ACTIVATION OF THE LEUKOCYTE INTEGRIN LFA-1 ON T LYMPHOCYTES

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JANUARY 1996

This thesis is Presented in Part Fulfilment
for the degree of Doctor of Philosophy
at the University of London

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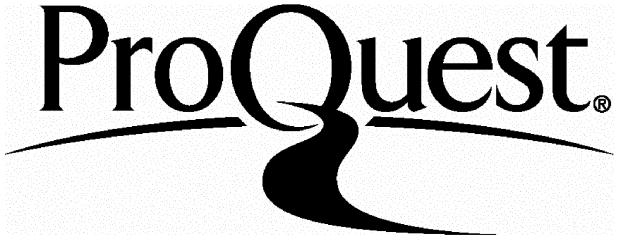

For My Parents, Jack and Sonia

ABSTRACT

Leukocytes, by dynamically altering the adhesive state of the integrin LFA-1 (lymphocyte function-associated antigen-1) on their surfaces, can perform many necessary immunological activities such as phagocytosis, cytolysis, lymphocyte activation and leukocyte migration. LFA-1 is not constitutively adherent for its ligands ICAM (intercellular adhesion molecule) -1, -2 and -3 but requires prior activation before it can bind. Activation is achieved by triggering other cell surface receptors, such as the T cell receptor (TCR), or with phorbol esters through a process referred to as "inside-out" signalling. It is also possible to directly activate LFA-1 from the cell exterior by manipulations with divalent cations such as Mg^{2+}.

In this thesis, the intracellular signals responsible for induction of LFA-1 activation on T cells are investigated with the finding that tyrosine kinases and the serine/threonine kinase protein kinase C (PKC) are involved in LFA-1 activation. Through the use of intracellular Ca^{2+} $[Ca^{2+}]_i$ mobilisers, such as ionomycin and thapsigargin, a role for $[Ca^{2+}]_i$ in LFA-1 activation was discovered. Ca^{2+} does not mediate this induction through the common Ca^{2+}-dependent signalling enzymes PKC or the serine/threonine phosphatase calcineurin but exhibits dependence on extracellular Ca^{2+} and cytoskeletal reorganisation.

Through the analysis of two distinct protocols for inducing LFA-1-mediated adhesion it is shown that stimulation with divalent cations, such as Mg^{2+} induces adhesion through high affinity receptors which can bind soluble ICAM-1 and are recognised by the activation reporter antibody 24. This form of adhesion has little dependence on major intracellular signalling mechanisms. In contrast, stimulation with the phorbol ester PDBu does not induce high affinity receptors, instead adhesion is facilitated through cell spreading which is dependent on PKC and $[Ca^{2+}]_i$. The relevance of these findings to physiological integrin stimulation is discussed.

ACKNOWLEDGEMENTS

No thesis would be complete without acknowledging all the people who made this work possible.....

Grateful thanks go to my long standing supervisor Nancy Hogg for those long nights of intense science and fruitful discussion, you have definitely given me my "scientific feet". Heartfelt thanks go to all members of the MacLab, past and present, for all the days of fun and friendship and for all their individualities which have made my stay in the lab such an interesting and educational one; Paul "Burger Dad" Hessian, Anna Randi, Jo Harvey, Carlos Cabañas, Jonathan Edgeworth, Rob Bennett, Sasha Annenkov for the older times, Martin Thiel, Clive Landis, Jo Porter, Conny Berlin, Rebecca "Crusty Pipe" Newton, Richard May, Paula Stanley for the more recent ones. Special thanks go to Alison McDowall for all her advice, secrets, cocktail and pizza nights and never-ending good temperament!

On more technical matters a big thankyou to Derek Davies, Clare Hughes and Simon Monard in the FACS lab for unimpeded advice on flow cytometry. Thanks also to Andrew Edwards and Peter Jordan for assistance with confocal microscopy.

Thanks to Doreen Cantrell, Enrique Rozengurt, Julian Downward, Patricia Warne and Fiona Watt for their help and advice on various aspects of this thesis particularly concerning the signalling areas.

And finally, a special thankyou to Kev for all his understanding throughout the whole of my PhD, especially during this extremely anti-social "last leg" phase.

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ABBREVIATIONS AND DEFINITIONS

Ab	Antibody
ADCC	Antibody-dependent cellular cytotoxicity
Ag	Antigen
APC	Antigen presenting cell
ATP	Adenosine triphosphate
BAPTA	Bis-(o-aminophenoxy)-ethane-N,N,N',N'-tetraacetoxy methyl ester
BSA	Bovine serum albumin
CD	Cluster of designation
CHO	Chinese hamster ovary
CO	Collagen
CTL	Cytotoxic T lymphocyte
DAG	Diacylglycerol
dBHQ	2,5-di(*tert*-butyl)hydroquinone
DMSO	Dimethyl sulphoxide
DTT	Dithiothreitol
EDTA	Ethylenediaminetetra-acetic acid disodium salt
EGTA	Ethylene glycol-bis (β-aminoethyl ether) N,N,N',N'-tetra-acetic acid
ELISA	Enzyme-linked immunosorbent assay
EN	Epiligrin
ER	Endoplasmic reticulum
F/P	Fluorescence/protein
FBS	Foetal bovine serum
FDG	Fluorescein di-galactopyranoside
Fg	Fibrinogen
FITC	Fluorescein isothiocyanate
fMLP	Formyl-methionine leucine phenylalanine
FN	Fibronectin
FX	Factor X

GDP	Guanosine diphosphate
GST	Glutathione S transferase
GTP	Guanosine triphosphate
HEPES	N-[2-Hydroxyethyl] piperazine-N'-[2-ethanesulphonic acid]
IC_{50}	Concentration giving half maximal inhibition
ICAM	Intercellular adhesion molecule
ICRF	Imperial Cancer Research Fund
IFN	Interferon
Ig	Immunoglobulin
IgSF	Immunoglobulin supergene family
IL	Interleukin
IMF	Integrin modulating factor
IP_3	Inositol trisphosphate
K_d	Dissociation constant
LAD	Leukocyte adhesion deficiency
LFA	Lymphocyte function-associated antigen
LIBS	Ligand-induced binding site
LN	Laminin
LPS	Lipopolysaccharide
mAb	Monoclonal antibody
MAdCAM	Mucosal addressin cell adhesion molecule
MAPK	Mitogen activated protein kinase
MHC	Major histocompatibility complex
MIDAS	Metal ion dependent adhesion site
min	minute
MIP-1β	Macrophage inflammatory protein-1β
MTT	3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Thiazolyl blue
NF-AT	Nuclear factor of activated T cells
NK	Natural killer

OD	Optical density
OP	Osteopontin
OPD	o-phenylenediamine dihydrochloride
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PDBu	Phorbol-12,13-dibutyrate
PHA	Phytohaemagglutinin
PI	Phosphatidylinositol
PIP_2	Phosphatidylinositol bisphosphate
PIP_3	Phosphatidylinositol trisphosphate
PKC	Protein kinase C
PLC	Phospholipase C
PMA	Phorbol-12-myristate, 13-acetate
PMSF	Phenylmethyl sulphonyl fluoride
$pp125^{FAK}$	Focal adhesion kinase
PTK	Protein tyrosine kinase
SDS	Sodium dodecyl sulphate
sICAM-1	Soluble ICAM-1
TCR	T cell receptor
Th	T helper
TM	Transmembrane
TNF	Tumour necrosis factor
TRITC	Tetramethylrhodamine isothiocyanate
TSP	Thrombospondin
VCAM	Vascular cell adhesion molecule
VLA	Very late antigen
VN	Vitronectin
vWF	von Willebrand factor
$[Ca^{2+}]_i$	Intracellular Ca^{2+} concentration

CHAPTER ONE

INTRODUCTION

1.1 THE ROLE OF LEUKOCYTES INTEGRINS IN THE IMMUNE SYSTEM

1.1a INTIMATE CELLULAR INTERACTIONS

The immune defence of the body is dependent on the efficient functioning of leukocytes (white blood cells). These are a group of motile cells which travel around the body in the circulation and must also be able to communicate with other cells through contacts mediated by molecules on their cell surfaces. Different cell types compose the leukocyte family and for ease they can be divided into two subfamilies. The myeloid cells are composed of the polymorphonuclear leukocytes (neutrophils, basophils and eosinophils) together with monocytes, macrophages and mast cells. The lymphoid cells compose both B and T lymphocytes (B and T cells). In general, the myeloid cells are part of the innate immune system and can directly attack and kill foreign micro-organisms by phagocytosis aided by complement-mediated opsonisation or antibody-dependent cellular cytotoxicity (ADCC). The lymphoid cells are the professional immune effector cells in that they require specialised recognition mechanisms with other cells in order to exert their effects and they are also responsible for providing immunological memory. Their interactions are mediated by unique cell surface receptors called the T cell receptor (TCR)/CD3 complex on T cells and the B cell antigen receptor, which is in fact surface immunoglobulin or antibody (Ab) molecules with associated proteins similar to CD3 proteins, on B cells. B lymphocytes are induced to proliferate and secrete Ab when stimulated by antigen (Ag) binding to cell surface immunoglobulin receptors. For full B cell activation, this signal alone is not sufficient and, an additional growth promoting switch is required which is usually in the form of soluble cytokines secreted from T lymphocytes. T lymphocytes can be subdivided according to their effector function; cytotoxic T lymphocytes (CTL) and helper T lymphocytes (Th cells). Both require specific recognition by their unique TCR/CD3 complex of processed antigen presented in conjunction with a specific major histocompatibility complex (MHC) on the surface of an antigen presenting cell (APC) or target cell. Individual responses occur when CTLs identify viral antigen products presented by MHC class I molecules through the TCR

together with another T cell surface molecule called CD8 and Th cells recognise processed peptides of exogenous antigen displayed by MHC class II molecules with additional help from the CD4 coreceptor (**see Figure 1.1**).

Upon successful recognition, the CTL secretes cytotoxic granules which kill the closely apposed virally infected cell. The CTL can then release from the target and complete another round of killing all within a 30 minute timespan (Perelson, et al., 1984). With the identification of novel cell surface receptors came the finding that a successful recognition and hence CTL response was not due solely to the interaction between TCR and Ag/MHC, but was also dependent on molecules termed LFA-1 (lymphocyte function-associated antigen-1), LFA-2 (CD2) and LFA-3 (reviewed in Springer, 1990). These molecules were so named because antibodies directed against them could inhibit the function of lymphocytes. CD2 and LFA-3 are both members of the immunoglobulin supergene family (IgSF) but LFA-1 is a member of another large family of adhesion molecules called the integrins. Monoclonal antibodies (mAbs) directed to LFA-1 were shown to inhibit CTL conjugation with and cytolysis of target cells (Krensky, et al., 1984). At this time it was not known whether LFA-1 was required simply to strengthen the adhesion between the two cells or whether it was also required as a costimulatory molecule like CD2.

Th cells recognise processed peptides of exogenous antigens in association with MHC class II molecules through their TCR together with a CD4 coreceptor. Once again, intimate cellular interaction is required to allow passage of the correct signals required for T cell proliferation and secretion of "help" in the form of soluble chemical mediators. LFA-1 is also necessary for this interaction as blocking mAbs have been shown to inhibit Ag-dependent T cell proliferation and induction of an Ab response (Davignon, et al., 1981). In these cases, LFA-1 is enhancing the adhesive strength of the interaction whereas the initial recognition step provides the specificity. LFA-1 is also important for innate immunity interactions as specific Abs will inhibit neutrophil ADCC and NK (natural killer) cell cytotoxicity (Kohl, et al., 1984).

1.1b TRANSENDOTHELIAL MIGRATION

In addition to these close intercellular interactions, lymphocytes and other leukocytes must also be able to leave the bloodstream and pass into the

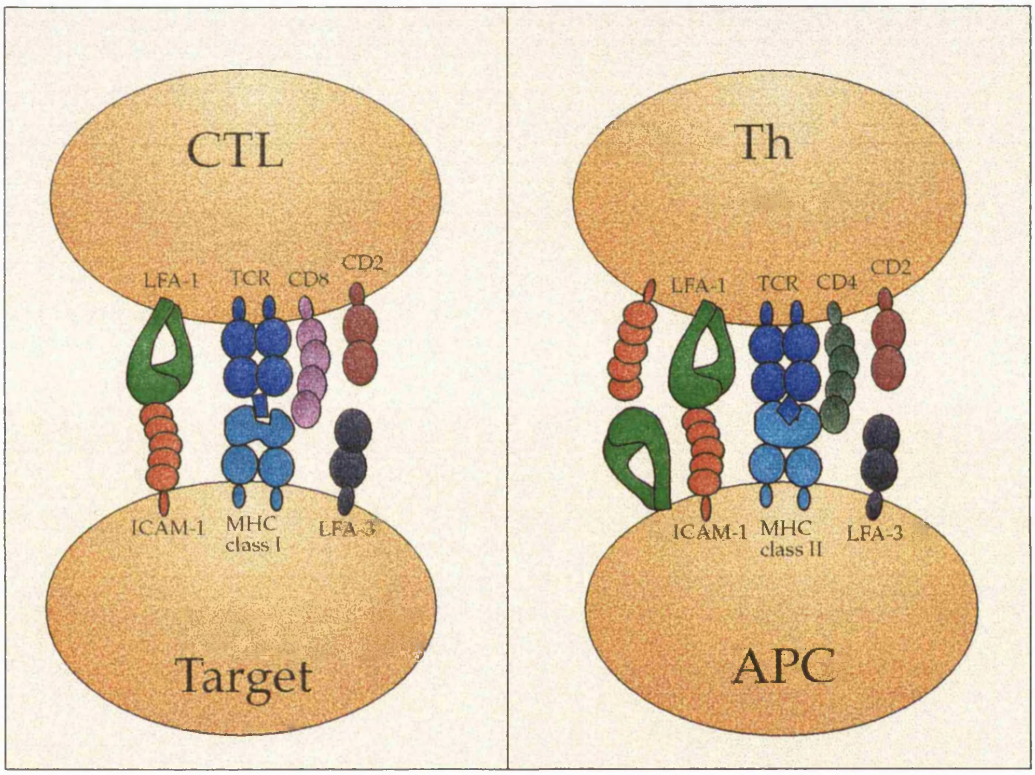

Figure 1.1: Schematic diagram of the cell surface molecules involved in T cell recognition and activation. In addition to specific recognition of Ag/MHC by the TCR/CD3 complex other accessory molecules such as CD4, CD8, LFA-1, ICAM-1, CD2 and LFA-3 are also required.

surrounding tissues. This allows trafficking to sites of infection during an inflammatory response and in the case of T and B lymphocytes it allows them to perform programmed recirculation cycles through the blood vessels and lymph in search of foreign micro-organisms. For both of these functions the method of leaving the blood stream is more or less the same. Cells transmigrate through the endothelial cells which line the blood vessels through a co-ordinated cascade involving various different families of adhesion molecules (for reviews see Hogg and Berlin, 1995; Stewart, et al., 1995). The sequence of events is such that cells roll on stimulated endothelium through the "Selectin" family of adhesion receptors, become arrested (through the β_1 integrin, VLA [very late antigen]-4, $\alpha_4\beta_1$), flatten onto the endothelial cells and finally transmigrate using β_2 integrins (**see Figure 1.2**). In the last year, it was demonstrated that α_4 integrins can participate in all phases of the adhesion cascade; $\alpha_4\beta_1$ and $\alpha_4\beta_7$ can mediate rolling and arrest by adhering to VCAM-1 (vascular cell adhesion molecule) and MAdCAM-1 (mucosal addressin cell adhesion molecule) and $\alpha_4\beta_1$ can also mediate transmigration. Following transmigration, cells proceed into the tissues to counteract foreign invaders or as part of their surveillance program. The importance of β_2 integrins in this process is evident from a human condition called LAD (Leukocyte Adhesion Deficiency, reviewed in Anderson and Springer, 1987) in which patients lack the β_2 subunits and hence have none or very little of the leukocyte integrins on their surfaces (Springer, et al., 1984). These patients present with recurrent bacterial infections, which can be fatal in childhood unless they are corrected by bone marrow transplantation. These symptoms result from defects in myeloid cell transmigration during inflammation. Lymphocyte-mediated effects are not so apparent presumably due to the ability of β_1 integrins on these cells to take over these functions.

The leukocyte integrin LFA-1, therefore, plays a central role in many cellular interactions of immune responses. From the compulsory motile nature of leukocytes it is imperative that they need to alternate between non-adherent (when circulating in the bloodstream) and adherent states (when associating with other cells). This is achieved by regulating the presence and adhesiveness of the integrins on their cell surfaces. Leukocyte integrins, such as LFA-1, are normally non-adherent until they become activated through various tightly controlled mechanisms (see section 1.4). This phenomenon, however, is not restricted to leukocyte integrins and has been particularly

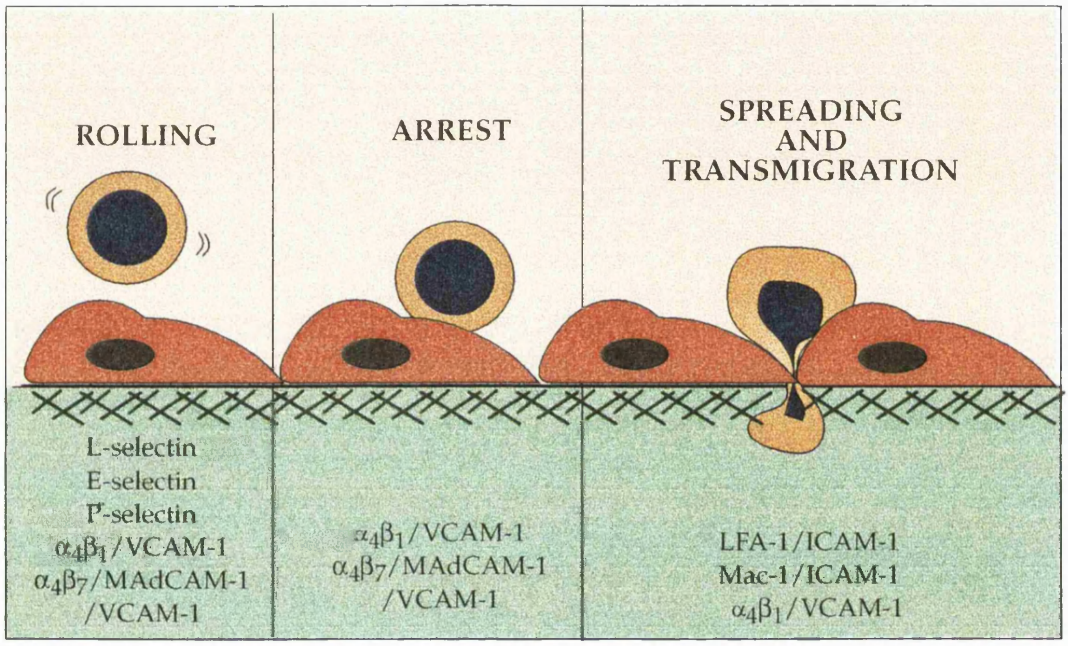

Figure 1.2: Schematic diagram of the leukocyte transendothelial migration process. Leukocytes roll along stimulated endothelium via selectin molecules (L-selectin on lymphocytes, E- and P- selectin on endothelium) and the integrins $\alpha_4\beta_1$ and $\alpha_4\beta_7$. Cellular arrest occurs when the α_4 integrins bind to their respective ligands. Leukocytes adhere firmly to the endothelium, flatten down and transmigrate historically using β_2 integrins but now α_4 can also perform this function.

well studied for the platelet integrin $\alpha_{IIb}\beta_3$. Studies using different integrins show that the activation process is dynamically controlled by various stimulants and is strictly dependent on the presence of divalent cations.

1.2 THE INTEGRINS

The term integrin was originally coined to describe cell surface molecules which could "integrate" the inner cell cytoskeleton with molecules present in the extracellular matrix. Integrins are expressed on virtually every cell in the body and form a large family of heterodimeric glycoproteins, composed of α and β subunits (Hynes, 1992). The α subunits vary between 120-180kDa and are noncovalently associated with β subunits (90-110kDa). An image of how the subunits might associate together was obtained from electron microscopic analysis of the platelet integrin, $\alpha_{IIb}\beta_3$ and then later the fibronectin receptor. Rotary shadowing showed that the subunits form a dimer with a thick globular head composed of the N terminus of both subunits with two, thin individual stalks extending from them to the membrane (Carrell, et al., 1985; Nermut, et al., 1988). To date there are now 16 α subunits and 8 β subunits which have been identified (Stewart, et al., 1995; see **Figure 1.3**). These could have the potential to associate into more than one hundred combinations but in fact there are only 22 characterised partnerships. Of these integrins, 13 are expressed on leukocytes (**Figure 1.3**- blue lines) although the β_2 and β_7 integrins are exclusive to leukocytes. Originally, it was easy to divide the integrins into subfamilies on the basis of their β subunit, which could associate with more than one α subunit. As increasing numbers of integrins have been discovered however, it has become evident that they no longer slot helpfully into discrete subgroups as many α subunits do not pair faithfully with just one β subunit. LFA-1 ($\alpha_L\beta_2$), however, is a member of one family, the β_2 integrins; which also contain Mac-1 ($\alpha_M\beta_2$), p150,95 ($\alpha_X\beta_2$) and a newly discovered member $\alpha_D\beta_2$. The majority of integrins recognise extracellular matrix molecules and soluble ligands (**Figure 1.3**) whereas some, and notably LFA-1, recognise other cell surface receptors which mostly belong to the immunoglobulin family. To date, LFA-1 can bind to three ligands ICAMs (intercellular adhesion molecules)-1, 2 and 3.

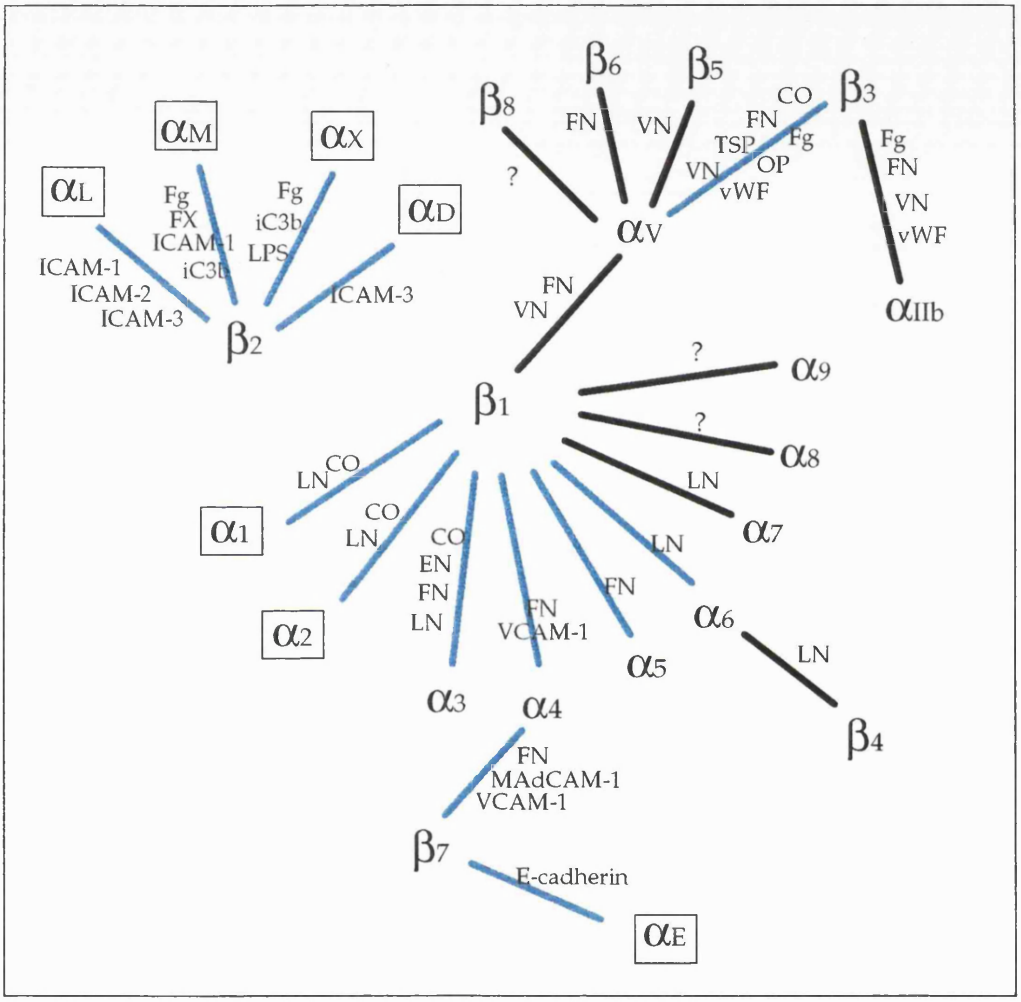

Figure 1.3: The integrin family of adhesion molecules. Lines denote which α subunit pairs with which β subunit and blue lines indicate integrin heterodimers which are expressed on leukocytes. Boxed α subunits indicate those which contain an I domain. The ligands for each heterodimer are typed adjacent to each line. Ligand abbreviations: ICAM (intercellular adhesion molecule), CO (collagen), EN (epiligrin), Fg (fibrinogen), FN (fibronectin), FX (factor X), LN (laminin), LPS (lipopolysaccharide), MAdCAM (mucosal addressin cell adhesion molecule), OP (osteopontin), TSP (thrombospondin), VCAM (vascular cell adhesion molecule), VN (vitronectin), vWF (von Willebrand factor).

INTEGRIN SUBUNIT STRUCTURE

The integrins all have a similar domain structure to LFA-1 which is shown in **Figure 1.4**. The α subunit is composed of a 1,063 amino acid extracellular domain, a 29 amino acid transmembrane domain and a 53 amino acid cytoplasmic tail. Present in the extracellular domain of the α subunit is a series of seven (or eight in some other integrins) short repeating domains of about 60 amino acids each (indicated by roman numerals in **Figure 1.4**). The last three of these domains (or four in non-I domain containing integrins) contain structural homology to the EF hand-type divalent cation binding motifs originally described in calmodulin. For some time it was not known if these domains would be able to bind divalent cations as each one lacked the last (-z position) of six metal co-ordinating residues. A recombinant $\alpha_{IIb}\beta_3$ protein fragment containing domains 4-7 has been shown to bind divalent cation in the absence of any added ligands (Gulino, et al., 1992) and the vitronectin receptor can also bind divalent cation (Smith and Cheresh, 1991), indicating that these cation binding sites can indeed function. As yet it is not clear whether the similar domains in LFA-1 can also perform this feat. Within the last couple of years, some of these putative cation binding domains have been found to contain ligand binding sites. Domains V/VI in LFA-1 (Stanley, et al., 1994) and domains within $\alpha_{IIb}\beta_3$ (D'Souza, et al., 1990) contain binding sites for their respective ligands ICAM-1 and fibronectin suggesting that divalent cation and ligand binding are tightly associated. This is probably not so surprising as the function of integrins is highly dependent on the presence of divalent cations which are necessary for proficient ligand binding function.

In only seven integrins ($\alpha_L\beta_2$, $\alpha_M\beta_2$, $\alpha_X\beta_2$, $\alpha_D\beta_2$, $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_E\beta_7$; see boxes in **Figure 1.3**) there is an additional region of approximately 200 amino acids called the I domain, so called as it is 'inserted' between the short homologous domains II and III (**Figure 1.4**). This domain is homologous to the A domain found originally in von Willebrand factor (vWF) and also in cartilage matrix protein and the complement regulatory proteins, factor B and C2. As the A domains were important as ligand binding sites within these proteins it was speculated that integrin I domains might also contain ligand binding sites. The importance of this domain in integrin function was initially demonstrated when it was found that epitopes for many antibodies which affected integrin function mapped to here (reviewed in Hogg, et al., 1994). A host of prolific research studies on the I domain was initiated with the

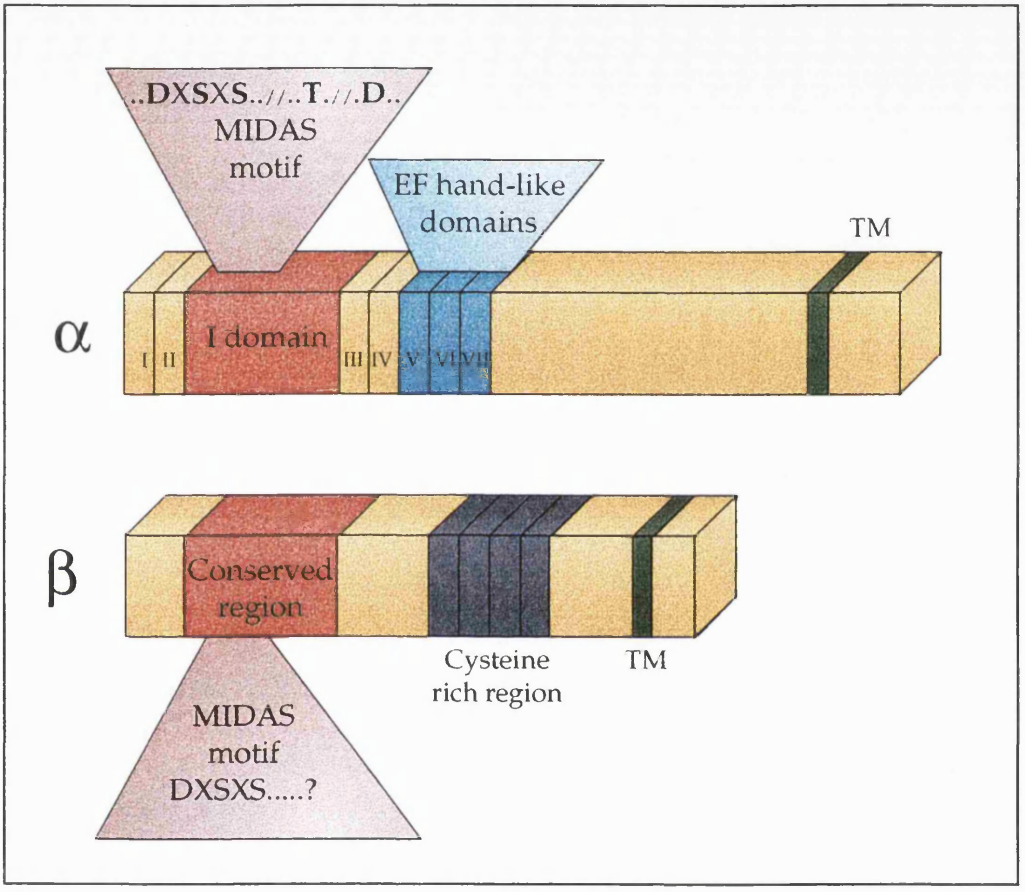

Figure 1.4: The αβ subunit structure of LFA-1. The N terminal portion of the α subunit extracellular domain is composed of 7 repeating domains (numbered in Roman numerals). The last three domains (V-VII) have structural homology to EF hand-like domains (blue). Between domains II and III lies the inserted or I domain (red) which contains the MIDAS motif (where X represents any amino acid). The β subunit has a cysteine rich region (purple) towards the transmembrane domain (TM) and N terminal to this a conserved domain (red) where secondary structure prediction indicates the presence of another MIDAS motif.

finding of a novel type of divalent cation binding site within this region of Mac-1 (Michishita, et al., 1993). Within the last year, ligand binding sites have also been found within isolated I domains of LFA-1 (Randi and Hogg, 1994), Mac-1 (Ueda, et al., 1994; Zhou, et al., 1994) and VLA-2 (Kamata and Takada, 1994; Tuckwell, et al., 1995). Further information about this domain was made evident when the crystal structures of the Mac-1 (Lee, et al., 1995) and, more recently, the LFA-1 I domain were determined (Qu and Leahy, 1995). Within the domain exists a Mg^{2+} co-ordination site composed of the sequence of amino acid residues DxSxS with contribution from two discontiguous residues T and D further downstream (see **Appendix II** for single and triple letter amino acid codes). This was a new cation binding motif termed a MIDAS (Metal Ion Dependent Adhesion Site) motif (see **Figure 1.4**). This gave further evidence to a very close association between divalent cation and ligand binding on the integrin subunits (reviewed in Bergelson and Hemler, 1995).

The β subunit is slightly shorter than the α, having a 701 amino acid extracellular portion, 23 amino acid transmembrane segment and once again a relatively short cytoplasmic tail of 45 amino acids. Within the extracellular portion, close to the transmembrane region, exists an area composed of four consecutive repeats of ~40 amino acids (see **Figure 1.4**). As there are eight cysteines per domain, this area is particularly cysteine-rich and as such is heavily disulphide bonded. Such bonding might be important for maintenance of the structure of integrins as shown for the β_3 subunit (Calvete, et al., 1991). Secondary structure prediction of a highly conserved domain in the N terminal portion of many β subunits has shown the presence of another MIDAS-like motif within it. Earlier studies showed that RGD (arginine-gycine-aspartic acid) peptide ligand could be cross-linked to this area (Smith and Cheresh, 1988) and together these findings suggest that this area within the β subunit can also function as a ligand and perhaps also a cation binding site (Lee, et al., 1995). A recent study showed that a fragment from the β_3 subunit of $\alpha_{IIb}\beta_3$ composing residues 118-131 could bind divalent cation (Cierniewski, et al., 1994). Thus several distinct areas on the integrins are involved in ligand binding, highlighting that the activation-induced ligand binding process might involved dynamic alterations in integrin structure.

1.3 LIGANDS FOR LFA-1 -THE ICAMS

Back in 1986 the first potential ligand for LFA-1 was described to be ICAM-1 (CD54) (Rothlein, et al., 1986). Direct evidence for this interaction came when purified ICAM-1 was shown to support binding of LFA-1-mediated cell adhesion (Makgoba, et al., 1988, Marlin and Springer, 1987). ICAM-1 is a member of the IgSF, composed of five Ig-like extracellular domains, and the LFA-1/ICAM-1 interaction was the first identification of an adhesion event between an integrin and an IgSF member. ICAM-1 has broad tissue distribution on myeloid, lymphoid and endothelial cells. ICAM-1 expression is inducible on endothelial cells following stimulation with inflammatory cytokines such as IFNγ (interferon), TNF (tumour necrosis factor) and IL-1 (interleukin). Although upregulation of ICAM-1 provides one mechanism for enhancing adhesion between LFA-1 and ICAM-1, it cannot explain the short term effects seen following normal stimulation methods as its inducible expression usually requires several hours. ICAM-1 is not a ligand exclusive to LFA-1 as it also binds Mac-1. Evidence for a further ligand for LFA-1 came from studies investigating adhesion mediated by various lymphocyte cell types. In these studies, cell adhesion could be blocked totally with LFA-1 mAbs but only partially with ICAM-1 mAbs suggesting that another ligand was also responsible for adhesion. The second ligand, ICAM-2, was cloned by transfection of the COS fibroblastoid cell line with an endothelial cDNA library (Staunton, et al., 1989) and was found to be another member of the IgSF, this time composed of only two extracellular Ig domains which had some homology to ICAM-1. Expression of ICAM-2 is more restricted than ICAM-1 but it is abundantly expressed on endothelium. Unlike ICAM-1, expression is constitutive and not induced by inflammatory cytokines. With ICAM-2 cloned and mAbs made against it, it was then found that adhesion between certain lymphocytes and their targets could still not be attributed to either ICAM-1 or -2 and the possibility of a further ligand for LFA-1 existed (de Fougerolles, et al., 1991). ICAM-3 was cloned in 1992 and found to contain five Ig-like domains similar to ICAM-1 and to be constitutively expressed on resting leukocytes (Fawcett, et al., 1992; de Fougerolles and Springer, 1992). A model of the three ICAM ligands is shown in **Figure 1.5**. ICAM-1 and -3 are the most homologous sharing 46% overall homology but 77% when domain two is compared. The two Ig-domain ICAM-2 molecule shows greatest homology to the two N terminal domains of ICAM-1. It is these two domains which contain the major binding sites on ICAM-1 and

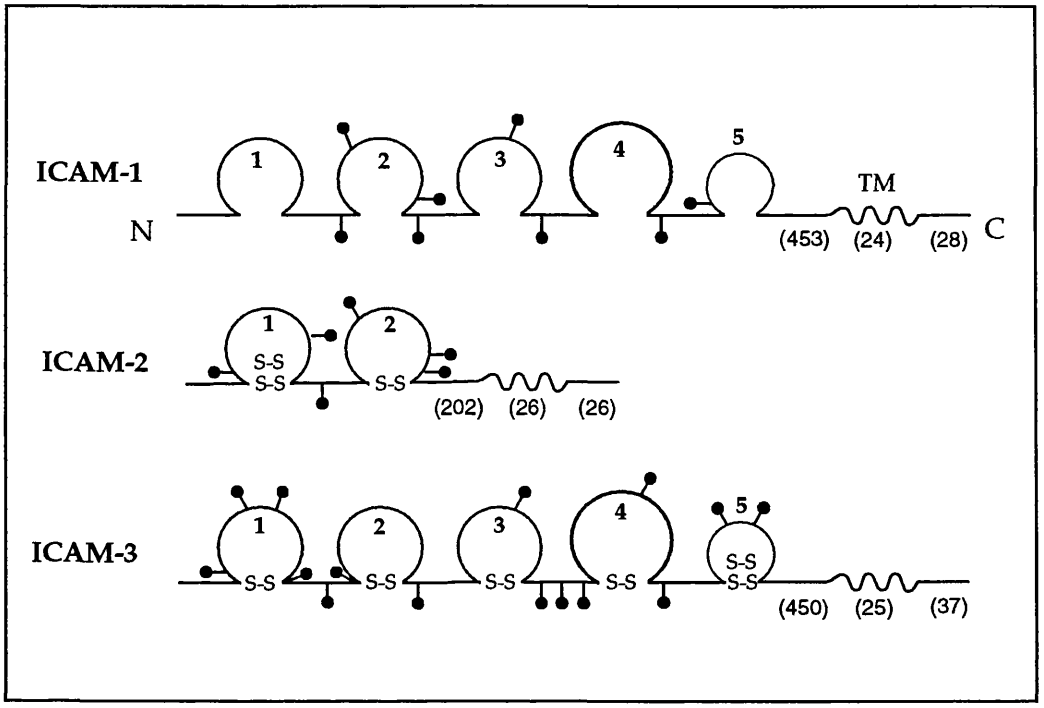

Figure 1.5: Comparison of the IgSF family members- the ICAMs.
The immunoglobulin domain structures of ICAMs-1, -2 and -3 are pictured. The length of each domain, e.g. extracellular, transmembrane and cytoplasmic, in amino acid residues is depicted in brackets underneath each structure. Dark circles on sticks represent glycosylation moieties and S-S represents disulphide bonds.

ICAM-3 for LFA-1 (Staunton, et al., 1990; Berendt, et al., 1992; Holness, et al., 1995,). Due to the differential tissue and cellular distribution, with ICAM-3 showing high constitutive expression on resting leukocytes, it is thought that ICAM-3 is involved in the initiation of an immune response and ICAM-1 involved later and during interactions with endothelium. Definitive proof of these ideas, however, requires further study.

1.4 THE ACTIVATION OF INTEGRINS

1.4a CELL TYPE SPECIFIC ACTIVATION

As previously mentioned integrins have the ability to exist in different states of competence to bind ligand. Several factors influence the ability of integrins to gain a competent ligand binding state. The cell type on which the integrin is expressed can influence its activity. For example, α_2 isolated from platelets and endothelial cells can bind collagen, but the endothelial cell form can also bind laminin and the 110kDa fragment of fibronectin (Kirchhofer, et al., 1990). Similar results were obtained for VLA-2 from melanoma cells which could bind both collagen and laminin whereas the same integrin from platelets, fibroblasts and T cells could only bind collagen (Elices and Hemler, 1989; Goldman, et al., 1992). These cell-type specific discrepancies in the ability of VLA-2 to bind ligands were further confirmed using α_2 transfection experiments where, depending on the cell type into which it was transfected, the integrin was capable of binding different substrates (Chan and Hemler, 1993). These integrins appeared to be identical by immunochemical analysis and N terminal sequencing and it was thought that some cell-type specific factor was influencing the ability to bind different ligands. In a similar way, endogenous LFA-1 on T cells requires prior activation before it can bind to ICAM-1, whereas LFA-1 transfected into the fibroblastoid cell line, COS is constitutively active for ligand and cannot be induced further by phorbol ester (Larson, et al., 1990). However, other studies have found discrepancies regarding the ability of the phorbol ester PMA to induce COS cell transfected-LFA-1 to bind ICAM-1. In one study PMA enhanced adhesion (Holness, et al., 1995) whereas in another there was no effect (C. Landis, personal communication). As well as cell-type specific differences, a cell can become competent to bind ligand through integrins by differentiation and maturation processes. The ability of Mac-1 on monocytes to bind fibrinogen is related to the state of cellular maturation and differentiation (Altieri, et al., 1988a).

These observations suggest that some sort of intracellular factors or signalling pathways present in different cell types can influence the behaviour of integrins. These factors may be constitutively present but require activation in certain cell types/differentiation states. Another possibility could be that in certain cell types, e.g. motile ones, the activation process is normally held in check by a repressor, whereas the absence of such a repressor in other cell types would lead to constitutive integrin activation. This could be as simple as the way in which the integrin gets anchored into the cell membrane, or cell-type differences in membrane lipid composition (Conforti, et al., 1990) or divergent cytoskeletal components in different cell types/states (Lub, et al., 1995).

1.4b STIMULUS INDUCED ACTIVATION- THE "INSIDE-OUT" SIGNAL

In a resting lymphocyte, LFA-1 is inactive and can be induced to bind ligand by various factors. This enhanced adhesion is mediated not through an increase in the number of integrin receptors present on the cell surface, although this does happen for Mac-1 and p150,95, but is due to an alteration in existing receptors. This phenomenon is not unique to the β_2 integrins and it is now well recognised for members of the β_1 and β_3 families. Over the last few years, the list of integrin activating stimuli has been expanding and the major ones are discussed below.

(i) Phorbol Esters

Very early studies documented that addition of phorbol esters to lymphocytes could cause morphological changes such as uropod formation, membrane ruffling and induction of homotypic aggregation before it was known which adhesion molecules were responsible for the association (Patarroyo, et al., 1982). It was later shown that phorbol ester-mediated lymphocyte aggregation was formed by the interaction of LFA-1 with one of its ligands ICAM-1 (Patarroyo, et al., 1985; Rothlein, et al., 1986; Rothlein and Springer, 1986). In the early days it was not clear which side of the adhesive interaction was being regulated. It was found that through inflammatory stimulation of endothelium, levels of ICAM-1 were dramatically upregulated. However for the relatively short-term effects on adhesion, it was obvious that control of adhesion lay with the LFA-1 side of the interaction (Dustin and Springer, 1989; van Kooyk, et al., 1989). Despite intense research effort since

then, it is not yet clear what is the exact mechanism stimulating integrin adhesion following phorbol ester stimulation. The best studied effect of phorbol ester is to activate protein kinase C (PKC) which is a major serine/threonine kinase and as such, a transducer of many mitogenic signals particularly in T cells. LFA-1 has been shown to undergo stimulation-induced phosphorylation on the β subunit following phorbol ester treatment and the α subunit appears to be constitutively phosphorylated (Buyon, et al., 1990; Chatila and Geha, 1988; Valmu, et al., 1991). With the finding that it is the β subunit cytoplasmic tail which is necessary to transmit the activation of LFA-1 (Hibbs, et al., 1991a) this initially seemed quite encouraging that phorbol ester might induce adhesive changes through direct phosphorylation on the integrin. However, further detailed mutagenesis studies demonstrated that mutation of the phorbol-ester induced phosphorylation site (S^{756}) on β_2 did not eliminate the ability of the integrin to be activated (Hibbs, et al., 1991b) thus phosphorylation of the subunit itself was not the stimulating factor. This does not rule out the possibility that phosphorylation of other intracellular proteins such as cytoskeletal proteins might be important, modification of which might induce changes in nearby or associated integrin molecules. It is also possible that phorbol esters might be exerting their effects through methods which do not directly induce changes in the integrin itself. These could be morphological changes which would enhance adhesion, such as cell spreading (see section 1.5).

(ii) TCR/CD3 triggering

Phorbol esters are pharmacological models which do not exist *in vivo*. A major breakthrough in analysing the induction of integrin adhesion on T cells came with the finding that stimulation of the TCR/CD3 complex with antibodies induced a transient increase in the adhesiveness of LFA-1 for ICAM-1 (Dustin and Springer, 1989; van Kooyk, et al., 1989). This provided a more physiological trigger for stimulating LFA-1 adhesiveness. The stimulation of adhesiveness was transient, peaking at 10-20 min and returning to basal state at 30 min- 2 hours. These kinetics corresponded well with the time course of the lytic cycle of a CTL, which can attach, release lytic granules and kill its target within the space of up to 30 min (Perelson, et al., 1984). Phorbol ester stimulation induced not a transient but a sustained activation of LFA-1 adhesiveness suggesting that these two methods of stimulation might act in a different manner (Dustin and Springer, 1989).

Adhesion caused by phorbol ester could be blocked by a general inhibitor of PKC, staurosporine, indicating that PKC was a major target for phorbol ester in this pathway. This inhibitor also caused partial inhibition of TCR/CD3 triggered adhesion suggesting that at least part of the mechanism of activation following this stimulant occurred through this same serine/threonine kinase. Around the same time the pathway of signalling events radiating from the TCR/CD3 complex following stimulation was beginning to be more fully elucidated. Within the last couple of years these pathways have become quite complex with intense research interest continually discovering novel molecules and associations within these cascades (reviewed in Cantrell, 1994; Weiss and Littman, 1994). A much simplified illustration of the major signals emanating from the TCR is shown in **Figure 1.6**. The earliest detectable event is the activation of protein tyrosine kinases (PTK) which leads on to various downstream events the more conventional of which is activating phospholipase Cγ1 (PLC) which triggers the phosphatidylinositol cascade. Phosphatidylinositol bisphosphate (PIP_2) is hydrolysed to release two products, diacylgylcerol (DAG) which activates PKC and inositol triphosphate (IP_3) which induces release of intracellular Ca^{2+} ($[Ca^{2+}]_i$) from stores. Ca^{2+} is an important second messenger within the cell and in T cells one of its major functions is to activate calcineurin which in turn activates the machinery for gene transcription within the nucleus. The early tyrosine kinase events can also induce activation of the MAPK (mitogen activated protein kinase) cascade via the ras and raf kinases. Activation of PKC has a dominant role in T cell signals so it is perhaps not surprising that activation of LFA-1 does rely on this kinase in part. The adhesion of other integrins to their ligands has also been shown to be induced following TCR/CD3 engagement; these include the β_1 integrins, VLA-4 ($\alpha_4\beta_1$) and VLA-5 ($\alpha_5\beta_1$) for fibronectin, VLA-6 ($\alpha_6\beta_1$) for laminin and VLA-2 ($\alpha_2\beta_1$) for collagen (Chan, et al., 1991; Shimizu, et al., 1990). Work is no doubt in progress to identify the mechanism leading to activation of these adhesion molecules and it will be interesting to see if it is different from the mechanism which stimulates LFA-1.

(iii) Other cell surface receptors

Following the initial finding that triggering the major receptor on T cells could induce an adhesive state of LFA-1 a whole flurry of studies reported

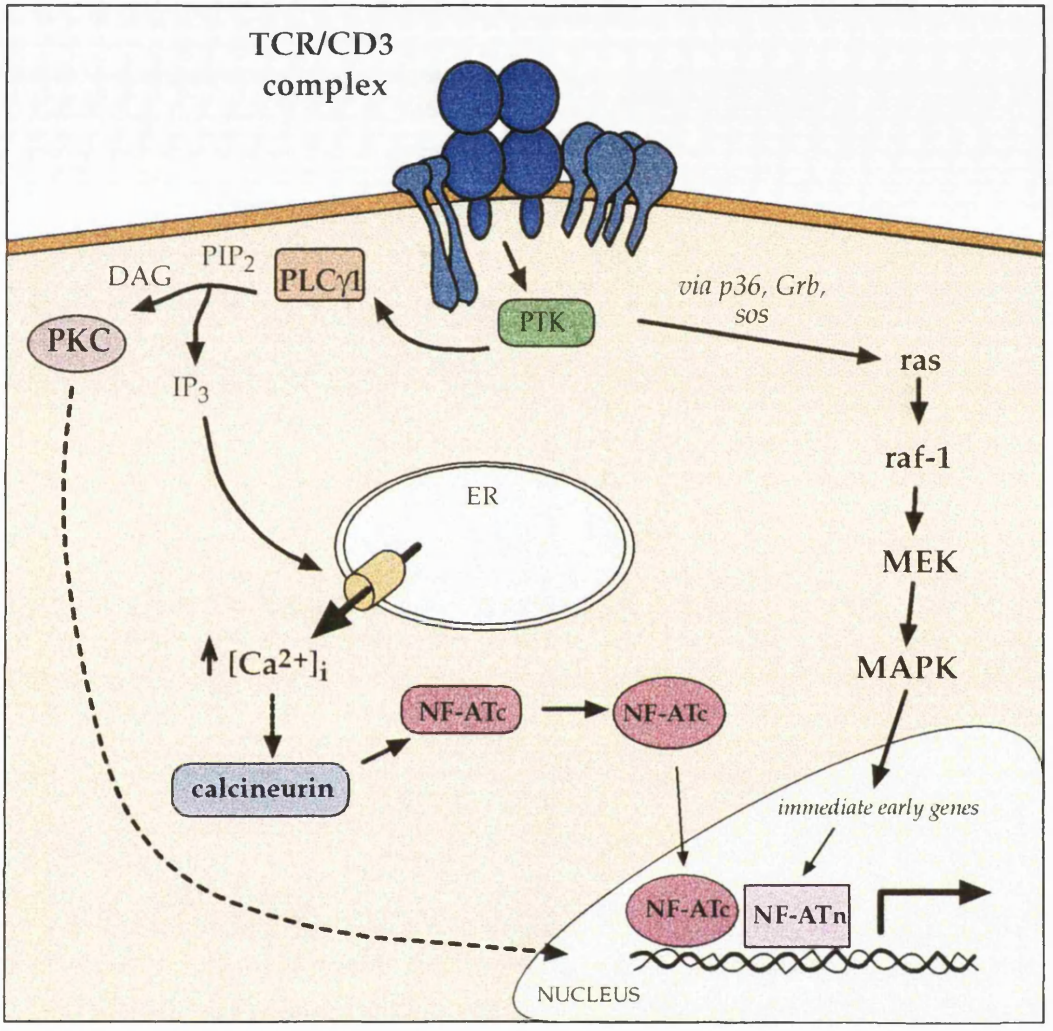

Figure 1.6: Schematic diagram of the early signalling events emanating from the T cell antigen receptor. The early tyrosine kinase induced events at the TCR/CD3 complex can be split into 2 pathways. The newer pathway induces activation of ras, raf and the subsequent MAP kinase cascade ultimately leading to alterations in nuclear events. The older pathway involves the phosphatidylinositol cascade where PIP_2 is hydrolysed into DAG and IP_3, the former activates PKC and the later binds to receptors on the endoplasmic reticulum (ER) which causes the release of Ca^{2+} from intracellular stores and subsequent increase in the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$). One of the well studied effects of $[Ca^{2+}]_i$ is activation of the serine/threonine phosphatase calcineurin which is thought to cause the activation and nuclear translocation of NF-AT (nuclear factor of activated T cells) which can then combine with a nuclear component of NF-AT (NF-ATn) to induce IL-2 gene transcription.

that triggering other T cell surface molecules could also achieve the same goal. The first one was the important coreceptor CD2 which was found to be able to induce a persistent activation of LFA-1 (van Kooyk, et al., 1989); this suggests, as with the phorbol ester-induced sustained adhesion, that LFA-1 can be differentially regulated. A whole host of other molecules were then identified on various cell types which could increase the adhesive ability of LFA-1 (Shimizu, et al., 1992; for review see Pardi, et al., 1992a). Even mAbs specific for ICAM-3, the third ligand for LFA-1, stimulate LFA-1 to bind to ICAM-1 (Campanero, et al., 1993) and this process is regulated by the cell-surface protein tyrosine phosphatase CD45 (Arroyo, et al., 1994). At this point it must be emphasised that any studies using mAb crosslinking as the stimulant and homotypic aggregation as the adhesion read-out should be viewed with caution until it is certain that observed effects are not due to a non-specific bridging effect of the mAb (reviewed in Harvey, et al., 1993). The aforementioned methods for inducing LFA-1 activation all result from intracellular signals which impose their results on the extracellular ligand-binding domain of the integrin. For this reason, these modes of activation are commonly referred to in the field as "inside-out" signalling events.

(iv) Lipids

A study in 1992, showed that Mac-1 on neutrophils could be induced to bind to one of its ligands, iC3b, by stimulation with a lipid termed IMF-1 (integrin modulating factor; Hermanowski-Vosatka, et al., 1992). This lipid was isolated from neutrophils following stimulation with various agonists. As these agonists could also induce Mac-1 binding activity with similar kinetics it was proposed that activation of neutrophils induced synthesis of IMF-1 which could then stimulate cell-bound integrins. This new integrin activator was not exclusive to Mac-1 as it could also stimulate LFA-1 on lymphocytes to undergo homotypic aggregation. A host of tests were carried out to try and identify IMF-1 and eventually, by process of elimination, it was hypothesised to be an unsaturated fatty acid. As IMF-1 could also induce isolated Mac-1 to bind iC3b the authors proposed that this lipid might be present in the cell membrane and act on the conformation of the receptor there. Unfortunately no further studies on IMF-1 have been published since then to try and shed more light on this matter. Further evidence for lipid regulation of integrins comes from an earlier study on the vitronectin receptor which showed that

this could be regulated by the lipid environment of the membrane into which it was inserted (Conforti, et al., 1990).

(v) Calreticulin

An idea for a molecule which might act directly on the integrins came with the finding that calreticulin, a Ca^{2+} binding protein known to be associated with the endoplasmic reticulum, would bind to a peptide sequence FGFFKR (Rojiani, et al., 1991). This sequence is highly conserved in all integrin α subunits and is located on the cytoplasmic domain adjacent to the transmembrane area (see **Figure 1.7**). This sequence is also present in the DNA binding domain of all steroid hormone receptors and calreticulin is able to inhibit their binding to target DNA response elements (Dedhar, et al., 1994) Calreticulin was directly linked to integrin activity by the finding that anti-sense oligonucleotides to calreticulin inhibited the collagen binding ability of Jurkat T lymphoblastoid cells following stimulation with activating antibodies and PMA (Leung-Hagesteijn, et al., 1994). Up until this point however, calreticulin and integrins had not been shown to associate *in vivo*. It has now been shown very recently that calreticulin co-immunoprecipitates with $\alpha_2\beta_1$ on Jurkat cells, and that this association is dependent on the integrin obtaining a competent ligand binding state by stimulation with either the anti-α_2 mAb JBS2, the anti-β_1 mAbs JB1 and B3B11 or the phorbol ester, PMA. Permeablisation of the cells to allow intracellular localisation of a calreticulin neutralising Ab prevented $\alpha_2\beta_1$ -mediated adhesion to collagen following stimulation with either the activating mAbs or PMA (Coppolino, et al., 1995). This now proves that an intracellular interaction with calreticulin is required by $\alpha_2\beta_1$ for successful activation-dependent interaction with collagen. It remains to be see if calreticulin plays a role in the activation of LFA-1 or other integrins.

(vi) New physiological triggers

The search is on for other triggers of integrin adhesion which might be more physiologically relevant. For example, during the cascade of transendothelial migration it is not known which stimulus triggers the activation of integrins allowing the leukocytes to firmly adhere to and migrate through the endothelial cell layer. Candidate molecules have been the selectin adhesion receptors as they are the first molecules to be engaged in the cascade when

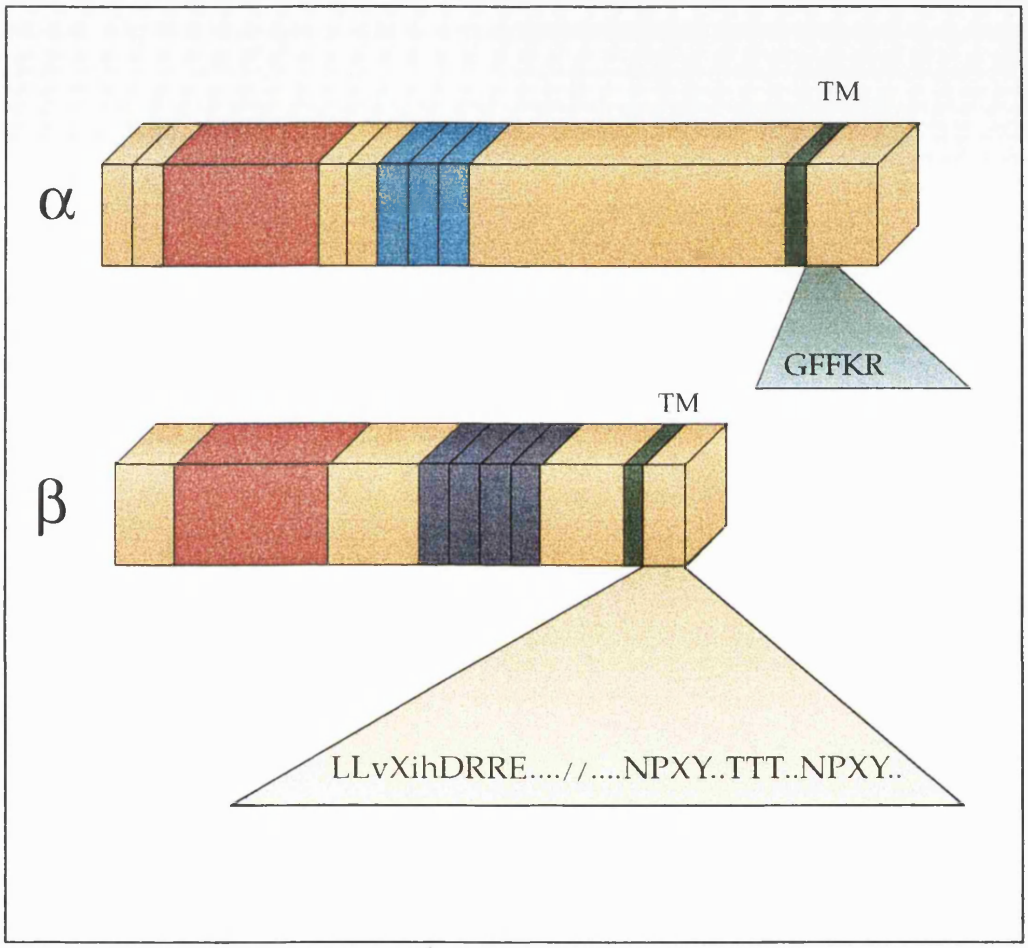

Figure 1.7: Important motifs in integrin cytoplasmic domains. Adjacent to the transmembrane domain in all integrin α subunits lies the GFFKR motif which binds the Ca^{2+}-binding protein calreticulin and is responsible for maintaining a default low affinity status. A similar motif LLvXihDRRE (where lower case represents less conserved amino acids and X is any amino acid) is present in all integrin β subunits and is thought to perform a similar function. The first NPXY motif is also through to control integrin affinity status. The TTT motif which nestles between the two conserved NPXY motifs in β_2 integrins is essential for regulation of LFA-1 activation.

they tether the cell during the rolling process. Although full details of the molecular events which occur in this cascade have just been elucidated during the last year or so, preliminary studies seem to suggest that triggering L selectin, by Ab crosslinking, stimulates an increased adhesiveness of Mac-1 for fibrinogen and albumin (Simon, et al., 1995). As yet unpublished observations are that stimulation of L-selectin through its natural ligand GlyCAM-1 activates LFA-1 to induce expression of the activation reporter epitope recognised by mAb 24 suggesting that this might also be a triggering mechanism for LFA-1 (S. Rosen, personal communication). The next few years will no doubt reveal other natural ligands which can stimulate the activity of the integrins.

Other candidate molecules for physiological triggers are the chemokines. These are a subset of cytokines, released near the endothelium during an immune response, which have chemotactic activity. Despite intense research into the topic of chemokine activation there are still few reports as to their effects on integrins. The chemokine MIP-1β (macrophage inflammatory protein) has been shown to be chemotactic for $CD8^+$ resting T lymphocytes (not $CD4^+$) and able to induce them to adhere to VCAM-1 and fibronectin, through VLA-4. Immobilisation of MIP-1β on proteoglycans could also stimulate these cells to bind ligand which provides a more physiological method for presenting chemokines to lymphocytes (Tanaka, et al., 1993). These areas of investigation will no doubt be major topics for future studies.

1.4c STIMULUS INDUCED ACTIVATION- FROM THE OUTSIDE

It is now well established that certain stimulants can activate LFA-1 through so called "inside-out" signals. There are a variety of factors which, when added to the extracellular environment, can also induce activation of integrins. These are thought to bypass normal intracellular signalling due to their direct effects on the integrins.

(i) Divalent Cations

As previously mentioned, the binding of integrin to ligand is strictly dependent on the presence of the correct divalent cations; therefore there has been intense research interest in trying to decipher their part in integrin activation. Integrins may be activated directly by incubation with the

divalent cations Mn^{2+}, Mg^{2+} and Ca^{2+}. Not all cations have similar effects. Mg^{2+} and Mn^{2+} can stimulate most of the leukocyte integrins whereas Ca^{2+} will negatively regulate ligand binding (Dransfield, et al., 1992a; Staatz, et al., 1989). The cation requirements for LFA-1 are such that incubation with mM concentrations of Mg^{2+} and μM concentrations of Mn^{2+} and will induce binding to ICAM-1, but for Mg^{2+} to exert its effects, cell-bound Ca^{2+} must first be removed (Dransfield, et al., 1992a). Mn^{2+} can also induce T cell LFA-1 to adhere to ICAM-3 (Landis, et al., 1994). Although these experiments are performed on cell-bound integrins, studies with other integrins have indicated that cations can still mediate these stimulatory effects in a cell-free context. For example, VLA-2 artificially inserted into liposomes could be induced to bind collagen through incubation with Mg^{2+} and Ca^{2+} inhibited this binding (Staatz, et al., 1989). Isolated Mac-1 binds to fibrinogen in the presence of Mn^{2+} (Altieri, 1991), Mn^{2+} can also induce the binding of purified $\alpha_4\beta_1$ to VCAM-1 and fibronectin (Mould, et al., 1994) and $\alpha_5\beta_1$ to fibronectin (Mould, et al., 1995). This suggests that cation-induced effects on ligand binding occur in the absence of intracellular signals. As previously mentioned, integrins have several potential cation binding sites, in both the MIDAS motifs of the α subunit I domain and the β subunit and the EF-hand like cation binding sites (see **Figure 1.4**). It is speculated that divalent cations regulate integrin adhesion by binding to these sites and either acting as a bridge to link ligand and receptor, or by causing a conformational change which exposes another site(s) on the integrin to which ligand can then bind. An alternative hypothesis has recently been put forward from studies on an isolated peptide motif, β_3 (118-131), from the β subunit of the platelet integrin $\alpha_{IIb}\beta_3$. In this study Ca^{2+} was found to bind to the isolated domain but upon RGD ligand binding, cation was displaced from the unstable ternary complex (D'Souza, et al., 1994). This provides a method to explain how distinct cations might differentially affect ligand binding. So why do some cations induce ligand binding and others seem to inhibit it? Originally it was thought that these opposing effects of Mg^{2+} and Ca^{2+} might be due to competitive cation binding to the same binding site. Regression analysis of cation binding to VLA-2 however showed that the relationship between Ca^{2+} and Mg^{2+} was non-competitive and the simplest explanation to explain this was that Mg^{2+} and Ca^{2+} were binding to distinct sites on the integrin (Staatz, et al., 1989). More recently a very detailed study measuring cation-induced ligand binding affinity has shown that the binding of Ca^{2+} to its high affinity site allows Mg^{2+} to bind with high affinity to a distinct ligand-competent site. However,

Ca^{2+} can also compete with Mg^{2+} to bind to the ligand competent site, which appears to bind both cations with lower affinity (Mould, et al., 1995). In this rapidly expanding field it will be interesting to see how the binding of divalent cations will affect the crystal structures of isolated cation binding domains, and if the sites which bind Mg^{2+} and Ca^{2+} can be identified.

(ii) Activating antibodies

With the continued ease of making mAbs and the increased knowledge about the activation of integrins, many mAbs have now been characterised to induce the ligand-binding state of integrins directly. One of the first activating antibodies to be demonstrated for LFA-1 was the α subunit specific, NKI-L16. This was found to induce homotypic aggregation of the B cell lymphoblastoid line, JY and a CTL clone, JS136 (Keizer, et al., 1988). Other antibodies have been characterised which can activate LFA-1 to bind ligand. One of these, KIM 127, binds to the common β_2 subunit and induces activation of LFA-1 and Mac-1 mediated adhesive events (Robinson, et al., 1992). Characterisation of a similar mAb called KIM 185 revealed that it could also induce LFA-1- and Mac-1- dependent adhesive events (Andrew, et al., 1993). The epitopes for these mAbs are both on the β_2 chain but do not crossblock. Another activating mAb MEM83, specific to the LFA-1 α subunit, causes induction of T cell mediated adhesion to ICAM-1 but not to the related ligand ICAM-3 which suggests that LFA-1 can exist in different activation states for both ligands (Landis, et al., 1993; Landis, et al., 1994). This mAb was mapped and found to interact with the I domain of LFA-1 indicating that this area is important for regulation of LFA-1-mediated adhesion (Landis, et al., 1993). Although none of these LFA-1 activating mAbs have yet been tested on isolated receptors, studies with mAb specific for other integrins have shown that these activating mAbs can mediate their effects in a cell free context. For example, mAbs P41 and Ab 62 can stimulate either fixed or detergent solubilised $\alpha_{IIb}\beta_3$ to bind to fibrinogen (O'Toole, et al., 1990) and the β_1 specific mAb TS2/16 can induce solubilised VLA-5 and VLA-2 to bind fibronectin and collagen respectively (Arroyo, et al., 1993). Isolated Mac-1 can be induced to bind the complement component iC3b with the β_2-specific mAb KIM 127 (Cai and Wright, 1995). These studies suggest that activating mAbs do not require intact cellular functions and thus probably work by directly altering the conformation of the integrin. These are laboratory tools which have proved extremely useful in the dissection of integrin activation.

(iii) Role of Ligand

A new phenomenon was proposed from experiments where incubation of the platelet integrin $\alpha_{IIb}\beta_3$ with an RGD peptide ligand could induce it to bind with higher affinity to fibrinogen (Du, et al., 1991). A similar scenario was demonstrated for the vitronectin receptor (Orlando and Cheresh, 1991). This could occur on isolated receptors which suggests that it is another extracellular phenomenon which does not require intact cellular events (Du, et al., 1991). Studies on the role of ligand in integrin activation events has been made much simpler with the identification of a whole host of unique mAbs which are specialist reporters in that they recognise epitopes exposed only upon activation of the integrin and some even recognise the ligand induced form. These tools have helped to assess that changes do occur in integrin receptors upon activation events.

1.5 ACTIVATION OF INTEGRINS- AFFINITY AND AVIDITY

Stimulants are required to induce integrins into a state capable of binding ligand. Originally it was assumed that these stimulants were working by inducing changes in the affinity (conformation) of integrins for their ligands. Over the last year or so it has become an emerging theme that integrin activation is not quite as simple as this and adhesion upregulation can also result from avidity (post-receptor occupancy) changes. It has become possible to measure these differences through the use of special monoclonal antibodies (mAbs) which recognise epitopes only expressed once the integrin has been activated. Use of the confocal microscope has also provided insight as to the distribution of integrin receptors throughout the cell following activation.

1.5a AFFINITY CHANGES IN INTEGRINS

Originally it was assumed that activation of an integrin induced a high affinity binding state. This change can most easily thought of as a change in the existing structure of the integrin which is most easily thought of as a conformation change. Information regarding these changes has become much more evident over the last few years with the production and characterisation of mAbs which can report active integrins by recognising

epitopes unique to their stimulated forms. Various stimulants have been shown to induce these reporter epitopes and the ones which act on isolated integrins give the greatest evidence for a conformational change occurring in the receptor. As the affinity of an integrin for ligand is measured by its ability to bind soluble ligand, it remains to be seen whether these changes in the receptor result in higher affinity binding conformers or some other alterations which aid adhesion through other means. Over the last few years, it has become obvious which stimulants do indeed alter the affinities of integrins and it is now being realised which mAbs can actually report this form.

(i) mAbs, divalent cations and ligand

Perhaps the most conclusive evidence that activated integrins exist in different conformations from their inactive counterparts came from the use of monoclonal antibodies (mAbs) which either activate the integrin directly (activators) or act as reporters of the active form (reporters). $\alpha_{IIb}\beta_3$ was the original prototype integrin used for many integrin regulation studies. Activating mAbs were found to induce this integrin to bind soluble ligand (O'Toole, et al., 1990) and the high affinity state is thought to be reflected by the expression of an activation reporter mAb called PAC-1 (Faull and Ginsberg, 1995; Shattil, et al., 1985). The β_1 activators 8A2 and TS2/16 have been shown recently to induce higher affinity states of $\alpha_5\beta_1$ for fibronectin and $\alpha_4\beta_1$ for VCAM-1 (vascular cell adhesion molecule-1) with dissociation constants (K_d) of 54 nM (Faull, et al., 1993) and 0.71 μM (Jakubowski, et al., 1995a) respectively. Activating mAbs will also induce the binding of soluble ligand to isolated receptors. For example, mAbs P41 and 62 cause $\alpha_{IIb}\beta_3$ to bind soluble fibrinogen (O'Toole, et al., 1990) and TS2/16 enhances binding of isolated $\alpha_5\beta_1$ and $\alpha_2\beta_1$ for fibronectin and collagen respectively (Arroyo, et al., 1993). A K_d of ~19 nM has actually been calculated for KIM127 induced-binding of purified Mac-1 to iC3b (Cai and Wright, 1995). Therefore, there is no doubt that these mAbs cause alterations in receptor affinity but it is debated whether they actually induce a conformational change *per se* or trap an active receptor which is one of the states observed on continual receptor 'flip-flop' between forms (Cai and Wright, 1995). Affinity changes in integrins have also been measured following stimulation with divalent cations. Mn^{2+} has been shown to induce VLA-4 ($\alpha_4\beta_1$) on T cells to bind VCAM-1 with an affinity of 33 nM (Jakubowski, et al., 1995) and isolated $\alpha_4\beta_1$ binds VCAM-1 with an apparent affinity of 5 nM (Mould, et al., 1994). As previously

discussed, ligand itself has been found to contribute to the induction of fully activated receptors. This was originally described for $\alpha_{IIb}\beta_3$ when it was found that ligand mimetic peptides could bind to the isolated receptor and induce expression of neoantigenic sites recognised by a mAb called PMI-1 (Frelinger, et al., 1988). Similar mAbs were discovered which could also report a conformation of integrin found following ligand binding, these antibodies are said to report LIBS (ligand-induced binding sites). Further studies then showed that addition of an RGD ligand mimetic peptide could induce a high affinity binding conformer of $\alpha_{IIb}\beta_3$ which could bind fibrinogen and mediate cellular aggregation in the absence of agonist stimulation. This suggested that the ligand itself could activate the integrin (Du, et al., 1991). It is hypothesised that this phenomenon occurs by ligand binding through an initial low affinity interaction which then stabilises the interaction to a more stable high affinity state. This has also been shown for LFA-1 in that interaction with ICAM-1 is required for full expression of the epitope recognised by the activation reporter antibody called mAb 24 (Cabañas and Hogg, 1993). In this case however, cells still require stimulation with an agonist such as phorbol ester or TCR/CD3 complex triggering. Therefore it seems that these stimulants which have actually been measured to induce affinity changes in integrins are all believed to act by stimulating the receptor from the cell exterior.

(ii) Intracellular control of affinity

Although affinity studies performed on isolated receptors suggest that changes can be induced independently of additional cellular factors, experiments performed on intact cells have shown otherwise. The metabolic inhibitors sodium azide and 2-deoxyglucose have been shown to inhibit induction of integrin affinity changes on intact cells (Dransfield and Hogg, 1989; O'Toole, et al., 1994). This means that these stimulants, although exerting their effects predominantly from the cell exterior, are still regulated by some cellular components. Whether this is simply a matter of intact membrane fluidity to ensure mobility of receptors or efficient anchoring of the integrin in the membrane is still not known. Recent mutagenesis studies have shown that substitution of certain motifs in the cytoplasmic domain of integrins can alter the affinity of the integrin, proposing that there is a degree of intracellular control on receptor affinity (reviewed in Williams, et al., 1994). Further indication that affinity changes have a component of intracellular

control has come from studies where overexpression of integrin cytoplasmic domains, by transfection into cells, resulted in the inhibition of receptor affinity (Chen, et al., 1994). This study explained these inhibitory effects by competition between the endogenous and transfected cytoplasmic tails for binding to intracellular moieties necessary to project affinity changes. Deletion of one site, the GFFKR sequence proximal to the transmembrane domain (see **Figure 1.7**), has been shown to induce $\alpha_{IIb}\beta_3$ into a constitutive high affinity state which is resistant to the effects of metabolic inhibitors (O'Toole, et al., 1994). The authors propose a model where it is assumed that the presence of this sequence maintains the integrin in a default low affinity state and binding of an unknown intracellular factor to this site, upon agonist-induced activation, might alter the conformation of the receptor presenting a high affinity binding site. Deletion of this sequence removes the default intracellular constraints and allows expression of the high affinity form. The β subunit has a similar homologous motif (LLvXihDRRE; where lower case represents less conserved amino acids and X any amino acid, see **Figure 1.7**) in an identical position close to the transmembrane domain and deletion of this has similar consequences on affinity (Hughes, et al., 1995). Further down the relatively short β subunit lies two highly conserved NPXY motifs, the first of which, when eliminated, inhibits the integrin affinity status (O'Toole, et al., 1995). Speculation of course would be that some kind of intracellular factors might bind to these sites and, in doing so, promote affinity changes. As already discussed, the intracellular Ca^{2+} binding protein, calreticulin has been shown to precipitate with peptides containing the GFFKR motif. This year more possibilities have arisen with the finding that the focal adhesion kinase (pp125FAK) and cytoskeletal protein paxillin bind to peptides containing the LLvXihDRRE motif (Schaller, et al., 1995); an area to which the actin-binding protein filamin has also been shown to bind on β_2 (Sharma, et al., 1995). Another cytoskeletal protein talin has been shown to colocalise with the first NPXY motif on β_1 (Lewis and Schwartz, 1995). It will probably not be long before it is known whether these *in vitro* interactions can alter the affinity of intact integrin receptors.

1.5b AVIDITY CHANGES IN INTEGRINS.

Cell adhesion through integrins can also take place in the absence of substantial numbers of high affinity receptors. In this case adhesion is facilitated through increases in the avidity of integrins for their ligands.

There are several components which contribute to increase the avidity of adhesive interactions. Clustering or microaggregation of low affinity receptors locally at the adhesive site could act to increase the overall binding strength of the interaction. In addition, events taking place following initial receptor-ligand interactions (post-receptor occupancy events) could induce alterations in cell morphology such as cell spreading. Benefits to the cell following spreading or flattening can most easily be envisioned as a streamlining mechanism whereby cells are less susceptible to shear stress-induced detachment. This has been shown to be the case for lymphocytes adherent to ICAM-1 in a flow system, where phorbol ester induced spread cells show a greater resistance to physiological shear than do non spread cells (Lawrence, et al., 1995). Spreading could also increase the area of the cell in contact with ligand enabling more integrin-ligand interactions (Singer, 1992). Evidence exists for the participation of leukocyte integrins in both receptor clustering and cell spreading and both of these are dependent on interaction with the cytoskeleton.

(i) Integrin clustering.

The majority of studies documenting avidity changes on integrin-mediated adhesion were made using phorbol esters as the agonists. Very early studies showed that the phorbol esters could cause cytoskeletal-dependent capping of LFA-1 on lymphocytes in the absence of cell-cell adhesion (Haverstick, et al., 1992) and concomitant co-localisation with the cytoskeletal protein talin (Burn, et al., 1988). Another β_2 integrin Mac-1 was shown to undergo phorbol ester-induced aggregation in the absence of ligand binding (Detmers, et al., 1987). The divalent cation Ca^{2+} has also been shown to induce clustering of LFA-1 on T cell lines and the authors propose that this is a prerequisite to ligand binding; an event which definitely requires the presence of Mg^{2+} (van Kooyk, et al., 1994). This clustered state of LFA-1, which is detected by mAb NKI-L16, does not appear to be present on naive T cells and the hypothesis for this is that the cytoskeleton imposes an active constraint on LFA-1 mobility within these cells as cytochalasin treatment can release receptors for clustering (reviewed in Lub, et al., 1995).

(ii) Cell spreading.

It is now well known that phorbol esters cause spreading of lymphocytes onto ligand (Lawrence, et al., 1995). Spreading results from the induction of actin polymerisation (i.e. the conversion of monomeric G actin to filamentous F actin). Aside from phorbol esters, more physiological stimulating methods such as TCR/CD3 crosslinking have been shown to cause cell spreading (Pardi, et al., 1992b) and actin polymerisation (Parsey and Lewis, 1993) and interaction of T cells with Ag-pulsed presenting cells caused cell flattening and concomitant fluxes in the intracellular Ca^{2+} concentration $[Ca^{2+}]_i$ (Donnadieu, et al., 1994). One of these same studies showed that alongside cell spreading, TCR/CD3 triggering caused the association of LFA-1 with the cytoskeletal proteins α-actinin and talin (Pardi, et al., 1992); once again indicating a close relationship between integrins and cytoskeletal elements. Monocytes undergo spreading onto endothelial cells as part of the transmigration process (Beekhuizen, et al., 1992).

(iii) Intracellular regulation of avidity

Recent studies using chimaeric receptors, composing the extracellular and transmembrane portions of $\alpha_{IIb}\beta_3$ joined to the cytoplasmic domains of LFA-1, have shown that stimulation of adhesion of transfected fibroblasts to fibrinogen required a TTT motif towards the end of the β_2 tail even when the receptors were locked into a constitutive high affinity state by deletion of the GFFKR motif (see **Figure 1.7**; Peter and O'Toole, 1995). Mutation of the TTT motif prevented cell spreading and focal adhesion formation. This motif was originally described to be the site required for regulation of adhesion of LFA-1 to ICAM-1 (Hibbs, et al., 1991). A study this year, attempting to revive the cytoplasmic tail phosphorylation hypothesis, has performed phosphorylation analysis of this region and claim that threonine phosphorylation on these residues might prove to be the key regulatory mechanism (Valmu and Gahmberg, 1995). The phosphorylation hypothesis was previously discarded as mutation of the major phorbol ester phosphorylation site (S^{756}) had no effect on its ability to induce adhesion (Hibbs, et al., 1991). It remains to be seen if cytoskeletal factors will be identified which bind to this region. The actin binding protein α-actinin binds to an area towards the N terminal region of the β_2 cytoplasmic domain just after the LLvXihDRRE motif (Pavalko and LaRoche, 1993). This stretch is distinct from that previously discussed,

however associations here might have some effect on these other areas. As new associations are found it will be possible to draw up a map of which factors affect both the affinity and avidity status of integrins.

1.5c THE SITUATION FOR LFA-1

Studies measuring affinity changes in LFA-1 have not been performed due to the difficulties in obtaining soluble ICAM-1 binding. Good evidence exists, however, that changes do occur in LFA-1 following activation. This has come through the development and analysis of unique mAbs.

(i) Activation induced changes in ligand binding ability

Although specialised mAbs have been characterised for LFA-1 it is still not clear whether they are affecting affinity or avidity changes. The fact that LFA-1 can exist in different forms once activated is evident from the following studies. The α_L activating mAb MEM83 can differentially affect LFA-1 binding to two of its ligands ICAM-1 and ICAM-3 by stimulating binding to ICAM-1 but not ICAM-3 (Landis, et al., 1994). This suggests that activation of LFA-1 produces at least two distinct forms or activation sites which can recognise different ligands. This same study demonstrated that two other LFA-1 specific mAbs, YTH 81.5 and 122.2A9, could inhibit binding of T cells to ICAM-3 but did not interfere with ICAM-1 binding. This provides further evidence for the existence of different activation states of LFA-1 on the cell membrane upon activation. Another activating mAb KIM185, specific for the β_2 subunit, appears to be inducing structural alterations in LFA-1 upon binding, as it can induce the presence of a previously absent epitope for another β_2 mAb, KIM127 (Andrew, et al., 1993).

(ii) The mAb NKI-L16

Further studies on the mAb NKI-L16, which was originally described as an LFA-1 activator, have found that it recognises a Ca^{2+}-dependent epitope on LFA-1. The authors propose that NKI-L16 reports a form of LFA-1 with intermediate activity which is necessary, but not sufficient, to induce LFA-1/ICAM-1 mediated adhesion (van Kooyk, et al., 1991). More recent investigation into the epitope for this mAb has found that it correlates with a Ca^{2+} bound, clustered form of LFA-1 and can be induced on all LFA-1

molecules of a particular cell line. Although Ca^{2+} alone is enough to induce NKI-L16 epitope expression on certain cell types Mg^{2+} is still necessary before adhesion to ICAM-1 takes place (van Kooyk, et al., 1994). Therefore it seems that NKI-L16 can recognise a form of LFA-1 with bound Ca^{2+} and this is a prerequisite stage before full activation leading to ligand binding.

(iii) The mAb 24

In our laboratory, a mAb called 24 was originally identified due to its cation dependence for recognition of LFA-1. This mAb is present on three of the β_2 integrins; LFA-1, Mac-1 and p150,95 but instead of being directed against the shared β subunit it recognises a common epitope on the α subunit. The divalent cations Mg^{2+} and Mn^{2+} stimulate expression of the 24 epitope and addition of Ca^{2+} causes its inhibition; this situation which parallels the cation requirements for binding of T cells to ICAM-1 suggests that mAb 24 reports a Mg^{2+}-dependent active state of LFA-1 (Dransfield, et al., 1992a). MAb 24 is thought to recognise a LIBS-type epitope due to the fact that T cells without prior recognition of ICAM-1 cannot be induced to express the epitope and microscopy indicates that this epitope is restricted to areas where LFA-1 on one cell is in contact with ICAM-1 on a neighbouring cell (Cabañas and Hogg, 1993). In summary, these two special antibodies seem to recognise divergent forms of LFA-1 which are regulated by binding of different divalent cations. This suggests that LFA-1 can exist in different forms depending on its divalent cation and ligand binding status and perhaps by the method of stimulation. Precisely how the different cations contribute to different activation states is not yet known but is beginning to become clarified.

1.6 INTEGRINS AS SIGNAL TRANSDUCERS

1.6a THE ROLE OF FOCAL ADHESIONS

It has become obvious over the last few years that integrins not only play an important role in adhesion and maintenance of cell-cell contact but they can also act as signal transducers in their own right. Initial studies of leukocyte signalling found that integrin ligation could induce activation of $[Ca^{2+}]_i$ flux, stimulation of the Na/H antiporter and induction of gene expression (reviewed in Juliano and Haskill, 1993). A major breakthrough in the integrin signalling field came with the discovery of focal adhesions. These are

important areas where the extracellular matrix links to the actin-filamented cytoskeleton through integrins. In addition to providing tensile strength (stabilising adhesion and regulating cell shape), these structures act as a framework for recruitment of a whole host of cytoskeletal proteins and signalling molecules which include the kinases, pp125^{FAK} (focal adhesion kinase), pp60^{src}, adapter proteins and the cytoskeletal protein, paxillin (for review see Schaller and Parsons, 1994). A cascade of tyrosine phosphorylation, initiating with autophosphorylation of pp125^{FAK}, emanates from the integrin upon stimulation. These phosphorylation events act to enhance the recruitment of other molecules to the focal adhesion and so downstream signalling pathways are generated (reviewed in Clark and Brugge, 1995). Integrin engagment links into the ras pathway and stimulates MAPK activation (Schlaepfer, et al., 1994). It is hypothesised that ligand recognition and engagement cross-links or clusters integrins and this is necessary to induce the activation of downstream biochemical events. A recent study found that association of the majority of these focal contact cytoskeletal components requires both receptor clustering and integrin occupancy by ligand (Miyamoto, et al., 1995). Classic focal adhesions are found in adherent cells and it is in these cells where most of the focal adhesion associated molecules have been identified. For motile cells such as leukocytes, it is not thought that they have these same classic focal adhesions (reviewed in Huttenlocher, et al., 1995). In these cells it is hypothesised that other forms of adhesive contacts are present such as filopodia and lamellipodia which have recently been characterised in fibroblasts. These alternative kinds of focal contact structure are induced by signalling events from various small guanosine triphosphate (GTP)-binding proteins (Nobes and Hall, 1995).

Studies linking leukocyte integrins with these same focal adhesion-characterised signalling proteins have not yet been elucidated in much detail. Perhaps this might not be so surprising considering that leukocytes have a very different lifestyle to these comparatively sedentary adherent cells. As integrins on leukocytes have been shown to perform costimulatory functions most of the signalling events through leukocyte integrins have been documented in this manner.

1.6b LEUKOCYTE INTEGRINS AND CO-STIMULATION

The importance of LFA-1 as a costimulatory molecule for T cells was shown when transfection of ICAM-1 into APCs restored the ability of T cells to respond to suboptimal levels of MHC class II molecules (Altmann, et al., 1989). Triggering LFA-1 functions as a potent co-stimulus for resting T cell proliferation but only when the TCR/CD3 complex is simultaneously engaged. LFA-1 co-ligation enhances the production of $[Ca^{2+}]_i$ flux, IL-2 production and IL-2 receptor expression (van Noesel, et al., 1988; Wacholtz, et al., 1989). This is not solely due to an adhesion strengthening role of LFA-1 as it performs a similar function on cells stimulated with PMA and ionomycin in solution (van Seventer, et al., 1990). In these early studies, crosslinking of LFA-1 on its own without CD3 ligation did not induce any of these activation events. It was then shown, however that on peripheral blood lymphocytes engagement of the LFA-1 α, but not β, subunit induced a transient increase in $[Ca^{2+}]_i$ and a concomitant increase in the production of inositol phosphates (Pardi, et al., 1989). More recently, mAb crosslinking of LFA-1 on T cells has been found to result in both tyrosine phosphorylation of the early signalling marker $PLC_{\gamma 1}$ and mobilisation of $[Ca^{2+}]_i$, consistent with a role for LFA-1 as a *bona fide* signalling receptor (Kanner, et al., 1993). In this study $pp125^{FAK}$ was not phosphorylated following LFA-1 engagement. A further study investigating the signals transmitted following β_2 integrin engagement on neutrophils has found that CD18 crosslinking enhances actin polymerisation and phosphatidylinositol trisphosphate (PIP_3) production (Löfgren, et al., 1993). LFA-1 is not the only integrin, or indeed molecule, which can act as a costimulator for T lymphocytes. For example, the IgSF family members, CD28 and CD2 and the β_1 integrin VLA-4 can also perform this function. It seems that these different molecules might not provide redundant functions in that they exert differential effects depending if the T cells are resting or Ag-primed. LFA-1 and VLA-4 are better at stimulating the proliferation of resting cells with CD28 and CD2 more productive on Ag-primed T cells (Damle, et al., 1992). A recent paper trying to investigate the downstream effects of VLA-4 co-stimulation has found that only the VLA-4 mAbs which induce co-stimulatory function can also induce tyrosine phosphorylation of a host of proteins which include phospholipase Cγ (PLCγ), $pp125^{FAK}$, paxillin, $p56^{fyn}$, $p56^{lck}$ and MAPK in T cells (Sato, et al., 1995). Studies on neutrophils have found that β_2 can stimulate tyrosine phosphorylation of paxillin when there is simultaneous activation of the cells with agonists such as TNFα

(Fuortes, et al., 1994; Graham, et al., 1994). So it seems that classic focal adhesion proteins such as pp125^{FAK} and paxillin might indeed participate in leukocyte functions after all. Activation of $\alpha_2\beta_1$ on Jurkat T cells has been shown to induce tyrosine phosphorylation of proteins within the 47-52kDa range and accumulation of GTP-bound p21^{ras}, suggesting that this integrin has the potential to induce downstream alterations in gene expression in the nucleus (Kapron-Bras, et al., 1993). It is already well established that β_1 integrin engagement on monocytes leads to the induction of immediate-early gene expression (Juliano and Haskill, 1993). Thus signalling following leukocyte integrin triggering has been well documented to induce signals which are known to affect their function as costimulators. Recent data linking them to the same classical focal adhesion-type molecules as adherent cell integrins suggests that despite their different anatomical locations they might have pathways in common.

1.7 MAJOR AIMS OF THESIS

The major aim of this thesis was to identify the intracellular factors which contribute to the activation of LFA-1 on T lymphocytes. This included trying to elucidate which downstream mechanisms followed induction of LFA-1 activation by well defined stimulants and also to try and identify novel players important as LFA-1 activators. The question was also asked to decipher how different stimuli achieve LFA-1 mediated adhesion and whether this differs between those "inside-out" signal inducers or those thought to act from the cell exterior.

CHAPTER TWO

MATERIALS AND METHODS

MATERIALS

2.1 INHIBITORS AND STIMULANTS

BAPTA/AM (Bis-(o-amino phenoxy)-ethane-N,N,N',N'-tetraacetoxy methyl ester)	Stock solution of 50 mM in DMSO	GIBCO BRL Life Technologies
CYTOCHALASIN D	Stock solution of 1mg/ml in DMSO	Sigma
dBHQ (2,5 di-*tert*-butylhydroquinone)	Freshly made stock of 1mM in DMSO	Sigma
FK506	Stock solution of 100mg/ml or 5mg/ml in methanol	Dr Neil Clipstone, Stanford, USA
HERBIMYCIN A	Stock solution of 1mM in DMSO	GIBCO BRL Life Technologies
IONOMYCIN free acid	Stock solution of 1mM in DMSO	Calbiochem
PDBu (Phorbol-12,13-dibutyrate)	Stock solution of 2mM in DMSO	Calbiochem
PMA (Phorbol-12-myristate, 13-acetate)	Stock solution of 2mM in DMSO	Calbiochem
Ro 31-8220 (Compound 3)	Stock solution of 10mM in DMSO	Dr Trevor Hallam, Roche Research Centre, UK
THAPSIGARGIN	Stock solution of 1mM in DMSO	Calbiochem

2.2 BUFFERS

The following buffers were all made and autoclaved by ICRF Central Cell Services.

PBS-A

NaCl	80g
KCl	2.5g
Na_2HPO_4	14.3g
KH_2PO_4	2.5g

To 10 litres in water, pH 7.2

RPMI 1640

RPMI powder	103.9g
$NaHCO_3$	37g
Penicillin	0.6g
Streptomycin	1g

To 10 litres in water, pH 7.0

L-BROTH

Bacto-Tryptone	10g
Yeast Extract	5g
NaCl	10g

To 1 litre in water

L- AGAR

Bacto- Tryptone	10g
Yeast Extract	5g
NaCl	10g
Agar	15g

To 1 litre in water

2xTY

Tryptone	16g
Yeast Extract	10g
NaCl	5g

To 1 litre in water

2.3 ANTIBODIES

Monoclonal antibody (mAb) 38 (IgG2a) which reacts with the LFA-1 α subunit and blocks the LFA-1/ICAM-1 interaction was originally described in our laboratory (Dransfield and Hogg, 1989). MAb 24 (IgG1) recognises a Mg^{2+} dependent epitope on three of the β2 integrins, LFA-1, Mac-1 and p150,95 although it is thought to react with the α subunit (Dransfield and Hogg, 1989). MAb 15.2 (IgG1) reacts with domain 1 of ICAM-1 and blocks the ICAM-1 side of the LFA-1/ICAM-1 interaction (Dransfield, et al., 1992a). All

the above mAbs were characterised in our laboratory and were purified on Protein A Sepharose and when relevant, fluorescein (FITC)-conjugated according to procedures described in Section 2.7. The anti-CD3 mAb UCHT1 (IgG1) was a kind gift from Prof. Peter Beverley (University College London) and where required was biotinylated according to a protocol described in Section 2.8. The anti-CD3 mAb G19.4 (IgG1) was a gift from Dr. Ernest J. Plata, Bristol-Myers Squibb, USA. The blocking ICAM-1 mAb RR1/1 (IgG1) was donated by Dr. Robert Rothlein, Boehringer Ingelheim, Ridgefield, USA (Rothlein, et al., 1986). The anti-ICAM-3 mAbs CBR IC3/1 (IgG1), CBR IC3/2 (IgG2a) and CBR IC 3/6 (IgE) (de Fougerolles, et al., 1994) were obtained from the fourth Leukocyte Typing Workshop. The anti β2 activating antibody KIM185 (IgG1) (Andrew, et al., 1993) was kindly given by Dr. Martyn Robinson (Celltech, UK). The mAbs 52U (IgG1) and 4U (IgG2a) were used as isotype-matched negative control mAbs. The secondary antibodies used for crosslinking were goat anti-mouse IgG1 (Nordic) and goat anti-biotin (Sigma). A secondary goat anti-human IgG Fc specific peroxidase conjugated antibody (Sigma) was used in ELISA assays. The FITC- conjugated secondary antibodies, goat anti-human IgG Fc specific and goat anti-mouse IgG Fc specific were obtained from Jackson Immunoresearch Labs.

2.4 PROTEIN CONSTRUCTS

A construct encoding a chimaeric form of ICAM-1 consisting of the five domains of ICAM-1 fused to the hinge and Fc regions of human IgG1 was kindly donated by Drs Tony Berendt and Alister Craig (Institute of Molecular Medicine, Oxford, UK). This construct was stably transfected into Chinese Hamster Ovary (CHO) cells by Paula Stanley (Leukocyte Adhesion Laboratory, ICRF) by $CaPO_4$ precipitation. The protocol for the production and isolation of the protein is described in Section 2.10. A similar recombinant construct of ICAM-3Fc was obtained from Dr. David Simmons, Institute of Molecular Medicine (IMM), Oxford, UK and protein was prepared by transient transfection in COS cells and purified by Alison McDowall (Leukocyte Adhesion Lab, ICRF). A control protein comprising the immunoglobulin superfamily member CD14 fused to a similar Fc tail of human IgG1 was donated by Dr. David Simmons (IMM, Oxford, UK; Randi and Hogg, 1994). A pGEX vector encoding a fusion protein of GST (glutathione S transferase) with the *Clostridium botulinum* toxin C3 exoenzyme

was obtained from Dr. Marc Symons (Onyx Pharmaceuticals, San Francisco, USA) and prepared as described in Section 2.23.

2.5 CELLS

T lymphoblast cells were grown up from leukocyte concentrate obtained from the South Thames Blood Transfusion Service as detailed in Section 2.12. The Jurkat T leukaemic cell lines J6 (ICRF Central Cell Services), JKHM1 (Dr Doreen Cantrell, Lymphocyte Activation Laboratory, ICRF), T Ag (Dr Neil Clipstone, Stanford, USA), Rubin (Dr David Simmons, Oxford, UK) and the EBV-transformed B lymphoblastoid cell line JY (ICRF Central Cell Services) were grown in RPMI 1640 with 10% FBS and passaged every 3 days. The S13.2 cell line obtained from Dr Lucy Wedderburn (Lymphocyte Development Laboratory, ICRF) was maintained in RPMI 1640 with 10% FBS, 4 mM glutamine (stock 200 mM, ICRF Central Cell Services) and 50 µM 2-mercaptoethanol (stock 50 mM in RPMI; Sigma)

METHODS

2.6 PURIFICATION OF MAbs ON PROTEIN A SEPHAROSE

Monoclonal antibodies (mAbs) were purified from ascites by affinity chromatography on Protein A Sepharose (Pharmacia). Approximately 10 ml of ascitic fluid was microfuged then diluted with an equal volume of 0.1 M phosphate buffer pH 8 and filtered through a 0.45 µm filter (Millipore). This was then loaded onto a Protein A Sepharose column which had been pre-equilibrated with 0.1 M phosphate buffer pH 8 and the column washed with phosphate buffer to remove unbound material. IgG subclasses were then sequentially eluted with 0.1 M citrate buffers of varying pH such that IgG1 was eluted at pH 6, IgG2a at pH 4 and IgG2b at pH 3. Eluted fractions were neutralised with 1 M Tris, pH 9 such that 2 ml of citrate pH 6 required ~80 µl, citrate pH 4 required ~375 µl and citrate pH 3 required 500 µl of Tris. Fractions containing antibody were pooled, protein concentration estimated using the BCA Protein Assay Reagent Kit (section 2.9) and then concentrated and stored as required. The column was regenerated with at least 10 volumes of 0.1 M Tris, pH 8.5 containing 0.5 M NaCl followed by at least 10 volumes of 0.1 M sodium acetate, pH 4.5 containing 0.5 M NaCl then finally re-equilibrated with 0.1 M phosphate buffer, pH 8 prior to re-use.

2.7 FLUORESCEIN ISOTHIOCYANATE (FITC) CONJUGATION OF MAbs

Conjugation of fluorescein to mAb was carried out essentially as described below. Purified mAb was placed in dialysis tubing and concentrated to approx. 2-3 mg/ml by immersing the tubing in Aquacide (Calbiochem) until the desired volume was reached. The concentrated mAb was then dialysed against several changes of carbonate/bicarbonate buffer, pH 9.5 (17.3 g of $NaHCO_3$ and 8.6 g of Na_2CO_3, to 1 litre with water). A stock of 10 mg/ml fluorescein isothiocyanate (FITC; Sigma, UK) was made in dimethyl sulphoxide (DMSO) and then 80 µg was added, dropwise whilst stirring, per ml of mAb solution. This was incubated for 3 hours at room temperature in the dark and then bound and unbound FITC was separated by size exclusion chromatography on a Sephadex G25 (Pharmacia) column. The FITC-conjugated mAb was eluted in PBS-A (phosphate buffered saline) containing 0.1% sodium azide with unbound FITC being retarded in the column. The OD (optical density) at 280 nm and 495 nm was obtained by spectrofluorimetry (Pharmacia) and the F/P (fluorescence/protein) ratio and protein concentrations were calculated according to the following equations;

$$F/P = \frac{2.87 \times OD_{495}}{OD_{280} - (0.35 \times OD_{495})} \qquad [IgG] = \frac{OD_{280} - (0.35 \times OD_{495})}{1.4}$$

2.8 BIOTINYLATION OF MAbs

Prior to biotinylation, mAbs were concentrated to 2-5 mg/ml and then dialysed into 0.1 M bicarbonate buffer (0.1 M sodium hydrogen carbonate), pH 8.4. N-hydroxysuccinimido biotin (Sigma) was made up to stock 20-40 mg/ml in DMSO then 150-200 µg of biotin ester was added per mg of antibody and the reaction incubated for 2 hours at room temperature with occasional agitation. Unbound biotin was removed by extensive dialysis against PBS-A at 4°C and the antibody stored at 4°C with addition of 0.02% sodium azide.

2.9 ESTIMATION OF PROTEIN CONCENTRATION

Protein estimation was calculated using the BCA Protein Assay Reagent Kit (Pierce, USA) using the microtitre plate method. 10 µl of BSA (bovine serum albumin) standard or unknown protein was added in triplicate to wells of an

Immulon 3 plate (Dynatech, USA). 200 µl of working reagent (50 parts reagent A to 1 part reagent B) was then added to each well. The plate was incubated at for 30 minutes 37°C and then the absorbance was read at 570 nm on a Titertek Multiskan plate reader. A standard curve for BSA protein concentration (x axis) against OD reading (y axis) was drawn up from which the unknown protein concentrations could be calculated from the absorbance values.

2.10 PREPARATION OF ICAM-1FC FROM CHO CELLS

Stable ICAM-1Fc-transfected CHO (Chinese hamster ovary)-K1 cells (made by Paula Stanley) were thawed and grown up in GMEM-S medium (see **Appendix II**) with 10% dialysed heat inactivated FBS (Gibco BRL Life Technologies) and selected with 100 µM methionine sulphoxamine (Sigma; from stock 100 mM in GMEM-S) by passaging and expanding every two days until approximately 1 litre of cell culture was obtained. On the final expansion, serum was decreased to 5% and confluent plates were then washed into serum-free GMEM-S supplemented with 2 mM sodium butyrate (N-butyric acid- sodium salt, Sigma) to allow enhancement of protein secretion. The cells were left for approx. one week to die and the supernatant was harvested. Particulate matter was removed by centrifugation at 2000*g* for 5 min and by filtration through a 0.22 µm filter (Millipore). Sodium azide (0.02%) was added to the supernatant which was then loaded onto a Protein A Sepharose (Pharmacia, Sweden) column at a rate of 1 ml/min. The column was then washed thoroughly with PBS-A/0.02% sodium azide to remove unbound material. The ICAM-1Fc was eluted from the column in 0.1 M citrate buffer, pH 3 and collected in 2 ml fractions. Those containing protein were then pooled and concentrated and the identity of ICAM-1Fc was made by SDS-PAGE (sodium dodecyl sulphate- polyacrylamide gel electrophoresis; section 2.21) and ELISA (enzyme-linked immunosorbent assay; section 2.11) and the concentration calculated using the BCA Protein Assay Reagent.

2.11 ELISA ASSAY TO DETECT THE PRESENCE OF ICAM-1FC

An ELISA was used to detect the presence of ICAM-1Fc in the supernatants and purified end product. A 96-well Immulon 3 plate (Dynatech, USA) was coated with 0.25 µg/ml mAb 15.2 (anti-ICAM-1) or mAb 52U (IgG1 isotype-matched negative control) and incubated overnight at 4°C in 50 mM Tris, pH

9. The plate was then washed 4 times in PBS-A and blocked with 10% FBS in PBS-A for 2 hours at room temperature. Samples of supernatant from the final tissue culture fluid of the ICAM-1Fc expressing CHO-K1 cells and purified material following affinity chromatography along with a sample of previously made ICAM-1Fc were added to wells in 50 µl volumes and incubated for 30 min at room temperature. Wells were washed 4 times in PBS-A prior to addition of 50 µl of goat anti-human IgG Fc specific peroxidase conjugated antibody (1/500; Sigma) for 30 min at room temperature. Wells were washed 4 times in PBS-A then 75 µl of OPD (o-phenylenediamine dihydrochloride) detection buffer (one OPD tablet, Sigma, was added along with 10 µl of H_2O_2 to 25 ml taken from the mixture between 20 ml of 0.1 M $NaHPO_4$ with 10 ml of 0.1 M citric acid) was added per well and the reaction allowed to develop. The reaction was stopped with 50 µl/well of 3 M H_2SO_4 and the colour changed was quantitated on a Titertek Multiskan plate reader by measuring the absorbance at 492 nm.

2.12 ISOLATION AND PROPAGATION OF T LYMPHOBLASTS

T lymphoblast cells were expanded from leukocyte concentrate obtained from the South London Blood Transfusion Service (Tooting, UK). Mononuclear cells were obtained by centrifugation at 600*g* over Lymphoprep® (Sodium metrizoate/Ficoll solution; Nycomed, Sweden) and then washed three times in RPMI 1640 (ICRF, Media Supplies). Cells were resuspended in RPMI 1640 with 10% FBS and 1 µg/ml PHA (Phytohaemagglutinin; Murex Diagnostics, UK) for 4 days in a 5% CO_2 humidifier. Cells were then maintained every 2 days by passaging in RPMI 1640 with 10% FBS and 20 ng/ml recombinant IL-2 (Interleukin-2, stock 20 µg/ml in RPMI, filter sterile; Eurocetus, USA) and used for experiments between days 10 and 14 of culture. Quiescent T cells were obtained by washing out IL-2 and using after 2 days. These cells are thought to resemble resting peripheral blood T lymphocytes (Cantrell and Smith, 1984).

2.13 MTT CYTOTOXICITY ASSAY

To assess the viability of cells, particularly after stimulant and inhibitor treatment, MTT assays were performed. MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Thiazolyl blue) is a tetrazolium salt which can permeate into cells. In the presence of active mitochondrial

dehydrogenase enzymes MTT is reduced to a dark blue insoluble product. This can be quantitated on a fluorescence plate reader by reading the absorbance at 570-630 nm. Cells which had been washed serum free were incubated with the desired concentrations of the stimulant or inhibitor on ICAM-1Fc-coated Immulon 1 plates. Each condition was analysed in triplicate wells. Cells without stimulant/inhibitor were included as a positive viability control and 50 μM digitonin (stock 10 mM in RPMI 1640) per well was included as a negative control. 10 μl MTT was added per well and the plates incubated for 3 hr at 37°C. The reaction was stopped by the addition of 150 μl per well of stop solution (40 mM HCl in isopropanol) and plates left in the dark overnight. The following morning the absorbance was read at 570-630 nm on a Titertek Multiskan plate reader. Cell viability was calculated as a percentage of the values of untreated cells after basal readings (cells in the presence of digitonin) had been subtracted.

2.14 ADHESION ASSAY

The method for quantitation of T cell adhesion to ICAM-1Fc protein has been previously described (Dransfield, et al., 1992a). The ICAM-1-Fc chimaeric protein was coated onto flat bottomed 96-well Immulon 1 plates (Dynatech, USA) at 0.24 μg/well in 50 μl of PBS-A (ICRF Media Supplies) overnight at 4°C. Adhesion assays to a similar construct of ICAM-3Fc and fibronectin were performed in an identical manner with the addition of 0.32 μg ICAM-3Fc and 1 μg fibronectin (Sigma) per well. The plate was then blocked with 2.5% BSA (Sigma) in PBS-A for 2 hours at room temperature and then washed 4 times with PBS-A and twice with the binding assay buffer to remove excess BSA. T cells or Jurkat cells growing in RPMI 1640 with 5% FBS were labelled with 1 μCi of ^{3}H thymidine (Amersham, UK), per ml of cells, overnight at 37°C then washed three times to remove excess thymidine and serum. Cells were resuspended into assay buffer, either RPMI 1640 for standard assays or HEPES buffer (20 mM HEPES (N-[2-Hydroxyethyl] piperazine-N'-[2-ethanesulphonic acid]); Sigma, UK, 140 mM NaCl, 2 mg/ml glucose, pH 7.4) for controlled cation assays. Stimulants (at double concentration) were made up in these buffers and added to the prepared 96-well plates (50 μl/well) to which 50 μl of washed cells ($2x10^5$ cells/well) were then added. For controlled divalent cation experiments various concentrations of the cation chlorides $MgCl_2$, $MnCl_2$ and $CaCl_2$ were made in HEPES buffer and the divalent cation chelators EDTA (Ethylenediaminetetra-acetic acid disodium

salt) and EGTA (Ethylene glycol-bis (β-aminoethyl ether) N,N,N',N'-tetra-acetic acid) were used at 1mM final concentration in HEPES buffer. For CD3 crosslinking experiments, cells were washed into RPMI 1640 and incubated with 10 μg/ml UCHT1, either in free or biotinylated forms, for 25 min on ice. Following three washes in cold RPMI 1640, cells were incubated for a further 25 min on ice with 10 μg/ml of either a secondary goat anti-mouse IgG1 antibody (Nordic) or a goat anti-biotin antibody (Sigma) to allow crosslinking. Cells were then washed a further three times to remove unbound antibody and then added to plates. Plates were incubated for 15 min on ice, to allow binding of any blocking or stimulatory antibodies, then spun at 40*g* for 1 min and the incubation was continued for 30 min 37°C. Plates were then washed gently four times with prewarmed RPMI 1640 and remaining adherent cells were harvested and counted on a betaplate counter (LKB Instruments Inc., Bromma, Sweden).

2.15 FLOW CYTOMETRY FOR ANTIBODY DETECTION

Flow cytometric analysis of mAb 24 and other antibodies was carried out by either direct or indirect immunofluorescence as described below. Prior to mAb labelling, cells were washed three times into assay buffer which was either RPMI for standard assays or HEPES buffer for controlled cations experiments. For direct labelling, $2x10^5$ cells/sample point were added to flexiwell plates (Dynatech, USA) and resuspended into 50 μl of RPMI or HEPES buffer containing the stimulants, inhibitors, or cations as required together with 10 μg/ml of FITC-conjugated mAbs 24 or 38 and incubated for 30 min at 37°C. Cells were then washed three times in ice cold FACS wash buffer (PBS-A with 0.2% BSA and 0.1% sodium azide) and maintained on ice until analysis. Fluorescence of live cells was analysed on a FACScan® flow cytometer (Becton Dickinson, Moutainview, USA) by adding 50 μl of 50 μg/ml propidium iodide (Sigma, UK) per tube just prior to acquisition of the sample. Dead cells will uptake propidium iodide, and can be separated from live cells due to their positive fluorescence (FL) in the FL2 channel. Data was acquired and analysed by the Cell Quest Program (Becton Dickinson, UK). For indirect fluorescence, primary unconjugated antibodies were added to cells at 10 μg/ml (unless otherwise stated) and incubated for 30 min on ice, followed by 3 washes with FACS wash buffer before a second incubation with a specific FITC- conjugated goat anti-mouse IgG (Jackson Immunoresearch

Laboratories, USA) for a further 30 min on ice. Cells were then washed in FACS wash buffer and analysed as described for direct fluorescence labelling.

2.16 FLOW CYTOMETRY FOR sICAM-1 DETECTION

Measurement of the binding of soluble recombinant ICAM-1Fc to T cells was based on that described in (Jakubowski, et al., 1995a) with some modification. T cells were washed 3 times into assay buffer and then resuspended into buffer containing stimulants, appropriate divalent cations if required, and the indicated concentrations of either sICAM-1Fc or the control protein CD14Fc. After a 30 min incubation at 37°C, cells were washed twice in ice cold FACS wash buffer and then incubated with 10 μg/ml FITC-conjugated goat anti-human IgG Fc specific antibody (Jackson Immunoresearch Laboratories, USA) for 20 min on ice. Unbound secondary antibody was removed by washing twice in ice-cold FACS wash buffer then fluorescence of live cells was detected using a FACScan® and data analysed using the CellQuest program.

2.17 FLOW CYTOMETRY FOR QUANTITATION OF CELL SURFACE RECEPTOR NUMBER

Cell surface receptor number was calculated using a calibrated microbead kit (Quantum Simply Cellular, Sigma, UK). This kit contains 4 populations of microbeads (with a similar size to lymphocytes) which each have a defined number of goat anti-mouse polyclonal immunoglobulins (determined by radioimmunoassay) covalently bound onto them. This value represents the Antibody Binding Capacity (ABC) of the beads. FITC-conjugated mAbs 24 and 38 (at saturating concentrations) were incubated with the microbeads under conditions identical for use of these mAbs in cell staining and data was acquired on a FACScan® flow cytometer. Median channel values for the cell- and bead-bound mAb fluorescence, obtained from the Cell Quest program, were entered into a Quickcal computer program (supplied with the kit). This program draws a standard curve corresponding to the bead fluorescence and ABC values for each particular sample. From this standard curve the unknown ABCs for cell-bound mAbs (of known fluorescence) can be calculated.

2.18 FLOW CYTOMETRY FOR CALCIUM FLUX

Flow cytometric analysis of intracellular Ca^{2+} fluxing was carried out on a FACStar^{PLUS} flow cytometer by Simon Monard and Derek Davies (FACS laboratory, ICRF). The principle of the assay involves loading the Ca^{2+} buffering agent Indo-1 into cells. Indo-1 is an acetoxymethyl ester which permeates the cell membrane and upon cleavage is retained within the cells and becomes active. When excited at wavelengths between 355-365 nm by an argon ion laser, Indo-1 emits a signal at 400 nm in its free form. If the Indo-1 has bound Ca^{2+} however it will emit a signal at 480 nm. These signals can be analysed by a FACStar^{PLUS} flow cytometer using LYSIS II software which calculates intracellular Ca^{2+} concentrations as a ratio of these two optically separated fluorescence signals. T cells in 5% FBS were labelled with 1 μM, per ml of cell volume, of Indo-1-acetoxymethyl ester (stock 1mM in DMSO; Molecular Probes) in the presence of 0.25 μl plurionic F127 (Molecular Probes) for 45 min at 37°C. Cells were then washed twice into serum free RPMI, resuspended to 10^6 cells/ml and retained at 37°C for the remainder of the experiment. As soon as the cells were placed onto the flow cytometer probe stimulants were added and any resulting intracellular Ca^{2+} flux was measured. A positive Ca^{2+} flux was always measured using the calcium ionophore ionomycin at the end of every experiment.

2.19 PRELIMINARY CALCULATION OF THE AFFINITY OF LFA-1 FOR ICAM-1.

Apparent affinities were calculated for LFA-1 based on the saturation curves for sICAM-1 binding following stimulation with 5 mM Mg^{2+}/1 mM EGTA in the presence or absence of 10 μg/ml mAb 24. The molecular weight of the recombinant ICAM-1Fc dimer was estimated to be 225 kDa by electrospray mass spectrometry kindly performed by Dr. Darryl Pappin (Protein Sequencing Lab, ICRF). This analysis was performed following dialysis of the protein into distilled water. This value is consistent with the presence of two homodimers of ~110 kDa each. For production of saturation curves and Scatchard plots the moles of bound ICAM-1 were calculated using the assumption that the secondary mAb, FITC conjugated goat anti-human IgG Fc (FITC-GaH), binds to cell bound ICAM-1 Fc in a 1:1 ratio. The moles of FITC-GaH Ab bound were determined from the F/P ratio of the antibody with the use of FITC-conjugated microspheres (Flow Cytometry Standards

Corporation). When analysed under standard parameter settings on the flow cytometer these microbeads provide a measure of the molecules of equivalent soluble fluorochrome (MESF) for each median fluorescent channel value. In this way, the MESF can be calculated at each sample point and then using the F/P ratio of the antibody the number of molecules of protein per molecule of FITC estimated. This value can be converted into Bound ICAM-1Fc by using Avogadro's number (6.022 $x10^{23}$ molecules per mole of solution) for the input cell number of $2x10^5$ per sample point. A Scatchard plot is then produced by plotting Bound/Free on the y axis against Bound on the abscissa x axis. The apparent dissociation constant (*Kd*) is 1/the slope of the line which is fit by linear regression analysis.

2.20 ADHESION ASSAY FOR IMMUNOFLUORESCENCE AND CONFOCAL MICROSCOPY

For immunofluorescence analysis, 13 mm round coverslips were coated with 0.72 μg of ICAM-1Fc in PBS-A overnight at 4°C. Coverslips were blocked with 2.5% BSA in PBS-A for 2 hr at room temperature then washed 4 times with PBS-A and twice with assay buffer (either RPMI or HEPES). T cells were washed 3 times into assay buffer before addition onto coverslips ($5x10^5$ cells/coverslip) in the presence of stimulants, cations where necessary and mAbs at 10 μg/ml. Cells were incubated for 10 min on ice, spun at 40*g* then incubated for 30 min at 37°C. Unbound cells were removed by four gentle washes in prewarmed RPMI or HEPES buffer (with the appropriate divalent cations). To prevent antibody-induced clustering of receptors, adherent cells were fixed with 1% formaldehyde in PBS-A or HEPES buffer for 20 min at room temperature prior to a second incubation with 10 μg/ml FITC-conjugated goat anti-mouse IgG (Jackson Immunoresearch Laboratories, USA) in PBS-A or HEPES buffer for 30 min at 4°C. For staining of intracellular polymerised actin, the prefixed adherent cells were permeabilised with 0.2% Triton X-100 in PBS-A or HEPES buffer for 10 minutes on ice, before incubation with 0.25 μg/ml TRITC (tetramethylrhodamine isothiocyanate)- conjugated phalloidin (Sigma, UK) for 20 min on ice. Cells were then washed twice and mounted on slides for either fluorescence or confocal microscopy. Confocal microscopy was performed with a laser scanning head (MRC-600, BioRad Laboratories) fitted onto a Nikon Optiphot Microscope.

2.21 SDS-PAGE

SDS/PAGE was performed essentially following the method of Laemmli *et al* (Laemmli, 1970). The polyacrylamide gel was composed of a separating gel made from 375 mM Tris, pH 8.8 (stock 1.5 M), 0.1% SDS (stock 20%) with 0.04% APS (ammonium persulphate; stock 10%) and 1/500 TEMED (N,N,N',N'- tetramethylethylenediamine, Sigma) with the appropriate volume of Easigel acrylamide/bisacrylamide stock solution (30% w/v acrylamide/0.8% w/v bisacrylamide; Scotlab, UK). The upper stacking gel was composed of 125 mM Tris, pH 6.8 (stock 0.5 M) with 0.1% SDS, 1/500 TEMED and 3% Easigel. Proteins were boiled for 5 min in sample buffer composed of 125 mM Tris, pH 6.8, 4% SDS, 27.5% glycerol (stock 50%) and 0.002% bromophenol blue (stock 1% w/v in ethanol) under either non-reducing or reducing (with 1% β-mercaptoethanol) conditions and electrophoresed in a BioRad tank with electrophoresis buffer (25 mM Tris/ 192 mM glycine, 0.1% SDS, pH 8.3) at 100 volts through the stacking gel and 180 volts through the separating gel. Standard high molecular weight markers (Amersham, UK) containing proteins in the range from 14 to 200kDa were run under identical conditions on the same gel. Proteins were visualised by staining with Coomassie Blue (0.5% w/v Coomassie Blue, 40% ethanol and 10% glacial acetic acid) and destained with 20% ethanol/10% glacial acetic acid.

2.22 ANALYSIS OF NF-AT ACTIVITY

The T cell hybridoma S13.2 has been stably transfected with a construct encoding carrying the NF-AT binding site tagged to the β-galactosidase gene (lac Z) reporter from *Escherichia coli*; this was kindly provided by Dr Lucy Wedderburn (Lymphocyte Development Lab, ICRF). The lac Z-NF-AT construct contains three copies of the NF-AT responsive sequence of the IL-2 gene promoter inserted within the minimal IL-2 promoter. Upon stimulation of T cell signalling pathways which trigger activation and nuclear translocation of the transcription factor NF-AT (nuclear factor of activated T cells), NF-AT will induce transcription of the IL-2 promoter and hence β-galactosidase activity. If β-galactosidase activity is turned on then a β-galactosidase substrate, fluorescein di-β D galactopyranoside (FDG) will be cleaved releasing fluorescein which is detected by flow cytometry. Prior to the assay, S13.2 cells were stimulated with 50 nM PDBu and 0.7 μM

ionomycin for 4 hours at 37°C. Cells were washed with PBS containing 10 mM HEPES, pH 7.3 and 4% FBS and then resuspended at 10^6 cells per 50 µl of the above mentioned buffer and placed in a water bath for 10 min at 37°C. 50 µl of prewarmed 2 mM fluorescein di-galactopyranoside (FDG; Sigma) in water was added and mixed rapidly and thoroughly. Cells were incubated for 75 sec at 37°C during which time the FDG loads into the cells by brief osmotic shock. Loading was stopped by the addition of 1 ml of ice cold PBS/HEPES/FBS and cells were then left on ice for 2 hours to allow cleavage of FDG. Cleaved fluorescein was then detected in live cells by propidium iodide gating and analysis on a FACScan flow cytometer as previously described.

2.23 PREPARATION OF C3-GST FUSION PROTEIN

(i) Transformation of Bugs

TOPP II bugs (Stratagene, UK) were made competent by Paula Stanley (Leukocyte Adhesion Laboratory) according to the following protocol. A 100 ml culture of bugs was grown exponentially and then spun at 2000*g* for 5 min at room temperature. The pellet was resuspended into 20 ml (per 50 ml bugs) of solution I (30 mM KOAc, 50 mM MnCl, 100 mM KCl, 10 mM $CaCl_2$, 15% glycerol, pH 5.8, filter sterile) and incubated for 30 min on ice. The bugs were then spun at 2000*g* for 5 min at 4°C and then resuspended into 4 ml (total volume) of solution II (10 mM MOPS (3-[N-Morpholino] propanesulphonic acid), pH 7, 75 mM $CaCl_2$, 10 mM KCl and 15% glycerol), stored overnight at 4°C then aliquoted and stored at -70°C. Competent bugs were then transformed by adding 50 ng of C3-GST in a pGEX-2T vector/100 µl of bugs with gentle pipetting. Bugs were left for 10 min on ice then heat shocked for 5 min at 37°C before addition of 1 ml prewarmed L-broth (ICRF Media Supplies). Bugs were incubated for 50 min at 37°C then 100 or 200 µl of culture was spread onto L- agar plates containing 50 µg/ml ampicillin (stock 100 mg/ml, Sigma) and left to grow overnight at 37°C. Single colonies were then picked and grown with aeration in 5 ml cultures of L-broth, containing 100 µg/ml ampicillin, overnight at 37°C then streaked onto L-agar plates containing 100 µg/ml ampicillin for a further overnight incubation at 37°C. A DNA minipreparation (miniprep) was then performed on single colonies to check for the presence of the C3-GST vector insert.

(ii) Miniprep

A 5 ml culture of bugs was grown up overnight at 37°C from a single colony in L-broth with 100 µg/ml ampicillin. 1.5 ml of bugs were then microfuged for a few min and all the supernatant was carefully discarded. The pellet was then resuspended into 100 µl of solution 1 (50 mM glucose, from 1M stock; 25 mM Tris, pH 8, from 1 M stock and 10 mM EGTA, pH 8, from 0.5 M stock) and left to stand for 5 min at room temperature. 200 µl of solution 2 (0.2 N NaOH, stock 10 N and 1% SDS, stock 20%) was then added to each tube and mixed gently by inversion before standing for 5 minutes on ice. Once cold, 150 µl of ice-cold solution 3 (3 M KOAc, stock 5 M and 11.5% glacial acetic acid) was added and the tube left to stand for a further 5 minutes on ice. Tubes were microfuged for 5 min at 4°C and then the supernatant was removed to a new tube. 200 µl of phenol (Fisons) and 200 µl of chloroform were added per tube and the solutions briefly vortexed before being microfuged for 1-2 min at room temperature. The top layer was retained to which a further 400 µl of chloroform was added, vortexed and microfuged once more for 1-2 min. The top layer was again retained and DNA was precipitated by addition of 800 µl of precooled ethanol and incubation for at least 15-20 min in a dry ice/ethanol bath . The DNA was obtained by microfugation for 10 min at 4°C, followed by; careful removal of all the ethanol, washing the pellet in 70% ethanol, once again removing all the ethanol and air drying on the bench. DNA was then resuspended in 50 µl of TE buffer (10 mM Tris pH 8, from stock 1 M and 0.1 mM EDTA from stock 1 M) and stored at 4°C until required.

(iii) Restriction Digest

The miniprep DNA was then tested in a restriction digest according to the following protocol. 15 µl Millipore pure water, 5 µl miniprep DNA (0.25-0.5 µg pure DNA is sufficient), 2.2 µl of buffer H (Boehringer Mannheim, Germany), 1 µl each of EcoR1 and BamH1 (Boehringer Mannheim, Germany) and 1 µl of RNase (Pharmacia) were combined in an eppendorf tube and incubated for 30 min at 37°C. 2 µl of dye (10X stock= 10% Ficoll- type 400, 25% glycerol, 10 mM Tris, 1 mM EDTA- pH 8, 0.25% bromophenol blue) was added per sample and run on a 1% agarose gel (1% agarose in TAE buffer with 1 µl ethidium bromide; Sigma) by electrophoresis at 90 volts in 1X TAE running buffer (50X= 2 M Tris, 5.7 % glacial acetic acid, 50 mM EDTA, pH 8)

with a 1kB ladder of markers (Gibco). The DNA fragments were visualised under UV light.

(iv) Preparation of C3 protein

To obtain C3-GST protein a 2 litre batch culture was made as follows. Four 50 ml cultures were inoculated in 2xTY medium containing 100 μg/ml ampicillin and grown by shaking overnight at 37°C. In the morning, each 50 ml culture was expanded to 500 ml with 2xTY plus 100 μg/ml ampicillin and grown for another hour at 37°C. 1 mM IPTG (Isopropyl β-D-Thiogalactopyranoside; stock 100 mM=25 mg/ml) was then added and induction of transcription and translation of the C3-GST construct was performed for 4 hours at 37°C. Bugs were then harvested, centrifuged at 1000*g* for 10 min and then the supernatant was drained completely. The pellet was resuspended into approx. 5 ml of Buffer A (50 mM Tris, pH 7.5 from stock 1 M; 50 mM NaCl from stock 5 M and 5 mM $MgCl_2$ from stock 1 M) plus 1 mM DTT (dithiothreitol) and 1 mM PMSF (phenylmethyl sulphonyl fluoride, Sigma; stock 50 mM in ethanol) per pellet from 500 ml of start bacterial culture. The bacteria were sonicated thoroughly on ice by 6 x 10 sec in a Soniprep 150 sonicator and then centrifuged at 6000*g* for 10 min at 4°C and the supernatant removed to universal tubes. 750 μl of a 50% slurry of glutathione sepharose 4B beads which had been pre-equilibrated with buffer A for 15 min at 4°C was then washed 3x into buffer A supplemented with 1 mM DTT and 1 mM PMSF and incubated with supernatant corresponding to 1 litre of original bacterial culture with rotation for 30 min at 4°C. Beads were then spun at 8*g* and washed 3x in ice-cold buffer A supplemented with 1 mM DTT and 1 mM PMSF to remove unbound protein. Beads were then washed once into Buffer A (with DTT in the absence of PMSF) and then resuspended into 200 μl Buffer A with DTT and transferred to an eppendorf tube to which a further 200 μl of the same buffer, used to wash any residuals remaining in the tube, was added. Beads were spun and then resuspended into 500 μl (per 1 litre of bacterial culture) of thrombin resuspension buffer (50 mM Tris, pH 8, 150 mM NaCl, 5 mM $MgCl_2$, 2.5 mM $CaCl_2$, 1 mM DTT) to which bovine thrombin (Sigma, UK) was added, at a final concentration of 1U per 100 μl, and the mixture was incubated overnight at 4°C. Following this beads were spun and the supernatant removed and retained on ice. Beads were washed with a further 500 μl of wash buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 5 mM $MgCl_2$) which was then pooled together with the initial thrombin

supernatant. Thrombin was removed by incubation with 10 µl (per litre of culture) of p-aminobenzamidine agarose beads (Sigma) while mixing for 30 min at 4°C. Beads were then spun for 2 min at 800*g*, the supernatant carefully removed, and dialysed for 6 hours at 4°C against 6 litres of dialysis buffer (20 mM Tris, pH 7.5, 2 mM $MgCl_2$, 150 mM NaCl, 100 µM DTT) to remove Ca^{2+}. The end product was then concentrated in prepared centricon 10 micro concentrators (Amicon, see **Appendix III** for preparation method) until the volume was approximately 500 µl. The protein was then aliquoted, snap frozen in dry ice/ethanol and stored at -70°C. The identification, purity and concentration of the C3 protein was investigated by SDS-PAGE and the BCA Protein Assay Reagent kit.

CHAPTER THREE

ROLE OF PROTEIN KINASES IN LFA-1 ACTIVATION

3.1 INTRODUCTION

The alterations which induce leukocyte integrins, such as LFA-1, to bind to ligand take place on pre-existing cell surface receptors, do not require *de novo* protein synthesis and thus occur via post-translational modifications. These alterations happen fairly rapidly and result from unknown intracellular signalling pathways. The components of cell surface receptor-triggered signalling pathways are now quite well understood and the major constituents are fuelled by phosphorylation and dephosphorylation events which can induce rapid and transient alterations in intracellular proteins. In T cells, activation of tyrosine kinase activity is the first detectable event measured following stimulation through the TCR/CD3 complex therefore it was relevant to examine whether these events were crucial to LFA-1 activation following TCR/CD3 crosslinking. As PKC is the major serine-threonine kinase in T cells (Berry and Nishizuka, 1990) and as it is also the major receptor for phorbol esters it was decided to examine whether this prime candidate was involved in LFA-1 activation. The function of these different types of kinase was investigated using intracellular biochemical inhibitors; herbimycin A for tyrosine kinase inhibition and Ro 31-8220 (also known as Compound 3) for inhibition of PKC. Although it is more likely that these kinases might be involved in the "inside-out" signal it is also possible that they might have an effect when cells are stimulated through external manipulations. For this reason, the effects of the inhibitors following stimulation with activating mAbs and divalent cations were also examined. LFA-1 activation on T cells was measured by two independent assays, by the ability of cells to bind to recombinant ICAM-1 (ICAM-1Fc) immobilised on plastic and to express the reporter epitope detected with mAb 24. mAb 24 is thought to serve as a reporter of LFA-1 activation (Cabañas and Hogg, 1993; Dransfield, et al., 1992b).

3.2 RESULTS

3.2a TIME COURSE OF LFA-1 ACTIVATION

To ascertain the correct stimulation times for induction of adhesion, T cells stimulated by either T cell receptor crosslinking (XLCD3) or the phorbol ester, PDBu were examined for their ability to adhere to ICAM-1 at various time intervals. The induction of T cell adhesion to immobilised ICAM-1 follows different kinetics depending on which stimulation protocol is used. As shown in **Figure 3.1** stimulation of T cells with the phorbol ester, PDBu, induces a sustained activation of LFA-1-mediated adhesion which peaks at 30 min and remains adhesive at 2 hours. The situation following stimulation by XLCD3 follows a different pattern in that adhesion is transient, peaking at 30 min but returning to a baseline level by 2 hours. This result confirms the kinetics shown previously (Cabañas and Hogg, 1993, Dustin and Springer, 1989). In all subsequent adhesion assays, incubations were performed at the 30 min time point where peak adhesion is seen. The kinetics for induction of mAb 24 epitope mirror those for adhesion (Cabañas and Hogg, 1993); therefore cells for flow cytometric analysis were also stimulated for this time interval.

3.2b THE PROTEIN KINASE INHIBITORS HERBIMYCIN A AND RO-31 8220.

An important insight into the characterisation of downstream events following TCR/CD3 engagement came from a study which highlighted the importance of tyrosine kinases as immediate effectors in the pathway (June, et al., 1990). This was found through the use of a benzoquinonoid ansamycin antibiotic called herbimycin A. Concentrations of around 1 μM inhibited TCR/CD3 mediated activation of the phosphatidylinositide cascade as measured by production of inositol 1,4,5-trisphosphate and $[Ca^{2+}]_i$ elevation. The inhibitor seems to be relatively specific to tyrosine kinase inhibition as it had only slight effects on PKC stimulated c-raf activity. It also inhibited TCR/CD3 mediated production of IL-2 but had no effect on IL-2 production induced by the pharmacological agents phorbol ester and calcium ionophore. Herbimycin A does not seem to be a classic competitive kinase inhibitor and appears to exert its effects by degradation of the kinase, at least this seems to be the case for inhibition of the src family kinase $p56^{lck}$ (June, et al., 1990). This probably explains why it requires 12-16 hr preincubation. Stimulation of

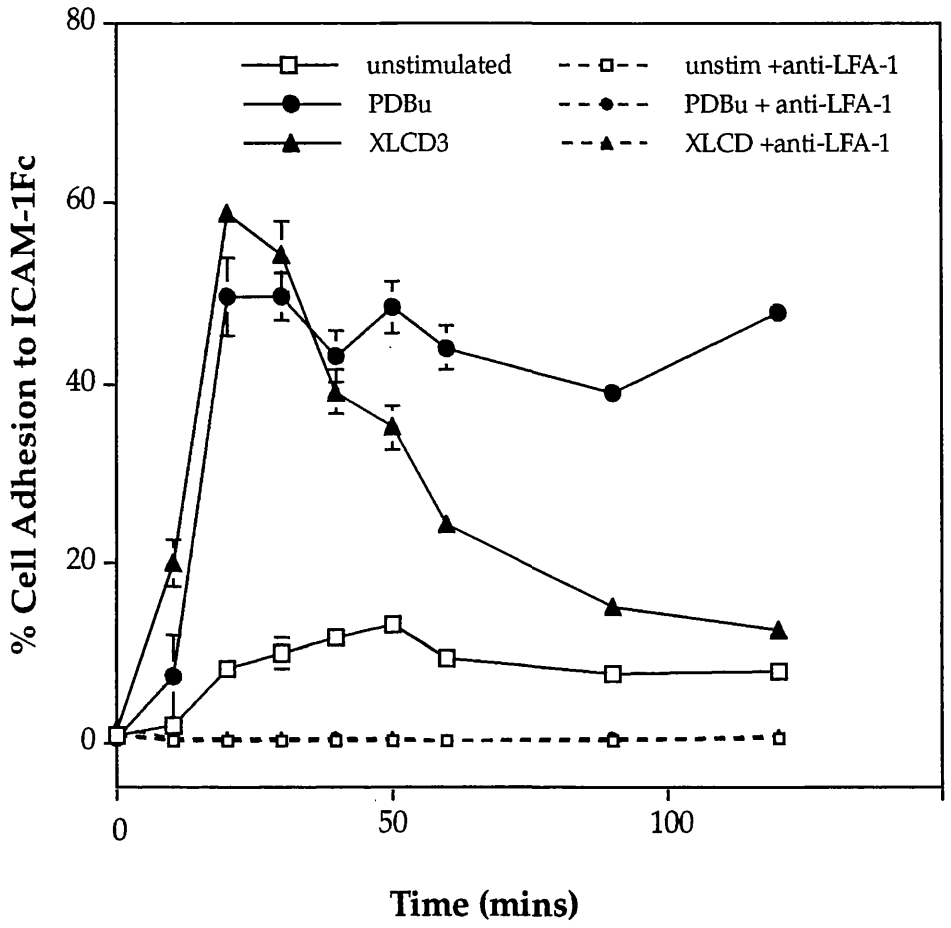

Figure 3.1: Time course of LFA-1-mediated adhesion of T cells to ICAM-1 following PDBu and XLCD3 stimulation. ^{3}H-labelled T cells were stimulated with either 50 nM PDBu or by preincubation with the anti-CD3 mAb UCHT1 (10 μg/ml) followed by crosslinking with a goat anti-mouse IgG1 secondary antibody and induced to bind to plastic-bound ICAM-1Fc for the indicated time points. Adherent cells were then harvested and counted. The LFA-1 function blocking mAb 38 (**anti-LFA-1**) was added to ensure that adhesion to ICAM-1 was mediated through LFA-1. Results are shown as means of triplicates +/- standard deviation and one representative experiment of three is shown.

the TCR/CD3 complex is the most physiological method, so far discovered, for inducing LFA-1 activation. Therefore it was of interest to examine the effects of Herbimycin A on this activation procedure. In the laboratory, TCR/CD3 triggering can be achieved by crosslinking Abs to the CD3 complex and is thought to mimic ligation which occurs naturally through interaction with Ag and MHC. Herbimycin A was titrated for its effects on T cell adhesion to ICAM-1 and shows a dose-dependent inhibition of TCR/CD3 induced adhesion (**Figure 3.2A**). The IC_{50} for herbimycin is ~70 nM and binding is totally inhibited at 1 μM. These concentrations are equivalent to those shown to inhibit TCR induced activation of tyrosine phosphorylation, $p56^{lck}$ activity and $[Ca^{2+}]_i$ mobilisation (June, et al., 1990).

The PKC inhibitor Ro 31-8220 was kindly donated by Dr. Trevor Hallam, Roche Pharmaceuticals where it was synthesised artificially by modification of the backbone structure of the microbial metabolites, staurosporine and K252a (Davis, et al., 1989). It has improved activity and is more selective for PKC than its parent compounds. It is thought to act by competing with ATP (adenosine triphosphate) for binding to PKC. Ro 31-8220 exhibits a dose-dependent inhibition of PDBu-induced T cell adhesion to ICAM-1, with an IC_{50} of ~0.5 μM and total inhibition seen in the 1-5 μM range (**Figure 3.2B**). Thus, both inhibitors can eliminate T cell adhesion to ICAM-1 at concentrations similar to those previously shown to be relatively specific for their respective targets. The inhibition of adhesion is not due to toxicity effects as trypan blue exclusion illustrated that cell viability remained intact (data not shown).

3.2c EFFECT OF INHIBITORS ON THE "INSIDE-OUT" SIGNAL

(i) TCR/CD3 -stimulated LFA-1 activation

Now that the inhibitors had been titrated for optimal use it was imperative to examine their effects on different LFA-1-activating procedures. As TCR/CD3 crosslinking is deemed to be the most natural stimulant for LFA-1, so far discovered, the effects of inhibiting PKC and tyrosine kinases were examined in more detail. As previously shown in the dose-response curve, and now also in **Figure 3.3A**, herbimycin A inhibits adhesion induced by Ab crosslinking of the TCR/CD3 complex. It also inhibits induction of the reporter epitope recognised by mAb 24 (**Figure 3.3B**). This result confirms that activation of LFA-1 following TCR/CD3 stimulation does rely on

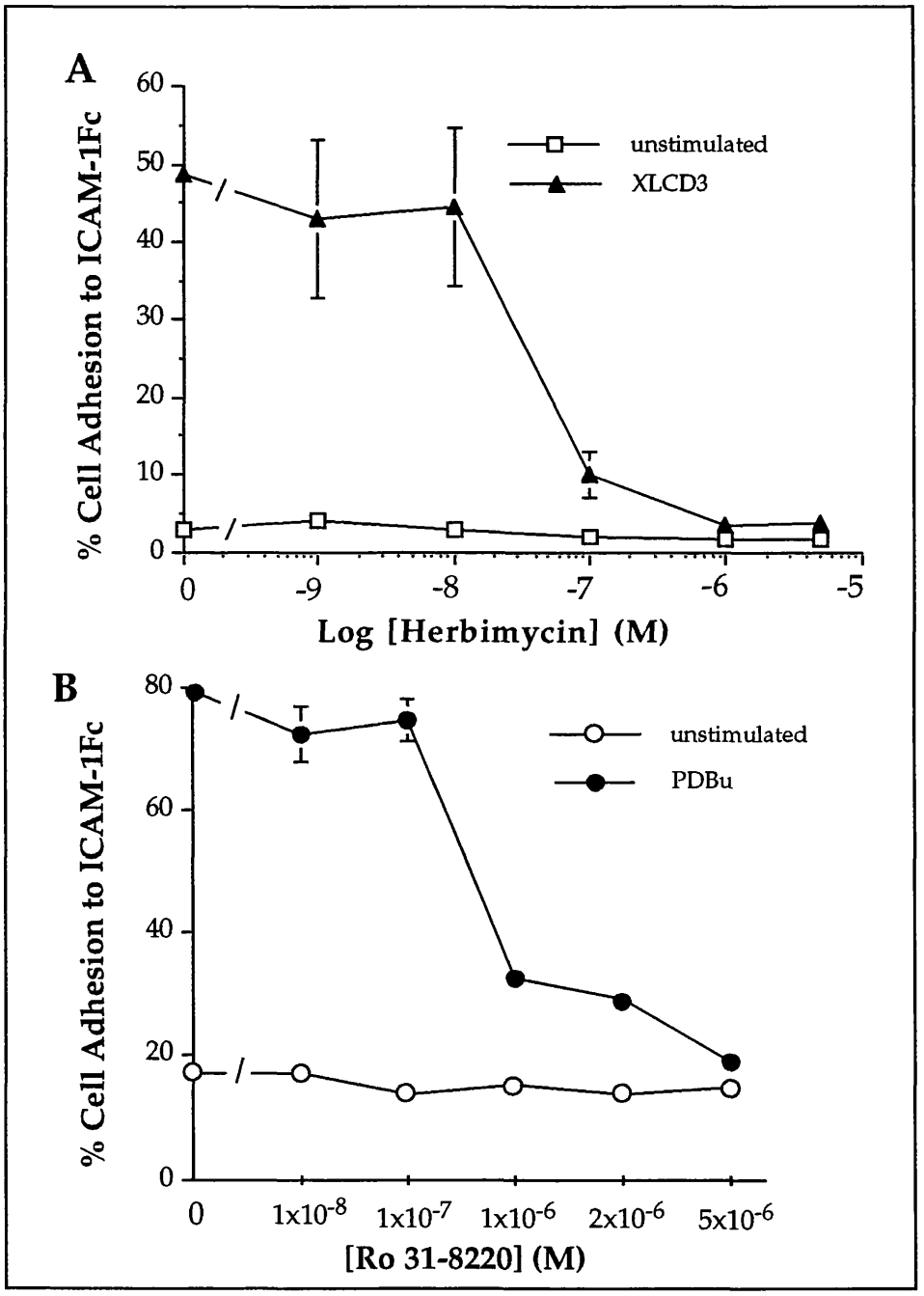

Figure 3.2: Titration of herbimycin A and Ro 31-8220 on stimulated T cell binding to ICAM-1. Cells were preincubated with indicated concentrations of either herbimycin A for 16 hr (**A**) or Ro 31-8220 for 30 min (**B**) before stimulation by UCHT1 crosslinking (**A**) or 50 nM PDBu (**B**) for 30 min at 37°C. Cells adherent to immobilised ICAM-1 following washing were counted. Data is expressed as means of triplicates +/- standard deviations and in both panels one representative experiment of 3 is shown. Both stimulated and basal binding were blocked by mAb 38 (anti-LFA-1).

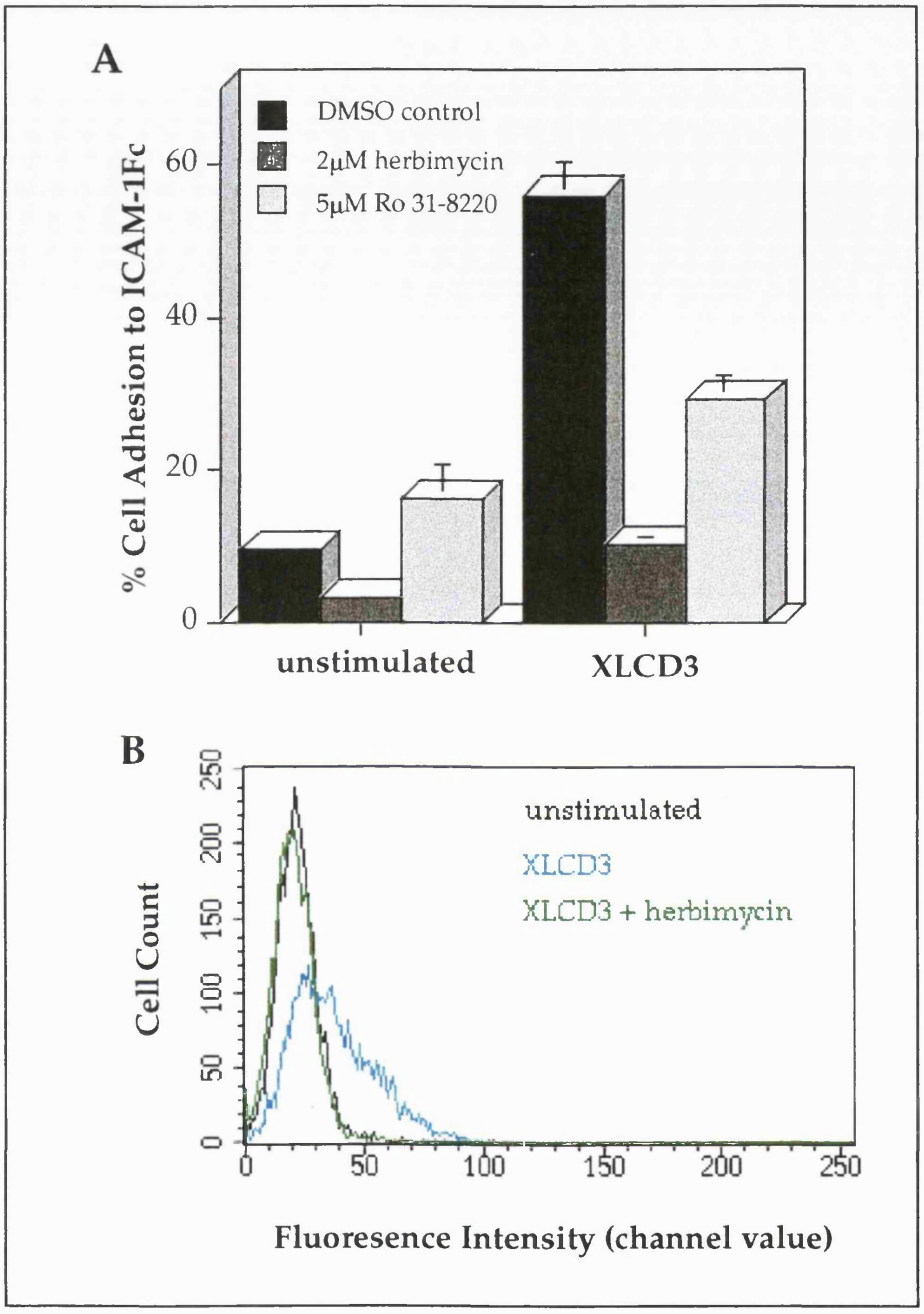

Figure 3.3: Effect of protein kinase inhibitors on XLCD3-stimulated LFA-1 activation. Cells were preincubated with 2 μM herbimycin A overnight or 5 μM Ro 31-8220 for 30 mins then washed and stimulated by UCHT1-crosslinking (XLCD3). After a 30 min incubation at 37°C, cells were analysed for adhesion to ICAM-1Fc-coated plastic **(A)** or for expression of the mAb 24 epitope **(B)**. In (A) results are expressed as means of triplicates +/-standard deviations and in both panels one representative experiment of 5 is shown. Adhesion was blocked with mAb 38 indicating specificity of adhesion between LFA-1 and ICAM-1.

tyrosine kinases, therefore the intracellular signals leading to LFA-1 activation could follow pathways already defined which also depend on tyrosine phosphorylation events (see **Figure 1.6**). Adhesion is partially inhibited by the PKC inhibitor Ro 31-8220 (**Figure 3.3A**) which confirms another report where the less-specific PKC inhibitor staurosporine exhibited similar effects. (Dustin and Springer, 1989). This suggests that although PKC is involved in the activation of LFA-1 it does not necessarily constitute the only road to integrin activation. Perhaps this is not surprising since the well characterised phosphatidylinositol pathway emerging from the TCR/CD3 complex forks into two and PKC is only on one arm of this pathway (see **Figure 1.6**). Unfortunately the effects of Ro 31-8220 on 24 epitope expression could not be examined as cells preincubated with the drug have a very high autofluorescence making detection in any of the flow cytometer fluorescent channels impossible.

(ii) Phorbol-ester stimulated LFA-1 activation

Phorbol esters, such as PDBu, have been used as a common means to induce homotypic cell aggregation mediated through integrins; therefore it was of interest to examine the effects of the inhibitors on activation of LFA-1 by means of this agonist. PDBu-induced T cell adhesion can be blocked to basal levels by the PKC inhibitor Ro 31-8220 as previously shown in Figure 3.2. and now in **Figure 3.4A** suggesting that PDBu is working through its suspected target PKC to induce activation of LFA-1. When the effect of herbimycin A is examined it is found to partially inhibit PDBu-induced adhesion (**Figure 3.4A**). This finding was initially quite surprising and suggested that, amongst other things, either tyrosine kinase events lie somewhere downstream of PKC activation or that perhaps the inhibitor was somehow non-specifically inhibiting PKC. In contrast to the adhesion results, herbimycin A has no effect on PDBu-induced 24 epitope expression (**Figure 3.4B**). This confirms that herbimycin A is not non-specifically inhibiting PKC as PDBu can still induce the ability of LFA-1 to express the mAb 24 epitope and that the herbimycin A-sensitive events must occur following expression of the activation epitope. Previous work in the lab has shown that induction of the mAb 24 epitope on LFA-1 requires a prior interaction with ligand ICAM-1; proposing that the epitope could be of the ligand induced binding site (LIBS)-type (Cabañas and Hogg, 1993). When this phenomenon is taken into consideration it would appear that herbimycin A must be exerting its effects

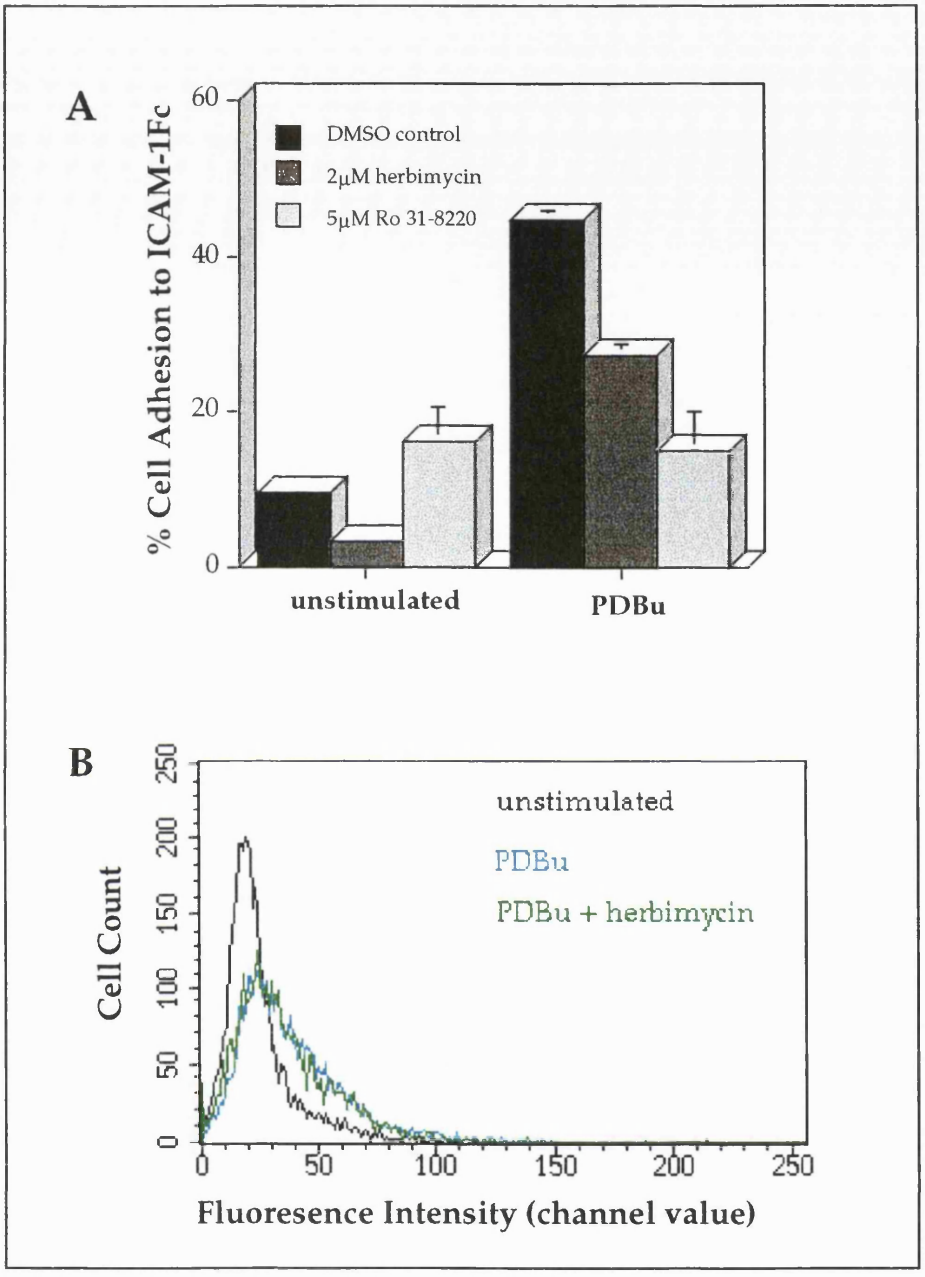

Figure 3.4: Effect of protein kinase inhibitors on PDBu-stimulated LFA-1 activation. Cells were preincubated with 2 μM herbimycin A or 5 μM Ro 31-8220 then stimulated with 50 nM PDBu for 30 min at 37° C after which time they were analysed for adhesion to ICAM-1Fc-coated plastic **(A)** or for expression of the mAb 24 epitope **(B)**. In (A) results are expressed as means of triplicates +/-standard deviations and in both panels one representative experiment of 5 is shown. Specificity of adhesion was shown by inhibition with mAb 38.

following some form of receptor/ligand engagement and the downstream tyrosine kinase events might be acting following receptor occupancy with ligand.

3.2d EFFECT OF INHIBITORS ON EXTERNAL STIMULI

The above studies indicate that tyrosine kinases and PKC are indeed involved intracellularly in the signalling mechanisms which activate LFA-1 following the common "inside-out" stimulants. Both with respect to the herbimycin A result above and for general interest it was relevant to examine whether these kinases are also important when LFA-1 is activated by stimuli thought to work from the cell exterior i.e. divalent cations and activating antibodies.

(i) Divalent cation stimulated LFA-1 activation

The divalent cations Mg^{2+} and Mn^{2+} can stimulate LFA-1 to bind to ICAM-1 and to express the mAb 24 epitope (Dransfield, et al., 1992a). Mg^{2+} only stimulates adhesion and epitope expression once Ca^{2+} is removed but Mn^{2+} can accomplish this without the requirement for chelating extracellular Ca^{2+}. When Mg^{2+}, in conjunction with EGTA to chelate Ca^{2+}, is used as stimulant adhesion is not inhibited by Ro 31-8220 indicating that activation does not require PKC activity (**Figure 3.5A**). This result would lend credence to the hypothesis that triggering integrins from the outside bypasses the intracellular signals which are normally stimulated through agonist-induced activation. This might be expected if cations are thought to bind directly to the integrin in order to exert their effects. Herbimycin A however causes a decrease in adhesion (**Figure 3.5A**). This decrease is not reflected in the ability of Mg^{2+} to stimulate mAb 24 epitope expression (**Figure 3.5B**) suggesting once again that herbimycin A does not affect the ability of LFA-1 to present an active conformation but does affect downstream events which are required to facilitate stable cell adhesion. Incase this was an unusual feature of using Mg^{2+}, activation with Mn^{2+} was also examined. A similar phenomenon is seen with Mn^{2+}, which induces PKC-independent but tyrosine kinase-dependent cell adhesion (**Figure 3.6A**). With identical results to Mg^{2+}, Mn^{2+}-stimulated mAb 24 epitope expression is not affected by herbimycin A (**Figure 3.6B**). Therefore, divalent cations appear to induce mAb 24 epitope expression independently of tyrosine kinases and PKC whereas cell adhesion has some dependence on tyrosine kinases.

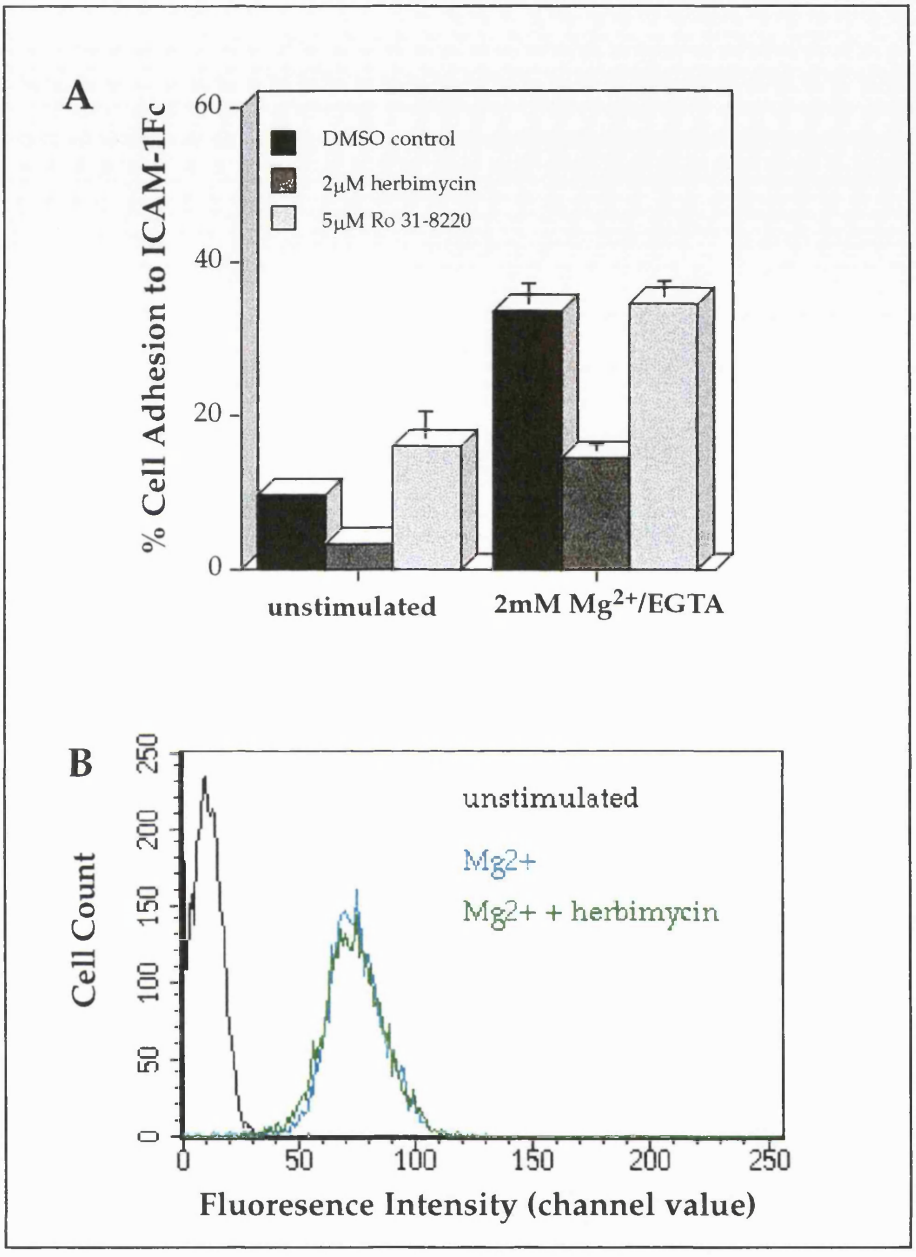

Figure 3.5: Effect of protein kinase inhibitors on Mg^{2+}-stimulated LFA-1 activation. Cells were preincubated with 2 μM herbimycin A or 5 μM Ro 31-8220 then washed into cation-free Hepes buffer before stimulation with 2 mM Mg^{2+}/1 mM EGTA for 30 min at 37° C. Cells were then analysed for adhesion to ICAM-1Fc-coated plastic **(A)** or for expression of the mAb 24 epitope **(B)**. In (A) results are expressed as means of triplicates +/- standard deviations and in both panels one representative experiment of 5 is shown. Adhesion was blocked with the anti-LFA-1 mAb 38 indicating specificity of binding for LFA-1 to ICAM-1.

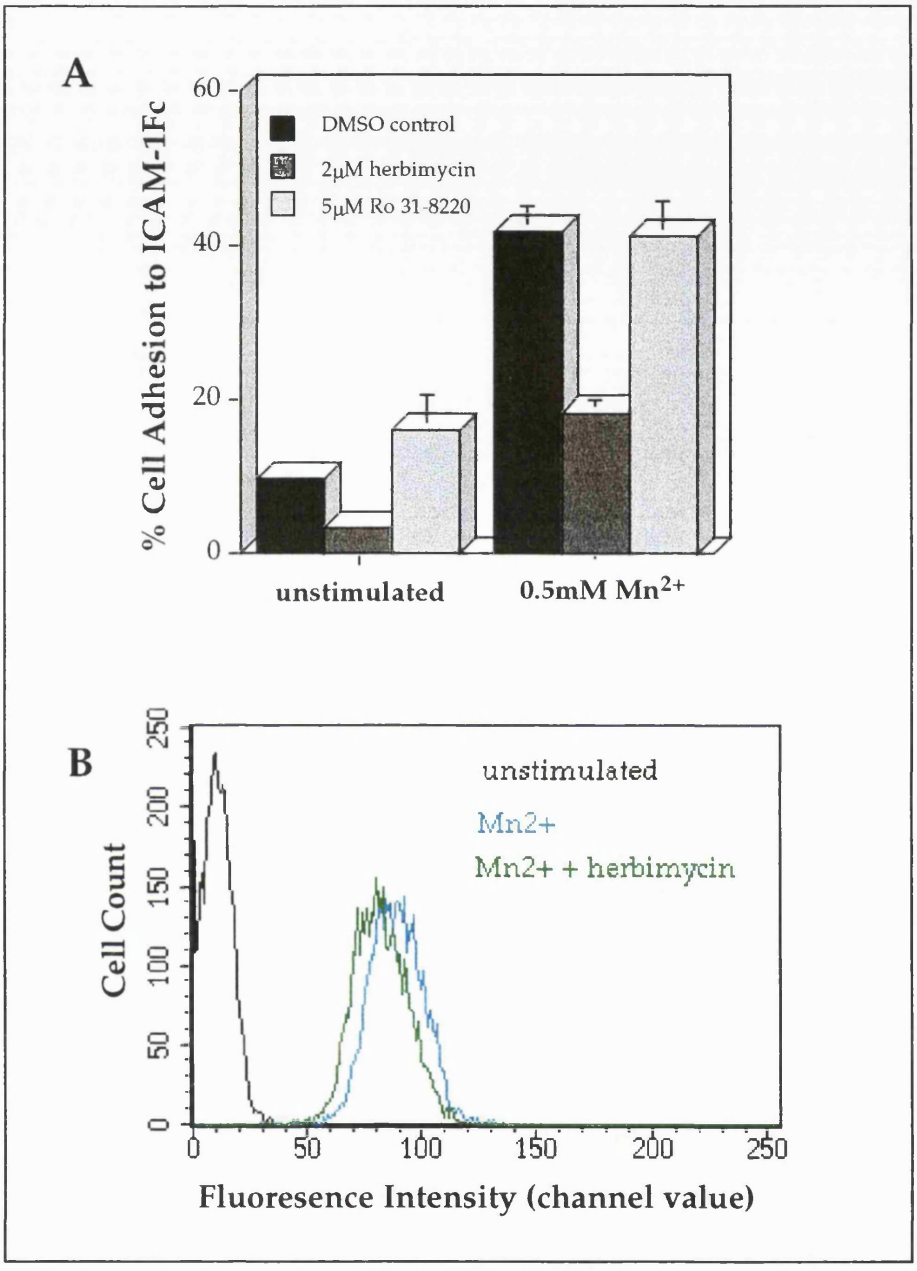

Figure 3.6: Effect of protein kinase inhibitors on Mn^{2+}-stimulated LFA-1 activation. Cells preincubated with either 2 μM herbimycin or 5 μM Ro 31-8220 were washed into cation-free Hepes buffer and then stimulated with 0.5mM Mn^{2+} for 30 min at 37° C after which time they were analysed for adhesion to ICAM-1Fc-coated plastic **(A)** or for expression of the mAb 24 epitope **(B)**. In **(A)** results are expressed as means of triplicates +/- standard deviations and in both panels one representative experiment of 5 is shown. Adhesion was blocked with the anti-LFA-1 mAb 38 indicating specificity of binding for LFA-1 to ICAM-1.

(ii) mAb stimulated LFA-1 activation

Several mAbs have now been characterised which induce the activation of LFA-1 (reported in Introduction; section 1.4c). One such mAb KIM185, directed to the common β_2 subunit, has been shown to induce the LFA-1-dependent homotypic aggregation of B lymphoid cells (Andrew, et al., 1993). This mAb was employed to induce activation of LFA-1 on T cells. When incubated with T cells, KIM185 is able to induce both adhesion to ICAM-1 and induction of mAb 24 epitope expression (**Figure 3.7**). Cell adhesion is not inhibited by preincubation with Ro 31-8820 but is with herbimycin A (**Figure 3.7A**). Once again, in contrast to the adhesion results, herbimycin A has no detrimental affect on the ability of KIM185 to induce mAb 24 epitope expression (**Figure 3.7B**). Another activating mAb MEM83, which is directed to the α_L subunit, can also induce T cell adhesion to immobilised ICAM-1 (Landis, et al., 1993). When this mAb was tested, a similar pattern of herbimycin A inhibition was seen for adhesion but not for mAb 24 epitope generation (data not shown). Therefore activating mAbs show similar results to divalent cations for the parameters examined.

3.2e SPECIFICITY OF HERBIMYCIN A

Due to this apparent broad spectrum inhibition of cell adhesion by herbimycin A it was important to check that it was not having a general inhibitory effect on downstream signalling events. As previously mentioned, herbimycin A was non-toxic to the cells due to their ability to exclude trypan blue dye. Intact cell viability was also confirmed from the flow cytometry experiments where results shown are for viable cells only as dead cells were eliminated by a propidium iodide gate. The inability to affect mAb 24 epitope expression also shows that the capacity of LFA-1 receptors to become activated remains intact in the presence of the inhibitor. Herbimycin A does not exert its inhibitory effects by decreasing the number of cell surface LFA-1 molecules; the fluorescence profile of mAb 38 staining, which recognises all LFA-1 molecules, is identical before and after 16 hr preincubation with herbimycin A (**Figure 3.8A**). Herbimycin A does not cause a general non-specific effect on downstream T cell signalling events as the calcium ionophore, ionomycin retains its ability to mobilise $[Ca^{2+}]_i$ following herbimycin A pretreatment (**Figure 3.8B**). The drug does specifically inhibit CD3-triggered $[Ca^{2+}]_i$ fluxing, as would be expected (**Figure 3.8C**). Therefore

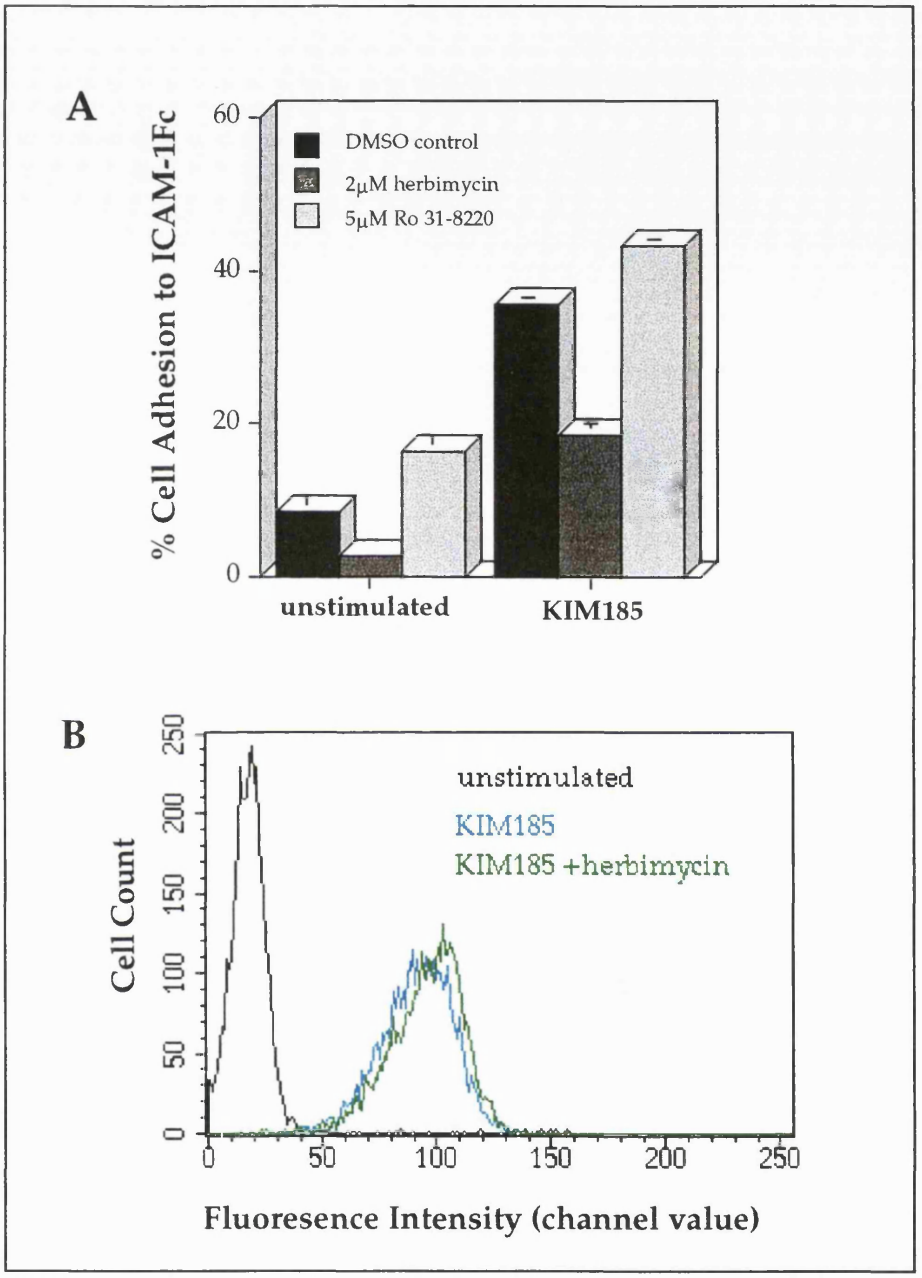

Figure 3.7: Effect of protein kinase inhibitors on KIM185-stimulated LFA-1 activation. Cells, preincubated with either 2 μM herbimycin A or 5 μM Ro 31-8220, were stimulated with 20μg/ml KIM185 for 30 min at 37°C after which time they were analysed for adhesion to ICAM-1Fc-coated plastic **(A)** or for expression of the mAb 24 epitope **(B)**. In (A) results are expressed as means of triplicates +/- standard deviations and in both panels one representative experiment of 3 is shown. Adhesion was blocked with the anti ICAM-1 mAb 15.2 indicating specificity of binding for LFA-1 to ICAM-1.

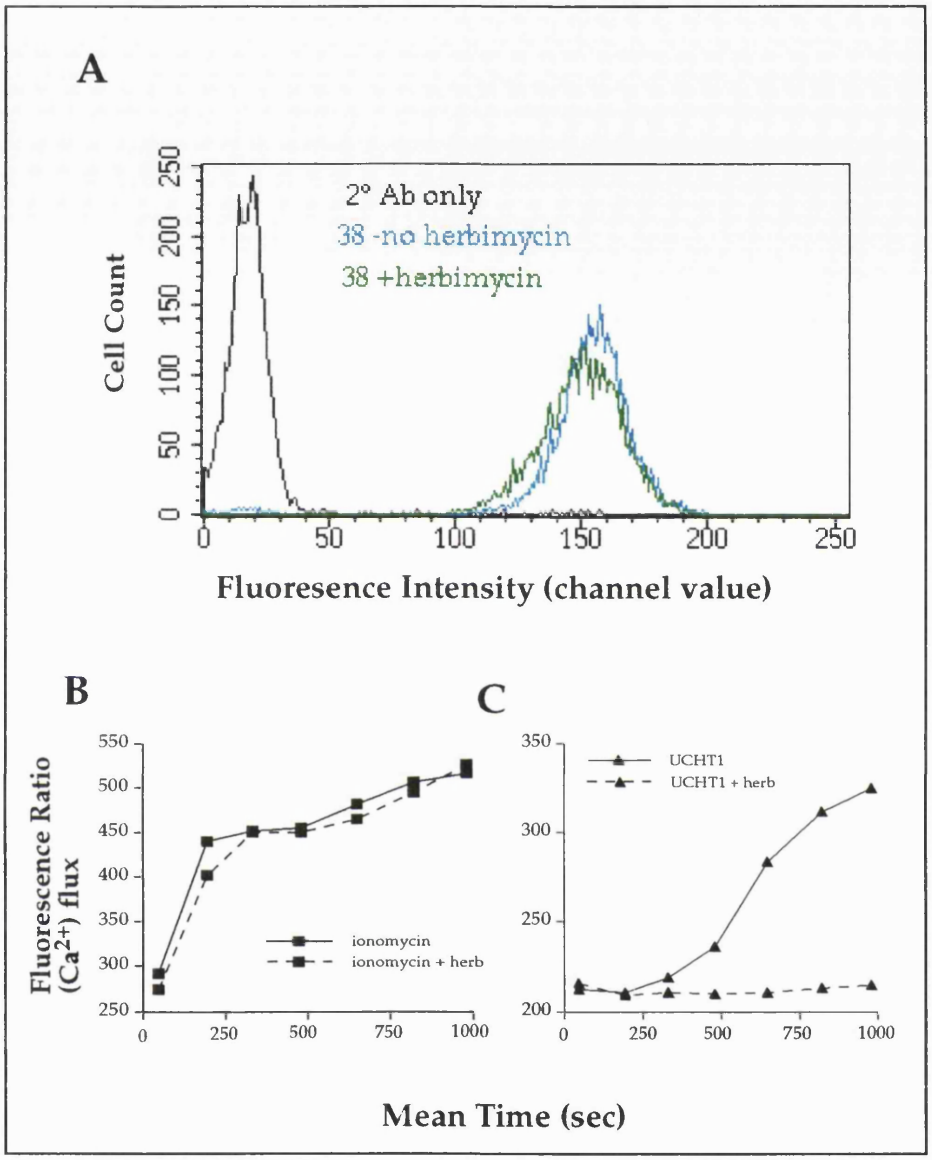

Figure 3.8: Does herbimycin A non-specifically affect downstream functions? Cells were preincubated with 2 μM herbimycin A for 16 hr and then analysed by flow cytometry for effects on cell surface expression of LFA-1, with 10 μg/ml of mAb 38 (**A**). The affect of herbimycin A on the ability of ionomycin (**B**) and UCHT1 (anti-CD3; **C**) to induce an $[Ca^{2+}]i$ flux in T cells was also examined by flow cytometry according to procedures described in Materials and Methods.

it seems that herbimycin A does not seem to be non-specifically inhibiting downstream signalling pathways.

3.2f IS THERE A DOWNSTREAM TYROSINE-KINASE PATHWAY?

Through analysis of the differential effects of herbimycin A on adhesion and mAb 24 epitope expression it appears that there is a tyrosine-kinase dependent pathway which occurs temporally after induction of the active receptor state. It is hypothesised that this might involve a post-receptor occupancy signal which is necessary to facilitate more stable cell binding perhaps by increasing the strength of the binding interaction or by inducing subtle changes in cell morphology which might enhance cell adhesion potential. It is well known that engagement of integrins can induce downstream tyrosine phosphorylation events and some of these events have been shown to contribute to morphological changes in cells (Sánchez-Mateos, et al., 1993). Initially it was decided to try and examine whether LFA-1 was associated with any specific tyrosine phosphorylation patterns following the time course of activation. This was performed by stimulating T cells with various agents known to cause activation of LFA-1, then immunoprecipitating LFA-1 from cell lysates followed by SDS-PAGE and Western Blotting. The presence of tyrosine phosphorylated proteins was then examined by probing blots with anti-phosphotyrosine Abs. Considerable effort was spent trying to identify bands which either changed over the stimulation time-course or appeared specific for LFA-1. Unfortunately, probably in part due to weekly variation of the primary cultured T cells, it proved extremely difficult to obtain consistent results showing different phosphorylation patterns over time. In addition, phospho-proteins which were detected showed an identical pattern to those immunoprecipitated by an anti-CD3 mAb (data not shown). Although this could suggest that the components of the CD3 complex and LFA-1 might link into the same intracellular signalling pathways it could also be an artefact of the immunoprecipitation procedure. However, a recent study looking at the pattern of tyrosine phosphorylated proteins following ICAM-3 and LFA-1 stimulation produced similar results; the phosphorylation patterns resembled those induced by CD3 triggering but to a lesser degree (Arroyo, et al., 1994).

A second approach to the problem was investigated in a collaboration with Dr Julian Downward and Patricia Warne (Signal Transduction Laboratory,

ICRF). As they had mAbs to many different intracellular signalling proteins, it was attempted to identify specific proteins which might undergo tyrosine phosphorylation following direct stimulation of the integrin with mAbs. Following stimulation through CD3 (with UCHT1), LFA-1 (β2, with KIM185) and β_1 (with TS2/16) the tyrosine phosphorylation of pp125^{FAK} and the cytoskeletal protein paxillin were monitored but with no obvious effects (data not shown). The activation of MAPK was also analysed which is readily observed by a molecular weight shift on an SDS-PAGE gel. Even though the shift was seen with immobilised anti-CD3 mAb, there was no MAPK activation seen with either of the integrin activating mAbs. At this time, MAPK was shown to be phosphorylated following plating of fibroblasts on fibronectin and laminin but not when they adhered to poly L-lysine (Chen, et al., 1994). However, we were unable to detect it with the mAbs which we employed. This could have been due to the specificity of the different activating mAbs used or alternatively that LFA-1 does not stimulate MAPK activation. Another possibility exists which is evident from signalling studies on neutrophils. Here β_2 integrins (in particular Mac-1) have been shown to induce tyrosine phosphorylation of paxillin and enhanced activation of a myelomonocytic-specific src family kinase p58*fgr* when cellular agonists such as TNFα, phorbol ester and fMLP are also present (Berton, et al., 1994; Fuortes, et al., 1994; Graham, et al., 1994). This suggests that for full signalling capacity a combination of different triggers may be required and this might also hold true for LFA-1. Therefore if the subject of LFA-1-induced signalling was to be pursued further, it would be pertinent to examine whether additional co-factors need to be included to detect any effects. A range of other mAbs should also be tested to address whether the lack of signalling is specific to certain mAbs.

3.2g LFA-1 ACTIVATION ON A T CELL LINE

Since the T blasts seemed to present donor variability and high basal levels of phosphorylation, analysis of activation-induced specific phosphorylation events was made difficult. Therefore it was decided to examine the prospect of using T cell lines. The T cell line Jurkat, which is an acute lymphoblastic leukaemic line, has been used extensively for many T cell signalling studies. In preliminary studies, a Jurkat subclone J6, was analysed for its LFA-1 activation characteristics. J6 cells stimulated with PDBu cannot be induced to adhere to ICAM-1 (**Figure 3.9A**). Stimulation by TCR/CD3 crosslinking also

failed to induce binding (not shown). This was initially quite disappointing and suggested that either LFA-1 was not present on these cells or was completely non-functional; however, upon further analysis it became evident that the situation was more complex than that. By phenotypic analysis it is obvious that the cells do have a substantial amount of LFA-1 on their cell surface (**Figure 3.9C**) although not to the extent seen on T cells (**Figure 3.9D**). Therefore the inability of Jurkat cell LFA-1 to become adhesive is not due to the lack of integrin. LFA-1 is functional on these cells when they are stimulated by manipulation of the extracellular concentration of Mg^{2+} (**Figure 3.9A**) and Mn^{2+} shows similar results. This demonstrates that the LFA-1 molecule itself does not have an intrinsic defect which prevents activation. It could be that these cells are faulty with regard to the intracellular machinery which is required to induce activation of LFA-1 following the "inside-out" agonists. Incase this was a problem unique to this particular Jurkat subclone, others were tested for their ability to be activated. The other lines, chosen for their ability to form mini-aggregates whilst growing in culture, were; JKHM1 (Jurkat stably transfected with the muscarinic receptor, obtained from Dr Doreen Cantrell, Lymphocyte Activation Laboratory, ICRF), TAg Jurkats, so called as they are transformed with large T Ag from SV40 (obtained from Dr Neil Clipstone, Stanford, USA) and Jurkat Rubin (obtained from Dr David Simmons, Oxford, UK). None of these lines could be induced to bind ICAM-1 using the "inside-out" agonists (data not shown). A recent review on LFA-1 also proposed this idea from work performed on leukaemic T cells which could not be activated with the phorbol ester PMA but could with the activating mAb NKI-L16 (Lub, et al., 1995). The authors suggest that this might be due to defective signalling pathways rather than from structural defects in LFA-1. The J6 Jurkats were then examined to see if they could be induced to adhere through other integrins which would indicate if the problem was specific to LFA-1 or not. The J6 Jurkat cells could be induced to bind to fibronectin following PDBu stimulation which suggests that their α_4 or α_5 integrins are able to be activated (**Figure 3.9B**). This could suggest that either LFA-1 and VLA-4/VLA-5 are activated through distinct pathways and in Jurkat cells the LFA-1 pathways is missing/defective or that they use similar pathways but for some reason there is a defect in the ability of LFA-1 to respond to signalling. Although this work with Jurkat cells was originally performed to try and see whether they would be suitable substitute cells for T blasts, the finding that certain integrins can be activated on them suggests

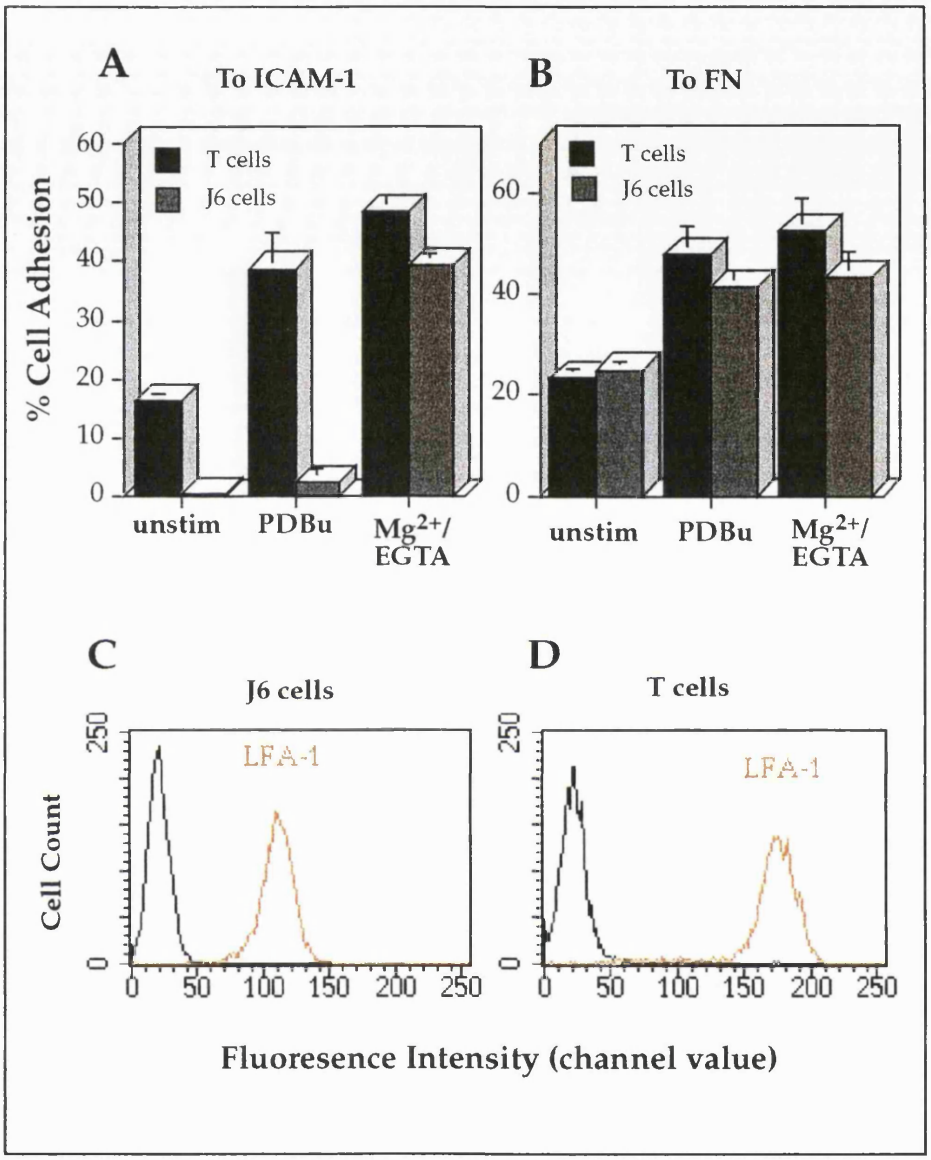

Figure 3.9: The characteristics of J6 Jurkat cell adhesion to ICAM-1 and fibronectin. T cells and J6 Jurkats were induced to adhere to ICAM-1 (**A**) or fibronectin (FN; **B**) with 50 nM PDBu or 5 mM Mg^{2+}/1 mM EGTA for 30 mins at 37°C prior to washing and counting. The amount of LFA-1 present on Jurkat (**C**) and T cells (**D**) was measured by flow cytometry with 10 µg/ml of the anti- α_L mAb, 38. Detection was made using a goat-anti mouse IgG secondary antibody (Jackson, Immunoresearch). One representative experiment of three is shown.

that they might be suitable models for gaining new insight into the mechanism of LFA-1 activation, providing that the defect can be identified.

3.3 DISCUSSION

Through the use of protein kinase inhibitors it is apparent that when cells are stimulated by the more physiological stimulus, CD3 crosslinking, both tyrosine kinases and PKC are involved in the activation of LFA-1. Due to the differential effects of herbimycin A on mAb 24 epitope expression and cell adhesion it appears that these two assays do not measure exactly the same activation status of LFA-1. The principal hypothesis would be that following the initial interaction with ligand, which is required to generate 24 epitope expression, further events occur (post-receptor occupancy) which are necessary to facilitate stable cell adhesion. This requires some involvement of tyrosine kinases as shown by herbimycin A inhibition. This could be something similar to that seen for β_1 integrins on a myelomonocytic cell line, U937 and an erythroblastoid cell line, K562 where induction of adhesion onto ligands fibronectin, VCAM-1 and collagen results in the generation of cell protrusions and colocalisation of the integrins with tyrosine phosphorylated proteins (Sánchez-Mateos, et al., 1993). Another idea would be that some relatively poorly understood signalling mechanism might signal into the cells from the integrin and then feed back positively on the integrin inducing more stable adhesion. It has recently been shown for $\alpha_V\beta_3$ and $\alpha_V\beta_5$ on renal epithelial cells that cells which exhibit increases in $[Ca^{2+}]_i$ facilitate increased binding to RGD peptide ligand attached to beads (Sjaastad, et al., 1994). To make definitive conclusions based on data obtained through the use of biochemical inhibitors it is desirable to have confirmatory data obtained through another experimental means. Unfortunately, further evidence of the nature of this putative pathway following LFA-1 engagement on T cells has not been obtained. To further pursue this issue, more defined conditions would need to be employed which might involve the addition of accessory stimulants or growth factors. These might be required before any tyrosine phosphorylation is detected.

Use of varying subclones of the leukaemic T lymphoblastoid cell line, Jurkat, did not prove to be fruitful as an alternative model for investigating LFA-1 activation and signalling. This would have had the benefit of producing more consistent results and also these cells are easier to manipulate than primary T

cells grown in culture. As LFA-1 could not be activated following conventional "inside-out" signalling methods, these cells must have a defect in their intracellular machinery which prevents LFA-1 activation. The LFA-1 is capable of being activated on these cells; extracellular manipulations with divalent cations can induce adhesion. As the Jurkats could be induced to bind fibronectin it suggests that the defect does not target all cell surface integrins and could be specific to LFA-1. Studies published around the same time as these discoveries found a similar phenomenon in that several T cell leukaemic lines could not be induced to bind to ICAM-1 but the Jurkats could be induced to bind fibronectin predominantly through VLA-4 (Mobley, et al., 1994; van Kooyk, et al., 1993a). In one of these papers, LFA-1 on Jurkat cells failed to express the NKI-L16 epitope which suggests that they are defective in their ability to undergo Ca^{2+}-dependent clustering if this feature is a reflection of epitope expression on Jurkat cells as implied in the Figdor laboratory (van Kooyk, et al., 1993a). This phenomenon has also been discussed in a recent review, where the authors propose that the inability of LFA-1 to be activated on leukaemic T cell lines is due to defective signalling pathways and not structural defects in the LFA-1 receptor itself. They propose that LFA-1 on these cells might be under tighter control by cytoskeletal elements (Lub, et al., 1995). Preliminary experiments show that these cells can be induced to bind to ICAM-3, which provides further evidence for the proposal that LFA-1 is differentially activated for binding to ICAM-1 and ICAM-3 and this discrepancy is evident in Jurkat cells. Even though LFA-1 does not function normally on these lines, they could prove useful in that, if the defect can be discovered, more can be learned about the regulation of LFA-1. Perhaps discovery of the intracellular signalling events will come from careful analysis of these lines which are missing a key signalling component. To obtain other T cell lines for use in LFA-1 activation and signalling analysis, attention should probably focus on the use of T cell clones.

CHAPTER FOUR

ROLE OF INTRACELLULAR CALCIUM IN LFA-1 ACTIVATION

4.1 INTRODUCTION

Over the last ten years, the signalling pathways present within T cells have been gradually discovered. One of the earliest pathways to be determined was the phosphatidylinositol cascade which results from the hydrolysis of PIP_2 by PLC. The two immediate substrates DAG and IP_3 split the pathway into two and the former activates the PKC arm and hence phosphorylation events and the latter induces mobilisation of ($[Ca^{2+}]_i$) down an alternative route (reviewed in Isakov, et al., 1986; see Figure 1.6). PKC and $[Ca^{2+}]_i$ are both very important second messengers which induce further downstream signalling events ultimately leading to induction of gene expression within the nucleus. Stimulation of both branches of the pathway will result in fully competent cell proliferation as pharmacological manipulation with phorbol ester and ionomycin, which activate PKC and $[Ca^{2+}]_i$ respectively, can induce cell cycle progression in the absence of the early TCR/CD3 triggered events (Truneh, et al., 1985). $[Ca^{2+}]_i$ becomes mobilised through a sequence of events which initiate with IP_3 binding to its receptor on the membranes of organelles which function as intracellular Ca^{2+} stores, such as the endoplasmic reticulum (ER) (for review see Putney and Bird, 1993). Upon binding, the channel is opened and Ca^{2+} effluxes from the organelle into the cell cytoplasm. This increase in $[Ca^{2+}]_i$ has a positive feedback effect on the levels of cytoplasmic Ca^{2+} in that it causes further influx of Ca^{2+} this time through the plasma membrane from the cell exterior. When $[Ca^{2+}]_i$ is imaged, this dual phase Ca^{2+} release is observed as a biphasic Ca^{2+} mobilisation curve with the initial rise due to release from intracellular stores and the subsequent plateau from the steady influx of Ca^{2+} across the plasma membrane (Merritt and Rink, 1987). In the absence of extracellular Ca^{2+} only the initial transient mobilisation curve is seen which is due to release of Ca^{2+} from intracellular stores. This positive feedback mechanism, whereby released Ca^{2+} stimulates Ca^{2+} influx, has been termed capacitative Ca^{2+} entry. It is not yet known precisely how this feedback mechanism is mediated but a recent report suggested that a novel small messenger, activated upon emptying of intracellular stores, somehow stimulates plasma membrane influx. This factor was named Ca^{2+} influx factor (CIF;

Randriamampita and Tsien, 1993). Other possible candidates to transduce the signal which mediates the influx are discussed in relevant reviews (Meldolesi, 1993; Putney and Bird, 1993). This overall net increase in $[Ca^{2+}]_i$ has many downstream effects which ultimately lead to control of gene expression. There has been much previous interest in the contribution of PKC towards LFA-1 activation but the role of $[Ca^{2+}]_i$ has somewhat taken a back seat. As $[Ca^{2+}]_i$ forms the basis of the other branch of the major phosphatidylinositol signalling pathway it was of interest to examine whether intracellular mobilisation of Ca^{2+} *per se* could induce activation of LFA-1. The easiest way to increase cellular cytosolic Ca^{2+} levels, without stimulating through cell surface receptors, is by treating cells with Ca^{2+} ionophores such as ionomycin. Ionomycin is a mobile ion carrier which transfers Ca^{2+} into the cell by virtue of the fact that it shields the charge of the transported ion which would otherwise be unable to penetrate the hydrophobic interior of the plasma membrane lipid bilayer. In order to examine the contribution of $[Ca^{2+}]_i$ to LFA-1 activation, the effect of the Ca^{2+} ionophore ionomycin on T cell adhesion and mAb 24 epitope expression was studied.

4.2 RESULTS

4.2a MOBILISATION OF INTRACELLULAR CALCIUM BY IONOPHORE INDUCES THE ACTIVATION OF LFA-1.

The calcium ionophore ionomycin is a cell permeant carrier which transports Ca^{2+} across the cell membrane into the cytoplasm where it can induce further Ca^{2+} influx across the plasma membrane. Ionomycin has been used in many T cell proliferation and signalling studies as it allows the contribution of Ca^{2+} to be studied in isolation from other signalling events which would normally be induced following cell surface receptor stimulated Ca^{2+} mobilisation. Ionomycin, in conjunction with phorbol ester, can stimulate T cell proliferation and IL-2 production when used at ~1 μM (Truneh, et al., 1985). Ionomycin was titrated and showed a dose-dependent effect on the binding of T cells to immobilised ICAM-1 with maximal binding achieved at ~0.7 μM (**Figure 4.1A**). Ionomycin could also induce a dose-dependent increase in expression of the epitope recognised by mAb 24 (**Figure 4.1B**). The dose response plots are not sigmoidal and exhibit bell shape curves. This could mean one of two things; either higher concentrations of ionomycin are toxic to T cells thus explaining lack of adhesion or that higher concentrations mediate

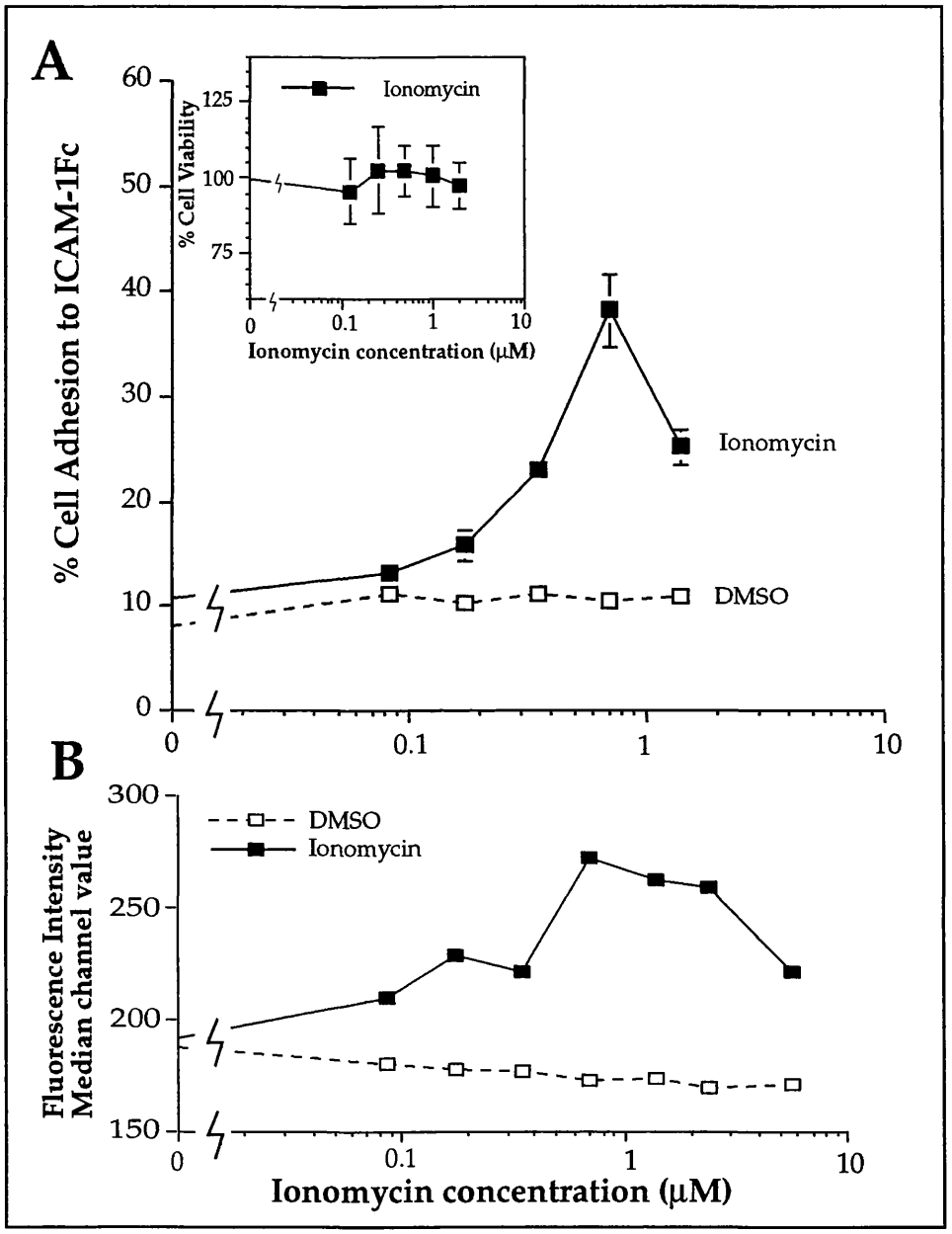

Figure 4.1: Dose response of ionomycin on LFA-1 activation. T cells were stimulated with the indicated concentrations of ionomycin or an equivolume control of DMSO and following a 30 min incubation at 37°C analysed for adhesion to ICAM-1 (**A**) and mAb 24 epitope expression (**B**). Adhesion was blocked by mAb 38 and data represents means of triplicates +/- standard deviation. The effect of increasing ionomycin concentration on cell viability was examined using an MTT assay where 100% viability represents untreated cells (**inset to A**). Data is expressed as means of triplicates +/- standard deviation.

de-adhesion and thus cells are actively de-adhering during this phase. In order to address this point, cell viability was examined upon ionomycin treatment by means of the MTT assay. This assay employs the tetrazolium salt MTT which is a yellow water-soluble substrate and upon reduction by cellular mitochondrial dehydrogenase enzymes it is converted to a dark blue product which can be read on a spectrofluorimeter. Only live cells with an active mitochondrial enzyme will perform the colour change. The range of ionomycin concentrations used in the LFA-1 activation experiments had no adverse effect on cell viability as shown by the MTT assay (**inset Figure 4.1A**). This results shows that the decline phase of the bell-shaped curve is not due to lack of cell viability and proposes that it might be due to active de-adhesion of cells. The mechanisms which lead to cellular de-adhesion through integrins are currently being investigated in other laboratories and it has been proposed that cAMP might play a role in this process (reviewed in Pardi, 1994). It will be interesting to see if there is any link between increasing $[Ca^{2+}]_i$ and cAMP in this process. Therefore it seems that mobilisation of $[Ca^{2+}]_i$, through the use of ionomycin, can induce activation of LFA-1. As ionomycin carries Ca^{2+} into the cell by plasma membrane penetration it was important to assess whether the increase in LFA-1 activation by ionomycin was due to its specific effects on Ca^{2+} mobilisation and not due to any coincidental non-specific effects which it might have on the cells e.g. membrane distortion effects.

4.2b THE ENDOPLASMIC RETICULUM Ca^{2+}-ATPASE PUMP INHIBITORS THAPSIGARGIN AND dBHQ INDUCE THE ACTIVATION OF LFA-1.

Intracellular Ca^{2+} can also be mobilised by treating cells with other pharmacological agents which act in a distinct manner to ionophores. The tumour promoter thapsigargin, which is a sesquiterpene lactone from the umbelliferous plant *Thapsi garganica*, and the synthetic compound 2,5-di(*tert*-butyl)hydroquinone (dBHQ) act by inhibiting the Ca^{2+}/ATPase pumps in the endoplasmic reticulum (ER) (reviewed in Thomas and Hanley, 1994. These pumps are normally required to maintain homeostasis by pumping Ca^{2+} into the ER, from the cytosol, in an ATP (adenosine triphosphate)-dependent manner. Inhibition of this pump facilitates unobstructed cytosolic $[Ca^{2+}]_i$ mobilisation by leakage from the ER (see **Figure 4.2** for diagram). The net effect of pump inhibition with these reagents is emptying of intracellular Ca^{2+}

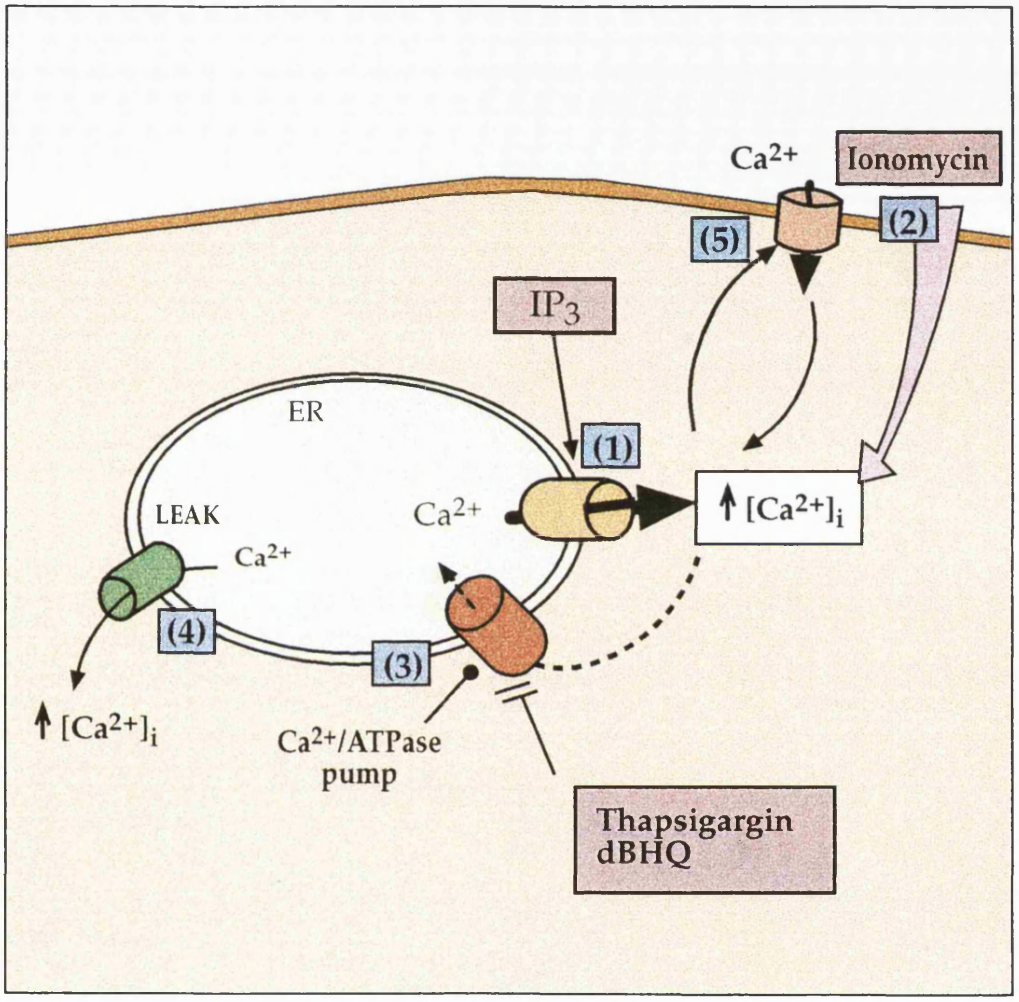

Figure 4.2: The capacitative Ca^{2+} entry pathway and mechansims of action of thapsigargin and dBHQ. Stimulus induced increases in cytosolic Ca^{2+} occur following IP_3 binding to receptors on the ER which allows Ca^{2+} to flow out from storage **(1)**. Ionomycin raises $[Ca^{2+}]_i$ without the requirement of IP_3 **(2)**. There are Ca^{2+}/ATPase pumps on the ER which maintain Ca^{2+} homeostasis by pumping cytosolic Ca^{2+} back into the store **(3)**. Thapsigargin and dBHQ both inhibit this pump and as result, Ca^{2+} is not pumped back into the store and any Ca^{2+} remaining in storage is lost through a Ca^{2+} leak **(4)**. The net effect is to empty intracellular stores and raise $[Ca^{2+}]_i$. This increase positively feeds back on the levels of Ca^{2+} by binding to plasma membrane Ca^{2+} channels and stimulating influx from the cell exterior **(5)**. This is called the capacitative Ca^{2+} influx and occurs following normal agonist, ionomycin, thapsigargin and dBHQ-induced Ca^{2+} release.

stores and resultant influx of Ca^{2+} across the plasma membrane via capacitative entry (Thastrup, et al., 1990). Thapsigargin and dBHQ were titrated for their effects on LFA-1 activation. Both showed a dose-dependent stimulation of T cell LFA-1 mediated adhesion to ICAM-1 (**Figure 4.3A**) and induction of the epitope recognised by mAb 24 epitope (**Figure 4.3B**). Similar to the effects of ionomycin, these compounds exhibited a bell-shaped response curve and once again the phase of decline was not due to loss of cell viability (not shown). The peak adhesion with thapsigargin was shown at ~5 μM and dBHQ at ~50 μM. When both agents were previously compared in other systems, dBHQ was also shown to have an optimal concentration much higher than that of thapsigargin. This is likely to be due to it being a more polar reagent than thapsigargin which makes it much poorer at penetrating the plasma membrane, hence explaining the requirement for a higher concentration (reviewed in Thomas and Hanley, 1994). Thapsigargin performed consistently better than dBHQ in inducing LFA-1 activation, perhaps due to the fact that it is more potent and selective in its activity. As a result of this, thapsigargin was used as the agent of choice in the majority of subsequent experiments. Since these agents work to increase mobilisation of $[Ca^{2+}]_i$ in a manner distinct from ionomycin, it confirms that the initial ionomycin-induced response must be specific to its effects on $[Ca^{2+}]_i$ and not due to coincidental non-specific effects. Even though thapsigargin and dBHQ both mediate mobilisation of $[Ca^{2+}]_i$ through inhibition of the ER Ca^{2+}-ATPase pump they have distinct structures making it unlikely that their effect on LFA-1 activation is some kind of artefact. Therefore mobilisation of $[Ca^{2+}]_i$ by three distinct agents can cause the activation of LFA-1, highlighting a role for $[Ca^{2+}]_i$ in the LFA-1 activation pathway.

4.2c LFA-1 ACTIVATION FOLLOWING $[Ca^{2+}]_i$ MOBILISATION REQUIRES EXTRACELLULAR Ca^{2+}

As the pharmacological agents studied raise cytosolic calcium levels not only by inducing release from intracellular stores, but also from the capacitative fluxing in through the plasma membrane it was important to define whether the observed effects on LFA-1 also required the secondary Ca^{2+} influx. This is normally investigated by analysing the response of Ca^{2+} mobilising agents in the presence and absence of extracellular Ca^{2+} ($[Ca^{2+}]_{out}$). The situation for analysing LFA-1 activation is slightly more complex as integrins require defined extracellular cation conditions to facilitate adhesion. As previously

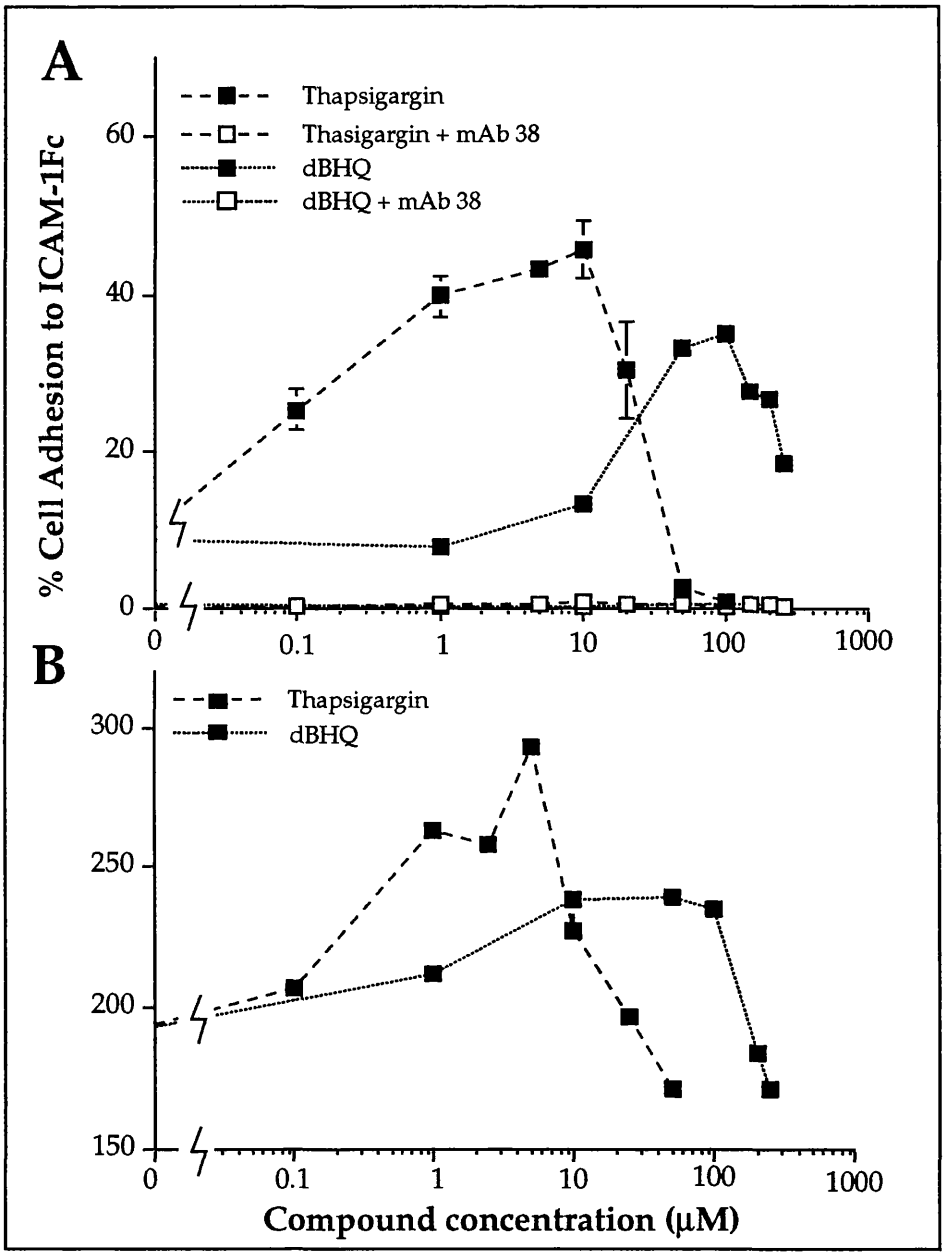

Figure 4.3: Dose response of thapsigargin and dBHQ on LFA-1 activation. T cells were stimulated with the indicated concentrations of thapsigargin or dBHQ and following a 30 min incubation at 37°C were analysed for adhesion to ICAM-1 (**A**) and mAb 24 epitope expression (**B**). In the adhesion assay, mAb 38 (anti-LFA-1) was added to confirm specificity of the interaction. Control cells which were incubated with an equivolume of the carrier DMSO showed basal adhesion and 24 epitope expression. In the adhesion assay data represents means of triplicates +/- standard deviations and for both **A** and **B** one representative experiment of three is shown.

discussed, LFA-1 is active in the presence of Mg^{2+} or Mn^{2+} but Ca^{2+} is inhibitory under some circumstances. Therefore to study the effects of ionomycin and thapsigargin with respect to their requirements for $[Ca^{2+}]_{out}$, LFA-1 activation was examined under carefully controlled cation conditions. When Mg^{2+} is titrated into cells which have been washed into cation free buffer, it will induce a dose-dependent increase in adhesion to ICAM-1 (see **Figure 4.4A**; **no stimulus -Ca^{2+}**), this increase is dramatically augmented when any remaining Ca^{2+} is chelated with EGTA (see Chapter 5). In the presence of exogenously added Ca^{2+} (**see Figure 4.4A; no stimulus +Ca^{2+}**) the enhancing effect of Mg^{2+} is lost. These values are the basal adhesion states seen in the presence of extracellular cations. When ionomycin-stimulated adhesion is examined it can be seen that in the absence of extracellular Ca^{2+} (**Figure 4.4A; ionomycin -Ca^{2+}**) there is no increased adhesion over the basal levels (compare **no stimulus -Ca^{2+}** curve); however in the presence of 1mM extracellular Ca^{2+}, ionomycin can stimulate adhesion over basal levels (compare **Figure 4.4A; ionomycin +Ca^{2+}** with **no stimulus +Ca^{2+}**). Therefore it is obvious that ionomycin can only stimulate LFA-1-mediated T cell adhesion to ICAM-1 when extracellular Ca^{2+} is present. The effects of varying extracellular cation concentration on thapsigargin-stimulated adhesion is shown in **Figure 4.4B**. Similar to the story with ionomycin, thapsigargin can only induce adhesion over basal levels when extracellular Ca^{2+} is present (compare **thapsigargin +Ca^{2+}** with **no stimulus +Ca^{2+}** curves). This shows that the ability of ionomycin and thapsigargin to stimulate LFA-1-mediated adhesion is dependent on the presence of extracellular Ca^{2+} and that simply mobilisation of Ca^{2+} from intracellular stores is not sufficient.

4.2d TITRATION OF EXTRACELLULAR Ca^{2+} ON ABILITY TO STIMULATE LFA-1 ACTIVATION

As extracellular Ca^{2+} is required to allow ionomycin and thapsigargin to induce LFA-1 activation, it was important to assess how much extracellular Ca^{2+} was actually needed. Titration of extracellular Ca^{2+}, in the presence of 0.5 mM Mg^{2+} required for integrin activation, resulted in a dose dependent increase in adhesion (**Figure 4.5**). The half maximal concentration of $[Ca^{2+}]_{out}$ required to facilitate thapsigargin and ionomycin-mediated adhesion was approx. 1.5 mM. This corresponds to the mM values of $[Ca^{2+}]_{out}$ required for activity of a plasma membrane Ca^{2+} channel (Mauger, et al., 1984) which

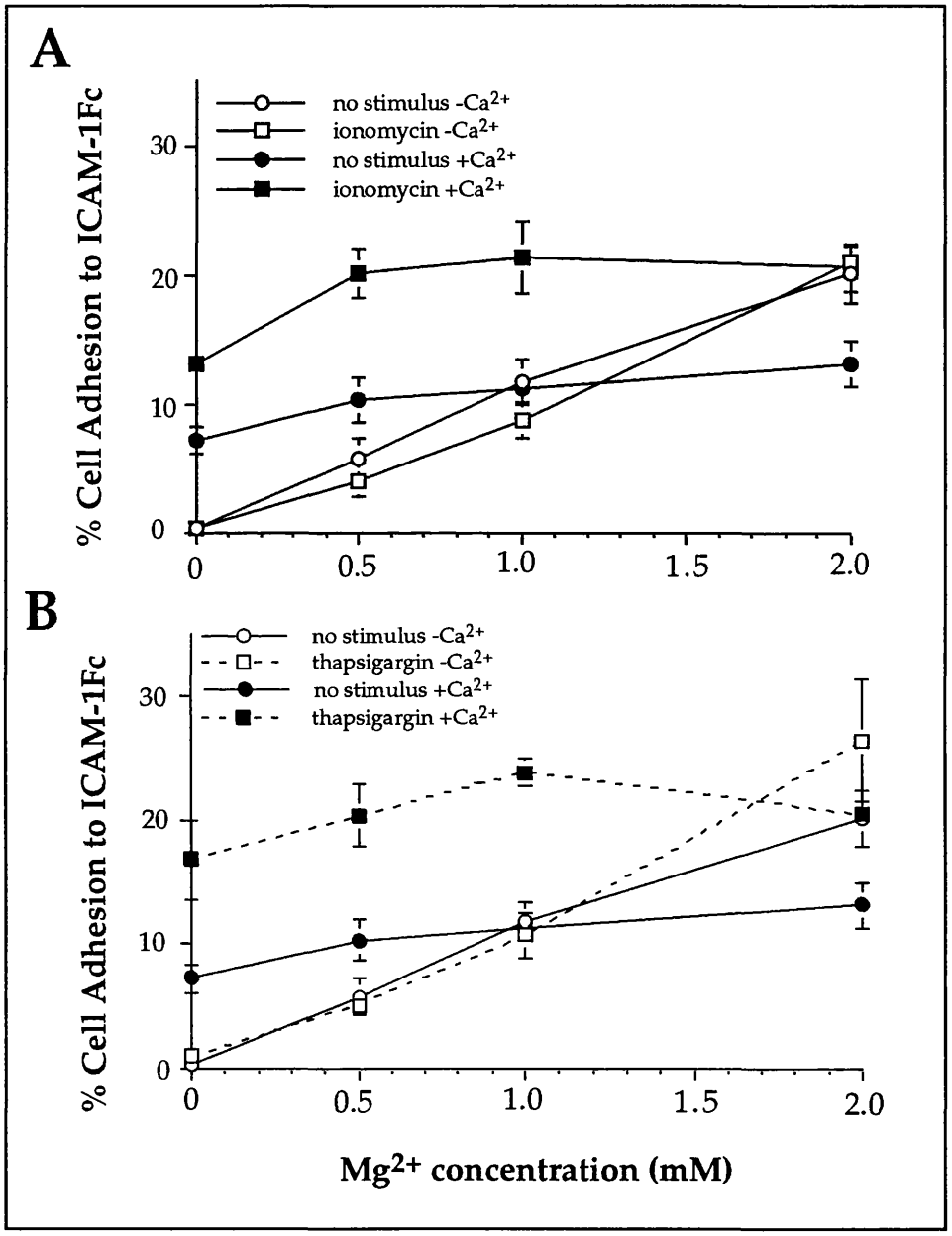

Figure 4.4: Effect of extracellular Ca^{2+} on ionomycin and thapsigargin-stimulated cell adhesion. T cells were washed into cation free HEPES buffer containing varying Mg^{2+} concentrations and stimulated with 0.7 μM ionomycin (A) or 5 μM thapsigargin (B) in the presence or absence of 1 mM Ca^{2+}. Following a 30 min incubation at 37°C, bound cells were counted. Data represents means of triplicates +/- standard deviations and one representative experiment of two is shown.

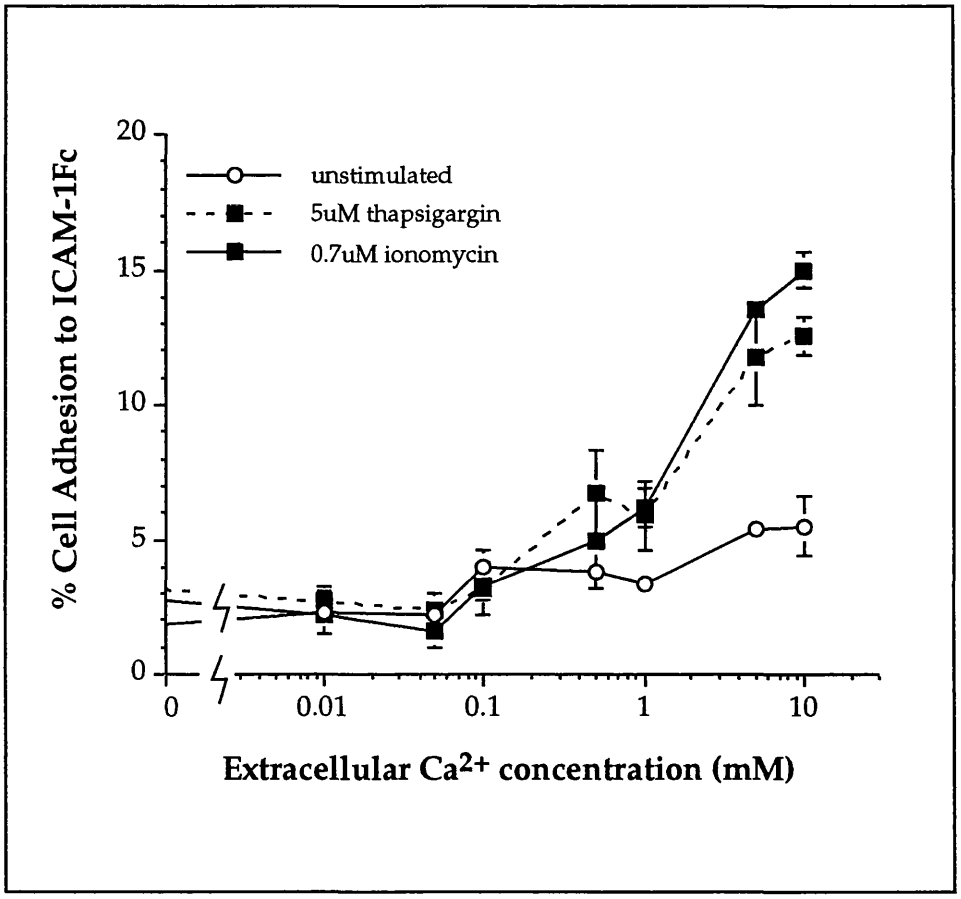

Figure 4.5: Titration of extracellular Ca^{2+} on ionomycin and thapsigargin-stimulated cell adhesion. T cells were washed into cation free HEPES buffer containing varying Ca^{2+} concentrations and stimulated with 0.7 μM ionomycin or 5 μM thapsigargin in the presence of 0.5 mM Ca^{2+}. Following a 30 min incubation at 37°C, bound cells were counted. Data represents means of triplicates +/- standard deviations and one representative experiment of three is shown.

confirms that capacitative Ca^{2+} influx is required for LFA-1 activation following ionomycin and thapsigargin stimulation.

4.2e EFFECT OF RO 31-8220 (THE PKC INHIBITOR) ON IONOMYCIN AND THAPSIGARGIN-STIMULATED LFA-1 ACTIVATION

Now that it was established that ionomycin and thapsigargin could stimulate LFA-1 activation and this was likely to require, in addition to release from intracellular stores, the capacitative influx of Ca^{2+} across the plasma membrane; it was important to try and establish where Ca^{2+} was having its effects in the LFA-1 activation pathway. One of the consequences of mobilisation of cytosolic Ca^{2+} is that it can feedback on PKC to enhance its activation (Isakov, et al., 1986). The importance of PKC in the activation of LFA-1 was previously highlighted in Chapter 3 therefore it was imperative to determine if the enhanced activity following Ca^{2+} mobilisation was a result of feedback activation of PKC. The effects of the PKC inhibitor Ro 31-8220 on ionomycin and thapsigargin- stimulated T cell adhesion to ICAM-1 were thus examined. Ro 31-8220 had no effect on ionomycin and thapsigargin stimulated LFA-1 activation even at 5 μM (**Figure 4.6**). To ensure that the inhibitor was functioning, it was tested for its effect on adhesion stimulated with PDBu. The inhibitor was functional as it could inhibit phorbol ester-stimulated adhesion at concentrations as low as 1 μM. The effects of the inhibitor on adhesion stimulated through the TCR/CD3 complex was also examined through the use of another mAb, G19.4. This mAb has T cell stimulatory properties without the requirement for extensive crosslinking. For LFA-1 activation studies it behaved similarly to crosslinking of CD3 with UCHT1 and anti-mouse IgG secondary Ab and therefore it was also used in LFA-1 activation analysis. G19.4-stimulated LFA-1-mediated adhesion was also inhibited by Ro 31-8220 indicating that the inhibitor was functional in these assays and therefore that ionomycin and thapsigargin are not mediating their activation effects through PKC in contrast to PDBu and CD3-stimulated adhesion. This result also places $[Ca^{2+}]_i$ further downstream from PKC in the activation pathway and proposes it as another proximal signalling molecule to LFA-1, in a temporal sense.

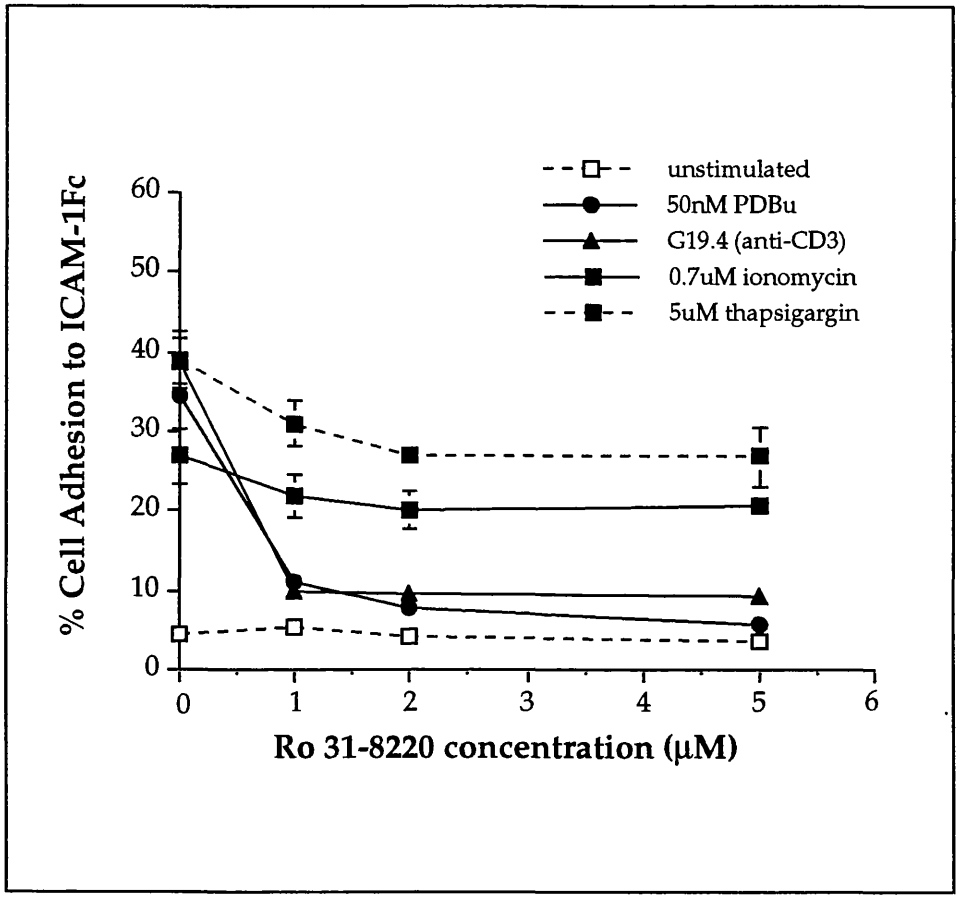

Figure 4.6: Titration of Ro 31-8220 on stimulated adhesion to ICAM-1. T cells were preincubated with the indicated concentrations of Ro 31-8220 for 30 mins at 37°C prior to washing in RPMI medium. Cells were then stimulated with 50 nM PDBu, 10 μg/ml G19.4 (anti -CD3), 0.7 μM ionomycin or 5 μM thapsigargin and induced to adhere to immobilised ICAM-1Fc for 30 mins at 37°C before unbound cells were removed by washing and bound cells counted. Data represents means of triplicates +/- standard deviations and one representative experiment of three is shown.

4.2f THE EFFECT OF THE IMMUNOSUPPRESSANT FK506 ON THE ACTIVATION OF LFA-1.

Now that a role for $[Ca^{2+}]_i$ mobilisation in LFA-1 activation was proven, and was not due to feedback effects on PKC, other potential downstream effects of Ca^{2+} leading to LFA-1 activation were sought. In T lymphocytes, one of the major enzymes stimulated following mobilisation of $[Ca^{2+}]_i$ is the serine/threonine phosphatase calcineurin which is regulated by Ca^{2+} and calmodulin. A critical role for calcineurin in the induction of T cell gene expression was originally found through the use of the immunosuppressive drugs cyclosporin A and FK506. These drugs were initially found to prevent the activation of specific transcription factors such as NF-AT (nuclear factor of activated T cells) which binds to sites on the IL-2 promoter and induces transcription (Mattila, et al., 1990). The drugs bind their endogenous intracellular ligands called immunophilins which are cyclophilin and FKBP (FK506 binding protein) respectively. A breakthrough in the link between their binding activities and effects on gene expression came when with the finding that calcineurin was the missing piece of the jigsaw (Clipstone and Crabtree, 1992). Binding of the immunosuppressant to the immunophilin prevents the activation of calcineurin (see **Figure 4.7**). Active calcineurin is thought to induce the nuclear translocation of NF-ATc (the NF-AT cytosolic subunit), perhaps following de-phosphorylation, which renders it free to complex with the nuclear subunit, NF-ATn. These subunits bind to the IL-2 promoter and drive IL-2 transcription (reviewed in Liu, 1993; Schreiber and Crabtree, 1992). As calcineurin has also been shown to be important for neutrophil motility on extracellular matrix proteins (Lawson and Maxfield, 1995) it seemed a pertinent Ca^{2+}-dependent enzyme which required further study. A role for calcineurin in LFA-1 activation was examined through the use of FK506 which was kindly donated by Dr. Neil Clipstone (Stanford, USA). FK506 is an antibiotic of the macrolide family which was isolated from *Streptomyces tsukubaensis*. FK506 was titrated and found to have no effect on ionomycin-stimulated LFA-1 activation measured either by adhesion to ICAM-1 (**Figure 4.8A**) or mAb 24 epitope expression (**Figure 4.8B**). The concentrations tested were in the same range as those previously shown to be sufficient for inhibition of T cell proliferation (Sawada, et al., 1987). Even at much higher concentrations, up to 100 ng/ml, there was no inhibition observed. In addition, FK506 had no effect on PDBu or TCR-stimulated adhesion to ICAM-1 or mAb 24 epitope expression. These results indicated

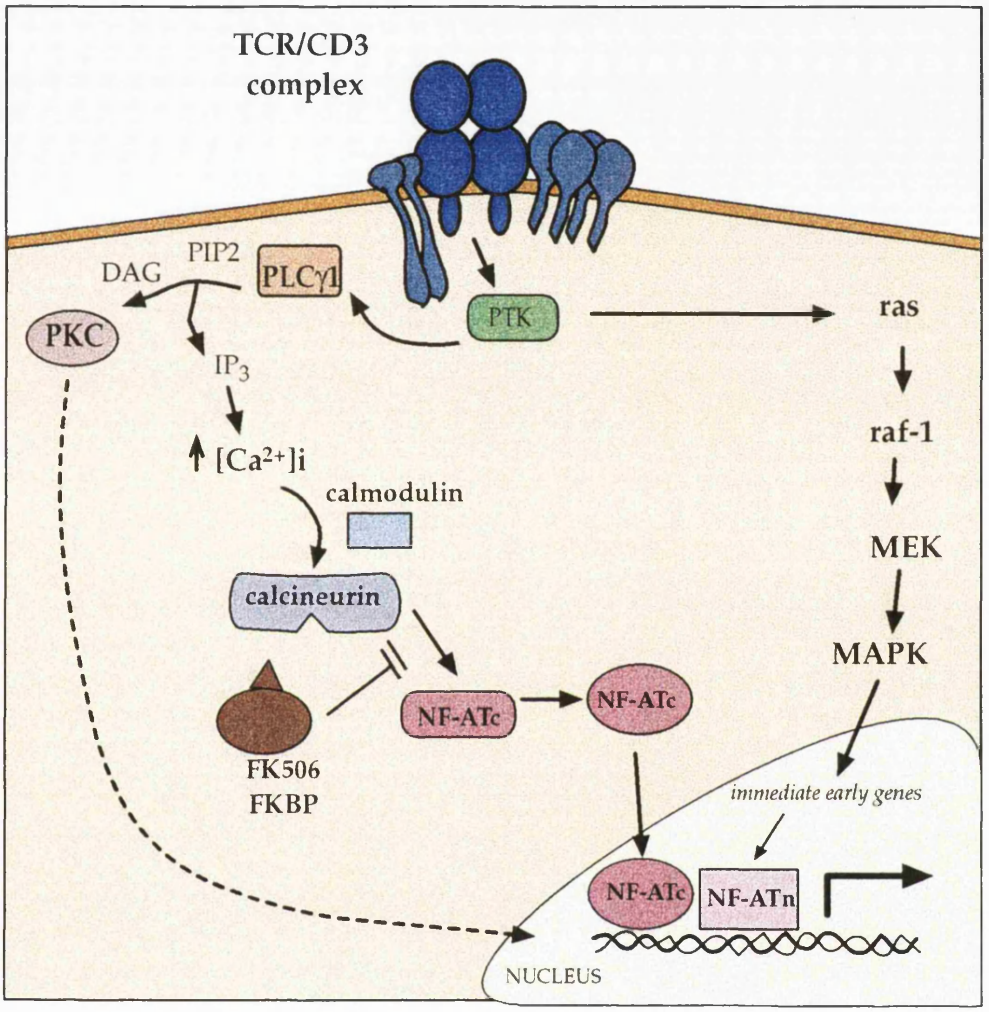

Figure 4.7: Schematic diagram of the mechanism of action of FK506. Stimulus induced increases in cytosolic Ca^{2+} activate calmodulin which both then activate the serine/threonine phosphatase calcineurin. This phosphatase is pivotal in inducing NF-AT dependent gene transcription and is hypothesised to work by dephosphorylating the cytosolic subunit (NF-ATc) and inducing its nuclear translocation. Within the nucleus NF-ATc can associate with the nuclear subunit (NF-ATn), which is activated following another intracellular signalling cascade, and together they can induce transcription. The immunosuppressant FK506 binds to its ligand, FK506 binding protein (FKBP), and this complex then inactivates calcineurin which results in inhibition of NF-AT dependent gene transcription. The activity of FK506 can be tested in the FDG assay which measures the NF-AT dependent transcription of the reporter gene β-galactosidase.

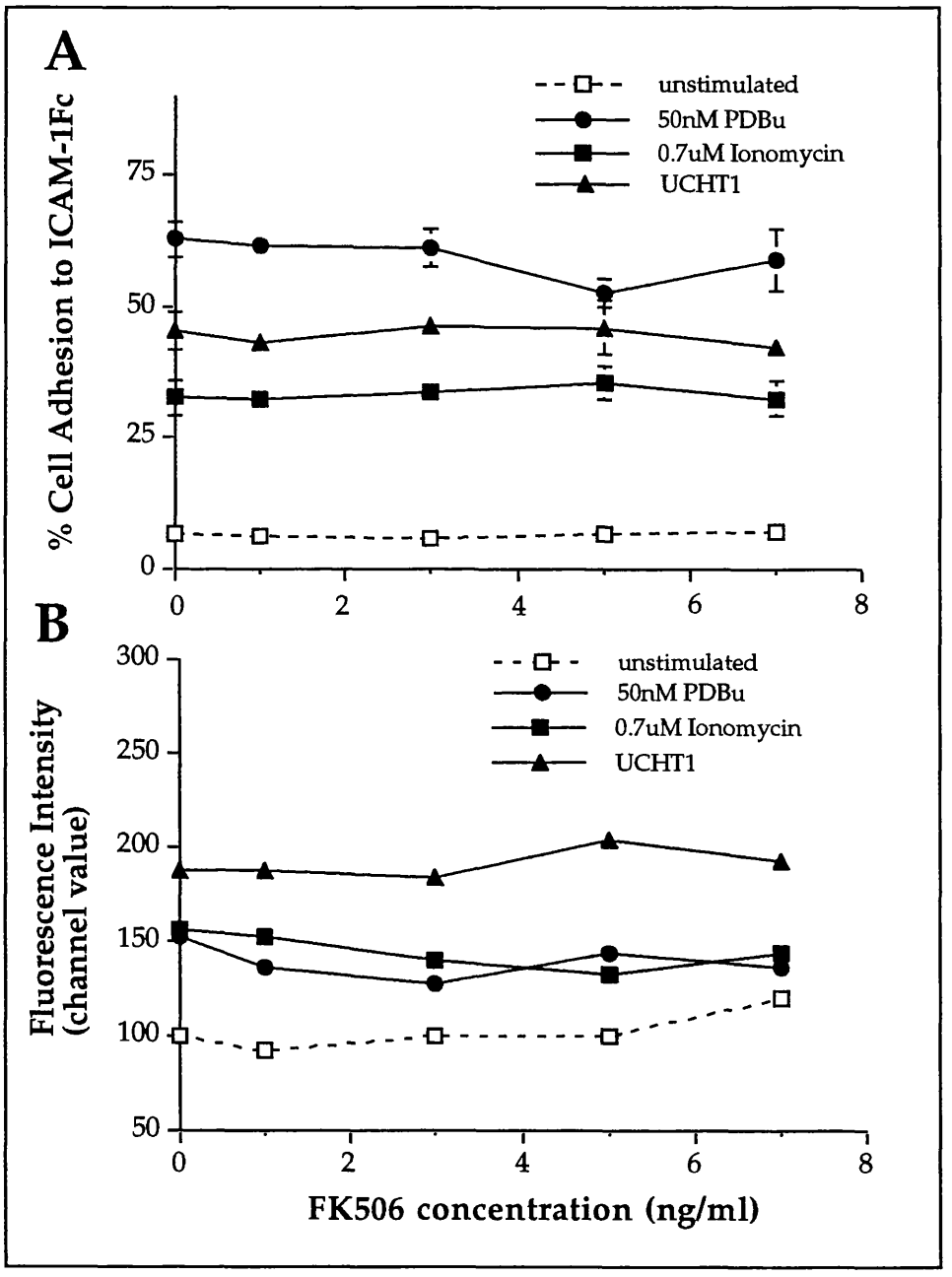

Figure 4.8: Effect of the immunosuppressant FK506 on activation of LFA-1. T cells were preincubated with the indicated concentrations of FK506 for 1 hr at 37°C prior to washing in RPMI medium. Cells were then stimulated with 50 nM PDBu, 10 µg/ml UCHT1 (anti -CD3) or 0.7 µM ionomycin for 30 mins at 37°C and then either induced to adhere to immobilised ICAM-1Fc (**A**) or examined for expression of the mAb 24 epitope (**B**). One representative experiment of three is shown.

that calcineurin had no role in LFA-1 activation following Ca^{2+} mobilisation nor was it involved following stimulation with phorbol ester or TCR/CD3 triggering. The possibility still existed however that this particular batch of FK506 was inactive as no positive results had been seen; therefore it required to be tested in a system known to be calcineurin-dependent.

4.2g USE OF A MURINE T CELL HYBRIDOMA TO TEST THE EFFICACY OF FK506

At a similar time to these experiments, Dr. Lucy Wedderburn in the Lymphocyte Development Laboratory was using a murine T cell hybridoma system which would allow testing of the FK506. The test system makes use of a T cell hybridoma stably transfected with a construct encoding three copies of the NF-AT binding site tagged with the lac Z or β-galactosidase reporter gene under the control of the IL-2 promoter. When provided with the appropriate stimulus which can activate NF-AT, such as phorbol ester and ionomycin, this construct is activated by NF-AT binding hence the β-galactosidase gene is transcribed. Addition of a fluorogenic substrate into the cells by brief hypotonic shock provides a readout for efficient transcription. In the presence of β-galactosidase the fluorescein di-β D galactopyranoside (FDG) substrate will be cleaved yielding a fluorescent product which can be detected by flow cytometry (Fiering, et al., 1990). Therefore, in theory, stimulation by phorbol ester and ionomycin will require calcineurin to mediate NF-AT binding and if the FK506 is active it should inhibit any β-galactosidase transcription and subsequent fluorescent change. This assay was employed to test the efficacy of the FK506 which had been used in the LFA-1 activation experiments. Following stimulation of the cells with 50 nM PDBu and 0.7 μM ionomycin, the fluorescent change and hence β-galactosidase transcription was seen in a proportion of the cells as expected (**Figure 4.9-green histogram**). Even at the lowest concentration tested (1 ng/ml) FK506 inhibited activation of NF-AT and hence transcription of the β-galactosidase construct and resultant fluorescent change (**Figure 4.9-purple histogram**). From this result it can be concluded that the FK506 is indeed functional and the lack of effect on LFA-1 activation means that none of the stimulants tested have their effects through calcineurin.

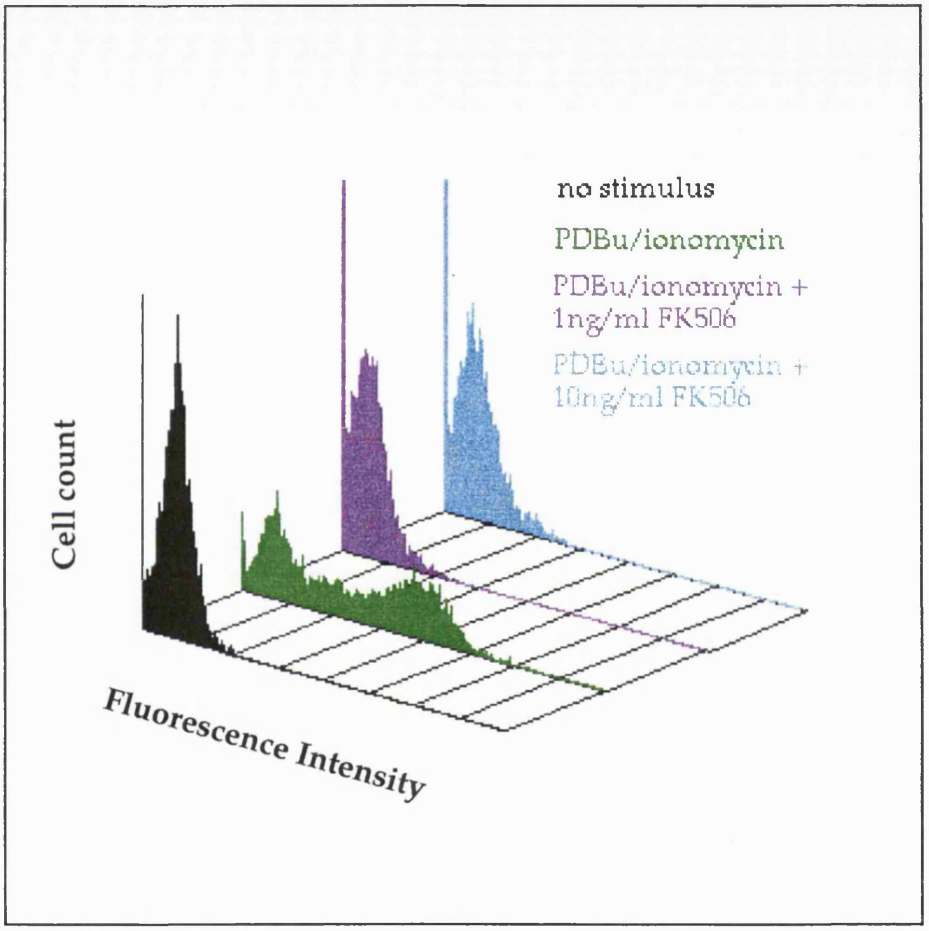

Figure 4.9: Effect of the immunosuppressant FK506 on activation of NF-AT construct and cleavage of FDG. S13.2 cells were preincubated with various concentrations of FK506 for 1 hour at 37°C before washing and stimulation with 50 nM PDBu and 0.7 µM ionomycin for 16 hours at 37°C. FDG was then loaded into cells by brief osmotic shock and the cells left for 4 hours on ice. FDG cleavage to fluorescein was recorded by flow cytometry. Black histogram represents unstimulated cells. All other histograms were stimulated with PDBu and ionomycin without (green) or with preincubation with 1 ng/ml FK506 (purple) and 10 ng/ml FK506 (blue). One representative experiment of two is shown.

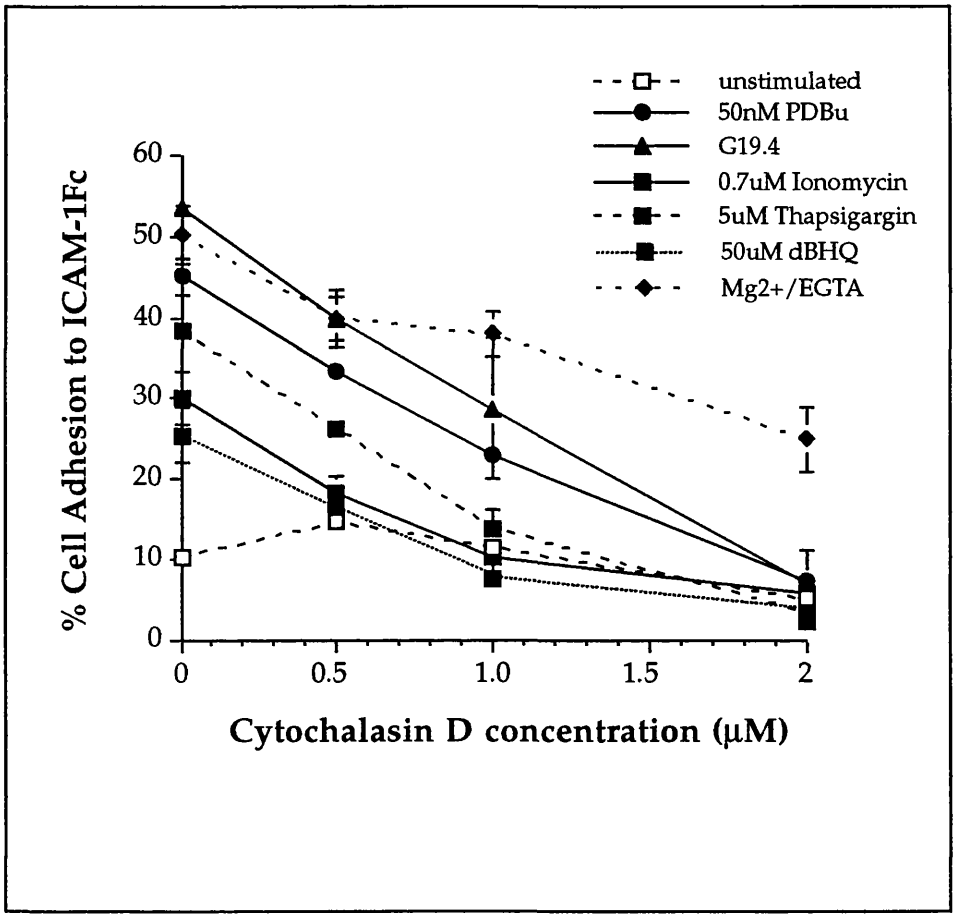

Figure 4.10: Effect of cytochalasin D on stimulated T cell adhesion to immobilised ICAM-1. T cells were stimulated with 50 nM PDBu, 10 μg/ml G19.4, 0.7 μM ionomycin, 5 μM thapsigargin, 50 μM dBHQ or 5 mM Mg^{2+}/1 mM EGTA in the presence of various concentrations of cytochalasin D and incubated for 30 mins at 37°C before washing and counting. Data represents means of triplicates +/- standard deviation and one representative experiment of three is shown.

4.2h ACTIVATION OF LFA-1 WITH THE Ca^{2+}-MOBILISING AGENTS IS SUSCEPTIBLE TO CYTOCHALASIN D

A role for the actin-based cytoskeleton in LFA-1-mediated adhesion was investigated through the use of the drug cytochalasin D which acts to inhibit actin polymerisation (see Chapter 5 for further results). Cytochalasin D from *Zygosporium mansonii* is one member of a group of fungal metabolites which can permeate cell membranes and inhibit actin polymerisation. This drug acts by binding to the barbed ends of actin filaments and preventing their polymerisation by blocking end on addition of actin monomers (Cooper, 1987). When cytochalasin D was titrated it was found to inhibit adhesion of T cells to ICAM-1 in dose-dependent fashion (**Figure 4.10**). Adhesion stimulated through the agonists ionomycin, thapsigargin and dBHQ, which mediate mobilisation of $[Ca^{2+}]_i$, was inhibited by 1 μM cytochalasin D. Cells stimulated by PDBu and G19.4 were also inhibited by cytochalasin D treatment emphasising the importance of cytoskeleton reorganisation in the activation of LFA-1. As a control to check that cytochalasin D was not mediating general non-specific inhibitory effects on the cells its effects were examined on cells stimulated from the cell exterior with 5 mM Mg^{2+} in the presence of 1 mM EGTA. **Figure 4.10** shows that there was much less effect of cytochalasin D on Mg^{2+}-stimulated adhesion confirming that cytochalasin D does not have a general knock-out effect on these cells. When cells were stimulated with the β_2 activating mAb KIM185, cytochalasin D was once more shown not to be generally inhibitory (data not shown). This result proposes a role for the cytoskeleton in the activation of LFA-1 following stimulation with agents inducing the 'inside-out' signal. In particular, those cells activated by the agents which cause $[Ca^{2+}]_i$ mobilisation appear to be particularly sensitive to its effects.

4.3 DISCUSSION

Through the use of distinct agents, ionomycin, thapsigargin and dBHQ, which have similar effects of increasing the intracellular cytosolic Ca^{2+} concentration, it is obvious that the event of mobilising $[Ca^{2+}]_i$ somehow causes induction of LFA-1 activation. As these agents have completely different structures and mechanisms of action it is unlikely that they are exerting their effects through secondary events such as alterations in the cell membrane. In addition to inducing release of Ca^{2+} from intracellular storage

organelles these agonists also induce the capacitative influx of Ca^{2+} across the outer cell membrane. By manipulation of the extracellular Ca^{2+} concentration, it was found that the effect of these Ca^{2+}-mobilising agents required sufficient extracellular Ca^{2+} and at a concentration which suggests the involvement of plasma membrane Ca^{2+} channels adding further evidence to the role of capacitative Ca^{2+} entry in LFA-1 activation. Concurrent with the findings in this report, a similar study was published which showed that ionomycin could stimulate LFA-1 activation as measured by the ability of a T cell clone to adhere to ICAM-1 transfectants (van Kooyk, et al., 1993b). Although the authors did not pursue the mechanism by which ionomycin might be exerting its effects they did show that stimulation of adhesion with mAbs to CD2, CD3 and MHC class II was dependent on the influx of extracellular Ca^{2+} across the plasma membrane. Thus is appears that $[Ca^{2+}]_i$ may play a dominant role in several of the methods to activate LFA-1. Around the same time another group found that stimulation of T cells with the calcium ionophores ionomycin and A23187 and the Ca^{2+}-ATPase pump inhibitors, thapsigargin, dBHQ and cyclopiazonic acid could induce them to bind fibronectin, collagen and laminin through β_1 integrins (Hartfield, et al., 1993). Stimulated adhesion occurred at concentrations similar to those used in this study. This group did not address the mechanism by which these agents and $[Ca^{2+}]_i$ might activate integrins. Therefore it seems as if the mobilisation of $[Ca^{2+}]_i$ is a general mechanism for the activation of various integrins and is not solely specific to LFA-1. The fact that thapsigargin is a better inducer than dBHQ might be due to the fact that it is a more specific and potent reagent or additionally dBHQ has been shown to have effects on various other aspects of the Ca^{2+} fluxing machinery. It has been shown to interfere with the ER Ca^{2+} leak and also to block capacitative Ca^{2+} entry (Foskett and Wong, 1992) thus it might be a poorer LFA-1 activator due to its inability to allow full potential mobilisation of cytosolic Ca^{2+}.

So what is all this cytosolic Ca^{2+} doing to induce activation of LFA-1? It was possible that the Ca^{2+} was acting as a positive feedback regulator of PKC and in this way enhancing adhesion. Stimulated adhesion with ionomycin, thapsigargin and dBHQ however was not inhibited by the PKC inhibitor Ro 31-8220 indicating that Ca^{2+} was not mediating its effects through the activation of PKC. This also suggests that the Ca^{2+} responsive part of the pathway lies downstream or parallel to PKC activation and identifies it as a proximal part of the pathway in the generation of active LFA-1. Investigation

of the effects of herbimycin A on the Ca^{2+}-induced adhesive process suggested, similar to the other stimulants in Chapter 3, that a tyrosine kinase pathway lay downstream of Ca^{2+} mobilisation but also of mAb 24 epitope expression making analysis of this effect quite difficult (data not shown). In an attempt to examine putative effectors downstream of Ca^{2+} it was decided to tackle a role for the Ca^{2+} binding protein calcineurin as this is an extremely important molecule for the efficient activation of T cells. This serine/threonine phosphatase is activated by binding of calmodulin and Ca^{2+}, and probably its best studied effect is on the induction of NF-AT responsive genes. The immunosuppressant FK506, which binds to an intracellular ligand and inhibits calcineurin activity, had no effect on the induction of LFA-1 mediated adhesion or expression of the mAb 24 activation reporter epitope. This drug was shown to be functional in a specially designed NF-AT responsive β-galactosidase assay confirming that calcineurin does not appear to be the major target for Ca^{2+} in the pathway to LFA-1 activation. Since novel protein synthesis is not required for the induced activation of LFA-1 it is unlikely that were calcineurin to have been involved in the first place that it would have acted through an NF-AT responsive mechanism. However, calcineurin does have other intracellular effects some of which have become especially evident recently in the field of cell motility. Inhibition of $[Ca^{2+}]_i$ fluxing and calcineurin have been shown to impair neutrophil migration by preventing cell detachment from the substrate which implicates these factors in the detachment process (Huttenlocher, et al., 1995, Lawson and Maxfield, 1995). It appears however, from this study, that calcineurin inhibition has no adverse affects on the LFA-1 activation process. Calcineurin is apparently becoming quite topical in adhesion studies as it has recently been shown to be involved in the binding of CHO cell $\alpha_5\beta_1$ to fibronectin (Pomiés, et al., 1995). This study showed that Abs to calcineurin could inhibit $\alpha_5\beta_1$, obtained from CHO cell lysates, binding to immobilised fibronectin. The effect was not thought to be a direct association of calcineurin with $\alpha_5\beta_1$ or through alterations in the phosphorylation/ dephosphorylation status of the integrin . It remains to be seen if calcineurin plays a role *in vivo* and on other cell types.

The adhesion was particularly susceptible to inhibition by cytochalasin D suggesting a role for actin polymerisation somewhere in the activation pathway. This might suggest that there were obvious morphological differences in the cells which had been stimulated through Ca^{2+} mobiliser-

stimuli, but; as will be shown in Chapter 5 there were no gross alterations in morphology seen following this activation pathway. This however does not rule out that more subtle changes in morphology might be important which were not detected in the confocal experiments. Ca^{2+} is known to influence the polymerisation of actin due to its interaction with actin binding proteins such as gelsolin. In a model uniting specific signalling events with the actin polymerisation/ depolymerisation cycle it was hypothesised that increases in $[Ca^{2+}]_i$ might contribute to actin disassembly (Stossel, 1989: Downey, et al., 1990). This would seem to contradict a role for Ca^{2+} in actin polymerisation but one explanation for this would be that a Ca^{2+}-induced cytoskeletal disassembly might be required to release LFA-1 from the cytoskeleton enabling it to be coupled to the activation apparatus or that this event might be a prerequisite for subsequent bouts of actin polymerisation which are required for LFA-1 activation. Careful microscopic experiments and studies on other T cell lines might eventually decipher if any of these possibilities are correct.

$[Ca^{2+}]_i$ might also be required for more immediate events at the plasma membrane. Titration of $[Ca^{2+}]_{out}$ showed that mM values were required for LFA-1 activation which is in keeping with the involvement of a Ca^{2+} channel on the plasma membrane. Perhaps actions associated with the use of this channel might influence plasma membrane proteins in the vicinity by alterations in membrane architecture or membrane fluidity and mobility. One early report on the platelet integrin $\alpha_{IIb}\beta_3$ indicated that it might itself function as a Ca^{2+} channel as it could induce $[Ca^{2+}]_i$ fluxing when inserted into liposomes (Rybak, et al., 1988). However no further studies have elaborated this finding and no other integrins have been shown to function as Ca^{2+} channels *per se.* , however it exists an interesting prospect. Even if the integrin itself is not a Ca^{2+} channel it is possible that if an integrin and Ca^{2+} channel were in close apposition on the membrane that fluxing through the channel might cause alterations and hence activation of the integrin.

One final word on the possible downstream effectors of $[Ca^{2+}]_i$ mobilisation lies with a molecule which is relatively new to the integrin field. The Ca^{2+}-binding protein calreticulin, which was originally thought to be an exclusive ER resident, has been found over the last few years to bind to a common FGFFKR motif initially described in nuclear hormone receptors. This motif is also highly homologous in all the integrin α subunits and is located adjacent

to the intracellular side of the transmembrane segment. Only this year, calreticulin has been shown to become associated with the integrin $\alpha_2\beta_1$ in Jurkat cells following PMA or β_1 activating mAb stimulation (Coppolino, et al., 1995). Thus it is an interesting hypothesis that the $[Ca^{2+}]_i$ mobilisation phenomena might be due to activation of calreticulin and hence integrin activation and this poses an interesting avenue for future research.

CHAPTER FIVE

A COMPARISON OF LFA-1 ACTIVATION INDUCED BY Mg^{2+} OR PDBU

5.1 INTRODUCTION

It is now well established that LFA-1 requires activation before it can bind to its ligands. In general stimulants which induce activation can be split into those agonists which are thought to go through the "inside-out" signal and those which exert their effects following addition to the cell exterior. The most common and widely used members of these different types of stimuli are phorbol esters and divalent cations respectively. Phorbol esters were first found to be important as inducers of integrin adhesion following early studies where they stimulated LFA-1/ICAM-1-dependent homotypic cell aggregation (Patarroyo, et al., 1985, Rothlein and Springer, 1986). The mechanism by which phorbol esters contribute to cell adhesion has not yet been fully elucidated. As phorbol esters are diacylglycerol analogues they are assumed to work through the activation of PKC and as shown in Chapter 3 PDBu-stimulated T cell adhesion to ICAM-1 is inhibited by a PKC inhibitor. The original idea that phorbol esters might be altering the phosphorylation of integrins, such as LFA-1, to mediate changes in their adhesiveness appeared to be discredited with the instrumental finding that elimination of the major phorbol ester-phosphorylation site on LFA-1, S^{756}, had no effect on the ability of the cells to adhere to ICAM-1. Rather, a string of three continuous threonines $T^{758\text{-}760}$ appeared to be important for regulation of adhesion along with F^{766} towards the C-terminal end of the cytoplasmic tail (Hibbs, et al., 1991). The phorbol ester-induced effect might not be a direct phosphorylation effect on the integrin itself but could act by an indirect mechanism such as phosphorylation of integrin-associated molecules and/or cytoskeletal proteins which might regulate integrin activity. Phorbol esters might also increase the avidity of the adhesive interaction by inducing receptor clustering which has already been shown for the β_2 family member Mac-1 (Detmers, et al., 1987) or indeed they might directly affect integrin affinity which has been shown for murine LFA-1 (Lollo, et al., 1993). In addition to mediating effects on integrins, phorbol esters could benefit adhesion by morphological changes such as spreading. This could reduce surface tension generated by shear stress in vascular flow (Lawrence, et al., 1995) or the

action of spreading could make more integrin available for an interaction with ligand (Singer, 1992).

LFA-1 can also be induced to bind ligand by incubation of cells with the divalent cations Mg^{2+} and Mn^{2+} (Dransfield, et al., 1992a, Dransfield and Hogg, 1989). Manipulation with the extracellular concentrations of divalent cations is speculated to circumvent physiological triggering procedures and directly permit integrins to bind ligand. This idea has arisen from studies on other integrins whereby cations have been shown to directly induce isolated or severely truncated receptors to bind ligand. For example, Mn^{2+} can induce isolated Mac-1 to bind fibrinogen (Altieri, 1991) and $\alpha_4\beta_1$ lacking the α subunit cytoplasmic domain can be induced to bind to VCAM-1 by Mg^{2+} (Kassner, et al., 1994). As discussed in the Introduction, divalent cations are thought to mediate these ligand binding effects by binding to several sites on the integrins. This binding might either directly co-ordinate binding of ligand and/or control access to an otherwise cryptic ligand binding site through an altered integrin conformation.

This chapter compares the effects of inducing LFA-1-mediated adhesion by these two different activation protocols, the phorbol ester, PDBu, and the divalent cation Mg^{2+}. Comparisons are made with respect to their abilities to induce both adhesion and mAb 24 epitope expression and also in their intracellular signalling requirements and the nature of the LFA-1 receptors present in both cases.

5.2 RESULTS

5.2a CATION REQUIREMENTS FOR Mg^{2+} AND PDBU-INDUCED ACTIVATION- ADHESION TO IMMOBILISED ICAM-1.

In order to define the correct cation conditions for induction of T cell adhesion to immobilised ICAM-1, following Mg^{2+} or phorbol ester stimulation, a detailed Mg^{2+} titration was performed. Mg^{2+}, when used at concentrations above 1 mM, was able to induce LFA-1-dependent adhesion in the presence of EGTA to chelate Ca^{2+} (**Figure 5.1A; EGTA**). Upon addition of 50 nM PDBu there was little enhancing effect seen over that with EGTA alone (**Figure 5.1A; EGTA +PDBu**). In confirmation of previous results in this study (Figure 4.4) and a previous one in this laboratory (Dransfield, et al., 1992a) addition of extracellular Ca^{2+} (1 mM) prevented the ability of Mg^{2+} to induce adhesion

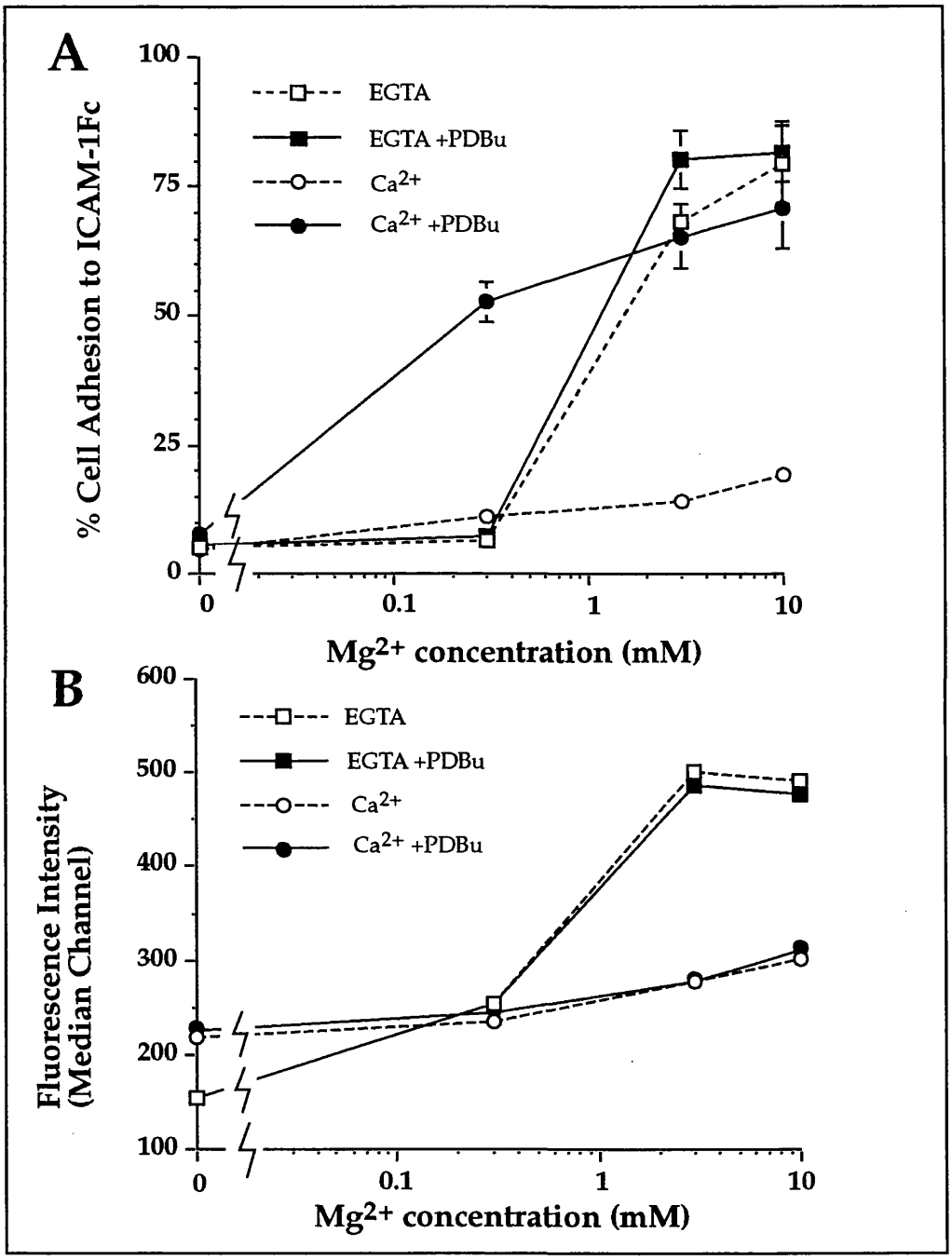

Figure 5.1: Comparison of the cation requirements for Mg^{2+} and phorbol ester induced LFA-1 activation. The cation requirements for LFA-1-mediated T cell adhesion to ICAM-1 (**A**) and mAb 24 epitope expression (**B**) were analysed as follows. Extracellular Mg^{2+} was titrated in Hepes buffer in the presence (**Ca^{2+}**) or absence (**EGTA**) of 1 mM Ca^{2+} and the same conditions were tested in the presence of 50 nM of the phorbol ester PDBu (**Ca^{2+} +PDBu**; **EGTA +PDBu**). For each experiment, data represents means of triplicates +/- standard deviation and one representative experiment of six is shown.

(**Figure 5.1A; Ca^{2+}**). This once again confirms that, in the absence of other stimuli, Mg^{2+} can only induce LFA-1-mediated adhesion when Ca^{2+} has been removed. The induction is much greater when Ca^{2+} is physically removed with a chelating agent.

When PDBu was used to induce adhesion it did not enhance adhesion seen with Mg^{2+} + EGTA; however, in the presence of extracellular Ca^{2+}, PDBu could enhance adhesion at suboptimal levels of Mg^{2+} (~0.3 mM Mg^{2+}) (**Figure 5.1A; Ca^{2+} +PDBu**). When extracellular Ca^{2+} was titrated it was found that there was a dose-dependent enhancing effect of Ca^{2+}, with half maximal adhesion reached at ~0.5 mM (data not shown). Cell adhesion was mediated by an LFA-1/ICAM-1 interaction as it was inhibited by both mAbs 38 (anti-LFA-1) and 15.2 (anti-ICAM-1) in all conditions (data not shown). Therefore, with respect to PDBu and Mg^{2+}, there are two distinct methods for inducing comparable amounts of LFA-1-mediated adhesion to ICAM-1, the PDBu protocol (using 50 nM PDBu in the presence of 1 mM Ca^{2+} and 0.3 mM Mg^{2+}) and the Mg^{2+} protocol (using 5 mM Mg^{2+} in the presence of 1 mM EGTA). In these two distinct activating procedures Ca^{2+} seems to play opposing roles.

5.2b CATION REQUIREMENTS FOR Mg^{2+} AND PDBU-INDUCED ACTIVATION- mAb 24 EPITOPE EXPRESSION.

To further investigate the characteristics of PDBu and Mg^{2+} on LFA-1 activation the next step was to analyse their effects on mAb 24 epitope expression. Similar to the adhesion result Mg^{2+}, at concentrations over 1 mM and in the absence of Ca^{2+}, induced high expression of the mAb 24 epitope on LFA-1 (**Figure 5.1B; EGTA**) and PDBu had no enhancing effect on this level (**Figure 5.1B; EGTA +PDBu**). As expected from previous studies addition of Ca^{2+} had a substantial inhibitory effect on the ability of Mg^{2+} to induce mAb 24 epitope expression (**Figure 5.1B; Ca^{2+}**; Dransfield, et al., 1992a) and this paralleled the adhesion results in Figure 5.1A.

When the PDBu protocol was assessed in the presence of Ca^{2+} there was no increase in mAb 24 epitope expression over that seen with Ca^{2+} alone (**Figure 5.1B; Ca^{2+} +PDBu**). Therefore, in this activating system, the positive effect of PDBu on cell adhesion is not mediated through an increase in the expression of the epitope recognised by mAb 24. These results show a discrepancy

between adhesion and 24 epitope expression (compare **Figure 5.1A** and **B**; **Ca^{2+} +PDBu**) and suggest that the Mg^{2+} and PDBu protocols do not promote adhesion by the same means. One explanation for this difference could be in the nature of the LFA-1 receptors present under both conditions. At this point reference should be made to the apparently contradictory result obtained here regarding the induction of 24 epitope expression compared to that previously presented in Chapter 3 (Figure 3.4). In Figure 3.4 it is obvious that PDBu induces a small increase in 24 epitope expression, although it is only a proportion of the shift seen with Mg^{2+}, Mn^{2+} or KIM185 (Figures 3.5, 3.6, 3.7). This phenomenon will be discussed in more detail in Chapter 6. For the present study it should be reiterated that when mAb 24 epitope expression is evaluated in cation controlled HEPES buffer that PDBu does not significantly enhance its expression over basal levels.

5.2c COMPARISON OF Mg^{2+} AND PDBU-INDUCED ACTIVATION-BINDING TO SOLUBLE ICAM-1.

To further define the characteristics of the LFA-1 receptors activated following the two different stimulating protocols and to shed some light on the possible reason for the discrepancy between adhesion and mAb 24 results, T cells were stimulated and examined for their ability to bind recombinant soluble (s)ICAM-1. The sICAM-1 is the same dimeric ICAM-1Fc protein which is used for the cell adhesion assays although in these experiments it is presented to the cells in soluble form. The sICAM-1 bound to LFA-1 following maximal Mg^{2+}/EGTA stimulation with saturated binding reached at ~400 μg/ml or ~1.8 μM (see Chapter 6, section 6.2g for details). This saturating concentration of sICAM-1 was incubated with either Mg^{2+} or PDBu-stimulated cells and analysed for its ability to bind to the LFA-1 molecules present on the cell membranes. Binding was detected using a FITC conjugated secondary Ab specific for the human Fc tail. **Figure 5.2** shows that sICAM-1 bound to Mg^{2+}-stimulated cells (**EGTA**) but not to cells stimulated according to the PDBu protocol (**Ca^{2+} +PDBu**). A control protein comprising another immunoglobulin supergene family member CD14 fused to the same human Fc tail did not bind to the cells under identical stimulating conditions and addition of mAb 38 blocked the binding of sICAM-1 to the Mg^{2+}-stimulated cells confirming the specificity of the reaction for LFA-1/ICAM-1 (data not shown). The ability to bind soluble ligand is considered a measure of increased affinity of integrin receptors; therefore it seems that Mg^{2+}

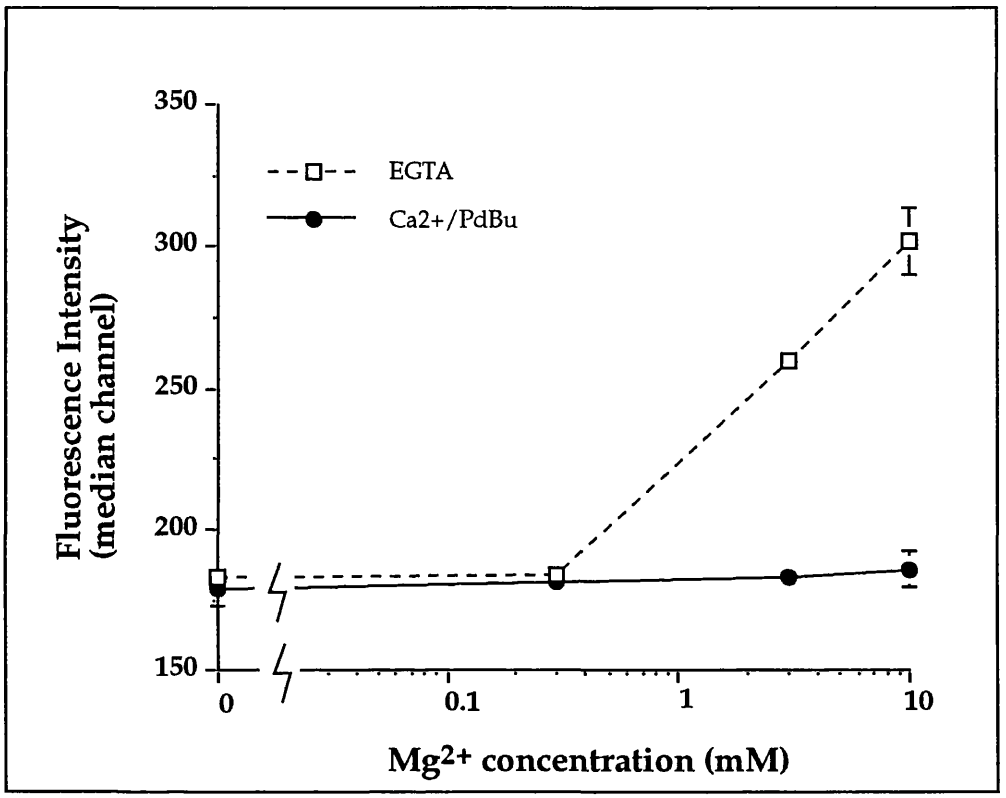

Figure 5.2: The ability of Mg^{2+} and phorbol ester to induce soluble ICAM-1 binding. The two activation protocols; Mg^{2+} in the presence of 1 mM EGTA (**EGTA**) and 50 nM PDBu in the presence of 1 mM Ca^{2+} (**Ca^{2+} +PDBu**) were examined for their ability to bind 400 µg/ml soluble ICAM-1Fc. This concentration of ICAM-1Fc was found to be saturating (See Chapter 6). Binding was detected by addition of a FITC-conjugated goat anti-human Fc specific antibody (10 µg/ml) and analysis on the FACScan. Results are expressed as means of triplicates+/- standard deviation of median fluorescence channel and one experiment of three is shown.

induces higher affinity LFA-1 receptors for ICAM-1 whereas PDBu does not. When mAb 24 epitope expression and sICAM-1 binding curves are compared (**Figures 5.1B and 5.2**) it is evident that 24 epitope is expressed under conditions which support sICAM-1 binding indicating that mAb 24 epitope expression correlates with the presence of high affinity LFA-1 receptors.

5.2d THE ABILITY OF sICAM-1 TO BLOCK Mg^{2+}- OR PDBU-STIMULATED ADHESION TO IMMOBILISED ICAM-1.

Although the phorbol ester protocol appeared not to induce detectable numbers of high affinity receptors, it was possible that a small number of high affinity receptors were present and that these had a critical role in initiating adhesion. Therefore it was important to investigate whether sICAM-1 could block the adhesion of T cells to immobilised ICAM-1 following stimulation by both protocols. Incubation of T cells with an optimal concentration of 400 μg/ml sICAM-1 could substantially inhibit Mg^{2+}-stimulated adhesion to immobilised ICAM-1 (**Figure 5.3; EGTA**). This illustrates that Mg^{2+}-stimulated cells have a dependence on high affinity LFA-1 receptors for adhesion to ICAM-1. In contrast, cells stimulated by the phorbol ester protocol were not inhibited by the addition of sICAM-1 (**Figure 5.3; Ca^{2+} +PDBu**). This confirms that phorbol ester stimulated T cells do not rely on even a small percentage of high affinity receptors for initial adhesion to ICAM-1 and adhesion must occur through other means.

5.2e MORPHOLOGICAL ANALYSIS OF Mg^{2+}- AND PHORBOL ESTER-STIMULATED ADHESION.

In an attempt to elucidate the means by which phorbol ester-stimulated cells adhered to ICAM-1, cells were subject to morphological analysis. Using TRITC-conjugated phalloidin to examine the distribution of polymerised actin, cell morphology was analysed by fluorescence microscopy. The majority of T cells which adhered to ICAM-1 via the Mg^{2+} protocol had a rounded appearance (**Figure 5.4A**), whereas the phorbol-ester stimulated cells appeared flattened and spread on ligand (**Figure 5.4B**). This observation suggests that when T cells are stimulated with phorbol ester, spreading is somehow instrumental in promoting LFA-1-dependent adhesion.

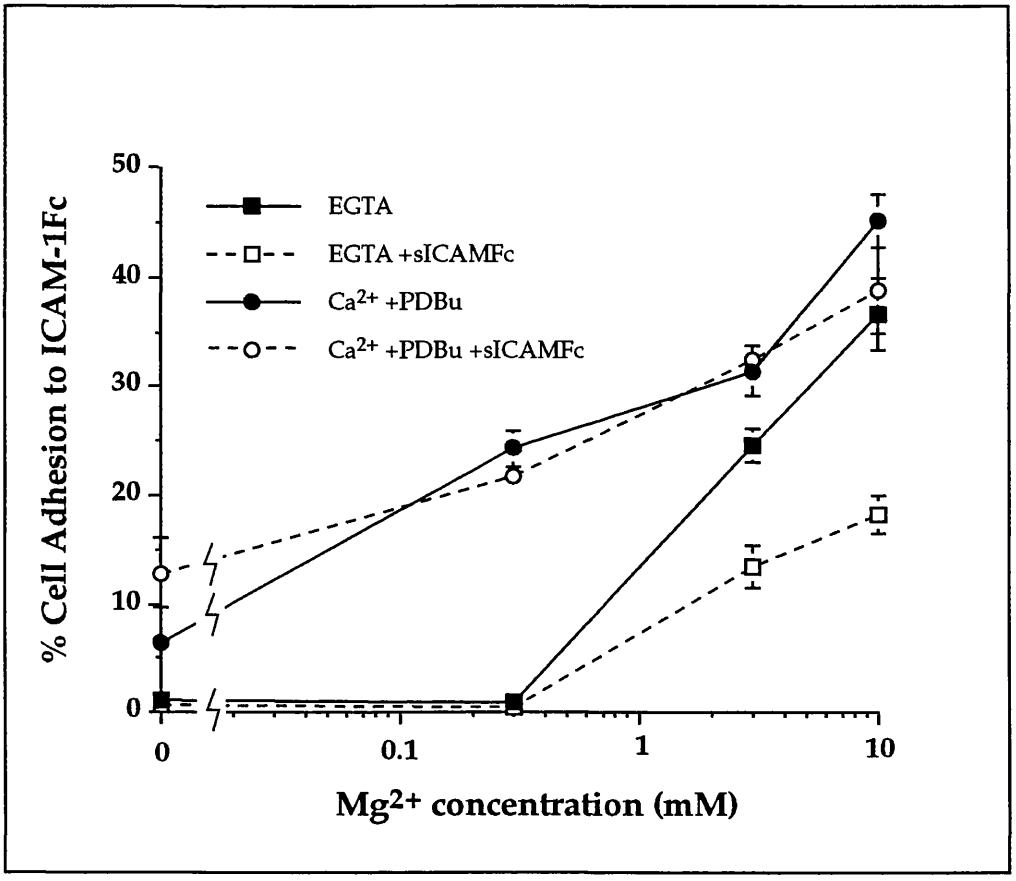

Figure 5.3: The effect of soluble ICAM-1 on Mg^{2+}- and phorbol ester-stimulated T cell adhesion to immobilised ICAM-1. Cells stimulated with the Mg^{2+} (**EGTA**) or phorbol ester (**Ca^{2+} +PDBu**) protocol were tested for adherence to immobilised ICAM-1 (0.12 µg/ml) in the presence or absence of 400 µg/ml sICAM-1Fc across a Mg^{2+} titration range. Results are shown as the mean of triplicates +/- standard deviation. One representative experiment of two is shown.

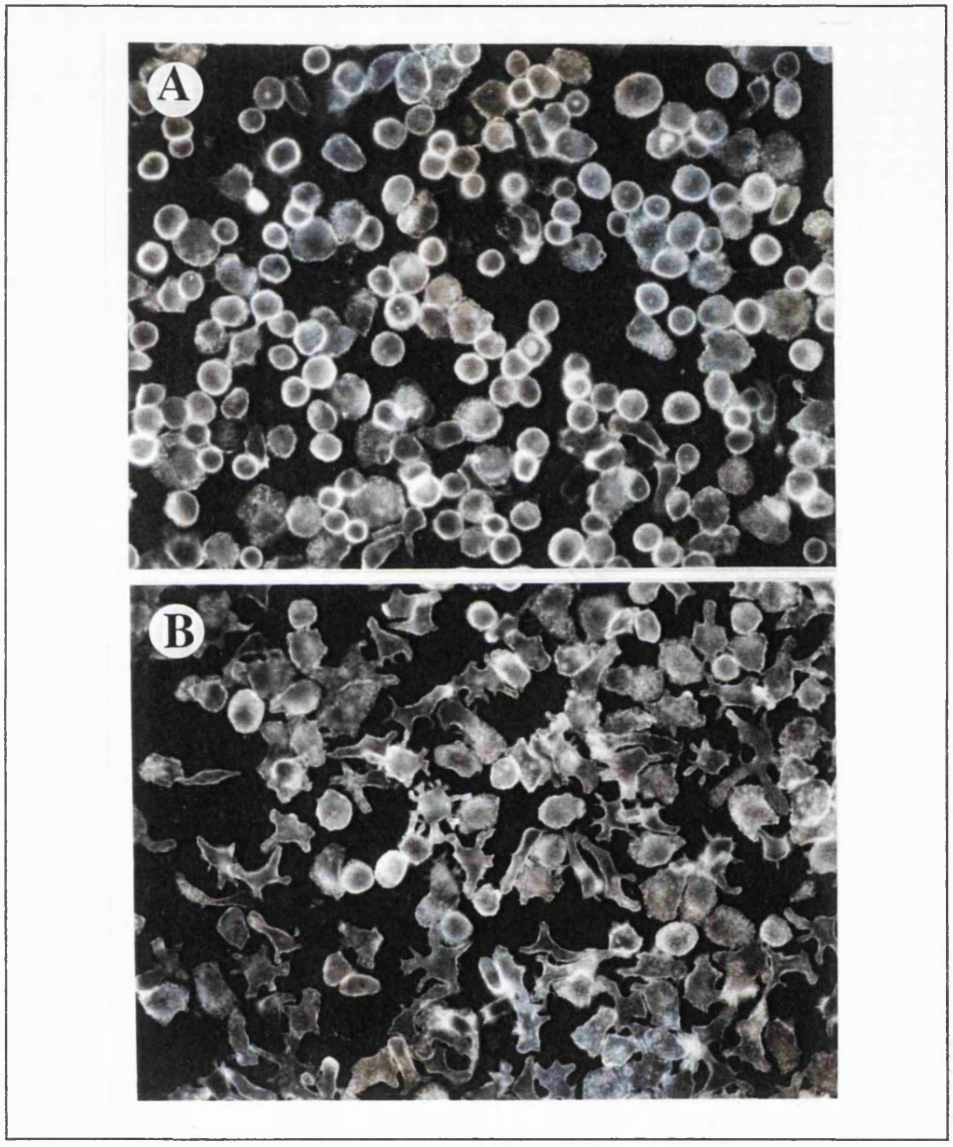

Figure 5.4: T cells adherent to ICAM-1 show different morphology following the Mg^{2+} or phorbol ester treatments. Cells were induced to adhere to ICAM-1Fc-coated coverslips under the Mg^{2+} (5 mM Mg^{2+}, 1 mM EGTA; **A**) or phorbol ester (50 nM PDBu, 1 mM Ca^{2+} +0.3 mM Mg^{2+}; **B**) protocols, then fixed, stained with TRITC-phalloidin and analysed by fluoresecence microscopy.

5.2f EFFECT OF CYTOCHALASIN D ON Mg^{2+}- AND PDBU-STIMULATED ADHESION.

Cell spreading is dependent on reorganisation of the cytoskeleton through actin polymerisation events. It was therefore pertinent to examine the effects of cytochalasin D, which inhibits actin polymerisation, on adhesion induced by the two different protocols. 2 μM cytochalasin D could inhibit the potentiating effect of phorbol ester on T cell adhesion, whereas there was much less effect on adhesion induced by the Mg^{2+} protocol (**Figure 5.5A**). This mirrors the earlier comparison that PDBu induced adhesion was much more susceptible than Mg^{2+} to the effects of cytochalasin D (Chapter 4; Figure 4.10) and confirms that these two different protocols show differential susceptibility to cytochalasin D. Cytochalasin D had no inhibitory effect on the ability of Mg^{2+} to induce expression of the mAb 24 epitope (**Figure 5.5B**) or sICAM-1 binding (data not shown). This confirms that cytoskeletal reorganisation leading to cell spreading is an essential component for cell adhesion of phorbol ester-treated cells but not for the induction of high affinity LFA-1 receptors from the cell exterior by Mg^{2+} treatment.

5.2g THE ROLE OF PKC AND $[Ca^{2+}]_i$ IN PDBU AND Mg^{2+}-STIMULATED ADHESION TO IMMOBILISED ICAM-1.

As the phorbol ester protocol included the addition of both PDBu and Ca^{2+} it was obvious to assess the requirement for both PKC and intracellular Ca^{2+} ($[Ca^{2+}]_i$) following the two different stimulating protocols. As previously shown in Chapter Three (Figure 3.4) the PKC inhibitor Ro 31-8220 was shown to inhibit phorbol ester stimulated adhesion to ICAM-1, however it was also important to assess its effects following this slightly different PDBu protocol. **Figure 5.6A** shows that adhesion stimulated through the phorbol ester protocol was inhibited by 1 μM Ro 31-8220, whereas there was no inhibitory effect on the Mg^{2+}-induced protocol. This confirms that PDBu operates through the activation of PKC and that for Mg^{2+}-stimulated cells adhesion is PKC-independent. A possible involvement for $[Ca^{2+}]_i$ was assessed using the acetoxymethyl ester BAPTA/AM. This compound is cell permeable in its ester form and once inside the cell hydrolysis yields BAPTA which is membrane impermeable Ca^{2+} chelator. In this way, BAPTA only mops up intracellular Ca^{2+} allowing the effect of decreasing $[Ca^{2+}]_i$ to be investigated. When the cells were pretreated with 50 μM BAPTA for 30 min prior to the adhesion assay the PDBu stimulation protocol was not very effective in

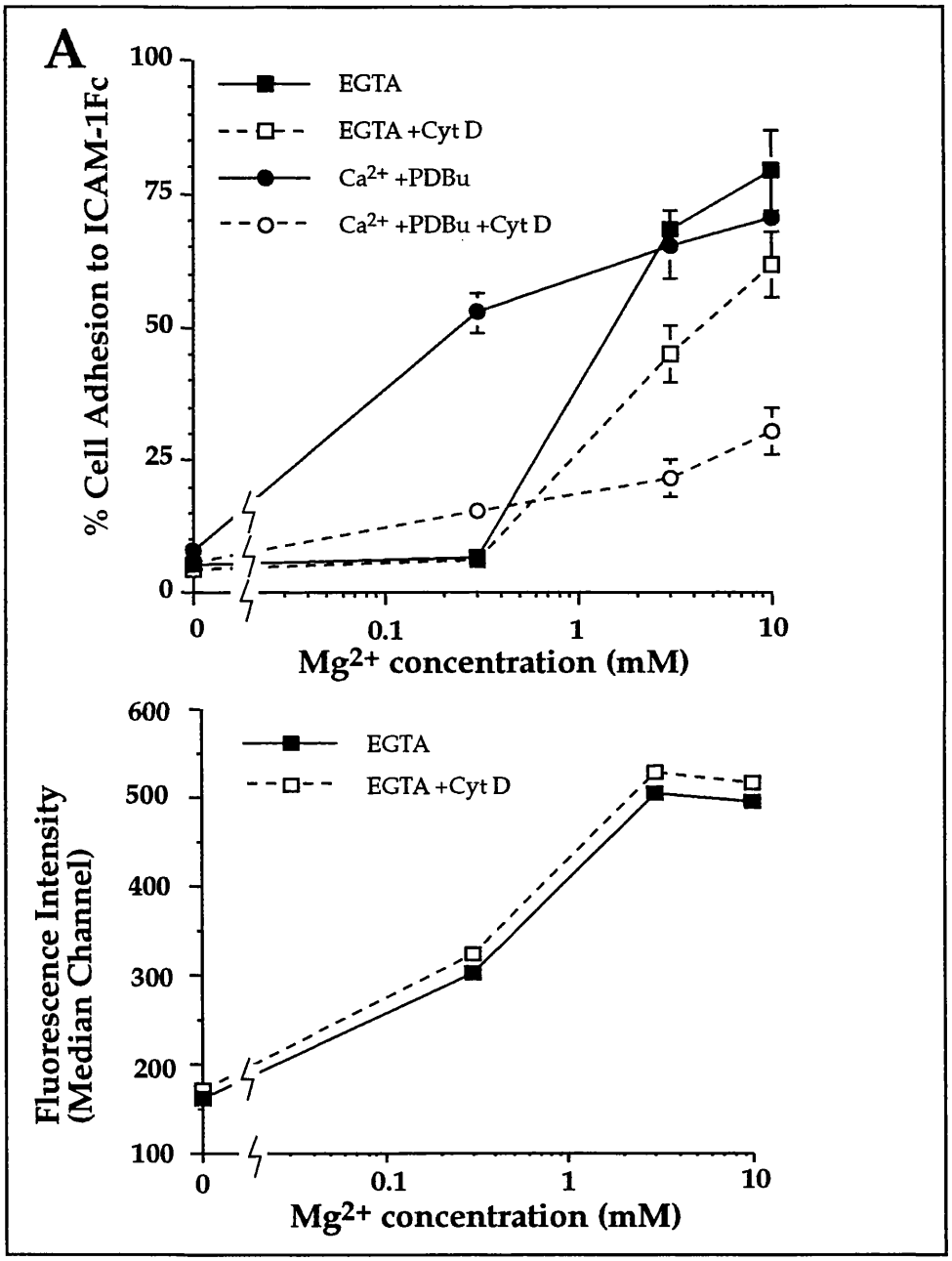

Figure 5.5: Adhesion induced by Mg^{2+} or PDBu shows differential susceptibility to cytochalasin D. Cells were analysed for adherence to ICAM-1Fc (**A**) or mAb 24 epitope expression (**B**) following the Mg^{2+} (**EGTA**) or phorbol ester (**Ca^{2+} +PDBu**) protocols in the presence or absence of 2 μM cytochalasin D (Cyt D). Data represents means of triplicates +/- standard deviation and for the adhesion assay, one representative experiment of five is shown and for the 24 expression assay one representative experiment of three is shown.

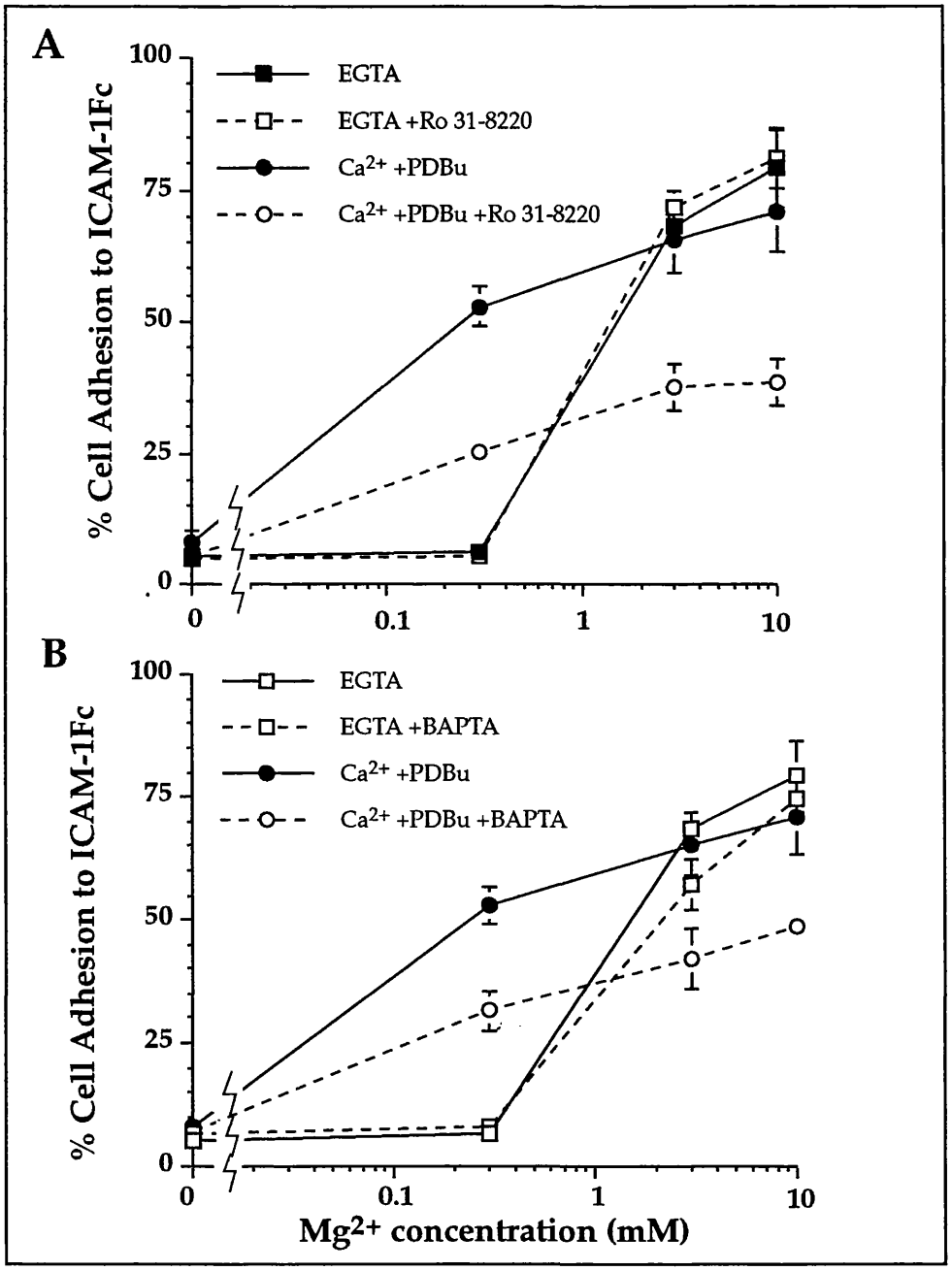

Figure 5.6: The effect of the PKC inhibitor Ro 31-8220 and intracellular Ca^{2+} chelator BAPTA on Mg^{2+}- and phorbol ester stimulated adhesion. Cells were pretreated with either 1 μM Ro 31-8220 (**A**) or 50 μM BAPTA (**B**) for 30 min at 37 °C before being induced to bind to immobilised ICAM-1 following the Mg^{2+} (**EGTA**) and phorbol ester (**Ca^{2+} +PDBu**) protocols. Data represents means of triplicates +/- standard deviation and one representative experiment of five is shown for each graph.

inducing adhesion whereas the Mg^{2+}-stimulated cells retained their ability to adhere (**Figure 5.6B**). When assessed microscopically the BAPTA-treated phorbol ester-stimulated cells exhibited a change from the normal spread morphology (**Figure 5.7A**) to a more rounded appearance (**Figure 5.7B**). These results indicate that Ca^{2+} plays an intracellular role to facilitate adhesion through cell spreading when cells are stimulated with phorbol ester.

5.2h ADHESION INDUCED BY CD3 CROSSLINKING- WHICH MODEL DOES IT RESEMBLE?

The two stimulatory protocols which have been examined in this Chapter have shown that LFA-1 mediated adhesion occurs through distinct mechanisms. Stimulation with the phorbol ester PDBu induces T cell adhesion to immobilised ICAM-1 by means of low affinity receptors and adhesion is facilitated through cell spreading which is dependent on actin polymerisation, PKC and intracellular Ca^{2+}. In contrast, a high concentration of the divalent cation Mg^{2+} induces a similar level of adhesion by direct induction of high affinity LFA-1 receptors. High affinity state is measured by the ability to bind sICAM-1 and mAb 24 epitope expression correlates well with this state. One caveat of the two activation methods which have been examined here is that they do not make use of cell surface receptors or agonists through which cells would normally be initially triggered *in vivo*. Therefore to assess the credibility of these model systems, it was imperative to examine similar parameters following stimulation of T cells by a more physiological stimulus, such as TCR/CD3 crosslinking (XLCD3). When cells were stimulated with an anti-CD3 mAb, UCHT1, they could not be induced to bind sICAM-1 (**Figure 5.8A**) in spite of the fact that they could adhere sufficiently to immobilised ICAM-1 (see **Figure 5.8B**). Titration of sICAM-1 up to 1000 μg/ml still failed to show specific binding to LFA-1. This shows that induction of T cell adhesion to immobilised ICAM-1, following TCR/CD3 triggering, does not occur through the induction of high affinity LFA-1 receptors in the first instance. As this method of stimulation is also susceptible to 2 μM cytochalasin D inhibition (see **Figure 5.8B**) and the cells display a spread morphology once adherent (data not shown) it appears that LFA-1-mediated adhesion via CD3 crosslinking resembles the phorbol ester model of activation in a number of ways. This raises a very interesting phenomenon in which stimulation through the most physiological stimulus does not appear to generate high affinity receptors and adhesion is instead

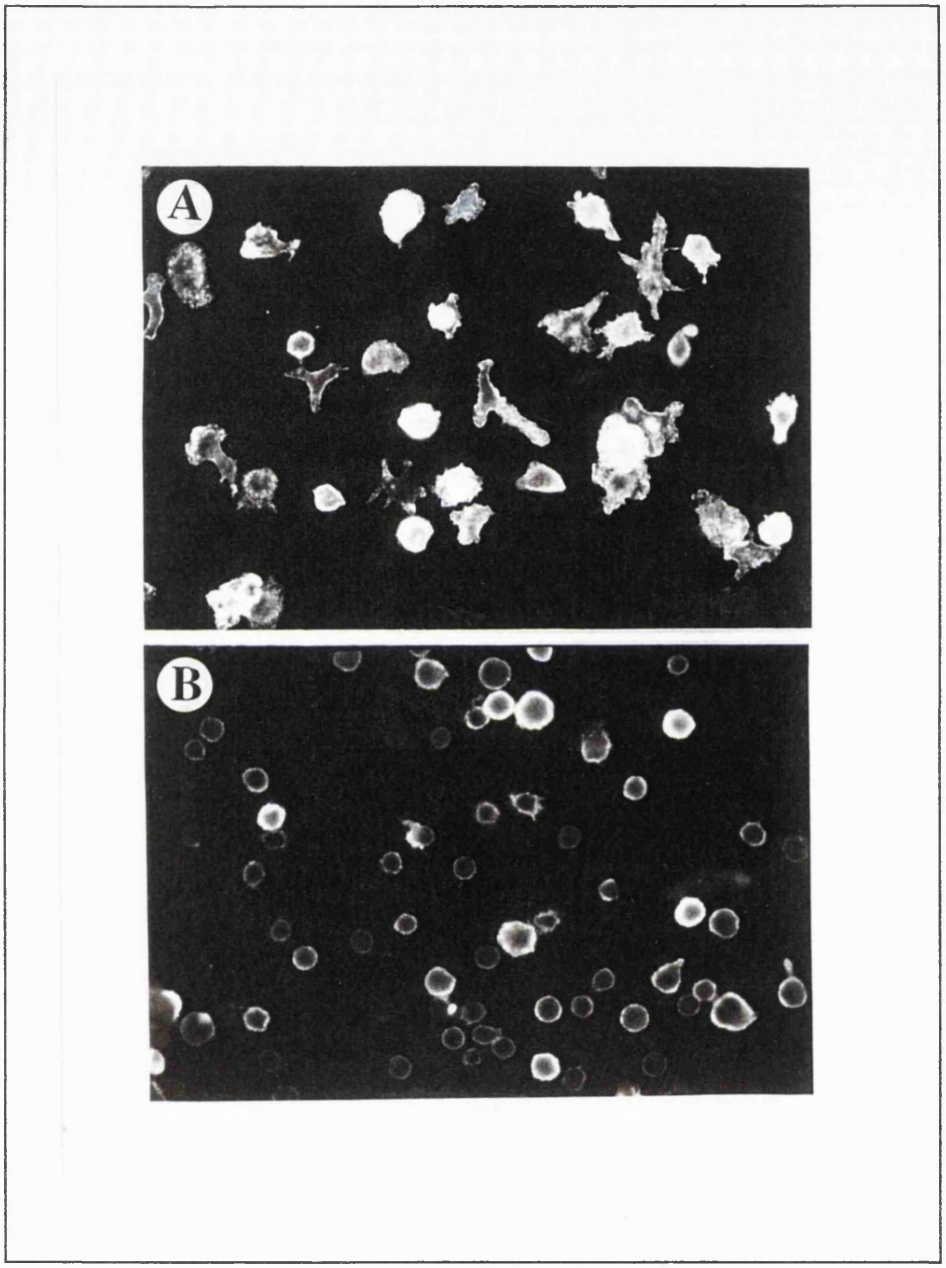

Figure 5.7: The inhibitory effect of BAPTA on PDBu-induced cell spreading. T cells were induced to adhere to ICAM-1 according to the phorbol ester protocol (HEPES buffer containing 50 nM PDBu, 1 mM Ca^{2+} and 0.3 mM Mg^{2+}) without (**A**) or with (**B**) pretreatment with the intracellular Ca^{2+} chelator BAPTA (50 μM) for 30 min at 37°C. Adherent cells were fixed, permeabilised and stained with TRITC-phalloidin and analysed by fluorescence microscopy.

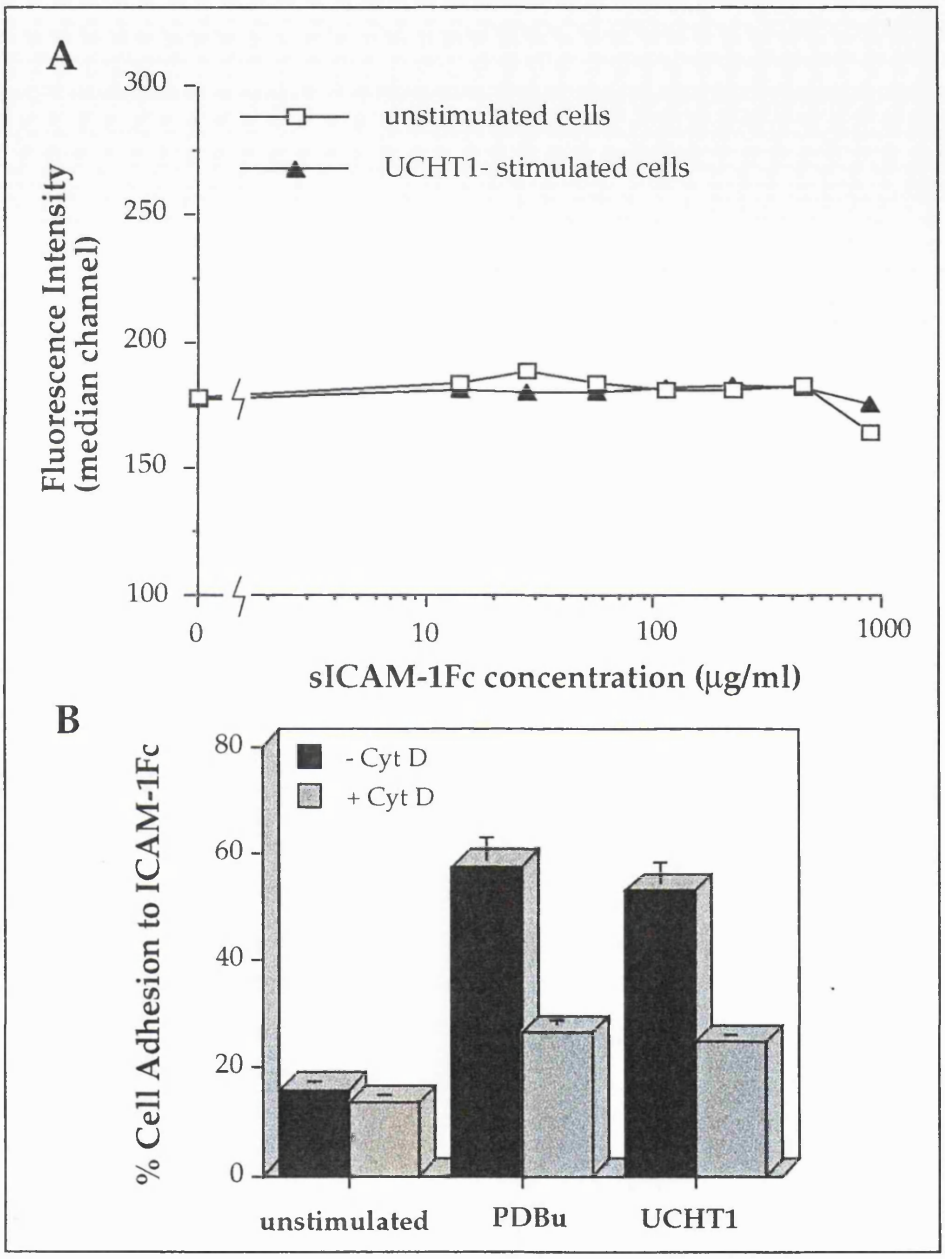

Figure 5.8: Triggering the TCR/CD3 complex induces cytochalasin-D sensitive adhesion to immobilised ICAM-1 but not binding of sICAM-1. (**A**) T cells were either unstimulated or incubated with 10 µg/ml of the CD3 mAb UCHT1 and examined for their ability to bind increasing concentrations of sICAM-1Fc. Results are shown as median fluorescence intensity. (**B**) T cells were induced to adhere to immobilised ICAM-1Fc by 50 nM PDBu or 10 µg/ml UCHT1 (in RPMI) in the presence or absence of 2 µM cytochalasin D (Cyt D). Data are expressed as means of triplicates +/- standard deviation.

dependent on intracellular events. These events lead to morphological changes such as spreading which are beneficial to the adhesive state.

5.2i ADHESION INDUCED BY THE INTRACELLULAR Ca^{2+}-MOBILISERS- WHICH MODEL DOES IT RESEMBLE?

The next obvious question to ask is what happens when cells are induced to adhere to ICAM-1 through the stimulants whose primary effect is to raise $[Ca^{2+}]_i$, such as ionomycin, thapsigargin and dBHQ? To review information presented in Chapter Four, these stimulants did not induce their adhesive effects by feedback activation of PKC but adhesion was particularly susceptible to cytochalasin D inhibition. This might suggest that they exert their affects by cell spreading although not in a PKC-dependent manner. When the morphology of the cells was examined by confocal microscopy the cells were found to be very round (**Figure 5.9; ionomycin B, thapsigargin C** and **dBHQ D**). They observed no gross morphological alterations from unstimulated cells especially with respect to spreading or flattening down onto ligand as is seen following PDBu stimulation (**Figure 5.9A**). This appears to be quite contradictory in that the cells are susceptible to cytochalasin D inhibition and yet they do not appear to be spread or morphologically altered once bound. When they were examined for their ability to induce binding of sICAM-1, none of the Ca^{2+}-mobilisers could induce binding even at the highest sICAM-1 concentration tested which shows good interaction with Mg^{2+}-stimulated cells (**Figure 5.10**). This results proposes that ionomycin, thapsigargin and dBHQ do not exert their affects on adhesion by inducing high affinity LFA-1 receptors and yet neither do they exhibit gross morphological changes when binding. The fact that adhesion is susceptible to cytochalasin D suggests that some kind of cytoskeletal alterations are taking place but not the same ones that occur following PDBu and XLCD3-stimulation. Cytochalasin D affects different cytoskeletal structures depending on the particular concentration used. When used at less than 2 μM cytochalasin D inhibits membrane ruffling but higher concentrations (greater than 2 μM) are required for inhibition of stress fibre formation (Yahara, et al., 1982). Due to the enhanced sensitivity of ionomycin and thapsigargin- induced LFA-1 activation to cytochalasin D compared to PDBu or XLCD3-stimulated adhesion, it could be that more subtle cytoskeletal effects are evident in cells following stimulation by these agents.

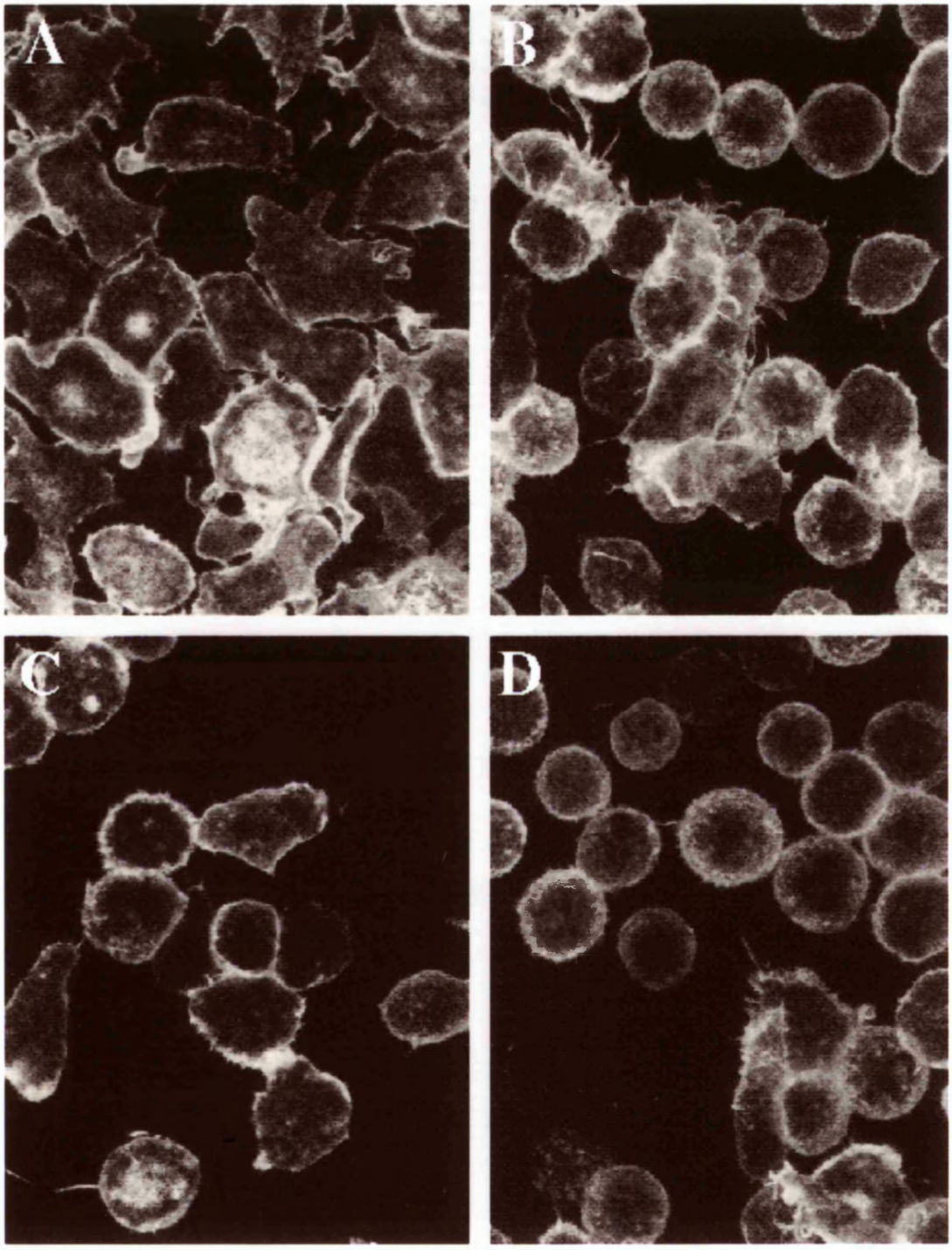

Figure 5.9: Analysis of the morphology of T cells stimulated to adhere to ICAM-1 following PDBu, ionomycin, thapsigargin and dBHQ treatments. T cells were induced to bind to ICAM-1 Fc-coated coverslips with 50 nM PDBu (**A**), 0.7 μM ionomycin (**B**), 5 μM thapsigargin (**C**) and 50 μM dBHQ (**D**) for 30 min at 37 C. Bound cells were fixed, permeabilised, stained with TRITC-phalloidin and analysed morphologically by confocal microscopy.

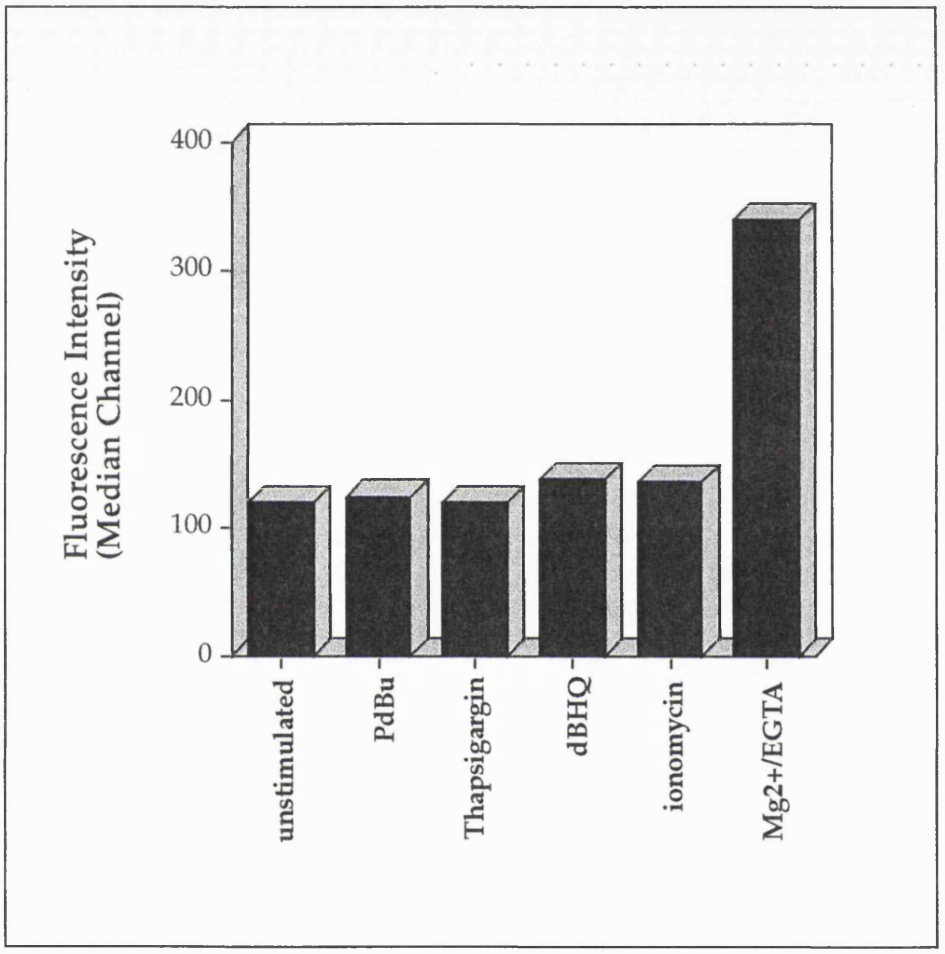

Figure 5.10: The $[Ca^{2+}]_i$ mobilisers ionomycin, thapsigargin and dBHQ do not induce binding of sICAM-1. T cells were stimulated with 50 nM PDBu, 5μM thapsigargin, 50 μM dBHQ, 0.7 μM ionomycin, or 5 mM Mg^{2+}/1 mM EGTA then analysed for their ability to bind 400 μg/ml sICAM-1Fc following a 30 min incubation at 37°C. Binding of sICAM-1 was detected using a FITC-conjugated goat anti-human Fc specific antibody and analysed by flow cytometry.

5.2j IS THE INDUCTION OF LFA-1-MEDIATED ADHESION DEPENDENT ON THE SMALL GTP-BINDING PROTEIN *RHO*?

In an attempt to shed some light on the matter and gain an understanding of additional intracellular mediators which might be responsible for stimulation by any of these individual protocols it was decided to examine a possible role for the small GTP- binding protein *rho* in the activation process. There are over 50 members of the ras superfamily of small GTP-binding proteins (reviewed in Hall, 1994). These are further divided into smaller subfamilies named ras, rho, rab, ARF and ran. These proteins all act as molecular switches depending on the status of their bound nucleotides; they are active in the GTP-bound state and inactive when GDP (guanosine diphosphate) is bound. Members of the individual subfamilies have different cellular effects. The ras family members control cell growth and differentiation by regulating the activity of the MAP kinase cascade. The rab and ARF subfamily regulate intracellular vesicular transport and ran controls protein and RNA transport across the nuclear membrane. The mammalian rho subfamily so far consists of five distinct proteins rho, rac, CDC42, TC10 and rho G. For some of these proteins there exists several isotypes. The rho subfamily has received intense interest recently with the finding that several of its members are responsible for organisation of the cytoskeleton and maintenance of cell shape. This was first found by microinjection of constitutively active rho into fibroblasts which caused the formation of stress fibres and focal contacts (Ridley and Hall, 1992). Introduction of active rac caused membrane ruffling (Ridley, et al., 1992) and much more recently active CDC42 induced filopodia formation in fibroblasts (Nobes and Hall, 1995). These different proteins are thought to act in a molecular cascade with CDC42 activating rac and subsequently rho. Since these proteins have morphological effects on cells they are good candidates to assess in integrin activation studies. During the course of this work a study appeared which documented the importance of rho in the LFA-1/ICAM-1-dependent homotypic aggregation of the JY B lymphoblastoid cell line (Tominaga, et al., 1993). This study utilised the *Clostridium botulinum* exoenzyme C3 transferase to inactivate rho and showed that it inhibited B cell homotypic aggregation. C3 transferase works by specifically ADP ribosylating rho at an asparagine residue (N^{41}) in the putative effector domain which presumably prevents interaction with downstream effector molecules (Narumiya and Morii, 1993). C3 also inhibits NK- and CTL-mediated cytotoxicity implicating a role for rhoA in either target recognition

or exocytosis of cytolytic granules (Lang, et al., 1992). Therefore it seemed an obvious move to test the effect of C3 on stimulation of T cell LFA-1 activation.

A construct encoding a C3 transferase GST fusion protein was kindly made available by Dr. Marc Symons, Onyx Pharmaceuticals, USA which was transformed into bacteria, grown up and purified. On preliminary testing, this enzyme was incubated with the T cells for 24 hr prior to the adhesion assay in the hope that it would passively diffuse into the cell cytoplasm or be taken up by pinocytosis as performed in the B cell homotypic aggregation assays (Tominaga, et al., 1993). The initial results showed that this enzyme caused no substantial inhibition of adhesion stimulated by either PDBu or PMA (**Figure 5.11**). Other reports in the literature have shown that C3 exoenzyme does not penetrate cytotoxic T or NK leukocyte membranes passively nor can it be induced to enter cells by stimulated pinocytosis. Instead it requires permeablisation or electroporation to get into the cells (Lang and Bertoglio, 1995). However, Tominaga and colleagues incubated their cells with the transferase for 24 hr and saw inhibition of JY cell adhesion. To check that the enzyme preparation was functional it was examined for its ability to inhibit JY cell homotypic aggregation following extracellular incubation. Results showed that it did have an inhibitory effect on JY cell adhesion but relatively little effect on inhibition of T cell homotypic aggregation (data not shown). This seemed to indicate that the preparation was functional and that either it could not traverse the T cell membrane as well as that of the B cell or that the enzyme was not involved in LFA-1 regulation on T cells. A previous report suggests that C3 cannot penetrate the cytotoxic T cell membrane; thus before rho is implicated or ruled out of LFA-1 activation on T cells, electroporation of C3 will be tried. Simultaneously the activity of the C3 preparation will be tested for its ability to ADP ribosylate rho in T cells in a ^{32}P[NAD] ADP ribosylation assay. These will be the avenues for future research on this topic.

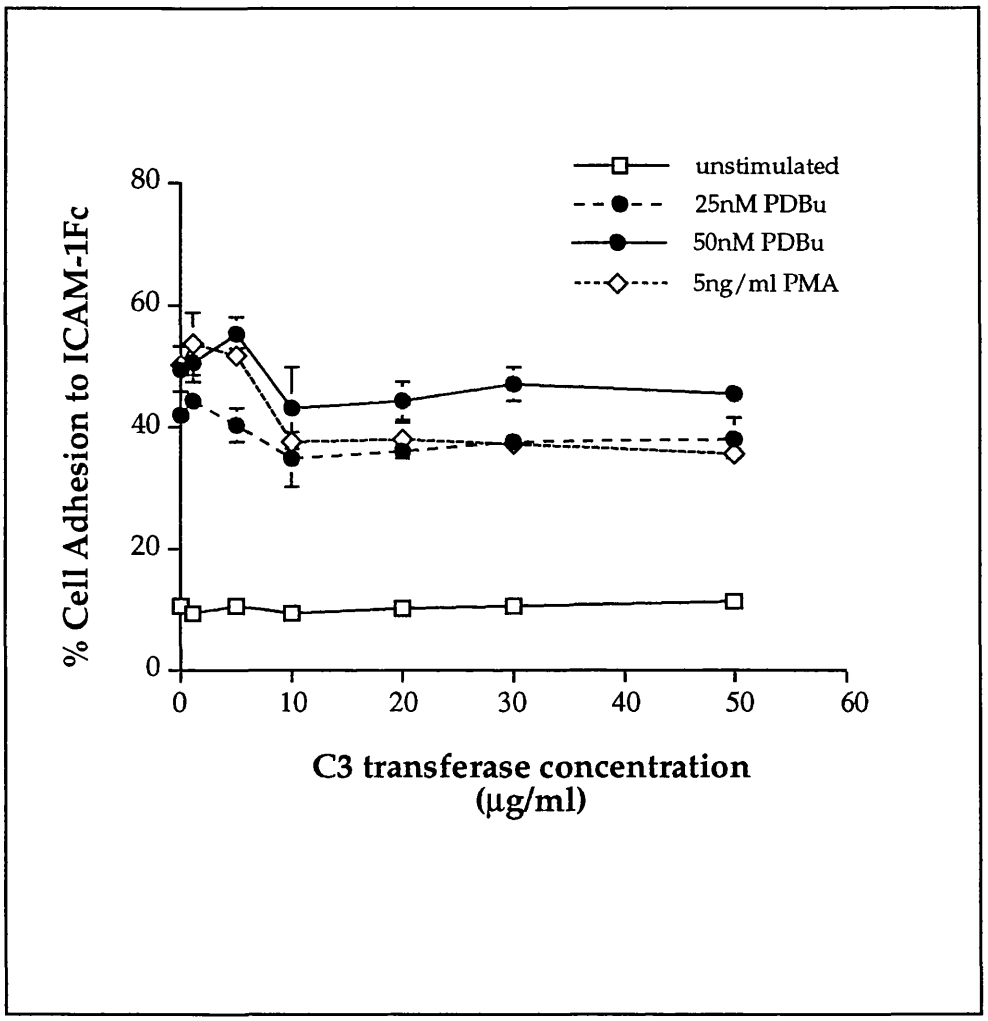

Figure 5.11: The effect of *Clostridium botulinum* C3 transferase on phorbol ester-induced adhesion to ICAM-1. T cells were pretreated with the indicated concentrations of C3 transferase for 24 hr prior to stimulation with either 25 nM PDBu, 50 nM PDBu or 5 ng/ml PMA. Cells were assessed for adhesion to ICAM-1Fc following removal of non-adherent cells. Data is expressed as means of triplicates +/- standard deviation and one representative experiment of two is shown.

5.3 DISCUSSION

Through a comparison of two distinct, but well recognised, laboratory models for stimulating T cell mediated adhesion to ICAM-1 it is shown that adhesion occurs by very different means. When stimulated with high concentrations (> 1 mM) of the divalent cation Mg^{2+}, in the absence of Ca^{2+}, adhesion is facilitated through direct induction of high affinity receptors and is not so dependent on major intracellular signalling events. In contrast, stimulation with the phorbol ester PDBu, in the presence of Ca^{2+}, induces adhesion through low affinity receptors. In this situation adhesion is facilitated by spreading which is dependent on cytoskeletal alterations, PKC and intracellular Ca^{2+}. When a more physiological stimulant, TCR/CD3 triggering, was tested for similar characteristics adhesion resembled that of phorbol ester-treated cells in that high affinity receptors were not induced and adhesion was facilitated through cytoskeletal events enhancing spreading. This result shows that stimulants which induce integrin activation do not always do so by direct induction of high affinity receptors.

The high affinity receptors generated by Mg^{2+} stimulation facilitate stable cell adhesion without the requirement for actin reorganisation and intracellular mediators such as PKC and $[Ca^{2+}]_i$. This result is consistent with the thought that Mg^{2+} exerts its effects by interaction with the extracellular portion of the integrin. This parallels many other studies where isolated integrin molecules or those with truncated cytoplasmic domains retain the ability to be stimulated by divalent cation (Altieri, 1991; Kassner, et al., 1994).

The assessment of high affinity receptors came through the use of sICAM-1 binding. It was previously thought that LFA-1 receptors were of such low affinity that it would be impossible to measure sICAM-1 binding. Success in this instance may be explained by the use of large concentrations of recombinant protein in order to detect binding. In fact since this work was performed a study was published illustrating sICAM-1-blocking of LFA-1-mediated adhesion to immobilised ICAM-1 but only at very high sICAM-1 concentrations (Meyer, et al., 1995). Alternatively, detection of sICAM-1 binding may have been facilitated by the dimeric nature of the ICAM-1 construct, due to disulphide bonding through a cysteine residue in the Fc tail, providing a greater opportunity for stable ligand interaction. This form of ICAM-1 appears to have physiological relevance in that the majority of native

membrane-bound and shed ICAM-1 has recently been demonstrate by several groups to exist in dimerised form (Miller, et al., 1995, Reilly, et al., 1995). The ability of Mg^{2+}-stimulated cells to bind sICAM-1 was mirrored by their ability to express the activation reporter epitope recognised by mAb 24. From this correlative expression, mAb 24 must recognise the high affinity LFA-1 receptor state. Further investigation into the characteristics of these receptors is presented in Chapter 6.

The nature of the receptors mediating adhesion to immobilised ICAM-1 was further examined by investigating the ability of sICAM-1 to block the adhesive interaction. This was performed to assess whether in the case of phorbol ester stimulation that there were perhaps a small percentage of high affinity LFA-1 receptors, undetectable in the assay system, which might have been responsible for mediating the initial adhesion. As soluble ICAM-1 could only block binding of Mg^{2+}-stimulated cells this result confirmed that high affinity receptors were responsible for adhesion following Mg^{2+} stimulation but not phorbol ester. As the phorbol ester PDBu-stimulated adhesion was not blocked by incubation with sICAM-1 it can be concluded that the receptors responsible for the initial adhesion are not of high affinity. Similar conclusions were reached in study of the effects of phorbol ester and the activating mAb 8A2 on $\alpha_5\beta_1$- mediated adhesion to fibronectin (Faull, et al., 1994). In this model the activating mAb could induce high affinity $\alpha_5\beta_1$ integrins but the phorbol ester PMA could not. In apparent contrast, another study found that PMA increases the affinity of 15-30% of cell surface murine LFA-1 in a competitive affinity assay using an anti-LFA-1 mAb (Lollo, et al., 1993). The reason for this apparent contradiction is not yet known but it could involve the activation state of the cells, species differences or the inclusion of extra Mg^{2+} in the Lollo *et al.* assay system.

Therefore in the absence of high affinity receptors, what is mediating the adhesion following phorbol ester stimulation? Using a variety of intracellular inhibitors it was found that phorbol ester stimulated adhesion had a requirement for cytoskeletal-induced cell spreading, PKC and intracellular Ca^{2+}. The exact interplay between these factors is not yet understood but another link between phorbol ester and the cytoskeleton comes from recent evidence using CHO cell transfectants where the TTT motif in the β_2 subunit cytoplasmic tail has a direct role in phorbol ester induced cell spreading through actin reorganisation (Peter and O'Toole, 1995). The $[Ca^{2+}]_i$

requirement could simply be required to ensure efficient activation of certain Ca^{2+}-dependent PKC isoenzymes or it could also potentially directly affect cytoskeletal changes through interaction with actin binding proteins (Stossel, 1989; Downey, et al., 1990). It is possible that these factors might converge on a specific signalling molecule or pathway for example on the small GTP-binding protein rho. Although preliminary studies showed no effect of the C3 transferase from *Clostridium botulinum*, this avenue must be further investigated especially as T cells are thought to be more resistant to passive entry of the exoenzyme. In addition to having an intracellular role, Ca^{2+} may also be required for other aspects of the adhesive event. Extracellular Ca^{2+} is important for clustering of LFA-1 receptors on the T cell membrane (van Kooyk, et al., 1994). Therefore in addition to cell spreading, it is possible that adhesion is also facilitated through the microaggregation of low affinity LFA-1 receptors. In this way the avidity of the interaction would increase through co-operativity between neighbouring receptors.

Mg^{2+} can only induce high affinity LFA-1 receptors when extracellular Ca^{2+} is removed from the medium by chelation. This phenomenon also applies to other integrins (Kirchhofer, et al., 1990; Masumoto and Hemler, 1993; Staatz, et al., 1989). One explanation for this apparent conundrum to that described above is that on a resting T cell, Ca^{2+} may occupy not only the sites involved in integrin clustering but also block access to or occupy the "active" high affinity site associated with Mg^{2+} which is revealed either through normal activation processes or artificially through Ca^{2+} stripping. In fact evidence for two Ca^{2+} binding sites on $\alpha_5\beta_1$ has recently been obtained where the authors propose that Ca^{2+} can compete with Mg^{2+} for binding to the Mg^{2+}-ligand competent site, but Ca^{2+} binding to a separate high affinity site can increase the affinity of Mg^{2+} for its ligand-competent site (Mould, et al., 1995). The question arises whether such exchange of divalent cation would occur *in vivo*. As the Mg^{2+}/Ca^{2+} ratio can increase significantly during tissue damage and this increase correlates with cell movement into wounds (Grzesiak and Pierschbacher, 1995) it is possible that regulation of integrin activation directly by cation may be possible under certain circumstances.

These two model systems provide two totally different methods for inducing T cell adhesion to ICAM-1. As these methods are not thought to be the most physiologically relevant it was important to assess adhesion stimulated following TCR/CD3 crosslinking to see which model, if any, that it

resembled. The characteristics of adhesion triggered through the TCR/CD3 complex were very similar to those observed following phorbol ester treatment in that cells were spread, adhesion was dependent on cytoskeletal reorganisation and cells could not be induced to bind soluble ICAM-1 (up to 100 μg/ml). Thus, this means of inducing T cell activation which might be considered to be more physiologically relevant, appears not to generate high affinity receptors, a finding which parallels the inability of CD3 triggering on T blasts to generate high affinity $\alpha_4\beta_1$ receptors for fibronectin (Jakubowski, et al., 1995a). This does not seem to be a golden rule as crosslinking the TCR on a T lymphoid cell line did induce a high affinity form of $\alpha_5\beta_1$ for fibronectin (Faull, et al., 1994). These differences require more investigation and analysis but perhaps might be explained by the nature of the integrin, its activation state or the cell type on which it is expressed. It will be pertinent to stimulate cells through their natural ligands rather than via crosslinking antibodies to conclusively decipher these points.

When adhesion was stimulated with intracellular Ca^{2+}-mobilising agents there was no induction of high affinity receptors for LFA-1. These cells however did not fit the conventional phorbol ester or CD3- models as morphological analysis revealed them to be rounded and not spread. Adhesion was, however, particularly susceptible to inhibition with cytochalasin D which suggests some kind of cytoskeletal involvement. Further analysis of the means of adhesion in this case will require careful morphological analysis of the receptors and cells using antibodies raised against different cytoskeletal and cell surface proteins. It remains to be seen if some subtle morphological changes are responsible for adhesion on these cells, such as membrane ruffling, or filopodia formation. It should be pointed out that cytoskeletal analysis is extremely difficult in lymphocytes due to their massive nuclear to cytoplasmic ratio. These cells are also extremely motile and as such do not display the conventional larger focal contact structures which are easily seen in fibroblasts. It will be interesting to study the adhesion of cells with simultaneous Ca^{2+} imaging to gain an idea of the temporal relationship between intracellular Ca^{2+} fluxing, cell adhesion and morphology.

CHAPTER SIX

ROLE OF ICAM-1 IN THE ACTIVATION OF LFA-1

6.1 INTRODUCTION

Consistently throughout the course of this work LFA-1 activation has been assessed by measuring T cell binding to ICAM-1 and analysis of mAb 24 epitope expression. This mAb was initially presumed to report active LFA-1 as induction of the mAb 24 epitope on LFA-1 paralleled adhesive ability (Cabañas and Hogg, 1993, Dransfield, et al., 1992a). It now appears from data presented in Chapter 5 that the situation is not quite as simple as this and mAb 24 epitope does not get induced to the same extent following distinct activators and instead its expression correlates strongly with the presence of high affinity receptors. This raises an obvious discrepancy regarding the small amount of mAb 24 which can be induced under certain circumstances by stimuli such as PDBu which are not thought to induce high affinity receptor expression (see Figure 3.4). If, following these stimuli, adhesion occurs through low affinity receptors then why do some LFA-1 receptors express the 24 epitope and what induces the expression of the epitope? In addition to the requirement for stimulation to induce activation, LFA-1 also requires prior interaction with ligand in order to be fully competent to bind ligand (Cabañas and Hogg, 1993). This suggests that induction of the 24 epitope might be a LIBS (ligand induced binding site). Therefore it was of interest to determine if the 24 expression seen in these studies was also dependent upon an interaction with ligand. A more detailed analysis of the characteristics of sICAM-1 binding to LFA-1 receptors was also carried out.

6.2 RESULTS

6.2a QUANTITATION OF THE PERCENTAGE OF LFA-1 CELL SURFACE RECEPTORS INDUCED TO EXPRESS THE mAb 24 EPITOPE.

Since expression of the mAb 24 epitope appeared to correlate with the presence of high affinity receptors it seemed pertinent to quantitate exactly how many LFA-1 receptors were induced to be activated following stimulation. This was performed through the use of a commercial calibrated microbead kit called Quantum Simply Cellular. Using the kit the total number of cell surface LFA-1 molecules was calculated to be ~270,000 using

	Mg^{2+} concentration (mM)	% of LFA-1 receptors which are 24 positive
EGTA	0	0.0 +/- 0.0
	0.3	1.1+/- 0.5
	3.0	14.4 +/- 4.7
	10.0	13.5 +/- 3.7
PDBu/Ca^{2+}	0	0.7 +/- 0.1
	0.3	1.0+/- 0.3
	3.0	1.8 +/- 0.6
	10.0	2.6+/- 0.6
Total LFA-1 receptor number per cell		268 424.8 +/- 56 940.1

Figure 6.1: Quantitation of the percentage of total LFA-1 receptors which express the mAb 24 epitope. The number of cell surface receptors recognised by mAbs 38 (total LFA-1) and 24 were calculated using calibrated microbeads supplied with the Quantum Simply Cellular Kit following stimulation of T cells with the phorbol ester (50 nM PDBu, 1 mM Ca^{2+}) or Mg^{2+} (1 mM EGTA) protocols. The proportion of 24 positive receptors was calculated as a percentage of the total number recognised by mAb 38. Data represent the means +/- standard deviation of seven independent experiments.

mAb 38 which is directed against all LFA-1 receptors (**Figure 6.1**). This is in good agreement with an earlier study on T cells where the number of LFA-1 molecules was calculated to be 250,000-300,000 receptors (Pardi, et al., 1989). LFA-1 is an extremely abundant molecule on T cells compared with the TCR which has a mere ~30,000 receptors per cell. When cells were stimulated with the Mg^{2+} protocol and the number of 24 positive receptors quantitated it was found that only 10-15% of total LFA-1 receptors were induced to express this epitope. This suggests that only a proportion of LFA-1 receptors are capable or are required to be activated to facilitate the adhesive process. Following the phorbol ester protocol, the number of 24 positive receptors was limited to only 1.0-2.6% of the total LFA-1 molecules. These values were similar to those present in the presence of Ca^{2+} without PDBu. Receptor numbers were calculated following subtraction of the values obtained in the presence of 1 mM EDTA which chelates both Mg^{2+} and Ca^{2+}. In summary, even though the Mg^{2+} protocol generates good 24 epitope expression, it is only present on a proportion of the total number of receptors and only ~1-2% of receptors on phorbol ester-treated cells express it.

6.2b DISTRIBUTION OF mAb 24 EPITOPE ON T CELLS FOLLOWING Mg^{2+} OR PHORBOL ESTER STIMULATION.

The exact distribution of the mAb 24 epitope positive receptors on T cells adherent to ICAM-1 was then examined by confocal microscopy. This technique allows the cells to be visualised in layers to obtain a more detailed picture of where epitopes are localised. Cells were also stained with TRITC-conjugated phalloidin to obtain a simultaneous analysis of cell morphology. T cells treated with phorbol ester, in the presence of 1 mM Ca^{2+} and 0.3 mM Mg^{2+}, displayed the characteristic spread morphology (**Figure 6.2A**). The small amount of 24 epitope expression, illustrated by the quantitation study, was confined mainly to the level of cell interface with immobilised ICAM-1 on the coverslip. A small amount was also present at higher sections through the cell, at cell-cell contact points (see thin arrows). This suggests that the proportion of 24 epitope which is present is restricted to areas of the cell which are in direct contact with recombinant ICAM-1 (coverslip) or cell surfaces which contain natural ICAM-1 (cell-cell points). These observations propose that 24 epitope expression may be ligand dependent. The Mg^{2+}-stimulated cells were observed to be slightly flattened onto the coverslip coated with ICAM-1 but at 2 μm above this level converted to a round

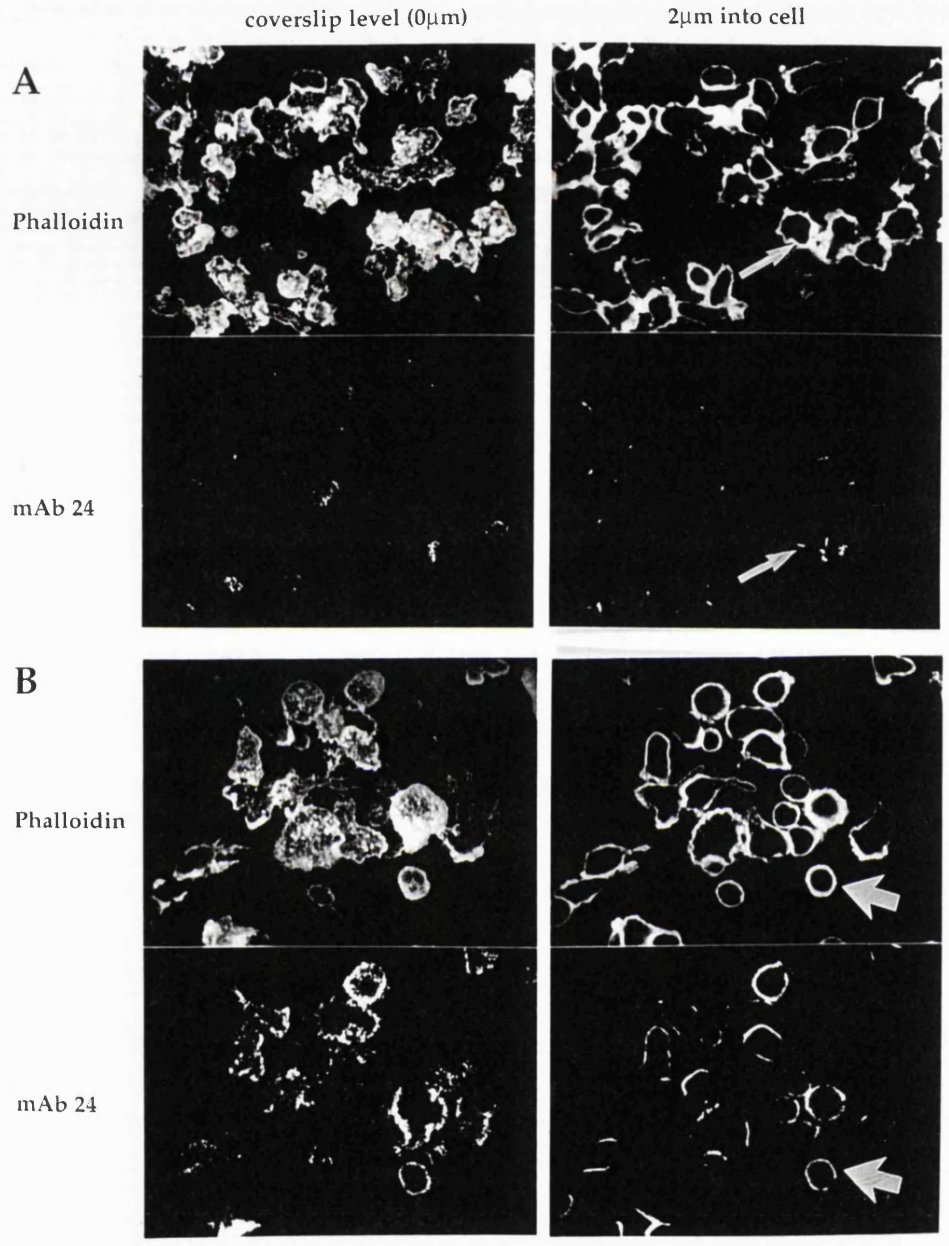

Figure 6.2: Distribution of the mAb 24 epitope by confocal microscopy following adhesion to ICAM-1 induced by Mg^{2+} or phorbol ester treatment. T cells were induced to adhere to ICAM-1Fc-coated coverslips in HEPES buffer following phorbol ester (50 nM PDBu, 1 mM Ca^{2+}, 0.3 mM Mg^{2+}; **A**) or Mg^{2+} (10 mM Mg^{2+}, 1 mM EGTA; **B**) treatments. FITC-conjugated mAb 24 and TRITC-phalloidin were visualised by confocal microscopy. The left hand panels show cells at the ICAM-1 interface (coverslip level) and the right hand panel depicts the same field but 2 μm into the cell from the coverslip. Thin arrows represent examples where 24 expression is observed at cell-cell contact points and thick arrows represent areas where there is expression at non contact points.

morphology (**Figure 6.2B**). The 24 epitope was expressed at the T cell/ICAM-1 coverslip interface and elsewhere on the cell at points of contact with other cells. This also suggested that the epitope was restricted to points of contact with ligand giving weight to the idea of 24 as a LIBS-type epitope. However it was notable that, following Mg^{2+}-stimulation, the 24 epitope was also distributed at sites on the cell which were not in obvious contact with immobilised ligand or other cell surfaces (see thick arrows). The 24 epitope expression was specific and not an artefact of adhesion as T cells adherent to fibronectin, through β_1 integrins, did not express the 24 epitope at the cell-fibronectin interface (data not shown). MAb 24 expression represents a proportion of activated receptors as the distribution of total LFA-1 molecules, examined with a non-blocking mAb YTH 85.1 (Landis, et al., 1994), was dispersed evenly over the entire cell surface (data not shown). Together these results indicate that following phorbol ester stimulation 24 epitope is induced on LFA-1 receptors when they come into contact with ICAM-1 and hence represents a LIBS-type of expression. Following Mg^{2+}-stimulation, in addition to ligand dependent expression, cells appear to express the 24 epitope in an ICAM-1-independent fashion. It is possible however that the areas expressing 24 epitope which are not in obvious contact with a source of ICAM-1 might have previously seen it at some point during the activation procedure even though they are now not in physical contact with it. It was therefore pertinent to examine the dependence on ICAM-1 for induction of 24 epitope expression.

6.2c RELATIONSHIP BETWEEN 24 EPITOPE EXPRESSION AND INTERACTION WITH LIGAND

In light of the confocal microscopy results and earlier results with 24 epitope expression it was important to address whether any 24 epitope expressed by the T cells was dependent on a prior interaction with ligand i.e. the LIBS effect. To perform this analysis cells were prevented from interacting with ICAM-1 by addition of the blocking anti-ICAM-1 mAb RR1/1 and induction of mAb 24 epitope expression was analysed as previously described. When cells are stimulated with low concentrations of Mg^{2+} (0.3 mM), RR1/1 shows a dose-dependent inhibition of 24 epitope expression (**Figure 6.3A**). The 24 epitope expression was completely eliminated by the addition of 50 µg/ml of RR1/1. Similar results were obtained for cells stimulated with the phorbol ester protocol (50 nM PDBu, 1 mM Ca^{2+} and 0.3 mM Mg^{2+}; data not shown).

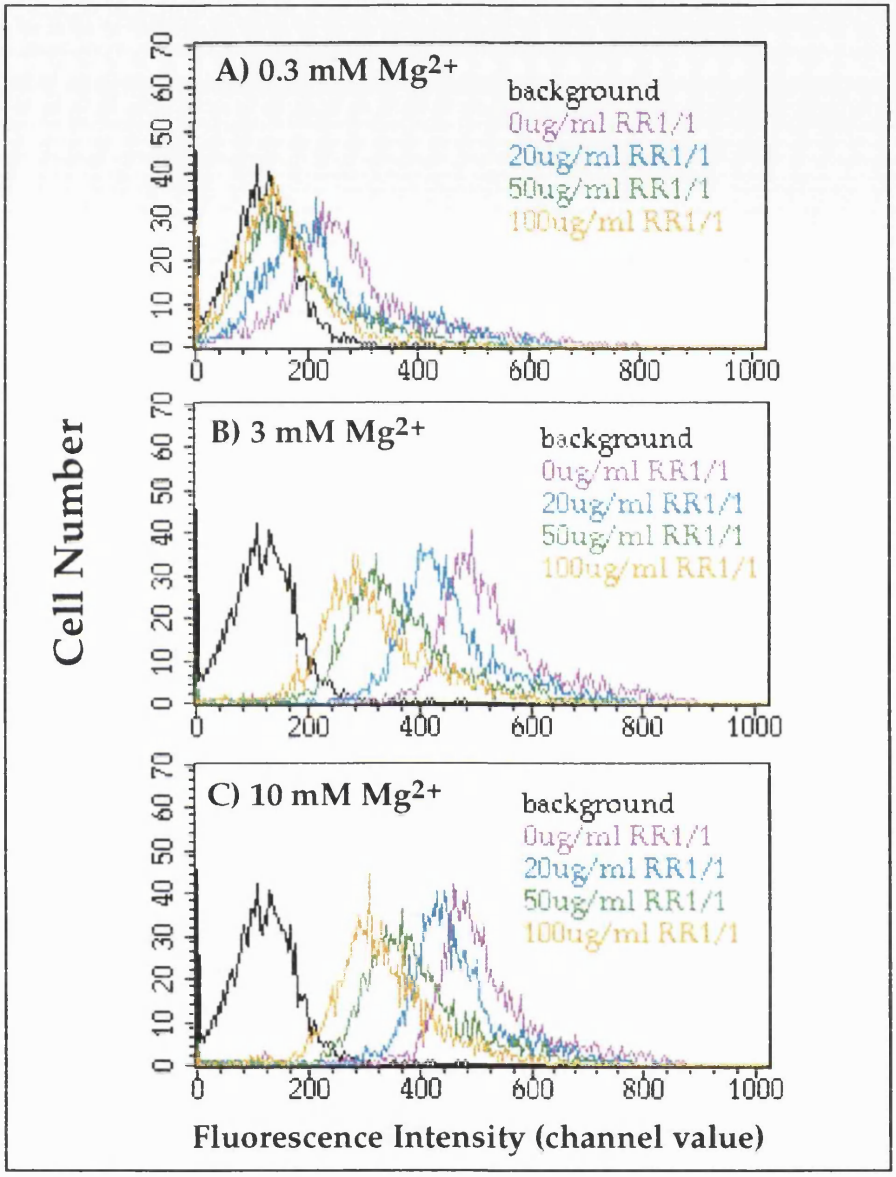

Figure 6.3: mAb 24 expression induced at low but not high concentrations of Mg^{2+} is dependent on a LIB effect with ICAM-1. T cells were preincubated with the indicated concentrations of the blocking anti-ICAM-1 antibody RR1/1 and examined for their expression of the 24 epitope following stimulation in low Mg^{2+} (0.3 mM Mg^{2+}, **A**) or higher Mg^{2+} (3 mM Mg^{2+}, **B** and 10 mM Mg^{2+}, **C**) conditions. 1 mM EGTA was included in all conditions to chelate Ca^{2+}. FITC-conjugated mAb 24 expression was analysed by flow cytometry.

This suggests that the small amount of 24 epitope expression seen when cells are stimulated through low Mg^{2+} concentrations or phorbol ester is induced following an interaction with ligand. It is known that low affinity receptors mediate the first contact with ICAM-1 (since sICAM-1 does not block phorbol-ester mediated adhesion) therefore this suggests that initial binding of these receptors induces some sort of alteration in LFA-1 which facilitates transferral to a high affinity receptor. This is hypothesised to work by ligand either stabilising a transiently active high affinity form or by inducing low affinity receptors to present a higher affinity form. These results provide evidence against 'selection' of a high affinity form and favour an 'induction' model if mAb 24 is indeed reporting high affinity receptors. These high affinity (24 positive receptors) might confer enhanced adhesive stability on the cell over the localised contact point. When cells were stimulated at higher concentrations of Mg^{2+} such as 3 mM (**Figure 6.3B**) or 10 mM (**Figure 6.3C**) RR1/1 once again shows a dose dependent decrease in 24 epitope expression. This time however, the expression was not totally eliminated even at higher doses of mAb RR1/1 (100 μg/ml). The lack of total inhibition at this concentration was not due to a deficiency of RR1/1 in fully reacting with all ICAM-1 molecules as the antibody showed saturated binding at all concentrations of RR1/1 tested (data not shown). Therefore it seems that when 24 epitope expression is induced in the presence of lower concentrations of Mg^{2+}, it requires prior interaction with ligand and therefore constitutes LIBS dependent expression. The situation appears more complex at higher concentration of Mg^{2+} where the expression is partly, but not wholly, dependent on ICAM-1. This result confirms the confocal findings which showed that mAb 24 epitope was not totally localised to areas on the cell which were in obvious contact with ligand and suggests that a component of 24 epitope expression, at high Mg^{2+} concentrations, can occur in the absence of interaction with ICAM-1.

6.2d CAN ICAM-3 SUBSTITUTE FOR ICAM-1 IN LIBS GENERATION FOLLOWING HIGH Mg^{2+} STIMULATION?

This finding does not prove that high concentrations of Mg^{2+} can generate 24 epitope expression in a totally LIBS-free way as there are two other ligands for LFA-1 present on T cells, ICAMs -2 and -3. As ICAM-3 is expressed at levels equivalent to ICAM-1 on these T cells, it was possible that the residual 24 epitope expression was due to a LIB effect with this second LFA-1 ligand.

The ICAM-3 mAbs CBR IC 3/1, CBR IC 3/2 and CBR IC3/6 (obtained from the Leukocyte Typing Workshop IV) were tested either singly or in combination together with mAb RR1/1 for their effects on 24 epitope expression. These mAbs have previously been shown to be efficient blockers of LFA-1/ICAM-3 interactions when used in combination but not singly (de Fougerolles, et al., 1994). The blocking combinations (CBR IC3/1 with CBR IC3/2 and CBR IC3/2 with CBR IC3/6) had no additional inhibitory effect on mAb 24 expression when used in combination with RR1/1 (to block the ICAM-1 LIBS) (**Figure 6.4**). These combinations of mAbs are functional as they block T cell adhesion assay to ICAM-3 (**Figure 6.5**) This shows that an interaction with ICAM-3 is not supplying an essential LIBS to promote the residual 24 expression left over following ICAM-1 blocking. These mAbs were also analysed in the absence of RR1/1 to see if ICAM-3 itself was responsible for LIBS dependent 24 epitope expression. There was no inhibitory effect of these antibodies on 24 expression which showed that at least for this system ICAM-3 is not supplying a LIBS interaction which is required for Mg^{2+}-generated 24 expression. Further evidence for the lack of ICAM-3 generated LIBS-high affinity receptors comes from the experiments performed at low Mg^{2+} concentrations where blocking ICAM-1 eliminates all the mAb 24 epitope expression. Therefore in low Mg^{2+} concentrations 24 epitope expression is totally dependent on the presence of an ICAM-1 encounter, whereas receptors stimulated with higher concentrations of Mg^{2+} (3-10 mM) are only partially dependent on ICAM-1, a result confirmed by confocal microscopy. This implies that high levels of the divalent cation Mg^{2+} can bypass ligand dependence and induce the 24 epitope directly. However before this is a concrete result it would have to be ruled out that the other, less well defined, LFA-1 ligand ICAM-2 was not supplying the LIBS. Unfortunately at the time of these experiments a blocking ICAM-2 mAb was not available so the experiment could not be performed. MAb 24 epitope expression present on the cell membrane at non cell contact points seems to suggest that high concentrations of Mg^{2+} can probably induce 24 epitope expression in the absence of ligand. It is thought that ICAMs-1, -2 and -3 are the sole ligands for LFA-1 and that there are no, as yet, undiscovered ones on haematopoietic cells (de Fougerolles, et al., 1994).

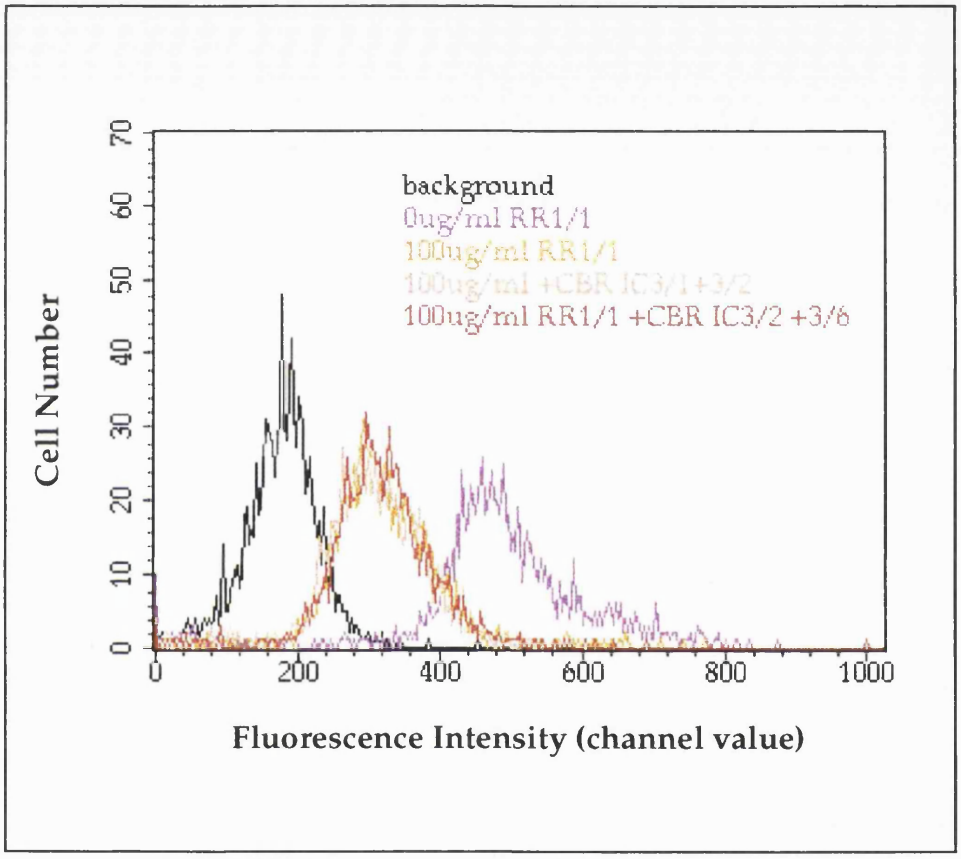

Figure 6.4: Can ICAM-3 provide a LIBS-induction of mAb 24 epitope expression? T cells were preincubated with a saturating concentration of the blocking anti-ICAM-1 antibody RR1/1 (100 µg/ml) and examined for their expression of the 24 epitope following high Mg^{2+} stimulation (3 mM Mg^{2+}, 1 mM EGTA). A role for ICAM-3 was examined by blocking ICAM-3 with combinations of the mAbs CBR IC3/1 with CBR IC3/2 and CBR IC3/2 with CBR IC3/6. FITC-conjugated mAb 24 expression was analysed by flow cytometry.

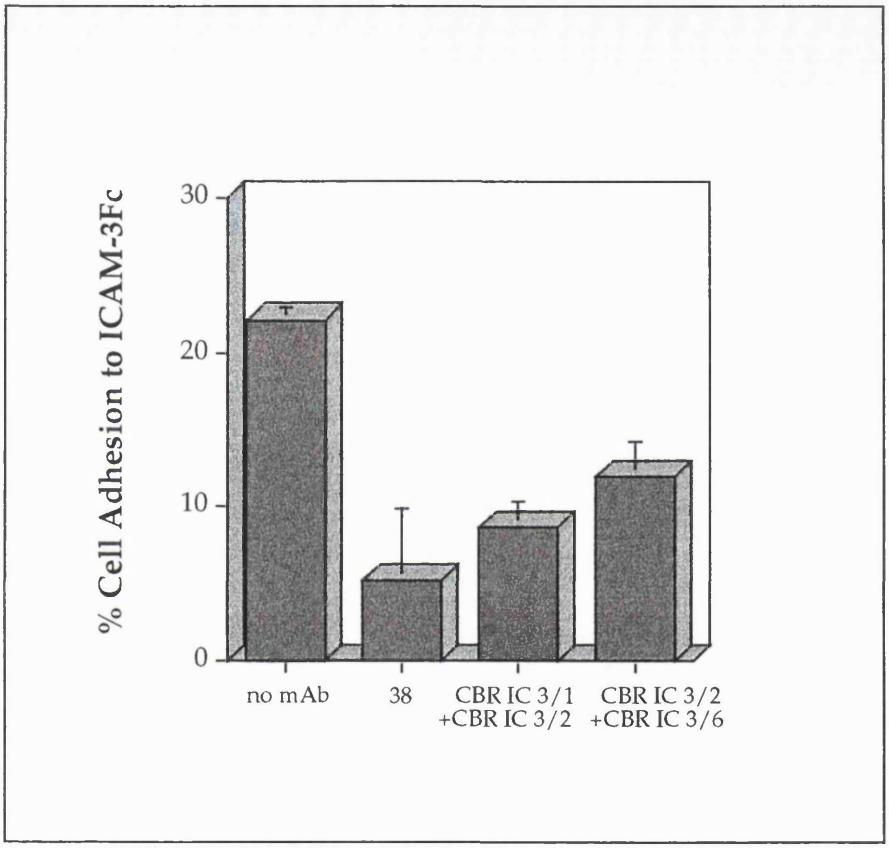

Figure 6.5: A combination of anti- ICAM-3 mAbs block T cell binding to ICAM-3. T cells stimulated with 50 nM PDBu were analysed for binding to recombinant ICAM-3 immobilised on plastic in the presence of 20 µg/ml of either mAb 38 (anti-LFA-1) or the combinations of anti-ICAM-3 mAbs CBR IC3/1 with CBR IC 3/2 and CBR IC 3/2 with CBR IC3/6. Unbound cells were removed by careful washing and cells remaining bound were harvestsed and counted. Data is expressed as means of triplicates +/- standard deviations.

6.2e DOES THE ADDITION OF sICAM-1 INCREASE mAb 24 EXPRESSION?

The true definition of a LIBS Ab is one whose epitope increases in expression following incubation of the receptor with ligand. For this reason an experiment was tried where sICAM-1 was added to T cells to see if it could increase expression of mAb 24. Addition of sICAM-1 to cells stimulated with 5 mM Mg^{2+}/EGTA had no significant enhancement on mAb 24 expression even at concentrations up to 1000 μg/ml (**Figure 6.6**). It was possible that, using this concentration of Mg^{2+}, the receptors might already be stimulated to their full activation capacity and hence might not be capable of being increased above this threshold by addition of ligand. To address this issue, cells were stimulated with a lower concentration of Mg^{2+} (1mM with EGTA) in an attempt to induce a suboptimal activation of the receptors. Unfortunately there was still no enhancing effect of adding in sICAM-1 (**Figure 6.6**). This experiment is hampered by the large amount of natural ICAM-1 on cell surfaces which inevitably competes with the recombinant ICAM-1 for adhesion to LFA-1. Therefore this experiment proved to be extremely difficult to perform. It was thought that perhaps cell surface ICAM-1 could be blocked by preincubation with mAb RR1/1 so that the contribution of sICAM-1 could be assess in isolation. Preliminary results show that this had no effect and it is likely that, even though preincubation with the blocking mAb coats cell surface ICAM-1, upon addition of sICAM-1 it might compete for binding to the mAb making it unavailable for any putative LIBS induction. This experiment remains inconclusive and must be assessed on a cell type, such as LFA-1-transfected COS cells, which has none or very low levels of endogenous ICAM-1.

6.2f ANALYSIS OF THE sICAM-1-BINDING RECEPTORS.

Results so far are consistent with the appearance of mAb 24 on cells following "inside-out" agonist-induced stimulation as a LIBS-dependent phenomenon. This suggests that following an initial interaction with ligand through low affinity receptors, ICAM-1 acts to stabilise the receptor inducing a high affinity form which is recognised by mAb 24. In order to further understand the relationship between ICAM-1 binding and 24 epitope-expressing LFA-1 receptors the following approach was investigated. Previous work in the laboratory has shown that binding of mAb 24 to cells inhibits several important lymphocyte functions such as Ag-specific T cell proliferation, NK

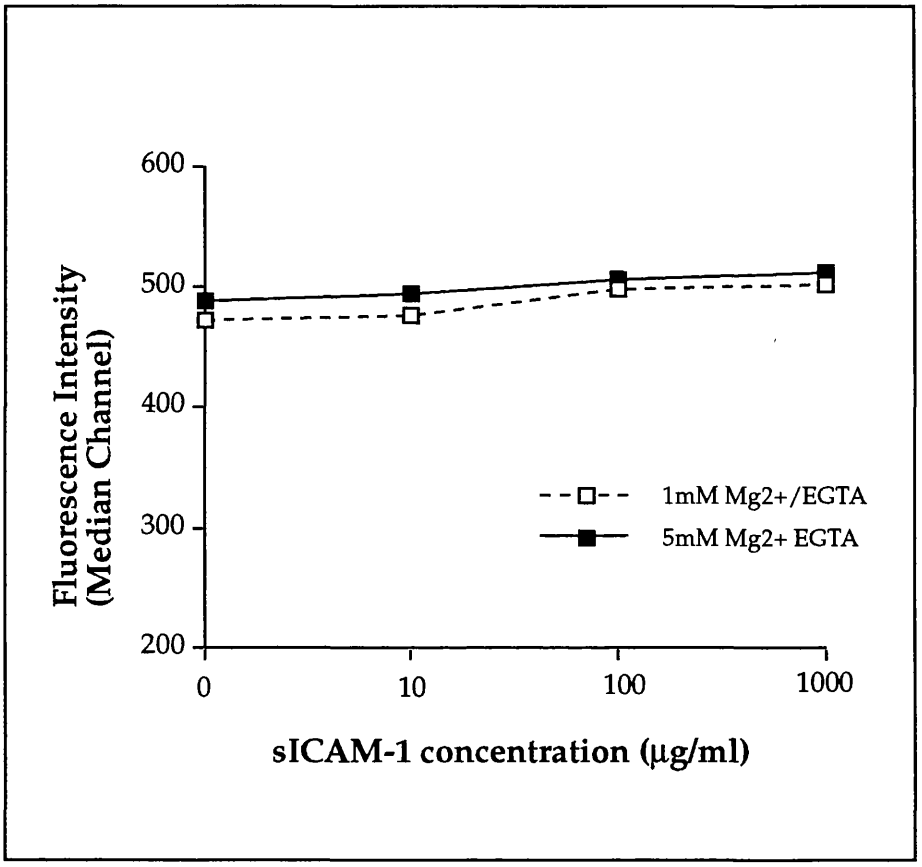

Figure 6.6: Can the addition of sICAM-1 increase mAb 24 epitope expression? T cells stimulated with either low (1 mM) or high (5 mM) concentrations of Mg^{2+} with EGTA were examined for their expression of the mAb 24 epitope following addition of the indicated concentrations of sICAM-1. FITC-conjugated mAb 24 binding was analysed by flow cytometry.

cell activity and Mac-1-dependent neutrophil chemotaxis to fMLP (Dransfield, et al., 1992b). The inhibitory effect is not due to induction of a negative signal or blocking of either the LFA-1 or Mac-1/ICAM-1 interactions but is due to the prevention of the deadhesion phase of integrin-ligand interactions. The hypothesis is that addition of mAb 24 "locks" receptors into an active conformation preventing disengagement from ligand and receptor recycling. Dynamic de-adhesive cycles are required for these active lymphocyte functions. Since mAb 24 is believed to lock or stabilise receptors in their active form it was of interest to investigate the effect of mAb 24 addition on the ability of cells to bind sICAM-1.

As discussed in Chapter 6, sICAM-1 does not bind constitutively to the T cells and only does so under certain conditions. sICAM-1 binds in a dose- and Mg^{2+}- dependent manner (see Figure 5.2). When the sICAM-1 dose response was studied in more detail by examination of the fluorescence histograms of soluble ICAM-1 binding it was found that LFA-1 receptors were quite heterogeneous in their ability to bind sICAM-1. **Figure 6.7** shows that when cells were stimulated with a high concentration of Mg^{2+} (5 mM), which is required for efficient binding of sICAM-1, very few receptors are positive at low concentrations of sICAM-1 (50 µg/ml, blue histograms; **A**) and many receptors show levels of fluorescence equal to those in the presence of mAb 38 (black line) which represents basal binding. As the concentration of sICAM-1 was increased to (100, 500 and 1000 µg/ml; **B**, **C** and **D**) more receptors became capable of binding sICAM-1 but the population as a whole showed a very heterogeneous binding pattern (blue histograms). Even at 1000 µg/ml (**D**) not all the receptors were capable of binding sICAM-1. This suggests that receptors are very heterogeneous in their ability to bind ICAM-1, with some binding well at low concentrations of ligand, others requiring much higher concentrations before binding and others being unable to bind at all. Addition of mAb 24 caused a dramatic enhancement of sICAM-1 binding (pink histograms- all graphs). The pattern of binding seemed to indicate that mAb 24 was allowing a greater percentage of receptors to register binding of sICAM-1. mAb 24 appears to exert its affects primarily on the population of receptors which were previously very poor binders of ICAM-1 (see solid arrow in D) with not so much effect on the receptors/cells which could already bind sICAM-1 well (see dotted arrow). This result seemed to suggest that LFA-1 receptors varied extensively in their capacity to bind sICAM-1 but that addition of mAb 24 could increase the proportion of receptors capable of

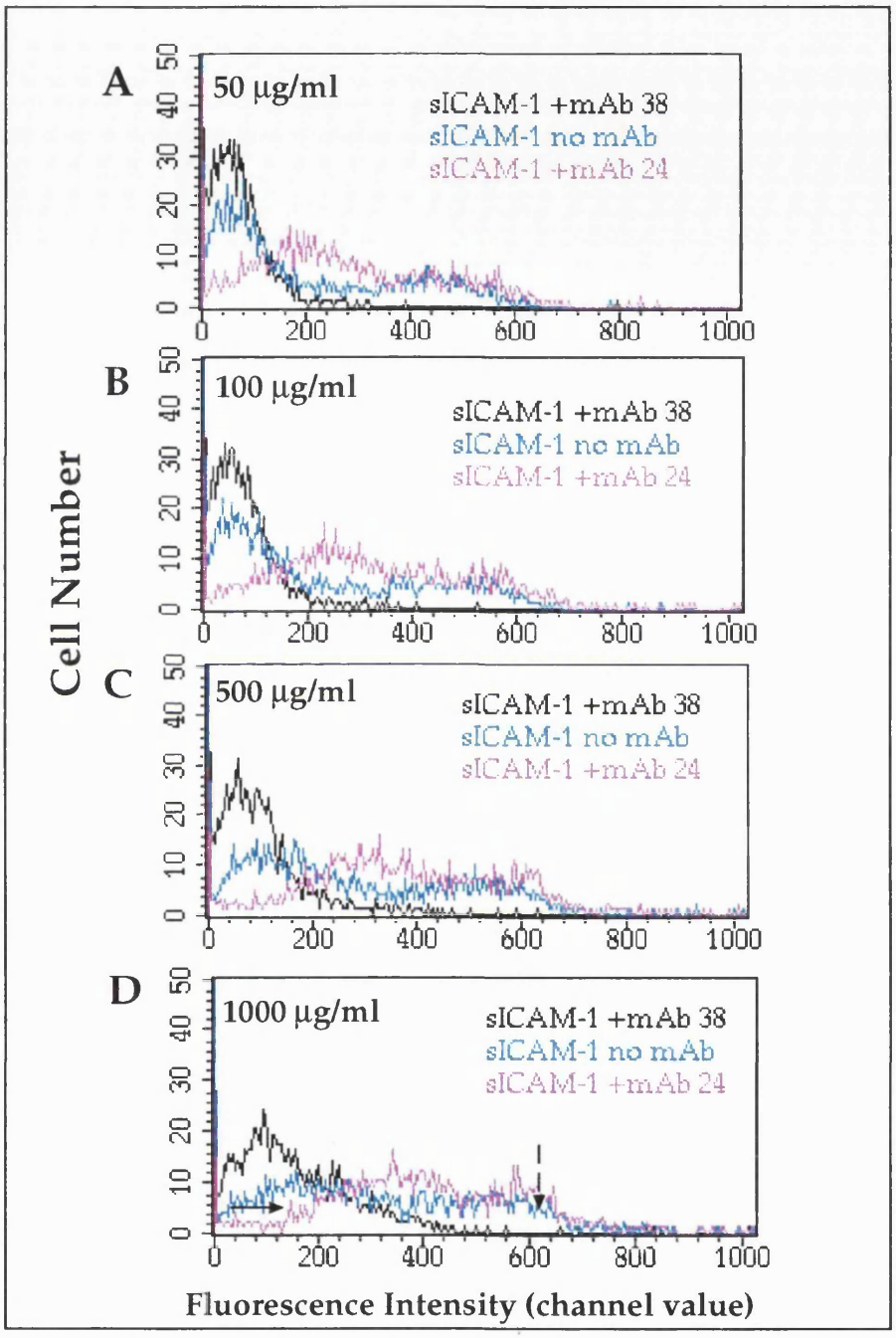

Figure 6.7: Soluble ICAM-1 binds to T cells in a dose-dependent manner and is enhanced by addition of mAb 24. T cells were stimulated with 5 mM Mg^{2+}/1 mM EGTA and incubated with 50 µg/ml (**A**), 100 µg/ml (**B**), 500 µg/ml (**C**) or 1000 µg/ml (**D**) of sICAM-1Fc in the presence of 10 µg/ml mAb 38 (black line), no added mAb (blue line), or 10 µg/ml mAb 24 (pink line) for 30 min at 37 °C. Bound ICAM-1 was detected with FITC-conjugated anti-human IgG specific antibody and analysed by flow cytometry.

binding soluble ligand. A similar effect was seen on cells which had been stimulated with the phorbol ester protocol (50 nM PDBu, 1 mM Ca^{2+} and 0.3 mM Mg^{2+}; **Figure 6.8**). As you will recall sICAM-1 is incapable of binding to receptors under this condition (**Figure 6.8**; blue line) but when mAb 24 is added into the reaction, sICAM-1 can now bind to a small proportion of receptors (**Figure 6.8A**; pink line). This result suggests that when the few initial LIBS interactions take place that addition of mAb 24 will stabilise this initially low affinity interaction allowing binding of sICAM-1 to be registered. An alternative explanation is that mAb 24 is inducing receptors to become competent to bind soluble ligand. This enhancement only occurs when Mg^{2+} is present in the medium because mAb 24 has no effect in the absence of Mg^{2+} (see **Figure 6.8B**). sICAM-1 could also be found to bind to LFA-1 following stimulation of T cells with TCR/CD3 triggering but only in the presence of mAb 24 (data not shown).

6.2g PRELIMINARY CALCULATION OF THE AFFINITY OF LFA-1 FOR ICAM-1.

As saturation had been achieved for binding of sICAM-1 to LFA-1 following Mg^{2+}-induced activation it was of interest to make a calculation of the dissociation constant or affinity of LFA-1 for ICAM-1. **Figure 6.9A** shows the dose-dependent effect of adding sICAM-1 to Mg^{2+}-stimulated receptors with saturation reached at ~1.8 μM. This graph shows specific binding of sICAM-1 with prior subtraction of non-specific binding measured in the presence of the blocking mAb 38. Addition of mAb 24 caused much more sICAM-1 to be bound (**Figure 6.9B**). In theory mAb 24 could affect these changes by either increasing the affinity of LFA-1 for ICAM-1 or by increasing the number of binding sites available. In order to check these proposals, the curves were subjected to a preliminary Scatchard analysis. The Scatchard plot for the curve in the absence of mAb 24 is shown in **Figure 6.9C** and in the presence of mAb 24 shown in **Figure 6.9D**. These plots show a single class of sites with the apparent dissociation constants (K_d) calculated to be 0.7 μM and 0.23 μM respectively. These values are roughly equal which suggests that the addition of mAb 24 does not increase the affinity of LFA-1 for ligand but rather increases the number of sites capable of binding to ligand. It should be stated that these are only preliminary measurements of apparent dissociation constants, a much more thorough evaluation is required before these values are totally proven.

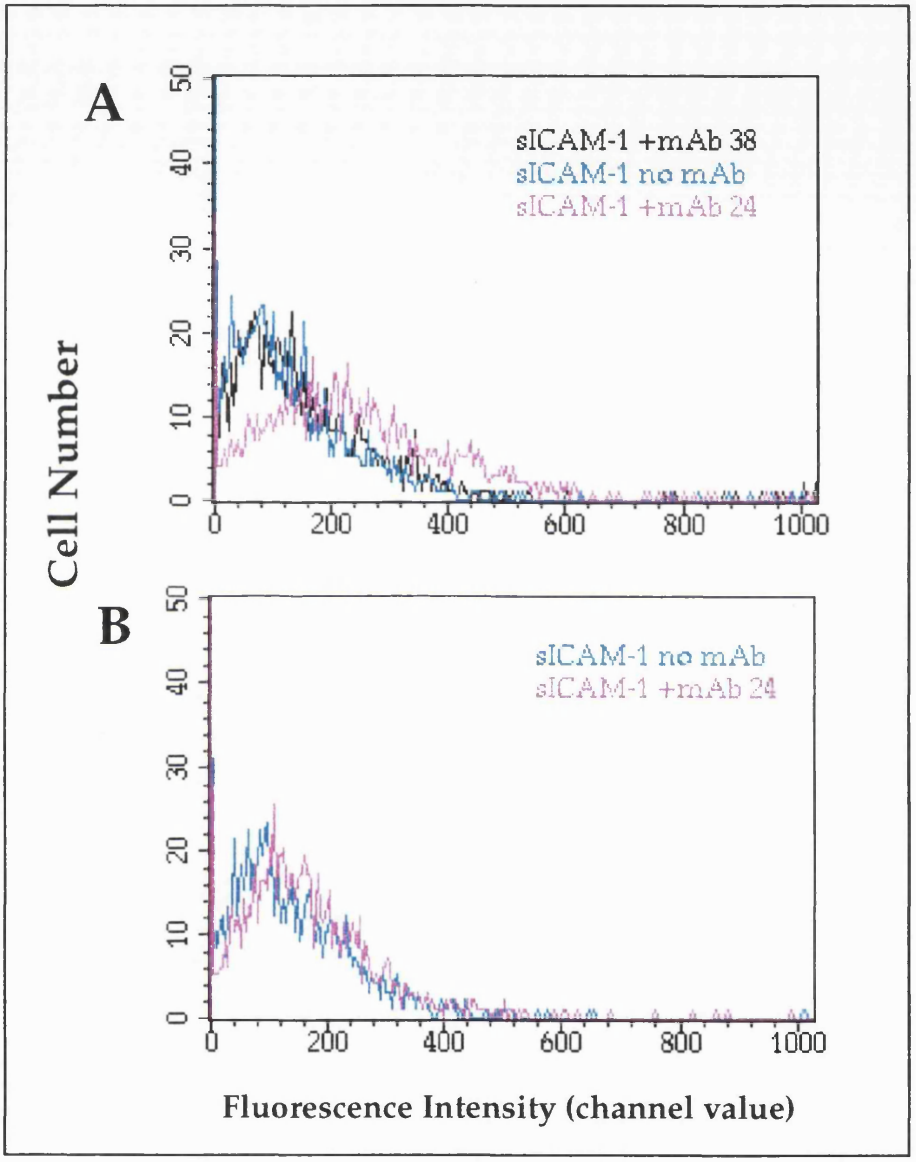

Figure 6.8: mAb 24 enhances sICAM-1 binding to phorbol ester-treated cells only in the presence of Mg^{2+}. T cells were stimulated with the phorbol ester protocol (50 nM PDBu, 1 mM Ca^{2+}) in the presence (**A**) or absence (**B**) of 0.3 mM Mg^{2+} and analysed for their ability to bind 1000 µg/ml sICAM-1 following a 30 min incubation at 37°C. sICAM-1 binding was detected with an anti-human Fc specific antibody and fluoresence intensity was analysed by flow cytometry. Data represents one representative experiment of two.

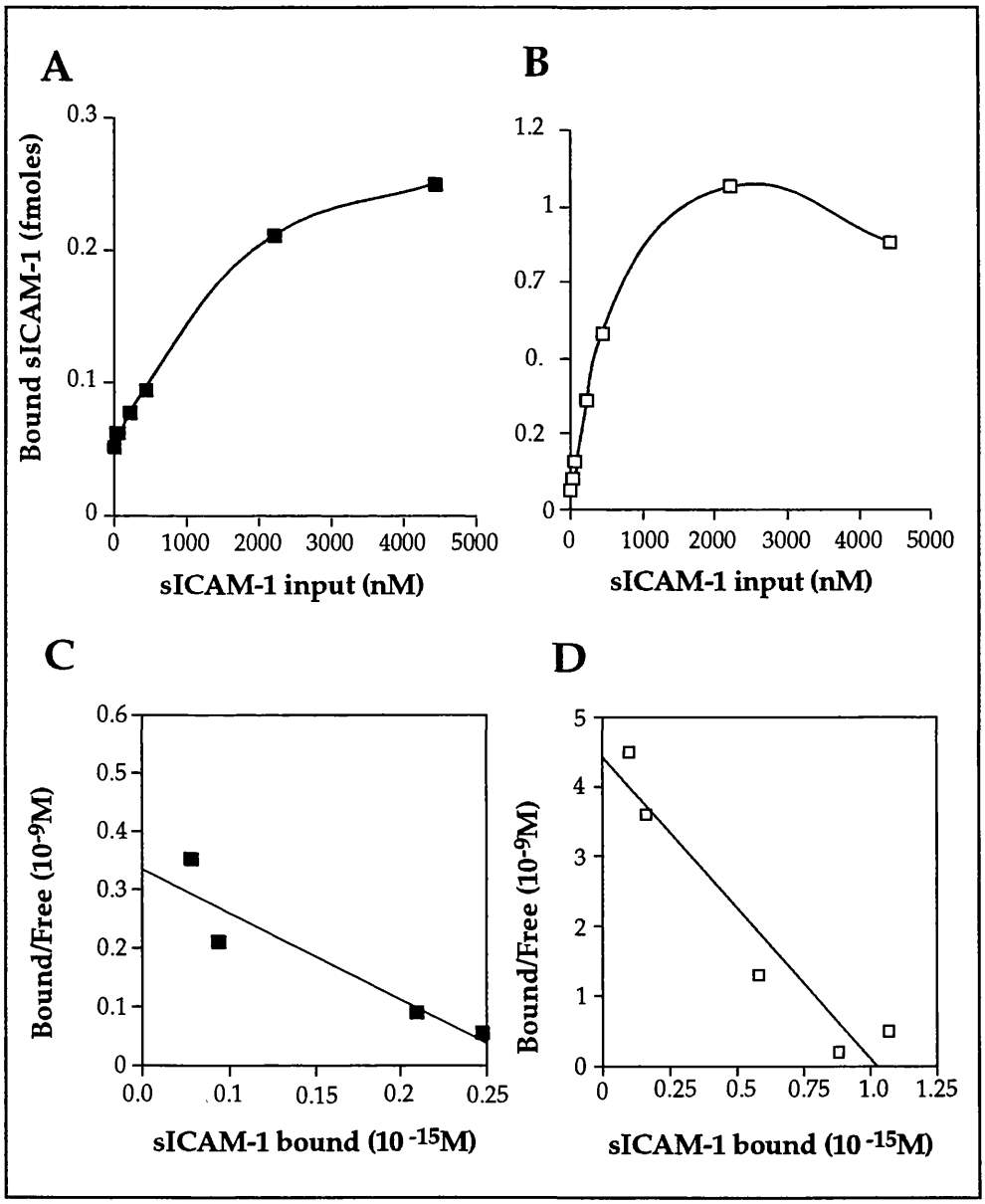

Figure 6.9: Saturation curves of sICAM-1 binding and preliminary Scatchard analyses. T cells were incubated with increasing concentrations of sICAM-1 and stimulated with 5 mM Mg^{2+} and 1 mM EGTA in the absence (**A**) or presence (**B**) of 10 µg/ml mAb 24. Binding of sICAM-1 was measured with a FITC-conjugated anti-human IgG Fc-specific antibody. Scatchard analyses were performed as described in the Materials and Methods and curves drawn by linear regression analysis for cells stimulated with 5 mM Mg^{2+}/EGTA in the absence (**C**) or presence (**D**) of mAb 24.

6.3 DISCUSSION

Through the quantitation of cell surface receptors which could be induced to express the mAb 24 epitope it was calculated that ~15% of receptors express this high affinity form when stimulated with high concentrations of Mg^{2+}. This suggests that only a proportion of LFA-1 receptors are capable or are required to be activated to facilitate productive ligand binding. This percentage is similar to the 15-30% of Mac-1 molecules which were shown to induce expression of another activation reporter epitope recognised by mAb CBRM1/5 on neutrophils following fMLP or PMA treatment (Diamond and Springer, 1993). Although these receptors compose only a percentage of the total number of cell surface Mac-1 molecules they are functionally responsible for Mac-1 mediated adhesion to fibrinogen and ICAM-1. Together these results confirm that integrin receptors are capable of existing in different functional states on the same cell surface.

In the presence of phorbol ester and Ca^{2+}, a small amount of mAb 24 epitope expression is evident. As seen from confocal microscopic analysis this amount is located at contact points with ligand ICAM-1. Antibody blocking analysis showed that this 24 epitope expression was dependent on an encounter with ICAM-1 and hence behaves like a LIBS-type epitope. Although experiments in Chapter 5 illuminated that high affinity receptors were not responsible for the initial adhesion of T cells to ICAM-1 following phorbol ester stimulation, 24 epitope was still expressed on these cells albeit to a minor extent. This suggests that an initial low affinity reaction with ICAM-1 generates a higher affinity receptor conformation which is recognised by mAb 24. It is plausible that this higher affinity structure is important for stabilising or strengthening the binding interaction in that particular area of the cell.

Following stimulation with high concentrations of Mg^{2+}, 24 epitope expression was found to be partially LIBS-dependent with respect to ICAM-1. It was possible that the ICAM-1-independent component was supplied by a LIBS interaction with the next well studied LFA-1 ligand ICAM-3 which is present to similar levels on these T cells. Blocking antibodies to ICAM-3 ruled out the possibility that ICAM-3 could supply a LIBS-dependent induction of 24 epitope expression. It remains undefined if the remaining LFA-1 ligand ICAM-2 contributes to the residual 24 epitope expression but

this seems unlikely from confocal microscopy examination. Following stimulation of T cells with high concentrations of Mg^{2+} mAb 24 epitope is not just confined to areas of the cell which are in obvious contact with a source of ligand; this suggests that Mg^{2+} can stimulate LFA-1 in a ligand-independent manner. This ligand independence has been confirmed with LFA-1 transfected COS cells which express the 24 epitope when stimulated with Mg^{2+} in the absence of ligand (C. Landis, C. Berlin; personal communication). Together these results suggest that Mg^{2+}, at high enough concentrations, can directly bring about a conformational change in LFA-1 favourable for high affinity ligand binding. As previously discussed, LFA-1 has potentially five cations binding sites (four in the α subunit and one in the β subunit; see Figure 1.4) but it is not yet known where Mg^{2+} is having its effect.

From a preliminary Scatchard analysis the apparent affinity of LFA-1 for ICAM-1, following Mg^{2+} stimulation, was calculated to be ~0.8 μM. A related study investigating the binding of a very similar recombinant dimeric construct of VCAM-1, attached to an Fc tail, calculated the affinity of T cell-expressed VLA-4 binding to be 33-70 nM (Jakubowski, et al., 1995). This is a much higher affinity than that calculated here for ICAM-1 and would probably be expected as VLA-4 appears to be more avid for VCAM-1 than LFA-1 is for ICAM-1 in that the VLA-4/VCAM-1 interaction is preferentially engaged under conditions of flow (Berlin, et al., 1995). A recent paper documenting the existence of ICAM-1 in both monomeric and dimeric forms calculated an affinity constant for the binding of isolated LFA-1 to dimeric ICAM-1 as 8 nM (Miller, et al., 1995). This is also a much higher affinity than that described here but that may be due to the use of isolated LFA-1 in contrast to cell-bound LFA-1. Perhaps a cell-bound location places constraints on molecules rendering them to bind less readily and with lower affinity to ligand.

A question which arises from this study is whether these potentially high affinity receptors, found here following stimulation with high concentrations of Mg^{2+}, might exist *in vivo* and if they did so would they have the potential to be blocked by soluble circulating ICAM-1 present in serum? The answer to the first part of this question awaits further study, but since the serum ICAM-1 level does not exceed ~400 ng/ml in healthy individuals and ~2 μg/ml in diseased states (Gearing and Newman, 1993), it is unlikely that circulating sICAM-1 would block potential high affinity receptors *in vivo* unless

produced locally at the required ~400 μg/ml (~1.8 μM) level. This situation is in accord with a recent study where sICAM-1 was shown to block LFA-1-mediated cell adhesion with an IC_{50} of 20-50 μM which is far higher than any documented serum concentration of ICAM-1 (Meyer, et al., 1995). In contrast to this, the serum level of soluble fibronectin (450-900 nM) has been shown to be capable of blocking high affinity $\alpha_5\beta_1$ receptors (Faull, et al., 1994).

Addition of mAb 24, which enhances the ability of LFA-1 receptors to bind sICAM-1, appeared not to be exerting its effects through any dramatic changes in the affinity of the LFA-1/ICAM-1 interaction. This suggests that the antibody is increasing the number of sites which are capable of binding to soluble ligand. It might achieve this through stabilising initially weak interactions with ligand enabling them to be registered in the assay or by inducing receptors to bind sICAM-1. MAb 24 is not an activating antibody *per se* as its addition to cells does not induce them to bind to immobilised ligand. Cells require some prior stimulation to express the 24 epitope in the first place before any enhancing effects are seen. Expression requires the presence of Mg^{2+} which suggests that a certain conformation of Mg^{2+}-occupied receptor is required before mAb 24 can recognise receptors perhaps even only weakly. A recent study investigating the effect of mAb KIM127 (anti-β_2) on the affinity of Mac-1 for the complement component iC3b has shown that although this mAb can induce inactive receptors to bind ligand it does not do so by altering the affinity of the receptor for ligand. The authors propose instead that the mAb might be acting by lowering the activation energy for the shift from an inactive to an active state (Cai and Wright, 1995). This might also be the case for mAb 24 although further detailed affinity studies will have to be performed to decipher whether or not this idea is validated.

CHAPTER SEVEN

DISCUSSION

The main findings from this thesis contribute to increase current knowledge of LFA-1 activation with regard to the intracellular events which induce it and the way in which activation is ultimately achieved.

7.1 INTRACELLULAR SIGNALS

Through the analysis of well known stimulants used to induce LFA-1 activation such as phorbol ester and TCR/CD3 triggering it is obvious that these mediate LFA-1 activation through previously characterised signalling pathways which have been elucidated following cell surface-receptor induced stimulation. Pivotal to these pathways is the serine/threonine kinase PKC and tyrosine kinases. Although tyrosine kinases appear to be involved at a very early stage of LFA-1 activation following TCR/CD3 crosslinking there is also evidence for their involvement later in the adhesive process following an initial receptor-ligand interaction which probably leads to LIBS generated high affinity receptors. This post-receptor signal is required for ensuring a more efficient and stable adhesion. The true nature of this signal will no doubt become elucidated as post-integrin ligation signalling is studied in more detail for the leukocyte integrins.

Use of various subclones of the leukaemic T cell line Jurkat failed to provide another means for analysing the stimulants which induce LFA-1 activation. These cells have a general defect in their ability to activate LFA-1 although other integrins such as those from the β_1 family retain the capacity to be activated. Further studies of this cell line will no doubt prove fruitful for analysing the particular defect in these cell lines which prevents LFA-1 from being activated. An interesting concept is raised from studies within another field where, in certain cells of transformed phenotype, the small GTP-binding proteins such as rho and rac are unable to mediate their typical alterations on cytoskeletal components due to the transformed nature of the cells (F. McCormick, personal communication). Perhaps deficiencies such as these will be the link to the defective signalling machinery associated with LFA-1 in these cell types.

7.2 THE ROLE OF $[Ca^{2+}]_i$

A novel second messenger, $[Ca^{2+}]_i$, was found to be important for inducing LFA-1 activation. Upon further analysis this was found to depend on extracellular Ca^{2+}. Although the downstream effectors of Ca^{2+} remain elusive, feedback activation of PKC and activation of the serine/threonine phosphatase calcineurin were eliminated from the equation. Adhesion induced by the Ca^{2+} mobiliser-stimulants, ionomycin, thapsigargin and dBHQ, was strongly susceptible to the effects of cytochalasin D suggesting that LFA-1 activation through these agonists is dependent on cytoskeletal alterations. Morphological analysis, however, revealed that cells triggered through these stimulants were not obviously spread as might be expected but were actually rounded. This does not resemble the situation when LFA-1-mediated adhesion is stimulated with phorbol esters and TCR/CD3 triggering.

The rounded morphology confirms a recent study where the simultaneous analysis of cell shape and Ca^{2+} fluxing showed three distinct phases during the course of T cell adhesion to an antigen presenting cell (Donnadieu, et al., 1994). Following the initial "scanning" phase of the T cell, an intracellular Ca^{2+} flux was observed which immediately preceded cell rounding up onto ligand (phase two). Some time following rounding, the intracellular Ca^{2+} oscillations subsided and cells spread out onto ligand (phase three). When cells were stimulated to adhere using ionomycin or thapsigargin the cells rounded but were unable to proceed onto the third phase characterised by a spread morphology. This suggests that the Ca^{2+} flux is enough to facilitate cell adhesion but, in the absence of other necessary signals such as PKC activation, spreading cannot occur. This observation parallels the results found here. The rounding up process might be important to release integrins from the cytoskeleton enabling them to be in a correct position for firm adhesion and subsequent morphological changes which take place in the presence of additional signals. Full investigation of this hypothesis would require simultaneous analysis of adhesion and Ca^{2+} imaging to gain an idea of the temporal kinetics of adhesion and $[Ca^{2+}]_i$ release. The localisation of various cytoskeletal components should also be studied to gain an impression of the association between integrin, $[Ca^{2+}]_i$ and the cytoskeleton. It is of interest that a previous study on $\alpha_V\beta_3$ and $\alpha_V\beta_5$ integrins found that adhesion to ligand was much stronger in cells which had been observed to flux Ca^{2+}

more readily (Sjaastad, et al., 1994). The authors propose that Ca^{2+} fluxing feeds back positively on the integrins to induce a more stable adhesion. This could also be a possible route for the observed Ca^{2+} effects seen here for LFA-1.

It could still be possible that Ca^{2+} is exerting its effects through the new player in the integrin activating field, calreticulin. This is a Ca^{2+}-binding protein which was originally thought to be strictly localised to the ER. It binds to the very homologous GFFKR sequence adjacent to the α subunit transmembrane sequence and in this way is speculated to alter the affinity of integrins. Very recently it has been shown to be associated with the $\alpha_2\beta_1$ integrin following stimulation with PMA or an activating mAb (Coppolino, et al., 1995). This is another avenue of study which might link the ability of Ca^{2+} to stimulate integrin adhesiveness.

7.3 AFFINITY V AVIDITY

Initially through the analysis of two different model systems used to induce LFA-1 activation it was shown in this thesis that stimulants induce T cell adhesion to immobilised ICAM-1 by very different means. Stimulation with the divalent cation Mg^{2+} induces high affinity LFA-1 receptors which are characterised by their ability to bind soluble ICAM-1. This form of LFA-1 is reflected by expression of the mAb 24 epitope. These high affinity receptors are sufficient to facilitate cell adhesion even when certain intracellular functions are disrupted. In contrast, stimulation with the phorbol ester, PDBu does not initially induce high affinity receptors and in this case adhesion is facilitated through cell spreading mediated through cytoskeletal alterations, PKC and intracellular Ca^{2+}. High affinity receptors are present in this situation following a LIBS-type interaction with ligand ICAM-1. Together these observations suggest that clustered or microaggregated low affinity receptors may co-operate to increase the overall avidity of cell-cell contact via LFA-1 and that such receptors could work together with higher affinity LFA-1 receptors, such as those generated by the LIBS interaction, to enhance adhesion of flattened T cells. Hypothetically, ligand could either induce these receptors by stabilising an active form which is alternately expressed following receptor "flip-flop" between states or it could actually cause an induction of the active receptor. As sICAM-1 did not block adhesion

stimulated by phorbol ester it is more likely that ligand is directly responsible for inducing an active form.

7.4 THE PHYSIOLOGICAL CONSEQUENCES

This raises the question as to what actually happens when cells are stimulated by other stimulants and of course following physiological triggering procedures. The divalent cation Mn^{2+} also induced soluble ICAM-1 binding suggesting that this route to stimulation generates high affinity receptors. At this point, the activating mAb KIM185 has not yet been tested for its ability to induce sICAM-1 binding but if it follows the characteristics of other activating antibodies it too probably works by inducing a high affinity form of integrin. Therefore, the stimulants which have been shown to induce high affinity receptors are the divalent cations Mg^{2+} and Mn^{2+}. These stimulants could be argued to lack physiological relevance so an important point is raised regarding affinity alterations in an "in vivo" physiological context. It remains to be seen whether there are physiological situations where the affinity of integrins are directly altered or whether this just results from experimental manipulations. In this study, stimulation with the phorbol ester PDBu, TCR/CD3 crosslinking and agents which induce $[Ca^{2+}]_i$ mobilisation, do not induce the binding of sICAM-1 and hence high affinity receptors. A pattern emerging from this list is that induction of adhesion through "inside-out" signalling does not occur by increasing the affinity of the integrin for its ligand. However, in this situation high affinity receptors can be induced following an interaction with ligand.

Would there conceivably be regulation of integrins by divalent cations *in vivo*? It is looking more likely that this might indeed happen as the concentration of Mg^{2+} and Ca^{2+} can shift dramatically during the wound process and wound fluid containing this altered ratio of cations allows cell migration to take place (Grzesiak and Pierschbacher, 1995). Therefore it remains conceivable that cation regulation may exist *in vivo*. This might occur in small pockets between actively adhering cells which might be thought of as protected environments where cations can exert paracrine effects. Closely apposed cells might be able to release some of the 30 mM of Mg^{2+} which is present intracellularly! Since there are as yet no other physiological stimuli which have been identified to trigger LFA-1 activation directly it remains a big question whether affinity regulation does actually occur *in vivo* or

whether in nature it occurs by a combination of ligand and cations? More natural means for regulating affinities are evident for several other integrins. ADP stimulates high affinity binding of factor X and fibrinogen to Mac-1 on neutrophils and monocytes (Altieri, et al., 1988, Altieri and Edgington, 1988) . More conclusive evidence for the existence of affinity regulation *in vivo* comes from an animal model. A soluble VCAM construct, injected into NOD (non obese diabetic) mice, was found to inhibit adoptively transferred autoimmune diabetes (Jakubowski, et al., 1995d). As soluble ligand binds to high affinity receptors this proposes the existence of high affinity receptors *in vivo* but it remains to be seen what the physiological stimuli for these receptors might be. Therefore in summary there is good evidence that affinity regulation might actually exist *in vivo* although, especially with respect to LFA-1, further conclusive evidence is awaited.

7.5 CONCLUSION AND FUTURE PROSPECTS

This thesis shows that activation of LFA-1 occurs through signalling mechanisms which may not be novel but rather are composed of molecules and pathways which are already characterised. One of the major findings is that various stimulants can induce LFA-1-mediated adhesion through very different means and that just because a stimulant will induce adhesion does not mean that it is functioning by increasing the affinity of the receptor for ligand. Indeed the stimulants which are used extensively to induce so called "inside-out" signalling events do so by altering cell morphology and not by inducing high affinity receptors. Future work should focus on trying to analyse which might be the physiological triggers for integrin activation and how they might act to induce adhesion i.e. through induction of high affinity receptors or other means. It would also be extremely interesting to elucidate the mechanism by which increases in $[Ca^{2+}]_i$ can induce LFA-1 activation and whether or not this acts through an altogether novel mechanism.

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APPENDIX I

SYMBOLS FOR AMINO ACIDS

A	Ala	Alanine
C	Cys	Cysteine
D	Asp	Aspartic acid
E	Glu	Glutamic acid
F	Phe	Phenylalanine
G	Gly	Glycine
H	His	Histidine
I	Ile	Isoleucine
K	Lys	Lysine
L	Leu	Leucine
M	Met	Methionine
N	Asn	Asparagine
P	Pro	Proline
Q	Gln	Glutamine
R	Arg	Arginine
S	Ser	Serine
T	Thr	Threonine
V	Val	Valine
W	Trp	Tryptophan
Y	Tyr	Tyrosine

APPENDIX II

GMEM-S MEDIUM CONSTITUENTS

GMEM-s medium was prepared using the following constituents in the order described under aseptic conditions

1.	Distilled water		400 ml
2.	10x GMEM	(Gibco)	50 ml
3.	7.5 % Sodium bicarbonate	(Gibco)	18.1 ml
4.	100x NEAA (non-essential amino acids)	(Gibco)	5 ml
5.	100x G+A (glutamate + asparagine)	(see below)	5 ml
6.	100 mM sodium pyruvate	(Gibco)	5 ml
7.	50x nucleosides	(see below)	10 ml
8.	5000 units/ml penicillin-streptomycin	(Gibco)	5 ml
9.	Heat denatured dialysed FBS	(Gibco)	to correct %.

100x G+A (Sigma)

600 mg glutamic acid
600 mg asparagine

To 100 ml in distilled water, filter sterilised and stored at 4°C.

50x nucleosides (all Sigma)

35 mg adenosine
35 mg guanosine
35 mg cytidine
35 mg uridine
12 mg thymidine

To 100 ml in distilled water, filter sterilised and stored at -20°C.

APPENDIX III

PREPARATION OF CENTRICON CONCENTRATORS

Centricon concentrators were prepared for antibody concentration by passivation with 2 ml of passivation solution (5% Tween-20 in distilled water). Concentrators were soaked over night at room temperature after which time the solution was discarded and the concentrator washed thoroughly with tap water. Any remaining passivation solution was removed by washing with 2 ml of water by spinning at 1000*g* until all fluid had passed through the filter. Washing was repeated once more and then the concentrator inverted and finally spun briefly to remove any excess liquid.

PUBLICATIONS ARISING FROM THIS WORK

The following references are publications which arose from this work. They are located in a pocket inside the back cover.

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Leukocyte integrins

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Lymphocytes, monocytes and granulocytes, which are collectively known as 'leukocytes', circulate primarily within the vascular system and lymphoid tissue but are found in essentially all tissues of the body. This mobile lifestyle necessitates the constant making and breaking of adhesive contacts with targets in their immediate environment. The adhesion receptors termed integrins, which are expressed in abundance by leukocytes, are well suited to carry out the transient interactions in which these cells engage. Major advances in the leukocyte integrin field this year have been the realization of the extensive roles for α4 integrins in leukocyte function, the solution of the crystal structure of an I domain and its identification as a major ligand-binding site, and the initial understanding of how divalent cations may function in an active integrin.

Current Opinion in Cell Biology 1995, 7:690–696

Introduction

The largest group of adhesion receptors are the αβ heterodimer family termed 'integrins'. They are found on most cell types and facilitate intercellular communication and cell–matrix interactions. Leukocytes are well endowed with integrin adhesion receptors. Of the 22 identified so far, 13 are expressed by leukocytes, although it is only the β2 and β7 integrins which are exclusive to these cells (Fig. 1). Of note is a newly identified member of the β2 integrin family, named αDβ2, found on a subset of T cells and on certain tissue macrophages in dog and man [1•]. On leukocytes, cell–cell contacts formed by integrins contribute to the activities of antigen presentation, cytotoxicity and phagocytosis. Integrins cooperate with the selectin adhesion receptors to guide T cells into lymphatic areas and in other tissues they direct leukocyte migration across endothelia in response to insult and injury. Leukocyte integrins undergo an activation process during which changes in affinity (conformation) and avidity (post-receptor occupancy) upregulate ligand-binding activity.

In this review we summarize information available this past year regarding leukocyte integrins, with particular emphasis on the β1 and β2 families.

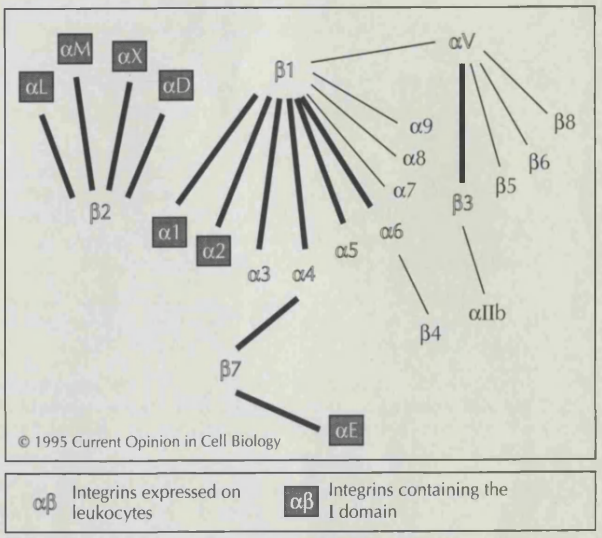

Fig. 1. The integrin family of adhesion receptors. Lines denote the αβ pairings, with thick lines denoting pairings between the integrins expressed on leukocytes. Those integrins that contain the ~200 amino acid inserted or 'I' domain are indicated. The β2 and β7 integrins are only expressed on leukocytes.

α4β1: integrin of the year

During an inflammatory response, a leukocyte must successfully navigate the endothelial barrier (Fig. 2). Neutrophils accomplish this task by the coordinated action of different sets of adhesion molecules. Selectin-mediated rolling along stimulated venular endothelium is followed by integrin activation which allows 'arrest', firm adherence and subsequent spreading and transmigration of the cells. Through studies with leukocytes other than neutrophils, it has become apparent that the use of selectins and integrins is not restricted to distinct stages, as the α4 integrins appear to be capable of participating in all aspects of the adhesion cascade. Under conditions mimicking blood flow, monocytes [2•] and

Abbreviations

ICAM-1—intercellular adhesion molecule-1; **I domain**—inserted domain; **IL**—interleukin; **LFA-1**—lymphocyte function-associated antigen-1; **mAb**—monoclonal antibody; **MIDAS**—metal ion dependent adhesion site; **VCAM-1**—vascular cell adhesion molecule-1.

ISSN 0955-0674

eosinophils [3•] roll on stimulated endothelium by means of the cooperative action of L-selectin and the integrin α4β1. Furthermore, cultured T cells can roll using α4β1 and α4β7 integrins independently of selectins [4••,5•]; however, as the rolling of freshly isolated T cells is mediated largely by P-selectin with support from α4β1 [6••], the former observation suggests that selectin-independence may vary with the state of T-cell activation. Endothelial transmigration has previously been considered to be dependent on αLβ2 (lymphocyte function-associated antigen-1, LFA-1) binding to one of its ligands, ICAM-1 (intercellular adhesion molecule-1), but *in vitro*, human monocytes can also use α4β1 to migrate across interleukin (IL)-1, tumour necrosis factor or lipopolysaccharide (LPS) stimulated endothelium [7] and *in vivo*, monocytic infiltration in a rat arthritis model utilizes both β2 and α4β1 pathways [8]. The role of α4 integrins in other *in vivo* models has been reviewed by Lobb and Hemler [9•]. It is apparent that there may be more to learn, as T-cell rolling along endothelium stimulated for 24 hours with IL-1 could not be attributed to any of the known adhesion molecules, implying that novel receptors have yet to be identified [10•].

As rolling can occur with resting leukocytes, the participation of α4 integrins seems to challenge the dictum that integrins on leukocytes require prior activation in order to bind ligand. The presence of two binding sites for α4 on VCAM-1 suggests another possibility. Binding of α4β1 to domain 4 of VCAM-1 requires cell activation or high levels of Mn^{2+}; however, adhesion to domain 1 appears to be activation-independent [11,12]. It is tempting to speculate that rolling may occur via binding to domain 1 with domain 4 engaged at the arrest stage. It should be stated however, that some studies failed to detect such differences in VCAM-1 domain recognition.

Activating adhesion: affinity versus cell spreading

One mechanism for controlling leukocyte adhesion is by regulating the activation state of integrins for their ligands, a process referred to as 'inside-out' signalling (reviewed in [13•]). The nature of the physiological triggers of integrin activation in the adhesion cascade is still not clear in spite of much recent interest in this topic. A subgroup of the cytokines termed chemokines are good candidates for this event as they have chemotactic and, in certain models, proadhesive functions. Other potential regulators could be the selectins. However, direct evidence that either of these classes of molecule causes integrin activation is still awaited.

A theme emerging from the literature this past year has been that the process of adhesion is not simply limited to the induction of high-affinity receptors, but that significant contributions come from events following

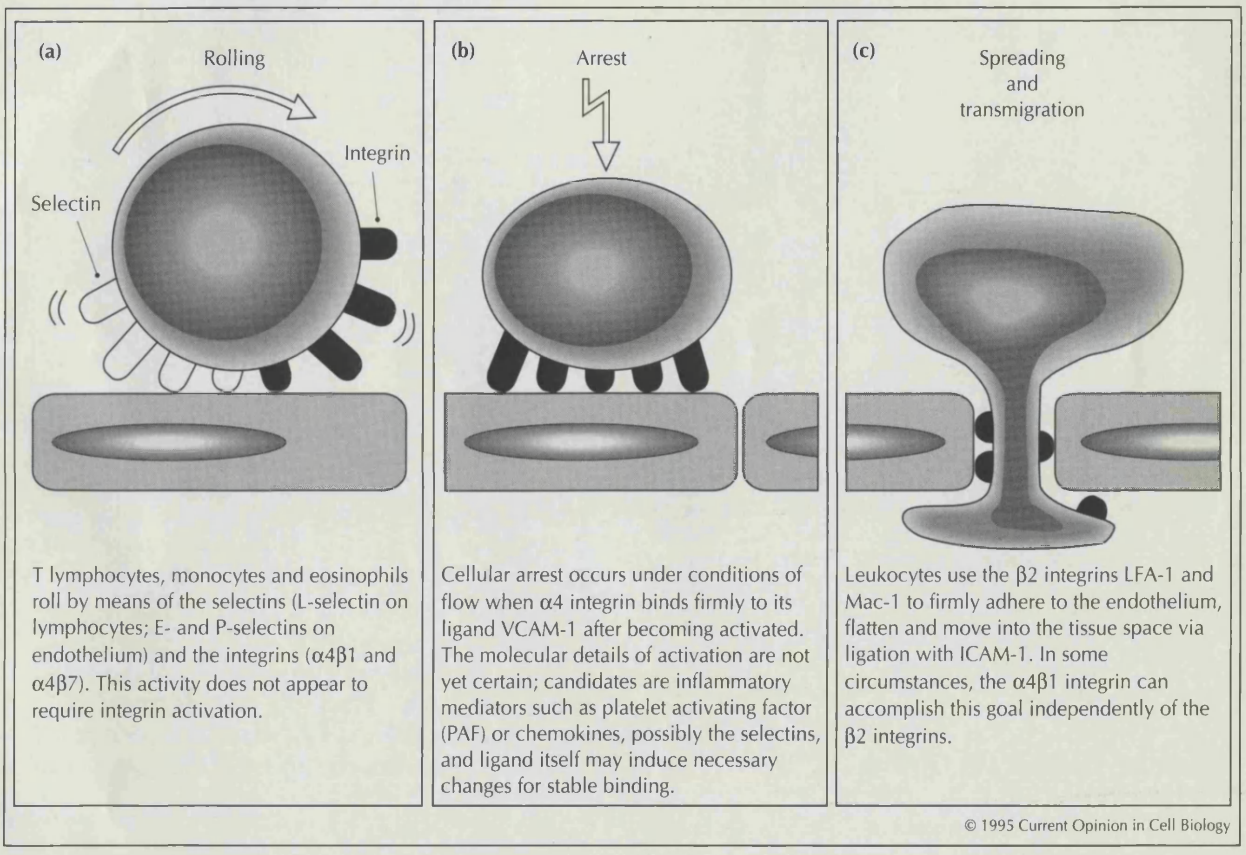

Fig. 2. A schematic depiction of the leukocyte adhesion cascade.

receptor occupancy, which direct cytoskeletal alterations such as receptor clustering and cell spreading. The stimulation of α5β1 on a T lymphoid line with an anti-β1 activating antibody will induce adhesion to immobilized fibronectin through increasing the affinity of the receptor for ligand. On the other hand, phorbol ester increases adhesion through cytoskeletal changes which induce cell spreading rather than by upregulating the receptor affinity [14•]. Similarly, LFA-1 on T lymphocytes binds to ICAM-1 through Mg^{2+}-induced high-affinity receptors which are recognized by an activation reporter monoclonal antibody (mAb) 24. Phorbol ester does not induce mAb 24 epitope expression and resulting adhesion is dependent on actin polymerization and cell spreading (our unpublished data). Integrin adhesion may also be regulated through receptor clustering [15], but whether clustering is a component of the adhesive process or a feature of fully differentiated cells is a question of interest. In summary, successful adhesion probably results from a balance of having sufficient high-affinity receptors and post-receptor events involving cell spreading.

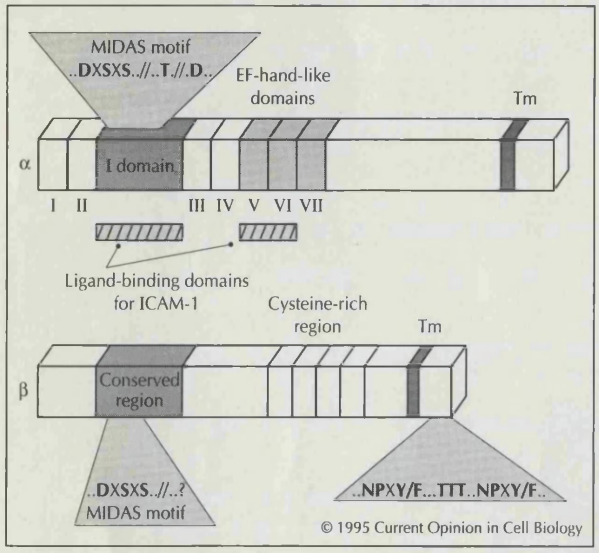

Fig. 3. The domain structure of integrin heterodimers. The α- and β-subunits both contain the highlighted MIDAS (metal ion dependent adhesion site) motif. Domains V–VII in I domain containing integrins (or IV–VII in others) have EF-hand-like cation-binding sequences with a conserved motif **D**X**D**X**D**XXX**D**XXX (see [29]; X is any amino acid). The positions of ligand-binding domains for ICAM-1 within αLβ2 are indicated. The β-subunit cytoplasmic domain insert shows the two conserved NPXY/F motifs present in all β-subunits except β4 and β8. The first NPXY motif has been shown to be important for affinity changes in β1 and β3. The TTT motif in β2 regulates adhesion through post-receptor events. Tm, transmembrane sequence.

Regulation through the tail end

It has been well documented that the cytoplasmic domains of integrin β1 and β3 subunits are required for cytoskeletal associations and signalling (reviewed in [16•]) but there has been little information concerning β2 integrins. New work using chimaeric receptors has shown that LFA-1-mediated adhesion is dependent on post-receptor events regulated through the β2 cytoplasmic domain, even when the receptor is locked in a state of constitutive high affinity [17•]. Mutation of three consecutive threonines (T758–760) in the β2 cytoplasmic domain abolished this adhesion, confirming earlier findings (see Fig. 3) [18]. As well as directing post-receptor events, sequences within the β-subunit cytoplasmic tail are now considered to alter integrin affinity. A conserved NPXY/F motif (single-letter code for amino acids; X is any amino acid) common to all β-subunit cytoplasmic domains (except β4 and β8) can regulate the affinity of β1 and β3 chimaeric receptors as measured by an activation reporter mAb PAC-1 [19•]. This NPXY sequence has previously been shown to be an internalization motif for the low-density lipoprotein receptor and Mac-1 [20]. Finally, it is interesting to speculate that the binding of intracellular factors such as cytoskeletal proteins to different sites might control either key post-receptor events or affinity changes. One potential factor could be the actin-binding protein filamin which directly associates with the β2 cytoplasmic domain at a site distinct from that where α-actinin binds [21•].

Leukocyte integrins as signal transducers

Much of the work on integrin signalling has made use of adherent cells and platelets where β1 and β3 integrins associate in focal adhesions, which are rich in kinases and cytoskeletal proteins (reviewed in [22••]; see also Yamada and Miyamoto, this issue, pp 681–689). Signalling through these integrins stimulates tyrosine phosphorylation (activation) of the focal adhesion kinase $p125^{FAK}$ and other associated molecules; however, the emergence of data connecting leukocyte integrins to these same events has been slow. This year, the cytoskeletal protein paxillin and the Src family kinase member $p58^{fgr}$ have been shown to become tyrosine phosphorylated in stimulated neutrophils through a β2 integrin dependent mechanism [23–25]. LFA-1 on a B-cell line is associated with unknown tyrosine-phosphorylated proteins [26] and on natural killer cells with tyrosine kinases as yet unidentified, but known not to be any of the major Src family members [27]. Triggering β1 integrins on monocytes causes the induction of immediate early genes such as those coding for the inflammatory mediator IL-1β. Recent work shows that the pathway responsible for this involves tyrosine kinase activation and phosphorylation of a pp76 protein [28]. Therefore it seems that integrins present on leukocytes do not engage the same downstream signals as integrins on other cell types and this perhaps reflects their own unique functions. As integrin-specific kinases are identified it will become apparent which signalling pathways are actually involved. One such kinase may be a serine/threonine integrin-linked kinase (ILK) directly associated with the β1 subunit (S Dedhar, personal communication).

Binding sites on integrins: the dynamic 'I' domain

Elucidating the structural basis of the integrin–ligand interaction continues to attract high interest. The ~200 residue sequence known as the inserted or 'I' domain is located in the α-subunits of seven integrins, which are all expressed on leukocytes (Figs 1,3). This I domain has previously been described in extracellular matrix proteins such as collagen and proteins involved in blood clotting such as von Willebrand factor where it is referred to as the 'A' domain. The use of chimaeric α-subunits and mapping studies have localized epitopes for mAbs affecting integrin function to this domain (reviewed in [29]). One of these mAbs, MEM-83, causes LFA-1 to bind to its ligand, ICAM-1, but not to the closely related ligand ICAM-3 [30]. Isolated I domains from LFA-1 [31], Mac-1 [32•,33] and VLA-2 [34,35] have been directly demonstrated to contain binding sites for the majority of their ligands. The I domain is also a target for pathogens with echovirus adhering to α2β1 [36] and the neutrophil inhibitory factor (NIF) protein of canine hookworm to one β2 family member Mac-1 (αMβ2) but not to the others, LFA-1 or p150,95 (αXβ2) [37•,38•].

A major achievement of the year was the determination of a high-resolution crystal structure of the Mac-1 I domain, the first part of an integrin to yield to physical analysis [39••]. The domain was revealed to be a classical 'Rossmann' dinucleotide-binding fold of the type previously observed in intracellular enzymes, displaying a row of six β-strands surrounded by seven α-helices (reviewed in [40•]). In the crystal structure, a Mg^{2+} coordination site termed the metal ion dependent adhesion site (MIDAS) is located at the 'top' of the fold and composed of residues DXSXS with contributions of two amino acids from discontinuous parts of the sequence (see Fig. 3). Mutation of the MIDAS-coordinating residues in the intact integrin prevents ligand binding [41]. The details of where ligand actually binds to this domain are still under investigation but peptides which disrupt intact Mac-1 binding to the complement component iC3b and NIF cover an overlapping β-strand sequence of ~20 residues which impinges on the MIDAS motif [32•,38•]. These peptides may block a direct ligand-binding site, or alternatively interfere with an essential conformational change occurring as a result of activation.

Liddington and colleagues [39••] have also highlighted the similarity in secondary structure between the I domain and a ~200 residue sequence highly conserved in β-subunits, which is also initiated by a MIDAS-like motif (reviewed in [42]). Mutations within this sequence in β1 (Asp131), β2 (Asp134, Ser134) [43•] and β3 (Asp119, Ser121, Ser123) [44] subunits also eliminate ligand binding. In the β-subunit of the platelet integrin αIIbβ3, the cation-binding MIDAS motif is directly implicated in ligand binding through cross-linking of the RGD peptide motif [45,46•,47••]. In a model which makes use of peptides, an unstable ternary complex that forms between divalent cation, integrin and ligand induces the release of cation. These interesting experiments suggest that receptor-bound cation is required initially to maintain conformation, but that ligand capture may generate further alterations within the domain and beyond. It can be speculated that these two MIDAS-containing domains, one in the I domain and one in the β-subunit, function similarly in ligand binding, but direct proof will require further mapping of binding sites in the isolated domains and most conclusively will come from analysis of receptor–ligand co-crystals.

Life beyond the I domain: more binding sites

As only seven integrins contain I domains, there must be binding sites elsewhere on the α-subunits. In α4β1, function-interfering mAbs map to a 52 amino acid sequence (α4:152–203) which surrounds the insertion site for the I domain [48•,49•]. This finding allows the speculation that in evolutionary terms, the I domains have inserted into a region of α-subunit which was already active in ligand binding. In αIIbβ3, additional ligand-binding sites have been identified further down the α-subunit in domain V [50], overlapping a sequence resembling the helix-loop-helix divalent cation binding motif known as the EF-hand motif (reviewed in [29]). Now an LFA-1 binding site for ICAM-1 has been located within domains V and VI, overlapping the EF-hand-like motif in domain VI [51]. ICAM-1 binding to this region is insensitive to chelating agents, suggesting that either divalent cation binds to another site or that it has a function only in the intact receptor.

The changes that occur in integrins as a result of activation and ligand binding have been detected by specific mAbs and named ligand-inducible binding sites (LIBS). Such sites have been mapped to the amino terminus (residues 1–6), the middle (422–490) and the carboxy-terminal end (602–690) of the β3 subunit [52•]. In fact according to the disulphide assignments of Calvete *et al.* [53], long-range bonds would be expected to juxtapose these sequences, forming a structurally constrained cluster of β3 sequence close to the cell membrane which is conformationally sensitive to integrin activation.

The ligands: old friends and new companions

The residues on ICAM-1 important for binding to LFA-1 are those surrounding residue Glu34 in the C/D β-strand (GIETP) and Gln73 of the F/G loop in the first domain. The prediction that this motif is common to all adhesion members of the immunoglobulin superfamily has been emphasised by the mutagenesis of ICAM-3 which highlights an homologous ALETSL sequence as well as Gln75 of the F/G loop [54,55]. Now the solved crystal structure of the first two domains of VCAM-1 [56•] shows the VLA-4 binding motif QIDSPL [57] to

be displayed as a prominent loop between β-strands C/D of domain 1 and repeated in domain 4, hence in an ideal position for ligand capture.

The recently cloned leukocyte-specific integrin, αEβ7, expressed by intraepithelial T cells, has now been shown to bind E-cadherin on keratinocytes, an interaction which may represent a mechanism for retaining T cells within this tissue [58•]. This first description of specific recognition between an integrin and a cadherin seemed less surprising when the solved crystal structures of N- and E-cadherin showed them to resemble Ig-superfamily-like molecules [59,60]. An unexpected ligand has been assigned to the little studied β2 integrin p150,95 which binds iC3b and fibrinogen. Lipopolysaccharide is well known to activate monocytes after binding to CD14 but the fact that p150,95 is also able to recognize lipopolysaccharide solves the longstanding mystery of the identity of the second monocyte receptor for endotoxin [61].

There is increasing information about the participation of integrins in *cis* interactions on the leukocyte surface of which the link between integrin-associated protein (IAP) and β3 integrins is a well known example. Similarly, when the tetraspan protein CD9 becomes expressed on a leukocyte, it is physically associated with the β1 subunit, and certain CD9 mAbs can influence β1 integrin activity [62,63]. Whether these multiple membrane spanning proteins (IAP and CD9) have functions in common remains to be seen but effort is directed towards identifying their possible role in signalling. The two β2 integrins LFA-1 and Mac-1 are found in a membrane complex together with plasminogen activator receptor (uPA-R) implying that localized proteolysis might be a part of β2 integrin mediated activities [64,65]. It is Mac-1 in particular which has these promiscuous tendencies and the most thoroughly investigated of its associations is that with FcRIIIb (CD16). The recognition of this FcR is mediated via a lectin-like site in the carboxy-terminal part of the Mac-1 α-subunit, an interaction mimicked by binding of Mac-1 mAb VIM12 [66]. Thus certain leukocyte integrins associate laterally with molecules on their membranes and in this way may act as important transducers of signals for a variety of receptors. For example, FcR-stimulated phosphorylation of paxillin in neutrophils requires Mac-1 [24]. These associations, which have only recently been appreciated, may rival in importance the more classic ligand-binding properties of these molecules.

Concluding remarks

The pace of new integrin discovery appears to be slackening, with effort now concentrated on exploring the many details of integrin function. This next year will bring more details of the changes which integrins undergo during the course of activation. In particular, the connection between conformational change, ligand and metal ion binding needs to be further understood. The rules of engagement of leukocytes with stimulated endothelium are becoming clearer, but some key issues do remain. The physiological factors responsible for integrin activation in this setting remain elusive and there are other receptors yet to be identified, particularly after prolonged stimulation of endothelium [10•]. It has become apparent that an important role for integrins is to affiliate with neighbouring receptors on the same cell, a feature which may be beneficial in terms of efficient signal transduction. Whether such signals are destined to trigger cytoskeletal change, cause transcriptional activation or co-stimulate by feeding into pathways activated by other receptors, needs further unravelling.

Acknowledgements

We are most grateful to our colleagues Alison McDowall, Clive Landis and Rebecca Newton for their careful reading of this manuscript; we thank Mike Gallatin, Shoukat Dedhar and Roy Lobb for permision to quote their unpublished results. This work was supported by the Imperial Cancer Research Fund.

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T Cell Adhesion to Intercellular Adhesion Molecule-1 (ICAM-1) Is Controlled by Cell Spreading and the Activation of Integrin LFA-1[1]

Mairi P. Stewart,[2] Carlos Cabañas,[2,3] and Nancy Hogg[4]

Many leukocyte integrins require activation before they can adhere to their ligands. For example, stimulation of T cells enables the integrin LFA-1 to bind to ligand. This study compares two well known protocols for inducing T cell LFA-1-mediated adhesion to intercellular adhesion molecule-1 (ICAM)-1. We show that treatment with high concentrations of the divalent cation Mg^{2+} induces a high affinity state of LFA-1, which is reflected in the binding of soluble ICAM-1 and correlates with the expression of the epitope recognized by mAb 24. The second stimulation protocol with the phorbol ester phorbol-12,13-dibutyrate (PDBu) does not induce a high affinity state of LFA-1, and in this situation, adhesion is dependent on cell spreading and intracellular events involving protein kinase C, $[Ca^{2+}]_i$, and actin polymerization. These low affinity LFA-1 receptors are responsible for the initial contact with immobilized ligand because, unlike the Mg^{2+}-stimulated receptors, adhesion is not blocked by soluble ICAM-1. Finally, we used a third method of inducing LFA-1-mediated adhesion by stimulation of T cells through the TCR/CD3 complex. This procedure, which is considered to be a more physiologic trigger for LFA-1 activation, resembles the phorbol ester protocol in that high affinity LFA-1 receptors are not induced and cell adhesion depends on involvement of the cytoskeleton and cell spreading. *The Journal of Immunology,* **1996, 156: 1810–1817.**

Interaction of the integrin LFA-1 (CD11a/CD18; $\alpha L\beta 2$) with its ligands intercellular adhesion molecules (ICAM[5])-1, -2, and -3 (CD54, CD102, CD50) is central to many leukocyte adhesive events (reviewed in Refs. 1 and 2). LFA-1 is maintained in an inactive form on resting leukocytes and becomes activated following signalling through other cell surface receptors such as the TCR/CD3 complex. Phorbol esters such as phorbol-12,13-dibutyrate (PDBu) cause formation of LFA-1/ICAM-1-dependent cell aggregates and have been used as another means of inducing integrin-mediated adhesion (3, 4).

The mechanism by which phorbol esters contribute to cell adhesion has not yet been fully elucidated. Their best studied effect is to activate protein kinase C (PKC), which in turn is responsible for key phosphorylation events in signal transduction (5, 6). Although phorbol ester treatment causes direct phosphorylation of both α and β subunits of the $\beta 2$ integrins (7–9), mutational analysis has shown that this phosphorylation is not necessary for PMA-stimulated adhesion to ICAM-1 (10). More generally, phosphorylation could alter interactions of the $\beta 2$ integrins with cytoskeletal proteins (8, 11) and/or promote adhesion by increasing the avidity of cell contacts through integrin clustering (12), which has been reported for a second $\beta 2$ integrin Mac-1 (CD11b/CD18) (13). Phorbol esters are also reported to cause an affinity increase in murine LFA-1 (14). Apart from effects on integrins, phorbol ester causes morphologic changes such as cell spreading. This could benefit the adhesive state by several means; it could reduce cell surface tension generated by shear stress of vascular flow (15, 16), be required for integrin clustering and potentially make more integrin available for interaction with ligand through a process of diffusion of receptors and ligand trapping (17). Cell spreading occurs following physiologic stimulation of T cells in that it is observed upon engagement of the TCR/CD3 complex (8, 18) and during T cell interactions with antigen-pulsed presenting cells (19).

It is also possible to directly convert LFA-1 to an active form using the divalent cations Mg^{2+} or Mn^{2+} (20, 21). Exposure to divalent cations is speculated to circumvent physiologic triggering procedures and directly bring about changes in the integrin, permitting ligand binding to occur. For example, addition of Mn^{2+} to isolated Mac-1 induces it to bind to fibrinogen (22), and Mg^{2+} can induce $\alpha 4\beta 1$ lacking an α subunit cytoplasmic domain to bind ligand (23). Divalent cations such as Mg^{2+} probably regulate ligand interactions through selective binding to several sites on integrins (reviewed in Refs. 2, 24, and 25) and are thought to directly associate with the ligand binding site and/or control access to a cryptic binding site(s) through altering the conformation of the integrin.

In this study we initially use two different models to analyze T cell adhesion to ICAM-1 and investigate characteristics of the LFA-1 receptor in each case. We show that phorbol ester-stimulated T cells adhere to immobilized ICAM-1 by means of low

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Received for publication October 19, 1995. Accepted for publication December 22, 1995.

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[1] This work was supported by the Imperial Cancer Research Fund (ICRF). C.C. is a recipient of a Postdoctoral Fellowship from the Ministerio Español de Educación y Ciencia and is a ICRF Visiting Research Fellow from Departmento de Bioquímica y Biología Molecular, Facultad de Medicina, Universidad Complutense, 28040 Madrid, Spain. We also gratefully acknowledge an Acciones Integradas grant (1993–1995) from the British Council and Spanish Ministry of Education and Science.

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[5] Abbreviations used in this paper: ICAM, intercellular adhesion molecule; sICAM-1, soluble ICAM-1; BAPTA-AM, bis-(*o*-aminophenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid, tetraacetoxymethyl ester; $[Ca^{2+}]_i$, intracellular calcium; PDBu, phorbol-12,13-dibutyrate; PKC, protein kinase C.

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affinity receptors and adhesion is facilitated though cell spreading, which is dependent on actin polymerization, PKC, and intracellular Ca^{2+} ($[Ca^{2+}]_i$). In contrast, a high concentration of the divalent cation Mg^{2+} induces a similar level of cell adhesion by direct induction of high affinity LFA-1 receptors as measured by the ability to bind soluble (s)ICAM-1. This high affinity receptor state correlates with expression of an epitope recognized by the activation reporter mAb 24. Mg^{2+}-stimulated adhesion has less dependence on the above-mentioned intracellular events.

Materials and Methods

Cells

T lymphoblast cells were expanded from unstimulated peripheral blood mononuclear cells by culture for 1–2 wk in RPMI 1640 medium containing recombinant IL-2 (20 ng/ml; Eurocetus, U.K. Limited, Harefield, U.K.) with details as previously described (21). Cells were used between days 10 and 14.

Monoclonal antibodies and other reagents

Purification and FITC-conjugation of mAb 24 has been previously described (20). Other mAbs used in this study were the LFA-1 α subunit mAb 38 (20), which blocks LFA-1 function, and the anti-CD3 mAb UCHT1 which was obtained from Dr. Peter Beverley (University College, London, U.K.). PDBu, phalloidin-TRITC and cytochalasin D were purchased from Sigma Chemical Co. (Dorset, U.K.). The intracellular Ca^{2+} chelator bis-(o-aminophenoxy)-ethane-*N,N,N′,N′*-tetraacetic acid, tetraacetoxymethyl ester (BAPTA-AM) was purchased from Life Technologies (Paisley, U.K.). The PKC inhibitor Ro 31-8220 was obtained from Dr. Trevor Hallam, Roche Research Centre. Stock concentrations of inhibitors were prepared in DMSO, and in every relevant experiment an equivolume of DMSO was added in the control sample.

T Cell adhesion to immobilized ICAM-1Fc

A dimeric form of an ICAM-1Fc chimeric protein consisting of the five domains of ICAM-1 fused to the Fc fragment of human IgG1 was prepared as previously described (26). The method for quantitation of T cell adhesion to ICAM-1Fc protein has also been previously described (27, 28). Flat-bottom 96-well Immulon 1 plates (Dynatech, Chantilly, VA) were precoated with 0.24 μg/well ICAM-1Fc in PBS-A overnight at 4°C, blocked with 2.5% BSA in PBS-A for 1 h at room temperature, and then washed four times in PBS-A and twice in 20 mM HEPES, 140 mM NaCl, 2 mg/ml glucose, pH 7.4 (HEPES/NaCl buffer). 5×10^7 cultured T cells were labeled with 200 μCi ^{51}Cr for 1 h at 37°C or 25 μCi of [^{3}H]thymidine overnight at 37°C and then washed three times in HEPES/NaCl buffer. Fifty microliters of the cell suspension (5×10^6 cells/ml) was combined on ICAM-1Fc-coated wells with 50 μl of HEPES/NaCl buffer containing titrations of Mg^{2+}, various combinations of 50 nM PDBu, 1 mM EGTA, 1 mM Ca^{2+}, and mAbs. For TCR/CD3 triggering, 10 μg/ml UCHT1 was added to radiolabeled cells, which were maintained in RPMI 1640. After incubation on ice for 20 min, plates were centrifuged at $40 \times g$ for 1 min, and the incubation was continued for 30 min at 37°C. Plates were washed gently four times with prewarmed RPMI, any bound cells were lysed in 1% Triton X-100, and the incorporated radioactivity was measured using a Betaplate counter (LKB Instruments Inc., Bromma, Sweden).

Immunofluorescence microscopy

For immunofluorescence analysis, 13 mm round glass coverslips were precoated with 0.72 μg of ICAM-1Fc in PBS-A overnight at 4°C. Coverslips were blocked with 2.5% BSA in PBS-A for 1 h at room temperature and then washed three times with PBS-A and once with HEPES/NaCl buffer. T cells were washed three times in HEPES/NaCl buffer before addition onto coverslips (5×10^5 cells/coverslip) in the presence of divalent cations, 1 mM EGTA or 50 nM PDBu and mAbs at 10 μg/ml. For staining of intracellular polymerized actin, prefixed adherent cells were permeabilized with 0.2% Triton X-100 in HEPES/NaCl buffer for 10 min, incubated with 0.25 μg/ml TRITC-conjugated phalloidin for 20 min, washed twice, and mounted for fluorescence microscopy.

Flow cytometry and assessment of soluble ICAM-1Fc binding

Measurement of the binding of soluble recombinant ICAM-1Fc to T cells was conducted in the following manner. T cells were washed three times into HEPES/NaCl buffer, resuspended into 50 μl of buffer containing titrations of Mg^{2+} with either 1 mM EGTA or 50 nM PDBu/1 mM Ca^{2+}, and incubated at 2×10^5 cells per well with the indicated concentrations of either sICAM-1Fc or the control protein CD14Fc (a gift from Dr. David Simmons, Oxford, U.K.) (29). After a 30-min incubation at 37°C, cells were washed twice in ice-cold PBS-A with 0.2% BSA and then incubated with 10 μg/ml FITC-conjugated goat anti-human IgG Fc specific Ab (Jackson Immunoresearch Labs, West Grove, PA) for 20 min on ice. Unbound secondary Ab was removed by washing twice in ice-cold PBS-A with 0.2% BSA, and then fluorescence of live cells was detected using a FACScan (Becton Dickinson, San Jose, CA) flow cytometer. For TCR/CD3 triggering experiments, T cells were maintained in RPMI 1640 and stimulated with 10 μg/ml UCHT1 for the 30-min incubation at 37°C and analyzed as described above. Flow cytometric analysis of the mAb 24 epitope was conducted as previously described (20). Briefly, purified FITC-conjugated mAb 24 (10 μg/ml) was added to T cells suspended in HEPES/NaCl buffer in the presence of divalent cations, EGTA and PDBu where appropriate. Following a 30-min incubation at 37°C, the cells were washed three times in ice cold PBS-A with 0.2% BSA before analysis as above.

Results

A comparison of two LFA-1-activating protocols: a) T cell adhesion to immobilized ICAM-1

To define the parameters that are important in the promotion of T cell adhesion, we determined the percentage of activated T cells adherent to immobilized ICAM-1 by means of the integrin LFA-1. The divalent cation Mg^{2+}, when used at concentrations above 1 mM, was able to induce LFA-1-dependent adhesion when EGTA was used to chelate Ca^{2+} (Fig. 1*A*; EGTA). There was little enhancing effect of additional treatment with PDBu (EGTA + PDBu). In confirmation of previous findings (21), the presence of 1 mM extracellular Ca^{2+} inhibited adhesion induced by Mg^{2+} (Fig. 1*A*; Ca^{2+}).

When PDBu was used to induce adhesion in the presence of Ca^{2+}, there was an enhancing effect on adhesion at suboptimal levels of Mg^{2+} (~0.3 mM Mg^{2+}) (Ca^{2+} + PDBu). This adhesion-potentiating effect of Ca^{2+} in the presence of phorbol ester is dose dependent, requiring ~0.5 mM Ca^{2+} for induction of half maximal adhesion (data not shown). The anti-LFA-1 (CD11a) mAb 38 or the anti-ICAM-1 (CD54) mAb 15.2 inhibited the adhesion of T cells in all the conditions, indicating the specificity of binding for LFA-1 to ICAM-1 (data not shown). Thus there are two distinct methods for inducing comparable amounts of LFA-1-mediated adhesion of T cells to ICAM-1; the Mg^{2+} protocol (using 5 mM Mg^{2+} in the presence of 1 mM EGTA) and the phorbol ester protocol (using 50 nM PDBu in the presence of 1 mM Ca^{2+} and 0.3 mM Mg^{2+}). In these two activating procedures, Ca^{2+} appears to have opposing roles.

A comparison of two LFA-1-activating protocols: b) expression of activation reporter epitope recognized by mAb 24

The active state of LFA-1 is reflected in the expression of an epitope detected with mAb 24, which serves as an LFA-1 activation reporter (28, 30). Therefore, the two LFA-1 adhesion-inducing protocols were assessed for expression of this epitope. Figure 1*B* illustrates that Mg^{2+}, at concentrations over 1 mM and in the absence of Ca^{2+} (EGTA), causes high expression of the 24 epitope on LFA-1 and PDBu does not alter this expression further (EGTA + PDBu). As expected from previous studies, the addition of Ca^{2+} had a substantial inhibitory effect on the expression of the Mg^{2+}-induced 24 epitope (Ca^{2+}) (21), which paralleled the adhesion results (Fig. 1*A*).

In the second activating procedure, PDBu in the presence of Ca^{2+} did not increase the expression of the mAb 24 epitope (Ca^{2+} + PDBu). Therefore, in this system the positive effect of PDBu on cell adhesion is not mediated through an increase in the expression of the epitope recognized by mAb 24. These results show a discrepancy between adhesion and 24 epitope expression

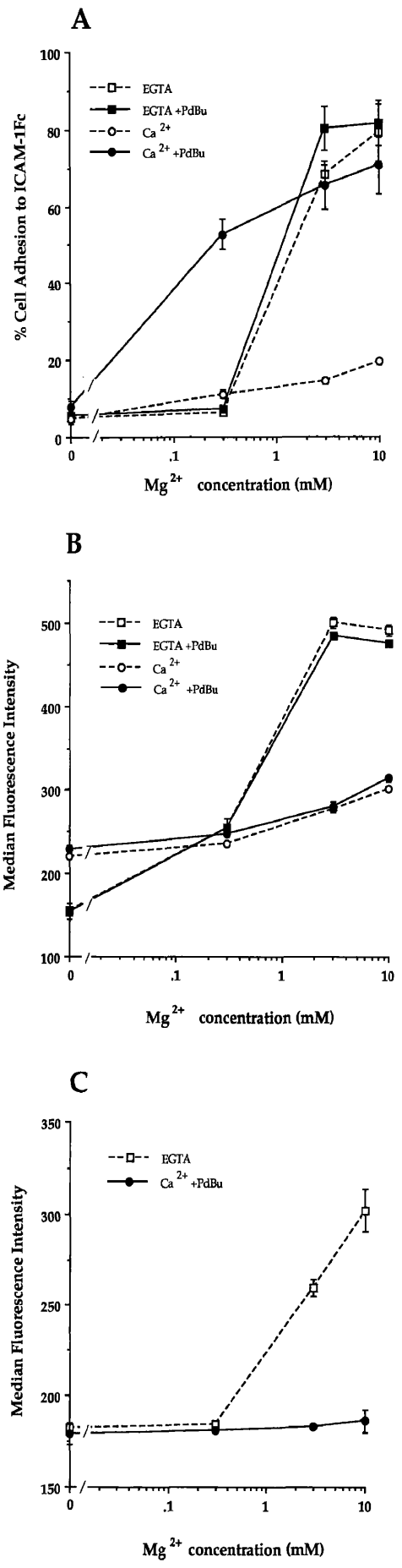

FIGURE 1. Two LFA-1 activation protocols were characterized for their ability to induce; (*A*) T cell adhesion, (*B*) mAb 24 activation epitope expression, and (*C*) soluble ICAM-1 binding, following Mg^{2+} titration. Titration of extracellular Mg^{2+} was carried out in the presence (Ca^{2+}) or absence (EGTA) of 1 mM Ca^{2+}, and the same conditions were tested in the presence of 50 nM of the phorbol ester PDBu (Ca^{2+} + PDBu; EGTA + PDBu). *A*, The adhesion assay was performed as described in *Materials and Methods* and results expressed as the percentage of adherent cells calculated from the total sample. Data represent means of triplicates ± SD, and one representative experiment of ten is shown. Specificity of the adhesion for LFA-1/ICAM-1 was shown by inhibition of adhesion with mAbs 38 (anti CD11a) and 15.2 (anti ICAM-1), not shown. *B*, mAb 24 epitope expression was analyzed as described and is expressed as median fluorescence intensity of triplicates ± SD. One representative experiment of six is shown. *C*, The two independent methods for inducing adhesion of cells to immobilized ICAM-1, following titration of Mg^{2+}, (EGTA; Ca^{2+} + PDBu) were examined for their ability to bind 400 μg/ml ICAM-1Fc as a soluble dimer. Results are expressed as median fluorescence intensity of triplicates ± SD. One representative experiment of three is shown.

(compare Fig. 1, *A* and *B*; Ca^{2+} + PDBu) and suggest that the Mg^{2+} and PDBu protocols do not promote adhesion by the same means, and one explanation for this difference could be in the nature of the LFA-1 receptors present under both conditions.

A comparison of the two LFA-1 activating protocols: c) binding to soluble ICAM-1

To further define characteristics of the LFA-1 receptors activated following the two different activating protocols, T cells were examined for their ability to bind recombinant soluble (s)ICAM-1. A sICAM-1Fc chimera in dimer form bound to LFA-1 following maximal Mg^{2+}/EGTA stimulation with saturating concentration reached at ~400 μg/ml or ~1.8 μM (M. P. Stewart and N. Hogg, manuscript in preparation). This concentration of sICAM-1 can bind to T cells when stimulated with the Mg^{2+} protocol, but the same saturating amount of sICAM-1 showed no binding to T cells stimulated according to the phorbol ester protocol (Fig. 1*C*). A control protein comprising the Ig superfamily member CD14 fused to the same Fc portion of IgG did not bind to the T cells, and mAb 38 blocked the binding of sICAM-1 to these cells indicating the specificity of the reaction for LFA-1/ICAM-1 (data not shown). As the binding of soluble ligand to receptor is considered a measure of receptor affinity, we conclude that high affinity LFA-1 receptors are induced by the Mg^{2+} but not by the phorbol ester protocol. When Figure 1, *B* and *C* are compared, it is evident that 24 epitope is expressed under conditions that support sICAM-1 binding, indicating that 24 epitope expression correlates with high affinity LFA-1 receptors.

Soluble ICAM-1 can block Mg^{2+}-stimulated adhesion to immobilized ICAM-1

Although the phorbol ester treatment protocol appeared not to induce detectable numbers of high affinity LFA-1 receptors, it was possible that a small number of such receptors had a critical role in initiating adhesion. Therefore, we next investigated the ability of sICAM-1 to interfere with the adhesion of T cells to immobilized ICAM-1. Incubation of T cells with saturating concentrations of sICAM-1 (400 μg/ml) could substantially inhibit Mg^{2+}-stimulated adhesion to immobilized ICAM-1, illustrating a dependence on high affinity LFA-1 for the adhesion process (Fig. 2). In contrast, the adhesion of phorbol ester-stimulated T cells was not inhibited by incubation with sICAM-1. This provides further evidence that phorbol ester-stimulated LFA-1 receptors are not in a high affinity state and suggests that adhesion occurs by other means.

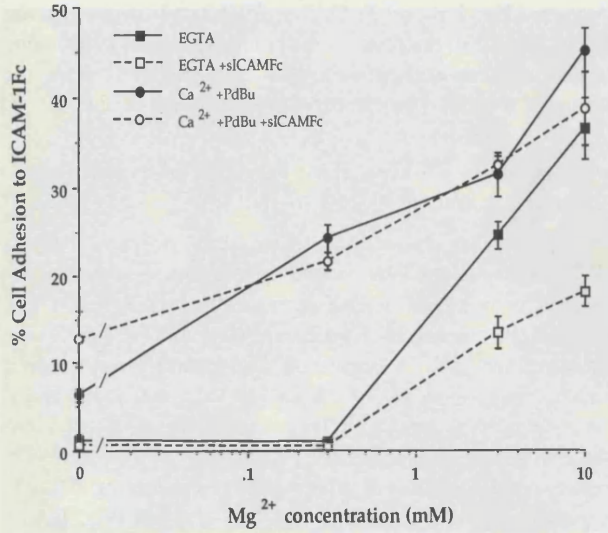

FIGURE 2. The effect of soluble ICAM-1 on Mg^{2+}- and phorbol ester-stimulated T cell adhesion to immobilized ICAM-1. Cells stimulated with the Mg^{2+} (EGTA) or phorbol ester (Ca^{2+} + PDBu) protocol were tested for adherence to immobilized ICAM-1 (0.12 μg/ml) in the presence or absence of 400 μg/ml sICAM-1Fc across a Mg^{2+} titration range. Adherent cells are expressed as a percentage of total cells added per well, and results are shown as the mean of triplicates ± SD. One representative experiment of two is shown.

Enhancement of LFA-1-mediated T cell adhesion to ICAM-1 by phorbol ester is accompanied by cell spreading

To determine whether an alteration in cell morphology could account for the potentiating effect by phorbol ester on adhesion, the distribution of polymerized actin in T cells was visualized by fluorescence microscopy using TRITC-conjugated phalloidin. The majority of T cells that adhered to ICAM-1 via the Mg^{2+} protocols had a rounded appearance (Fig. 3*A*), whereas the phorbol-stimulated cells were flattened and spread on ligand ICAM-1 (Fig. 3*B*). This observation suggests that when T cells are stimulated with phorbol ester, spreading is instrumental in promoting LFA-1-dependent adhesion.

Mg^{2+}- and phorbol ester-induced T cell adhesion show differential susceptibility to cytochalasin D

Since cell spreading is dependent on reorganization of the cytoskeleton, we examined the effect on cell adhesion of cytochalasin D, an agent that inhibits actin polymerization (31). Figure 4*A* shows that 2 μM cytochalasin D abolished the potentiating effect of phorbol ester on T cell adhesion, whereas there was much less effect on adhesion induced by the Mg^{2+} protocol. In addition, cytochalasin D had no effect on the ability of Mg^{2+} to induce expression of the 24 epitope (Fig. 4*B*) nor did it have an inhibitory effect on sICAM-1 binding (data not shown). Therefore, cytoskeletal reorganization leading to cell spreading is an essential component for cell adhesion of phorbol ester-treated cells but not when a high affinity form of LFA-1 is induced from the exterior of the cell by Mg^{2+} treatment.

Role of PKC and $[Ca^{2+}]_i$ in T cell adhesion to ICAM-1

As the phorbol ester PDBu and the divalent cation Ca^{2+} were used in combination to stimulate adhesion, we examined a role for PKC and $[Ca^{2+}]_i$ in this event using intracellular inhibitors. When phorbol ester-stimulated T cells were pretreated with 50 μM of the intracellular Ca^{2+} chelator BAPTA, they exhibited a change from the normal spread morphology (Fig. 5*A*) to a rounded appearance

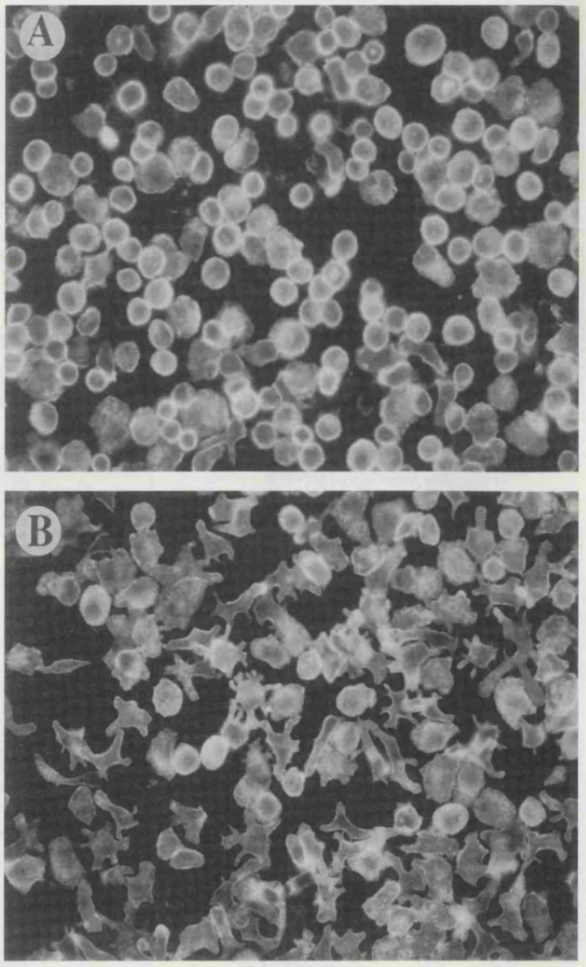

FIGURE 3. T cells adherent to ICAM-1 show different morphology following the Mg^{2+} or phorbol ester treatments. Cells were allowed to adhere to ICAM-1-coated coverslips under the Mg^{2+} (5 mM Mg^{2+}, 1 mM EGTA), *A*, or phorbol ester (50 nM PDBu, 1 mM Ca^{2+}, 0.3 mM Mg^{2+}), *B*, treatments, then fixed, stained with TRITC-phalloidin and analyzed by fluorescence microscopy.

(Fig. 5*B*) and when tested in an adhesion assay were substantially inhibited in adhesion to ICAM-1, whereas Mg^{2+}-stimulated cells were unaffected (Fig. 5*C*). These results indicate that Ca^{2+} plays an intracellular role to facilitate adhesion through cell spreading when cells are stimulated with phorbol ester. We next investigated the PKC inhibitor Ro 31-8220 (32), which significantly inhibited phorbol ester-induced T cell adhesion to ICAM-1 with maximal effects at 1 μM (Fig. 5*D*). As both the inhibitors of PKC and $[Ca^{2+}]_i$ did not interfere with adhesion induced by Mg^{2+}, this indicates that the phorbol ester and not the Mg^{2+}-stimulated adhesion is dependent on intracellular events involving PKC and Ca^{2+}.

Activating adhesion by TCR/CD3 triggering does not induce high affinity LFA-1 receptors

The two methods for inducing adhesion described in this paper are laboratory models that do not utilize the cell surface receptors through which cells would normally be initially triggered in vivo. Therefore, as a third procedure, we investigated the sICAM-1 binding characteristics of T cells, which are stimulated by Ag-specific TCR/CD3 complex engagement, a method that is used to induce binding of T cells to immobilized ICAM-1 and is considered to be more physiologically relevant. Figure 6 shows that sICAM-1 did not bind to T cells triggered by an anti-CD3 mAb,

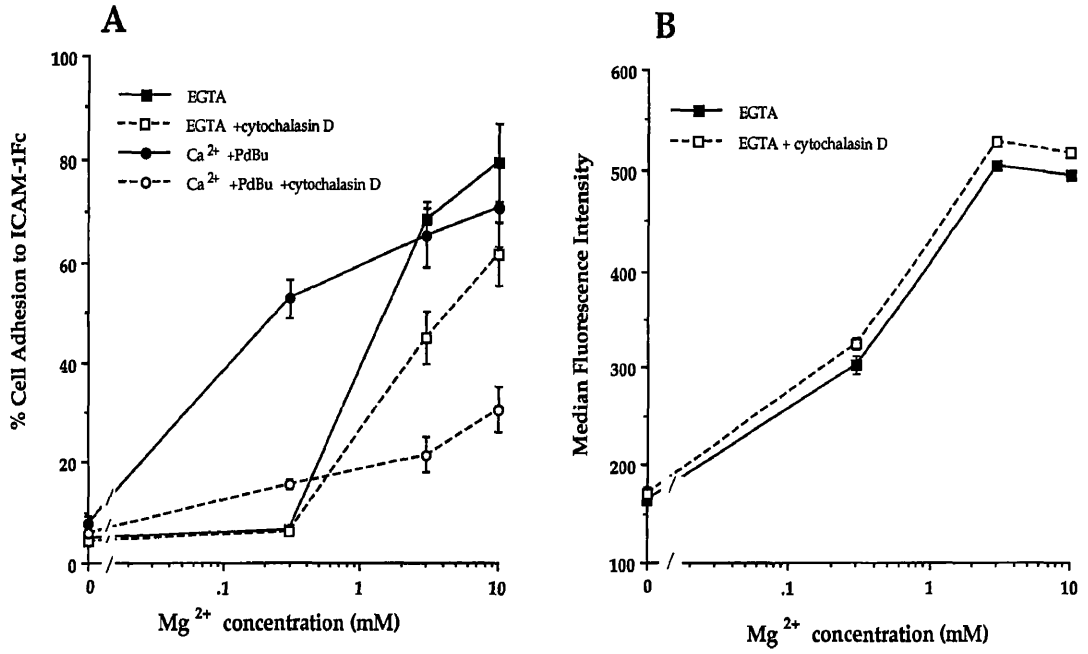

FIGURE 4. *T cell adhesion to ICAM-1 induced by the Mg^{2+} or phorbol ester protocols shows different susceptibility to inhibition by cytochalasin D. The adhesion assay (A) was performed as described in the presence or absence of 2 μM cytochalasin D. Data represent means of triplicates ± SD, and one representative experiment of five is shown. B, Expression of the mAb 24 epitope induced by Mg^{2+} treatment was analyzed in the presence of 2 μM cytochalasin D. One representative experiment of three is shown.*

although they adhered to immobilized ICAM-1 (see inset). Further titration of sICAM-1 up to 1000 μg/ml still failed to show specific binding to LFA-1. The result suggests that this route of stimulating T cell adhesion does not generate high affinity receptors in the first instance. Cells stimulated through TCR/CD3 also resemble phorbol ester stimulation in that they are susceptible to 2 μM cytochalasin D (see inset) and that they exhibit a spread morphology (data not shown).

Discussion

This study was undertaken with the aim of determining critical parameters involved in the adhesion of T cells to immobilized ICAM-1 mediated by LFA-1. Through the use of two different model systems, we find that stimulation with high levels of Mg^{2+} causes the induction of LFA-1 receptors, which can bind sICAM-1 with high affinity and express the mAb 24 epitope. In contrast, adhesion stimulated by the other activating protocol, using the phorbol ester PDBu, is not mediated by high affinity LFA-1 receptors and is instead dependent on cell spreading and intracellular events involving actin polymerization, PKC, and $[Ca^{2+}]_i$. The adhesion that is brought about by triggering through the TCR/CD3 complex resembles the phorbol ester protocol in that it does not induce high affinity LFA-1 receptors and adherent cells are dependent on the cytoskeleton for spreading.

For the first time sICAM-1 is demonstrated to bind to human LFA-1, an achievement that may be explained by the use of large amounts of protein to detect binding. From the plateau or saturation point on the binding curve, the amount of sICAM-1Fc required for saturation was estimated to be 400 μg/ml or ~1.8 μM (Stewart et al., manuscript in preparation). The binding of a similarly engineered construct of VCAM-1 to VLA-4 occurs with a higher affinity of 33–70 nM (33). This difference in affinity is probably not surprising given that the VLA-4/VCAM-1 interaction is preferentially engaged over the LFA-1/ICAM-1 interaction under conditions of flow (34). Alternatively, detection of sICAM-1 binding may have been facilitated by the dimeric nature of recombinant sICAM-1 providing greater opportunity for stable ligand interaction. This form of ICAM-1 appears to have physiologic relevance in that the majority of native membrane-bound, and shed ICAM-1 has recently been demonstrated to exist in dimerized form (35). The ability of Mg^{2+}-stimulated cells to bind sICAM-1 was mirrored by their ability to express the activation reporter epitope recognized by mAb 24. From this correlative expression, we conclude that mAb 24 is recognizing the high affinity LFA-1 receptor state. Future investigation will focus on defining the precise relationship of sICAM-1 and mAb 24 binding to activated LFA-1.

Further analysis of the LFA-1 receptors on T cells mediating adhesion to immobilized ICAM-1 was made by examining the ability of sICAM-1 to interfere with the process of adhesion. Soluble ICAM-1 inhibited Mg^{2+}-stimulated adhesion, confirming that high affinity receptors were responsible for adhesion in this situation. This raises the question as to whether such receptors would be found physiologically and, if so, whether they could potentially be blocked by soluble ICAM-1 in serum. As the serum sICAM-1 level does not exceed ~400 ng/ml in healthy individuals and ~2 μg/ml in diseased states (36), it is unlikely that circulating sICAM-1 would block potential high affinity receptors in vivo unless produced locally at the required ~400 μg/ml level. This situation is in contrast to serum level of soluble fibronectin (450–900 nM), which has been shown to be capable of blocking high affinity $\alpha5\beta1$ receptors (37).

As the phorbol ester PDBu did not induce sICAM-1 binding nor 24 epitope expression by LFA-1, it can be concluded that these receptors are not in a high affinity state. Similar conclusions have been reached by Faull and colleagues through analysis of PMA-stimulated T cell adhesion to fibronectin. In their model, β1 activating mAb 8A2 succeeds but PMA fails to generate high affinity $\alpha5\beta1$ integrins as measured by binding of soluble fibronectin (37). In contrast, PMA increases the affinity of 15 to 30% murine LFA-1 for sICAM-1 in a competitive affinity assay using an anti-LFA-1 mAb (14). It is not certain why there is a discrepancy with our data, but the explanation might involve the activation state of the

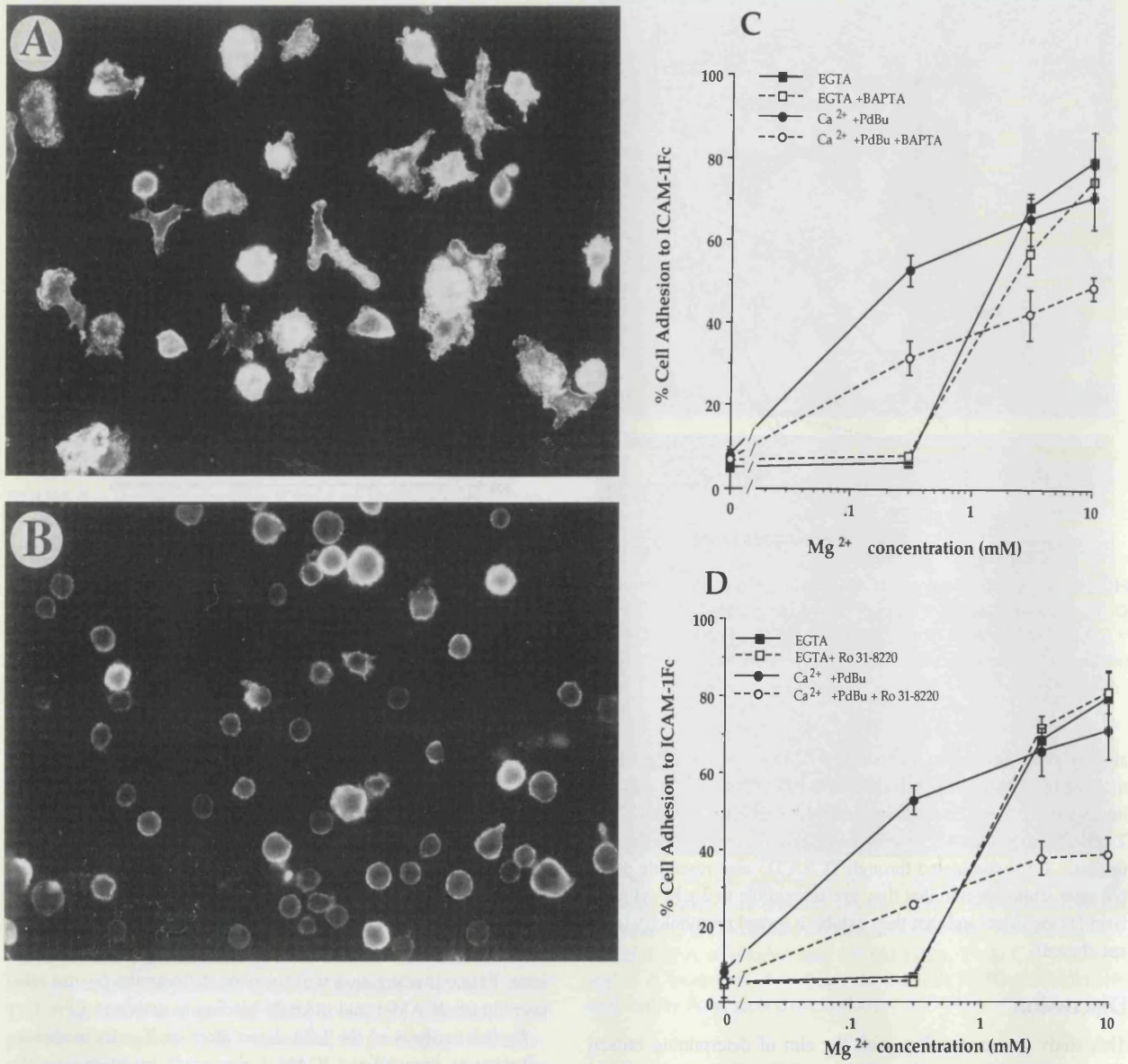

FIGURE 5. Inhibitory effect of the intracellular Ca^{2+} chelator BAPTA and the PKC inhibitor Ro 31-8220 on phorbol ester-induced T cell spreading and adhesion to ICAM-1. T cells were induced to adhere to ICAM-1 according to the phorbol ester protocol (HEPES buffer containing 50 nM PDBu, 1 mM Ca^{2+}, and 0.3 mM Mg^{2+}) without (*A*) or with (*B*) pretreatment with the intracellular Ca^{2+} chelator BAPTA (50 μM) for 30 min at 37°C. Adherent cells were fixed, stained with TRITC-phalloidin, and analyzed by fluorescence microscopy. T cells pretreated with 50 μM BAPTA (*C*) or 1 μM Ro 31-8220 (*D*) for 30 min at 37°C were allowed to adhere to immobilized ICAM-1 in HEPES buffer under the two activating protocols, and the percentage of cells adherent after a further 30 min at 37°C were counted. Data represent means of triplicates ± SD, and one representative experiment of five is shown.

cells, species differences, or the inclusion of extra Mg^{2+} in the assay system of Lollo et al. (14). This general deficiency of sICAM-1 binding to PDBu-stimulated cells raised the question as to the nature of the LFA-1 receptors mediating the adhesion in this situation. Two possibilities were that a small number of high affinity receptors might be pivotal in securing this adhesion or, alternatively, that adhesion was dependent upon another form of active receptor not recognized by mAb 24. As soluble ICAM-1 did not affect phorbol ester-mediated adhesion, it was evident that a low affinity form of LFA-1 was primarily responsible for this adhesion.

The adhesion of PDBu-stimulated T cells is critically dependent on cell spreading involving PKC, $[Ca^{2+}]_i$, and actin polymerization. The exact interplay between these intracellular factors is not yet known, but a link between phorbol ester and the cytoskeleton comes with recent evidence using cell transfectants, which shows that the TTT motif in the $\beta 2$ subunit cytoplasmic domain has a direct role in phorbol ester induced cell spreading through actin reorganization (38). The requirement for $[Ca^{2+}]_i$ could be simply because it is necessary for certain Ca^{2+}-dependent PKC isoenzymes (reviewed in Ref. 6), but it could also potentially, directly affect cytoskeletal rearrangements through its interaction with actin binding proteins (39, 40). Having an intracellular role does not exclude the possibility that calcium could also be required for other aspects of the adhesive event. Extracellular Ca^{2+} is important for clustering of LFA-1 molecules on the T cell membrane (41). Therefore, in addition to a role for cell spreading, these observations also suggest that clustered or microaggregated low affinity LFA-1 receptors may cooperate to increase the overall avidity of cell-cell contact in the absence of high affinity receptors.

The high affinity LFA-1 receptors generated by Mg^{2+} stimulation facilitate stable cell adhesion to ICAM-1 without requirement

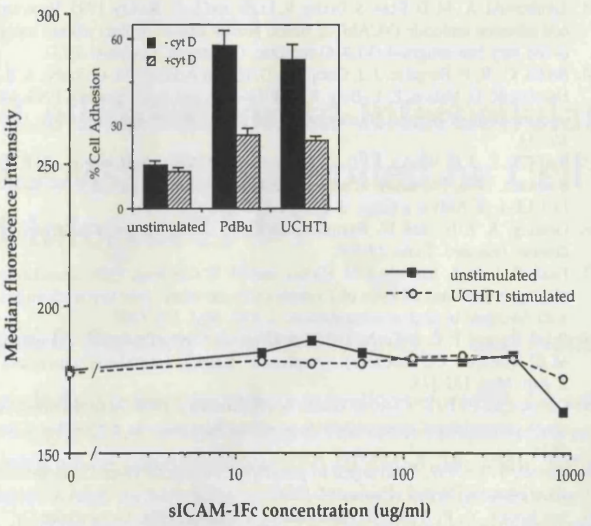

FIGURE 6. Triggering the TCR/CD3 complex induces cytochalasin D-sensitive adhesion to immobilized ICAM-1 but not binding of sICAM-1. T cells were incubated with 10 μg/ml of the CD3 mAb UCHT1 and examined for their ability to bind 400 μg/ml sICAM-1Fc. Results are shown as median fluorescence intensity. Inset: T cells were adhered to immobilized ICAM-1Fc following UCHT1 and PDBu treatment $\pm$ 2 μM cytochalasin D. Data are expressed as means of triplicates $\pm$ SD.

for actin reorganization and intracellular mediators such as PKC and $[Ca^{2+}]_i$. A previous study focussing on $\alpha 4\beta 1$ has shown that Mg^{2+} can support cell adhesion in the absence of the integrin cytoplasmic tail and hence cytoskeletal connection (23). We show here the first evidence that Mg^{2+}-stimulated cell-bound LFA-1 can still facilitate adhesion in the absence of cytoskeletal reorganization. These observations are consistent with the ability of Mg^{2+} to induce high affinity receptors from the cell exterior.

Mg^{2+} can only induce high affinity LFA-1 receptors when extracellular Ca^{2+} is removed, a finding that also applies to other integrins (28, 42–44). An explanation for this could be that on a resting T cell Ca^{2+} may not only occupy sites involved in integrin clustering, but also block access to or occupy the "active" high affinity site associated with Mg^{2+}, which is revealed either through normal activation processes or artificially through Ca^{2+} "stripping." In fact, evidence for two Ca^{2+} binding sites on $\alpha 5\beta 1$ has recently been obtained (45). The authors propose that Ca^{2+} can compete directly with Mg^{2+} for binding to the Mg^{2+}-ligand competent site, but Ca^{2+} binding to a separate high affinity site can increase the affinity of Mg^{2+} for its ligand-competent site. Such exchange of divalent cation may represent part of the normal regulation of integrin activation (21, 46). It is also of interest that the Mg^{2+}/Ca^{2+} ratio can increase significantly during tissue damage, and this correlates with cell movement into wounds (47). Therefore regulation of integrin activation directly by cation may be possible under certain circumstances.

Results from the use of these two model systems have shown us that LFA-1-mediated adhesion can result from direct stimulation of high affinity receptors or through cell spreading and low affinity receptors. This raises the obvious question as to what happens when T cells are stimulated through their physiologic receptors as would be evident during the course of an immune response. The characteristics of adhesion triggered through the TCR/CD3 complex are very similar to the phorbol ester model in that no sICAM-1 binding can be detected (up to 1000 μg/ml), adhesion is sensitive to cytochalasin D, and the cells are spread. Thus, this means of inducing T cell activation, which might be considered as more physiologically relevant, appears not to generate high affinity receptors, a finding that parallels the inability of CD3 triggering on T blasts to generate high affinity $\alpha 4\beta 1$ receptors for fibronectin (33). In contrast, cross-linking the TCR on a T lymphoid cell line did induce a high affinity form of $\alpha 5\beta 1$ for fibronectin (37). These differences in the ability to induce affinity changes require further investigation but may be explained by the nature of the integrin, its activation state, or the cell type on which it is expressed. It will also be pertinent to stimulate cells through their natural ligands rather than via cross-linking Abs to conclusively decide these points.

In summary, we have used the binding of soluble ICAM-1 and expression of an activation reporter epitope to analyze features of two commonly used models of LFA-1-mediated adhesion to immobilized ICAM-1. We show that in addition to high affinity LFA-1, there is a role for spreading in T cell adhesion. It is probable that in vivo a balance between these two factors is a feature of successful adhesion to ligands such as ICAM-1. Studies are awaited to determine whether physiologic triggering through natural ligands acts by increasing the affinity of LFA-1 for ligands or if adhesion occurs through these other means.

Acknowledgments

We thank our colleagues in the Leukocyte Adhesion Laboratory for helpful discussion and critical reading of the manuscript. We are grateful to Derek Davies for help with FACS analysis and to Martin Thiel for invaluable assistance with computer software.

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