0.7 μM (118). Lymphocytes are known to contain Mn++ dependant enzymes in mitochondria (119), and lymphocytes are also known to store a significant portion of the Mn++ in blood (120, 121). It has been hypothesized that Mn++ release may provide "a rapid mechanism for the activation and recruitment of multipotent leukocyte adhesive properties" (103).

While it would seem that the cytoplasmic domain of LFA-1 β is most likely responsible for cytoplasmic linkage resulting in "high avidity" LFA-1 (see below), the α chain's cytoplasmic domain remains a candidate for modulating LFA-1's affinity. Indeed there is one recent report in which the α chain of a β3 integrin was shown to influence the affinity of the
integrin complex for its ligand (122). In this report truncation of the α chain cytoplasmic domain resulted in an increase in the affinity of the integrin for its ligand, and the authors speculated that the cytoplasmic domain of the α chain might facilitate binding to some cytoplasmic moiety which maintains the integrin complex in the low affinity state. The authors also speculate that cell activation may result in the modification of this moiety such that the integrin complex is converted to the high affinity state. The location of the divalent cation binding site on the α chain combined with the effect of Mn++ on integrin affinity also supports the notion that the α chain may be involved in affinity regulation.

One very recent report has suggested that the avidity of β2 integrins can be modulated by a small molecular weight lipid present in stimulated but not in unstimulated human neutrophils (116). This molecule has been termed Integrin Modulating Factor-1 (IMF-1), and it appears to be an unsaturated fatty acid or isoprenoid acid. While the Mac-1 assays used in this study are cell adhesion assay and therefore measure avidity, certain aspects of the data strongly suggest that the effect of IMF-1 is to increase the affinity of β2 integrins for their ligands. In particular, when the authors immobilized purified Mac-1 onto polystyrene, treated the immobilized Mac-1 with IMF-1, and then measured the ability of untreated or IMF-1 treated, immobilized Mac-1 to bind to C3bi coated erythrocytes, they observed the IMF-1 treatment to greatly increase the binding ability of the absorbed Mac-1. IMF-1 activity could be found in lipids from neutrophils stimulated with PMA, TNF, PAF, and fMLP but not unstimulated neutrophils.

How IMF-1 fits in with more conventional concepts of integrin regulation, such as protein phosphorylation and cytoskeletal interaction, is unclear at this point. However, this report does add credence to reports in the literature concerning integrin conformational changes hypothesized to be indications of activation. With respect to LFA-1, two other groups have characterized 'activated' forms of LFA-1 through the use of mAb. Figdor et al. have raised a mAb, NKI-L16, which binds to an epitope of LFA-1 dependant on divalent cations and cellular activation (45, 46, 47). This Ab also facilitates LFA-1:ICAM-1 mediated homotypic
aggregation of cell lines, presumably by stabilizing an activated LFA-1 conformation. Based on their data, this group has postulated the existence of three distinct conformational forms of LFA-1 on the cell corresponding to distinct stages of activation (48). Dransfield and Hogg have similarly characterized an LFA-1 epitope dependant on active metabolism and divalent cations through the use of their mAb, Ab24 (49). The 24 epitope is also known to be induced by Mn++ (105). A similar situation exists with Mac-1, for which an antibody has been raised recognizing an epitope found only on activated cells (117). In addition, for the integrin IIb/IIIa, evidence for the presence of an activation epitope has been published (123).

3. The regulation of cell:cell adhesion by the regulation of the cell surface distribution of adhesion molecules

Logically, the most obvious way that avidity can be increased without increasing affinity or expression level is by changing the distribution of adhesion molecules on the surface of the cell. High local concentrations of adhesion molecules at the sites of cell:cell contact would increase the avidity of adhesion. The theoretical dissociation rate of cells adhering via evenly spaced adhesion molecules would be much more rapid than that for cells adhering via clustered adhesion molecules, as illustrated in Figure 25. The difference between randomly distributed receptors and clustered receptors with respect to cell adhesion has been verified experimentally (124). This report demonstrates that clustered receptors in general mediate more avid cell:cell adhesion than do evenly distributed receptors.

Despite the potential for distribution to significantly affect cell:cell adhesion, relatively little is known about the actual cell surface distribution of adhesion molecules, and what effects this distribution has on adhesion. In the case of the ICAM-1:LFA-1 adhesion system, the distribution of these molecules on the cell surface is likely regulated, as will be discussed below. With respect to the distribution of adhesion molecules on the cell surface, LFA-1 and ICAM-1 will be discussed separately, starting with LFA-1.
The primary line of evidence suggesting that the distribution of LFA-1 has significant functional consequences comes from the analogy with Mac-1, which of course shares a common β chain with LFA-1. For Mac-1 an aggregation of receptors, preceding any ligand binding, has been shown to occur with phorbol ester stimulation (51). This aggregation of receptors in the plane of the membrane was determined to correspond to increased ligand binding capabilities. Several lines of evidence suggest that the cytoskeleton plays a role in the distribution of LFA-1 on the cell surface. Firstly, cytochalasin B, an inhibitor of actin
polymerization, inhibits LFA-1 mediated cell adhesion (Figures 7, 8, 10, 11). Secondly, LFA-1 has been reported to associate with talin in a PKC dependant fashion (54, 55). Talin is a protein believed to provide a linkage between the actin cytoskeleton and integrin molecules (58, 63). The cytoplasmic domain of integrin β chains is believed to facilitate their cell surface localization in conjunction with the actin cytoskeleton (discussed below).

Phorbol esters, which upregulate LFA-1 avidity, stimulate the PKC mediated phosphorylation of a spectrum of proteins in the cell in addition to LFA-1 β (125, 126). Indeed Hibbs et al. recently reported that the serine phosphorylation of LFA-1 β could be separated from the phorbol ester induced, LFA-1:ICAM-1 mediated adhesion (127). Since phorbol ester induced LFA-1:ICAM-1 mediated adhesion is dependant on a functional actin cytoskeleton in addition to PKC mediated phosphorylation events, it seems likely that phosphorylation of protein(s) distinct from LFA-1 β link the actin cytoskeleton to the LFA-1 complex, thus facilitating the high avidity form of LFA-1.

There is one report in the literature about a poorly characterized protein found associated with LFA-1 in the cytoplasm (66). The protein is a likely candidate for the regulation of LFA-1 especially since the authors feel that the protein is only transiently associated with the LFA-1 complex. This 86 kd protein may reflect either an LFA-1:cytoskeletal link, or a mechanism by which the affinity of LFA-1 is modulated.

Since the phosphorylation of LFA-1 β is not required for the phorbol ester induced increase in the avidity of LFA-1, a model in which some other molecule, such as talin or the 86 kd LFA-1 associated protein, links LFA-1 β to the actin cytoskeleton seems likely. In support for the talin hypothesis, talin is known to be a PKC substrate (64).

For β1 integrins the cytoplasmic domain of the β chain appears to facilitate a link between the integrin complex and the cytoskeleton. Solowska et al. (128) demonstrated that the cytoplasmic domain of avian β1 integrin was responsible for the localization of integrin molecules to focal contacts, though this region of the molecule did not seem to be important for ligand binding. Marcantonio et al. (129) argued that the β1 region of the cytoplasmic domain
closest to the membrane is necessary for localization of avian $\beta_1$ integrin to focal contacts. The cytoplasmic domains of the integrin $\beta$ chains are highly conserved (127). By analogy the $\beta_1$ data provides evidence that the cytoplasmic domain of LFA-1 $\beta$ is also responsible for an integrin:cytoskeleton link.

Except for the T28 transfectants, I could not detect any change in the distribution of LFA-1 after stimulation with PMA. These observations were originally somewhat discouraging; however, fluorescence microscopy might not be a sufficiently sensitive technique for characterizing functionally significant changes in adhesion molecule distribution. Phorbol ester stimulation may induce relatively subtle changes in LFA-1 distribution, such as dimerization, which would require electron microscopy to visualize. The Mac-1 study employed electron microscopy, and indeed the degree of receptor aggregation was not extensive. Using fluorescence microscopy, the ICAM-1 transfected T28 cells were clearly observed to exhibit more punctate LFA-1 staining following PMA induced aggregation. This effect was not observed in any of the cell lines, including the untransfected T28 cells, prior to the ICAM-1 transfected T28 lines; therefore, this phenomenon is likely an example of mutual capping due to the extremely high level of ICAM-1 expression on the T28 transfectants. While this effect may be relevant in some respect, it does not likely indicate that the change LFA-1 distribution preceded aggregation or was involved in stimulating aggregation. It is possible that for LFA-1, as for Mac-1, electron microscopy is required to detect the microclustering of receptors, prior to ligand engagement, stimulated by phorbol esters.

The cell surface distribution of ICAM-1 may also strongly influence ICAM-1:LFA-1 mediated cell adhesion. In fact one of the most striking observations from my work was the punctate ICAM-1 spots on A20 cells. It is likely that the expression of punctate ICAM-1 on A20 cells is responsible for their observed aggregation in culture. An ICAM-1 distribution similar to that observed on A20 cells has also been reported on a human T cell line exhibiting homotypic aggregation in culture (50), and as on the A20 cells, LFA-1 did not co-localize with ICAM-1.
Interestingly, the punctate distribution of ICAM-1 on A20 cells is efficiently maintained even when the cells are disaggregated and incubated with cytochalasin B, which destroys the actin cytoskeleton. In another system, namely the human KG1a myelomonocytic cell line, treatment with cytochalasins also does not destroy punctate ICAM-1 localization (G.J. Dougherty, personal communication).

The punctate ICAM-1 staining on A20 cells and the punctate ICAM-1 staining patterns reported by others prompted us to hypothesize that the cytoplasmic domain of ICAM-1, through linkage to intracellular proteins, may be responsible for controlling ICAM-1's cell surface distribution. There is only one reference in the literature to studies on the cytoplasmic domain of ICAM-1 (56); however, this report was not definitive and we wanted to conduct our own study on the cytoplasmic domain of ICAM-1. We therefore transfected either complete ICAM-1 or ICAM-1 without it's cytoplasmic domain onto T28 and L cells and determined both the function of the two versions of ICAM-1 and their cell surface distribution. The results of these experiments showed that the cytoplasmic domain of ICAM-1 is functionally important, while not absolutely necessary, especially for adhesion between unstimulated cells. This observation, together with the data on ICAM-1 cell surface distribution suggested that the cytoplasmic domain of ICAM-1 may determine the molecule's cell surface distribution.

Despite the functional importance of the cytoplasmic domain of ICAM-1, analysis of ICAM-1 cell surface distribution with or without the cytoplasmic domain failed to define a role for the cytoplasmic domain of ICAM-1 in determining it's cell surface distribution. The distribution of ICAM-1 with or without it's cytoplasmic domain seemed consistent, exhibiting a somewhat patchy distribution in both forms. However, as for LFA-1, the fluorescence microscopic analysis used in this study may not be sufficient to demonstrate functionally significant differences in the cell surface distribution of adhesion molecules.

It has been hypothesized that ICAM-1 may co-cap with other molecules in the plane of the membrane (G.J. Dougherty, manuscript in preparation). This hypothesis is based on the observation that ICAM-1 localizes to uropods of several cell lines exhibiting homotypic
aggregation, and on these cell lines LFA-1 does not mutually cap. The term co-cap is used in the literature to represent the association of two molecular species in the plane of a single cell's membrane; mutual capping represents the association of two molecular species, each in the membrane of an individual cell, when the two cells are in contact with each other (53). Since ICAM-1 and LFA-1 are not observed to mutually cap, the capping of ICAM-1 is hypothesized to be mediated by co-capping. ICAM-1 has been observed to co-cap with CD44 as well as with CD43 on KG1a cells (G.J. Dougherty, manuscript in preparation). The hypothesis that CD44 and ICAM-1 associate in the plane of the membrane remains to be rigorously demonstrated or disproven; the results presented in this thesis are not decisive with respect to this hypothesis. The L cells used in this study were strongly CD44 positive, and the staining pattern for CD44 and both versions of ICAM-1 transfected into these cells were very similar (Figures 23 and 24). This observation might suggest that ICAM-1 can associate with CD44 with or without its cytoplasmic domain. However, the cytoplasmic domain of ICAM-1 appears to be functionally important in facilitating ICAM-1:LFA-1 mediated adhesion, and there are two possible explanations for this observation. The first explanation is that the cytoplasmic domain of ICAM-1 might be necessary for its interaction with CD44, and the second is that the cytoplasmic domain of ICAM-1 might provide a direct link with the cytoskeleton. Both of these explanations imply that the cytoplasmic domain of ICAM-1 facilitates some form of ICAM-1 aggregation in the plane of the membrane, which likely requires more sensitive techniques such as electron microscopy to demonstrate.

In addition to modulating the aggregation of LFA-1 molecules in the plane of the membrane, the actin cytoskeleton is likely involved in LFA-1 mediated functions in a more mechanical sense. PMA activation of neutrophils in suspension induces a change in shape from spherical to bipolar (23), presumably due at least in part to the actin cytoskeleton. In addition, when neutrophils, rolling under physiological sheer stress on an artificial CD62 and ICAM-1 substrate, are stimulated by either phorbol ester or fMLP, their rolling velocities slow to a stop, then the strongly adherent cells begin to flatten and spread (23). This kind of
alteration of cell shape favoring adhesion, and also facilitating other biological processes such as extravasation, is presumably due at least in part to the actin cytoskeleton. An attempt to characterize this phenomenon in our experimental system with cytochalasin B was not completely satisfying. The results of this experiment is shown in Figure 13. While cytochalasin B did reproducibly induce an alteration in cellular morphology, decreased flattening of cells on a sICAM-1 coated substrate was not demonstrable. In addition, through either cytoskeletal linkage or associations in the plane of the membrane, adhesion molecules might be physically braced and better anchored in the membrane. With such linkages adhesion molecules might be better able to mediate cell:cell adhesion. This mechanism might be more prominent with high affinity binding or under high sheer velocities.

Summary: towards a better understanding of LFA-1:ICAM-1 mediated cell adhesion

By exploring the use of sICAM-1 as an inhibitor of cell adhesion we have gained insight into the intricacies of adhesion regulation. While sICAM-1, at least in its monovalent form, has proven unsatisfactory for use in inhibiting in vivo immune responses, it has proven useful as a probe for the mechanisms regulating cell adhesion mediated by ICAM-1 and LFA-1. Through the studies presented in this thesis, we have addressed the mechanisms which we feel are important in the regulation of ICAM-1:LFA-1 mediated cell adhesion.

The regulation of the level of adhesion molecules expressed on the cell surface is the most obvious and well characterized mechanism regulating adhesion mediated by LFA-1 and ICAM-1.

It is very likely that affinity modulation is a mechanism for regulating ICAM-1:LFA-1 mediated cell adhesion. Mn++ is believed to increase the affinity of LFA-1 for ICAM-1, demonstrating the potential for alterations in the extracellular conformation of integrins to modulate the affinity of the interaction between integrin and ligand. As discussed above, more direct evidence for functionally significant changes in integrin conformation exist in the
literature. The cytoplasmic domain of LFA-1 α remains a good candidate for mediating this kind of 'inside out' signalling resulting in integrin affinity modulation.

It is possible that LFA-1:ICAM-1 mediated cell adhesion is also regulated by the regulation of the cell surface distribution of these molecules. This mechanism is largely overlooked in the literature, yet it has great potential for modulating adhesion. Both PKC mediated phosphorylation events and the actin cytoskeleton appear to be involved in the regulation of LFA-1 avidity apart from expression levels or affinity; this LFA-1 regulation is likely effected through changes in the cell surface distribution of LFA-1. The cytoplasmic domain of LFA-1 β is a good candidate for a cytoplasmic link, but the details of this link as well as its effects remain unclear. With respect to ICAM-1, cell surface distribution can be differentially regulated, and this distribution is also hypothesized to play a significant role in cell adhesion. The cytoplasmic domain of ICAM-1 remains a good candidate for the regulation of ICAM-1 function through modulation of the distribution of ICAM-1 on the cell surface.
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