Utilization and Regulation of Integrins by Lymphoid Cells To adhere to Fibronectin and Collagen

By

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UTILIZATION AND REGULATION OF INTEGRINS BY LYMPHOID CELLS TO ADHERE TO FIBRONECTIN AND COLLAGEN

BY

DWAYNE G.P. STUPACK

A Thesis/Practicum submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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LIST OF ABBREVIATIONS USED

BSA	Bovine Serum Albumin
CEA	Carcino-Embryonic Antigen
CHI	Cyclohexiimide
COL	Collagen
CS1	Connecting Segment 1 (alternatively spliced) of FN
DMP	Dimethyl Pimidelate
DMSO	Dimethyl Sulfoxide
DMF	Dimethyl Formamide
EDTA	Ethylene-diamine-tetracetic acid
FBS	Fetal Bovine Serum
FN	Fibronectin
FN-15	The 15kDa digestion product of FN containing RGD
FN-40	The 40kDa digestion product of FN containing LDV
FN-120	The 120kDa digestion product of FN containing RGD
GFFK	Glycine-Phenylalanine-Phenylalanine-Lysine residues
GlyCAM	Glycolated Cell Adhesion Molecule
HFN	Human Fibronectin
HFNR	Human Fibronectin Receptor
HSPG	Heparin Sulfate Proteoglycan
ICAM	Intercellular Adhesion Molecule
IGSF	Immunoglobulin Superfamily
LB	Luria Bertani Broth
LDV	Leucine-Aspartate-Valine residues
LFA	Lymphocyte Function Associated Antigen
LIBS	Ligand-Induced Binding Site
LN	Laminin
LPAM	Lymphocyte-Peyer's Patch Adhesion Molecule
MAdCAM	Mucosal Addresion Cell Adhesion Molecule
NCAM	Neural Cell Adhesion Molecule
NET	Tris Buffered Saline, .05% NP40 v/v, 5mM EDTA
NIRS	Nonimmune Rabbit Serum
PBS	Phosphate Buffered Saline
PBSA	Phosphate Buffered Saline, BSA 1% wt/v.
PBSAz	PBSA with .05% sodium azide wt/v
PECAM	Platelet/Endothelial Cell Adhesion Molecule
PMA	Phorbol Myristyl Acetate
PMSF	Phenylmethylsulfonyl Fluoride
RGD, R-G-D	Arginine-Glycine-Aspartate residues
RHAMM	Receptor for Hyaluronic Acid, Motility-inducing Molecule
SLx	Sialyl Lewis x antigen
TB	Transblotting Buffer
TBSA	Tris-Buffered Saline, BSA 5% wt/v.
TBSE	Tris-buffered Saline, EDTA 1mM.
TBST	Tris-Buffered Saline, 1% Tween-20 v/v.
TSA	Tris-Saline solution A (for blot development)
TSC	Tris-Saline solution C (for blot development)
VCAM	Venous Cell Adhesion Molecule
VN	Vitronectin
VNR	Vitronectin Receptor

Abstract

Lymphocytes present in the circulation may be recruited into specific tissue compartments as part of routine trafficking, or in response mediators or inflammation. Upon extravasation, lymphocytes are exposed to a wide variety of extracellular matrix constituents, including the glycoproteins collagen and fibronectin. Interaction with these components may have immunological consequences, as exposure to extracellular matrix components can influence cytokine expression, nuclear transcription factor expression, antibody production, and can act comitogenically to activate lymphocytes.

A variety of lymphoid populations were examined to determine if they could interact with collagen and fibronectin. Variation was observed in the adherence of different cell lines and lymphocyte populations, suggesting that adherence characteristics might be clonal properties. Importantly, many cell lines, and most lymphocyte populations examined, could increase adherence upon cellular activation with phorbol ester. This suggested that integrin mediated adhesion might be a regulatable process.

To examine aspects of integrin activation, a panel of monoclonal antibodies raised against the ß1 integrin were used as functional probes. Antibodies were grouped into three categories; (1) those that blocked attachment to ligand, (2) those that had no effect, and (3) those that activated adherence, analogous to (but distinct from) phorbol ester treatment. Competitive mapping experiments suggested that at least three dissociable epitope clusters were present on the Jurkat ß1 integrin bound by the activating monoclonal antibodies. One of these activating monoclonals, designated B3B11, was also found to be reactive with a transformed bacterial clone in a ß1 integrin/T7 capsid protein epitope display library. No other activating monoclonals (n=6), including two which bound ß1 integrin co-competitively with B3B11, were reactive with the bacterial clone, designated FPG10.B3B11. The pTope plasmid isolated from this clone was observed to bear an inserted DNA sequence encoding 35 amino acids with complete identity to ß1 integrin

sequence 635-669. Thus, one activating epitope influencing the regulation of ß1 integrin is located in a membrane proximal region of the molecule. The location of other putative regulatory sites, and their interactions with the B3B11 site, may be important targets for future studies for molecular/therapeutic manipulation of integrin function.

1.0 INTRODUCTION

1.1 Adhesion is Crucial to the Existence of Metazoans

Adherence is the unifying theme in the development, differentiation and persistence of multicellular organisms. Cellular adherence to neighboring cells or to cellular products is a requirement for maintenance of the physical architecture of any metazoan, and is integral to tissue segregation and organ structure. Thus, adhesion must be an evolutionarily ancient process (Williams and Barclay, 1988). The extracellular matrix glycoproteins collagen and laminin appear to predate cnidaria (Shenk and Steele, 1993). Intercellular adhesion mechanisms described in free-living prokaryotes, such as cyanobacteria and rodophyta, suggest that adhesion may predate the eukaryotic lineage (Stanier, 1974).

It is clear that the disruption of normal adherence processes can make key contributions to a variety of pathological disease states (Ryan and Majno, 1983, Kishimoto et al, 1989, Wardlaw et al, 1990, Coller et al, 1991, Weller et al, 1992). Conversely, the healing of wounds induced by pathogens or other sources of injury, such as physical trauma, require adhesive processes to initiate, facilitate, and complete tissue repair (Ryan and Majno, 1983, Cheresh, 1991a, Grinnell, 1994).

In many cases, the physical architecture of an organism progresses through a series of different stages of morphogenetic development. Drosophila provides a well studied invertebrate example of this phenomena. Interference with a variety of diverse adhesive processes, as a result of mutation or recombination, can result in characterized lethal mutants at different Drosophila life stages (Brown et al 1989, Nose et al, 1992, Wessendorf et al 1992). Similarly, adhesive processes are central to the proper development of the early blastocyst and trophoblast stages in mammals (Takeichi, 1990),

and even the initial fertilization of oocytes themselves (Gahmberg et al, 1992, Cheng et al, 1994). Within a developing fetus, cell-specific differentiation, cellular migration or dispersion, cellular aggregation, axonal outgrowth, and cellular responsiveness to paracrine factors may be all dependant upon directed, specific adherence processes (Hynes and Lander, 1992).

1.2 Adhesion is a Central Process within the Immune System

The concept of adhesion being crucial to development and maintenance of a multicellular organism can be extended beyond mechanical considerations to include defensive strategies. Even in simple animals such as the water flea, specialized cells analogous to human leukocytes exploit adhesion mechanisms as a means of ingesting and destroying (phagocytosis) perceived microbial threats (Metchnikov (1883), as related by Ryan and Majno, 1983). Higher animals respond similarly to localized injury (Conheim, 1889), although active recruitment of defensive cells to affected sites is known to be augmented by local chemical messengers (Devreotes and Sigmund, 1988). In addition to stimulation of phagocytosis, these chemical messengers may modify the capacity for recruited leukocytes to adhere to other cells and to extracellular matrix components, thus modulating the capacity of leukocytes to enter into, and remain within, affected sites.

In addition to local inflammatory responses, higher animals maintain a capacity to perceive and to respond to pathogens in a systemic manner. The systemic reaction is initiated by leukocytes, and is maintained to a large extent by a highly specialized subset of leukocytes, the lymphocytes. In order to effect function, lymphocytes depend upon mechanisms of adhesion. An example are immunoglobulins, secreted by B lymphocytes (Edelman, 1970). Immunoglobulins can act to strengthen adhesion of phagocytic cells to antigenic pathogens via opsonization. This process neatly couples antigenic recognition with basic adhesion-based defensive strategies. Similarly, it is well documented that

certain subtypes of lymphocytes (Tc, NK, K) can function cytolytically, destroying cells which are senescent, transformed, or infected with intracellular pathogens (Wraitch, 1987). The recognition of these cells as targets is dependant upon specific adhesive mechanisms, not all of which are antigen-specific (Young and Liu, 1988).

The interaction of helper T lymphocytes with antigen, under restricted conditions requiring multiple cell:cell adhesion mechanisms (Dustin and Springer, 1991), may trigger the release of a cascade of interactive cytokines from lymphocytes and subsequently from nonlymphoid tissues. In this respect, lymphocytes may be viewed as acting analogous to a decentralized endocrine organ. Lymphocytes are capable of secreting paracrine, autocrine and intercrine factors to attempt to optimize the host for defense against perceived threats (Miyake et al, 1991).

1.3 Adhesion and Lymphocyte Distribution

In the performance of their various functions, lymphocytes traverse between the vasculature and the tissues. Lymphocytes may extravasate as a function of normal daily trafficking, or alternatively, in response to a localized inflammatory reaction. It seems evident that in order to extravasate, adhesive processes are required. However, when lymphocytes are isolated from peripheral blood, they maintain a largely nonadherent phenotype with respect to each other, extracellular matrix components, and other cell types. This observation may be rationalized as follows: in fulfillment of their dichotomous roles (circulating vs tissue-borne) lymphocytes must, unlike many cell types, carefully and appropriately regulate their capacity to adhere. During extravasation, lymphocytes will first encounter endothelium, but subsequently will traverse into the tissues where it will encounter extracellular matrix and tissue-resident cell types (Shimizu and Shaw, 1991, Dustin and Springer, 1991).

The nature of the adhesive interactions within this milieu may be crucial to

subsequent immune responses. Interaction with collagen *in vitro* can influence the quantitative and qualitative antibody response of isolated B lymphocytes (Kemshead and Askonas, 1978). Similarly, T cell clones can be shown to readily secrete cytokines in response to collagen in a manner that is not antigen-specific (Ofusa-Apiah et al, 1989a and 1989b). Isolated T cells have been demonstrated to attach weakly to collagen, and to attach to fibronectin with increasing efficiency over time (Arencibia and Sundqvist 1989).

Despite these indications that lymphocytes may functionally interact with the extracellular matrix, and the potential implications these interactions might have in a potential immune response, the processes involved in these interactions occur are not well understood.

1.4 The Current Investigation

In order to better understand the processes inherent to lymphocyte interaction with extracellular matrix, several basic concerns need to be addressed. The foremost concern is the demonstration of specific interactions between lymphocytes and extracellular matrix. Preliminary studies had suggested that lymphocytes may interact with collagen coated surfaces (Wilkins and Ofusa-Apiah, unpublished observations). The nature of the receptors which might influence these processes had not, however, been determined. The precursor frequency of adherent cells suggested that the process was not antigen receptor-specific.

Within the past decade, a family of receptors which have cellular adhesion as a linking theme has been characterized on the surface of many cell types, including lymphocytes (Carderelli and Pierschbacher 1986, Hemler et al 1986, Hynes, 1987). These receptors, the integrins, have also been implicated in the adhesion of thymocytes (Carderelli and Pierschbacher, 1986) lymphocytes and monocytes (Klingemann and Dedhar, 1987), and lymphoid cell lines (Bernardi et al, 1986) to fibronectin.

The objective of these investigations has been to examine lymphoid adhesion to extracellular matrix *in vitro*. Both lymphoid cell lines and purified lymphocytes were used in the studies. The lymphoid lines provided the utility of homogenous cell populations in ready supply. The purified lymphocyte populations were used to assess the potential physiological relevance of observations established using the cell lines. In respect to this, the Jurkat cell line proved to be very useful in a collagen adhesion model. Jurkat cells attach weakly to collagen, but could be activated, or induced to attach strongly to collagen, through a variety of means. This observation parallels results obtained using activated T lymphocytes. Since lymphocytes isolated from the periphery are only weakly adherent, they must undergo phenotypic change to become adherent, and attach to extracellular matrix. It was not known whether this phenotypic change required *de novo* synthesis of adhesion receptors, or alternatively, whether receptors which might already present on the surface of lymphocytes are functionally regulated.

The current study implicates members of the integrin family of adhesion receptors, principally those belonging to the ß1 family, as the primary (with respect to strength of contribution) extracellular matrix receptors on the surface of lymphocytes. The expression of these receptors on peripheral lymphocytes appears regulated at both the level of expression, as a potential correlate of cellular activation/maturity, as well as at the level of functionality of expressed integrin complexes. Distinct signals can be shown to differentially influence these different processes *in vitro*.

In order to characterize the observed functional activation of the ß1 integrin receptor, a panel of monoclonal antibodies generated against ß1 integrins was used as molecular probes. These antibodies were used alone and in combinations to elucidate specific contributions of regions of the ß1 integrin to integrin heterodimer activation on lymphocytes. The results obtained suggest that (1) integrin functional regulation may be multifactorially influenced, and (2) interaction with several, experimentally dissociable sites

on the integrin heterodimer can contribute to activation.

These observations are consistent with the possibility that discrete, integrin-adhesion activating signals may originate external to the lymphocyte plasma membrane. It is clear, however, that functional activation may result from signals originating cytoplasmically or potentially even from within the plane of the membrane. Thus, the results of these investigations may prove valuable both in explaining regulatory phenomena observed in integrin adhesion systems, as well as providing a rational basis for future studies examining putative intramolecular associations influencing regulatory sites of the ß1 integrin subunit.

2.0 REVIEW OF THE LITERATURE

2.1 Several Adhesion Mechanisms Exist in Mammals

The Adhesion Receptors

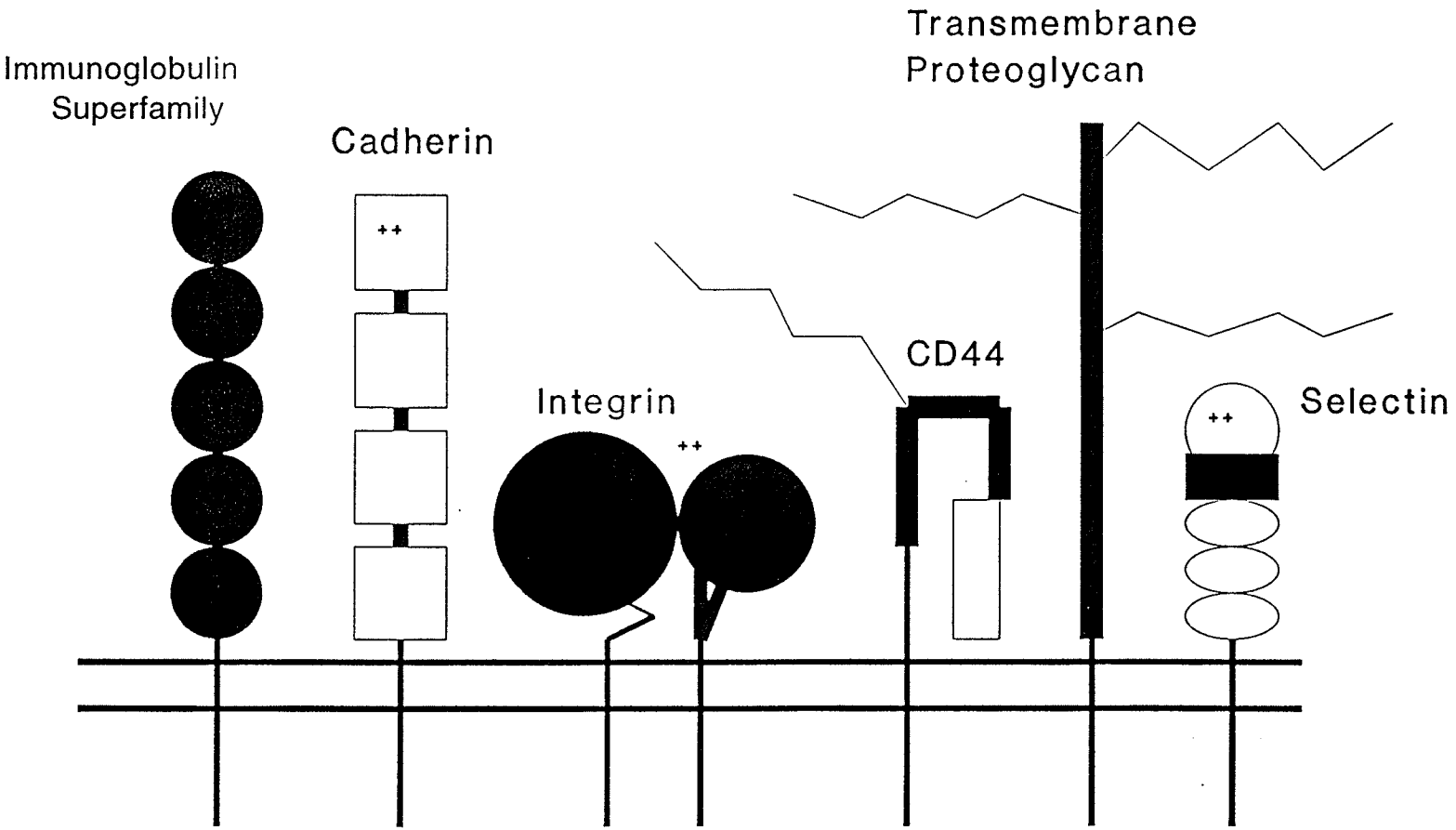
Within recent years, a number of different families of adhesion mechanisms have been identified as contributing to the interaction of cells with other cells or with extracellular glycoproteins. At least three distinguishable families of glycoproteins, the cadherins, the immunoglobulin superfamily, and the integrins are capable of mediating attachment to protein components within the extracellular matrix or upon adjacent cell surfaces (Springer, 1990a). In addition, several families of receptors which mediate protein:carbohydrate interactions with extracellular matrix or cell surface ligands have been identified, including chondroitin and heparin sulfate proteoglycans (HSPGs), syndecans (Elenius et al, 1990), hyaluronan receptors such as CD44 (Jalkonen and Jalkonen, 1992, Peach et al, 1993) and RHAMM (Turley, 1989), and selectins (Lasky et al, 1992).

These protein:protein and protein:carbohydrate interactions work in concert to mediate complex cell behaviours such as motility, extravasation, and wound contraction (Hynes and Lander, 1992). Many of these receptors can transmit signals into the cell. The interaction of the various signals, in conjunction with cell-specific factors and chemical stimuli, contribute to the observed behaviour of a specific cell type within a given milieu.

Each of the three described protein:protein type adhesion receptor families functions in a unique and specific fashion. Many (if not all) cells possess receptors from several of these families. Figure 2.1 illustrates important structural features of these identified adhesion systems.

The first group of adhesive glycoproteins listed are the cadherins, of which twelve have been characterized. These molecules mediate cell:cell adhesions via homotypic

Figure 2.1 Adhesion Receptors. Schematic diagram of the structure of selected cellular adhesion receptors. Small filled circles represent Immunoglobulin-type repeats, the IGSF member depicted is ICAM-1, and is based on EM derived images. Some IGSF molecules are not transmembrane, but instead are phosphatidyl inositol-linked (GPI-linked). Open boxes represent cadherin-type domains. The membrane distal domain possesses the calcium-binding domain (++ depiction) and adhesive site. The [?¿] and ß integrins are depicted as adjacent globular domains (resembling quotation marks, "), as seen in EM-derived images. The average integrin dimer is estimated to bind 3.6 divalent cations, as depicted by the ++ symbol. The ligand binding region comprises regions of both subunits. The hyaluronan-binding protein CD44 is depicted with a chondroitin-sulfate covalent attachment (thin zig-zag), as is common on lymphocytes. The link homology (hyaluronan-binding) region is depicted as an elongate open box, while the proteoglycan core type region is depicted as thick, filled line. The proteoglycan core motif is similarly depicted on the membrane proteoglycan depicted, ryducan (a syndecan-relative). Matrix binding is accomplished by multivalent interaction with the heparin and chondroitin sulfate polysaccharide groups. Both CD44 and some proteoglycans have forms which are GPI-linked. The short consensus complement-type repeats within the selectins are depicted as open ovals. Three are depicted here in the L-selectin model. Adjacent to the repeats is the EGF-like domain (filled rectangle) followed by the terminal lectin-like domain (pseudo-hemisphere). The terminal domain possesses the SLx-binding and calcium-binding sites.


Immunoglobulin
Superfamily
Cadherin
Transmembrane
Proteoglycan
CD44
Integrin
Selectin
++
++
++

cadherin associations (Takeichi, 1990). For example, N(neural)-cadherin bearing cells will aggregate with other N-cadherin bearing cells, but not T-(trophoblastoid) or E-(epithelial) cadherin bearing cells. In these cellular interactions, the cadherin present on one cell functions directly as the ligand of the cadherin on the other. This system of adhesion provides a mechanism of promoting aggregation of distinct and segregated cell types based upon cadherin expression. In order to promote these associations, cadherins require calcium. The calcium ions have been shown to be bound by the cadherin corresponding to the active ligand binding site. This site has been mapped to the most membrane-distal (N terminal) of three or four "cadherin type" repeated amino acid sequences.

The cadherins play a crucial role in morphogenetic events as early as the primary cell division following fertilization (Takeichi, 1990). The expression of cadherins in mature cells appears to be regulated at a constant level; decrease or lack of E (epithelial) cadherin expression has been demonstrated to influence the metastasis of tumors (Vleminckx et al, 1991). Conversely, increase in cadherin expression appears to correspond to terminal stages of keratinocyte differentiation (Hodivala and Watt, 1994).

The second group of adhesion molecules are members of the immunoglobulin superfamily (sometimes abbreviated IGSF). These adhesion molecules are also type I transmembrane or phosphatidyl-inositol linked glycoproteins comprised principally of several (two to seven) immunoglobulin-like domains which exist in the unpaired state. The immunoglobin-like domains include those which are immunoglobulin constant domain-like, immunoglobin variable domain-like, and fibronectin type-III domain-like. The latter group has recently been shown to possess a high degree of structural homology to the other principal groups (Leahy et al, 1992, Huber et al, 1994, Dickinson et al, 1994). Examples of these molecules include the various forms of NCAM (neural cell adhesion molecule)(Frelinger et al, 1986), the related ICAMs (intercellular adhesion molecules 1,2 and 3)(Staunton et al, 1988, 1989, 1990, Landes et al, 1994), VCAM-1 (vascular cell

adhesion molecule) (Osborn et al, 1990), MAdCAM (mucosal addressin adhesion molecule)(Hu et al, 1992), CD2, CD4, CD8 (Bierer and Burakoff, 1987, 1991), CD22 (Sjoberg et al, 1994), CD31/PECAM (Delisser et al, 1994), CEA (carcinoembryonic antigen)(Benchimol et al, 1987) and PVR (polio virus receptor)(Bernhardt et al, 1994). These adhesion molecules act both as receptors and ligands through an active binding region located principally in the terminal immunoglobulin repeat, although alternative domains may also be involved. The CD31 and NCAM molecules can function as receptors for cell surface heparin sulfate proteoglycans. This activity appears to be cell type specific, as these same receptors may also function as mediators of homophilic-self interactions similar to those performed by cadherins, but in a calcium-independent manner (Saffell et al, 1994, DeLisser et al, 1994). Additionally, MAdCAM possesses a 37 amino acid region between two immunoglobulin domains which is rich in O-glycosylation sites. This region has been identified as a sulfate-dependant ligand of L selectin (Briskin et al, 1992). Many of the IGSF members (ICAM- 1,2,3, VCAM-1, MadCAM) serve as ligand/binding structures for members of the integrin superfamily, discussed below.

The variety of interactions which can involve members of the IGSF raises questions regarding the distinction between receptor and ligand. At the purest molecular level, it is not currently understood which molecule binds which, and independant signal transduction events have been associated with both cell types in coligation studies (Hynes, 1992). Therefore the "ligand" and "receptor" roles are not absolutely defined. To address this, alternative terms have been proposed, including cognate and anti-cognate, and receptor and counter-receptor (Springer, 1990). Although this observation is conceptually important, the adaption of these terms has been slow; ICAMs, VCAM and MAdCAM are still termed "ligands" in the bulk of the literature.

The third family of protein-binding receptors are the integrins. Integrins are heterodimeric (α/ß) type I transmembrane glycoproteins with short cytoplasmic domains.

There are currently no less than 15 identified α subunits and 8 identified ß subunits, which may assemble into at least twenty-one known heterodimers (Hynes, 1992, Shaw et al, 1994)

The ligand binding site is comprised of regions of both the α and ß subunits (Smith and Cheresh, 1989, 1991a, 1991b, DeSousa et al, 1990, Loftus et al 1990, Yamada, 1991, Diamond and Springer 1993). The specificity of ligands bound by different integrin complexes is determined by particular α/ß combinations.

Integrins bind to at least two major classes of identified ligands. The majority of identified integrin complexes interact with extracellular matrix glycoproteins, including such ligands as fibronectin, collagen, laminin, vitronectin, thrombospondin and fibrinogen as a partial list (Ruohslahti, 1991, Hynes, 1992). Certain integrins (α4ß1, α4ß7, αEß7, αLß2, αMß2) also bind to cell surface adhesion molecules of the immunoglobulin superfamily, notably VCAM (Rice et al, 1990, Taichman et al 1991) the ICAMs (Diamond et al, 1991, Staunton et al, 1989, 1990) and MadCAM (Strauch et al, 1994). Few integrins are specific for a single ligand, most integrins may bind several (Hynes, 1992). Conversely, most ligands bound by integrins have sites to interact with other integrin and nonintegrin receptors. (Cheresh et al, 1989, Takada et al, 1989a, Pulido et al, 1991, Von der Mark et al, 1991, Wayner et al, 1991).

The amino acid sequence of integrins suggest no common structure related to known proteins, with the notable exception of the EF-hand type consensus sequence (the divalent cation-binding sequence first identified in calmodulin), one of which is present in the ß integrins (Hu et al, 1992) and three or four of which are present in the α integrins (Tuckwell et al, 1992). It is likely that these domains are functional; radiolabelled cations can be demonstrated to bind to integrins (Smith and Cheresh, 1992) and to influence attachment to ECM. The presence of different cations may influence ligand specificity of some integrin complexes. (Kirchhoffer et al, 1990b, 1991, Ignatius and Reichardt 1988,

Greziake et al, 1991). Further, it is clear that certain antibodies bind to integrins only in the presence of calcium, suggesting recognition of cation-dependant epitopes (van Kooyk et al, 1991, Kouns et al, 1990).

The second broad group of cellular adhesion receptors, which mediate binding via protein:oligosaccharide interactions, include two general classes of cellular receptors. One class of cellular receptor reacts with extracellular carbohydrate moieties via cellular glycoproteins, often lectins (Stahl et al, 1987). An example of this class of adhesion receptors are the selectins, type I transmembrane glycoproteins with short cytoplasmic domains. Selectins have been observed on the surface of hematopoietic as well as endothelial cells; selectins have not yet been found in other tissues (Lasky and Rosen, 1991). Three selectins are currently known, and are comprised of a calcium-dependant, amino terminal, type C lectin-like domain, followed by an epidermal growth factor (EGF)-like domain, and a variable number of short consensus repeats of the type common to complement protein receptors (Law, 1988). The function of the complement repeats is unknown, but have been suggested to function in possible oligomerization of the receptors.

The lectin-like domain is absolutely required for binding of selectins to their major ligand, the carbohydrate tetrasaccharide structure classified as sialyl Lewis X antigen (SLx). Different selectins bind different forms of this antigen with varying affinities, although some common forms are bound by all selectins (Lasky et al, 1992). The physiological ligands of the selectins are not well characterized. It is clear that not all glycoproteins bearing SLx are recognized. This may reflect particular local protein conformations (permissive to glycoconjugate binding) being present in only certain glycoproteins. Alternatively, it is also possible that a specific peptide sequence within the SLx bearing protein may be required as a coligand to stabilize binding. It is also possible that sialic acid residues in many SLx bearing proteins may be modified and thus be unrecognizable. One manner this could occur is via acetylation of carbon seven or carbon

nine on the sialic acid moiety (Sjoberg et al, 1994). The carbohydrate recognized by the selectins may be conjugated to proteins via different means. The 50kDa GlyCAM molecule, which has been called mucin-like, binds to L selectin via O-linked, sulfated, (non-sialyl Lewis X) moiety (Lasky et al, 1992). A similarly O-linked SLx bearing ligand for L-selectin is CD34 (Baumhueter et al, 1993). Both of these proteins are expressed within lymph node. L-selectin, a glycoprotein, also functions as a ligand for both E and P selectins (Lawrence and Springer, 1992). Other, less characterized ligands have been found for E and P selectins. P selectin can bind to an integrin (LFA-1, αLß2) at an undefined site (Gahmberg et al, 1992), as well as to a 250/120 kDa (nonreducing/reducing conditions) ligand which has yet to be further characterized (Moore et al, 1992). E selectin has been identified to bind to a 150 kDa ligand present on mouse neutrophils (Lenter et al, 1994). The differentiation of selectin ligands, though not understood, is likely to involve the EGF domain (Camerini et al, 1989, Kansas et al, 1994). It is not known if the EGF domain binds to ligand directly. It is possible that two different adhesive interactions are required, one by the lectin domain and one by the EGF-like domain, to elicit binding. A second required interaction would assist in explaining selectin specificities. Alternatively, the EGF-like domain may influence the conformation of the adjacent lectin domain, and therefore influences binding specificity in a more subtle manner.

All members of the carbohydrate-binding group of receptors do not require lectin-like domains, however. Well studied examples on non-lectins include the hyaluronan binding glycoproteins CD44 and RHAMM. These proteins appear to act to regulate cellular motility. The level of CD44 expression, as well as the expression of alternatively spliced forms of CD44 (Peach et al, 1993), have been associated with increased cellular motility and/or metastasis. Similarly, high levels of CD44 expression appear to identify memory lymphocytes in vivo, which are known to traffick to a greater extent (Sanders et al, 1988). The binding of the common ligand hyaluronan by the second receptor, RHAMM, triggers

protein-tyrosine kinase activity (Turley, 1989, 1992). This activity has been demonstrated to be required for cellular migration, and has a direct influence on the stability of focal adhesions (Hall et al, 1994), areas of intimate contact between cells and substratum (Burridge et al, 1987). These molecules may interact with hyaluronan through a region of protein homologous with link protein (Thomas et al, 1992), although other areas of these molecules may also be important (Peach et al, 1993, Mackay, et al 1994). Other nonlectin proteins which may bind proteoglycans include the previously mentioned IGSF members PECAM and NCAM, which can mediate heterotypic cell:cell adhesions in certain cell lines. These cell:cell adhesions are sensitive to the addition of heparin sulfate, suggesting membrane HSPG as a candidate counter-receptor on the target cell.

The second type of protein:carbohydrate interaction involves the attachment of cell-associated carbohydrate with extracellular glycoprotein ligands. This type of receptor is typified by membrane bound chondroitin or heparin sulfate proteoglycan (HSPG) adhesion receptors, which include the syndecans (Carey et al, 1992, Saunders et al, 1989), glypican (David et al, 1990) and cerebroglycan (Stipp et al, 1994). The extracellular matrix binding function of the syndecans, and probably the other HSPGs, resides in the heparin sulfate and chondroitin sulfate glycan groups attached. It is possible that some other glycoproteins interact with the extracellular matrix via similar means. The capacity of a variant CD44 to bind to the extracellular matrix component fibronectin has been associated with covalent modification to include a chondroitin sulfate group (Jalkonen and Jalkonen, 1993). The HSPG receptors interact with heparin binding domains principally on glycoprotein components of the extracellular matrix. For example, the CD44-chondroitin sulfate variant binds to the heparin binding domain on the carboxyl terminus of the glycoprotein fibronectin. This same location is bound by HSPGs of the syndecan family, of which four have been identified. The syndecans are grouped into a family based upon conserved transmembrane and cytoplasmic domains and homologous extracellular domains. Many of

the HSPG molecules may also bind the cytokine basic fibroblast growth factor. The capacity for syndecans to localize in regions of focal adhesion, to act as "coreceptors" for extracellular matrix components such as fibronectin and collagen, and to bind cytokines suggests strongly that these molecules contribute to cellular regulatory and response processes. In a similar manner, the expression of cerebroglycan in the brain tissues correspond exactly with the passage of mitosis and the advent of neural migrations. After migration, there is a loss of cerebroglycan expression, suggesting that cerebroglycan is intimately involved in migratory processes of developing neural tissue (Stipp et al, 1994).

Although many known adhesion receptors belong to the described "families" (or groupings), this list is not exhaustive. Many very well characterized adhesion receptors, such as the thrombin/von Willebrand factor receptor GPIb/XI (Murata et al 1991, Lopez, 1994) the thrombospondin receptor gp88/CD36(Huang et al, 1991, Asch et al, 1993), DPP-IV (Gorrell, 1994, dipeptidyl protease IV, CD26, a collagen receptor) and the ß1-4 galactosyl transferase (a laminin receptor) (Evans et al, 1994) do not neatly fit into one of the described families of related molecules. These ungrouped molecules clearly also play key roles in specific adhesive processes, often colocalizing at cellular sites of adhesion and modulating signalling events (Olurundare et al, 1993, Huang et al 1991, Gorrell et al, 1994). Since it is a common event for a single ligand (especially a component of the extracellular matrix) to be bound by several receptors which belong to the same or different families in a noncompetitive, and often cooperative, manner, it is possible for a great variety of different signals to be transmitted internally in response to ligand binding. A great variety of signals are potentially possible as permutations of the expression and function of different cellular receptors. The "summation," or interaction of these varied extracellular signals through specific and common cellular signalling pathways, must clearly be important in the final response of a cell to its environment.

The Extracellular Matrix

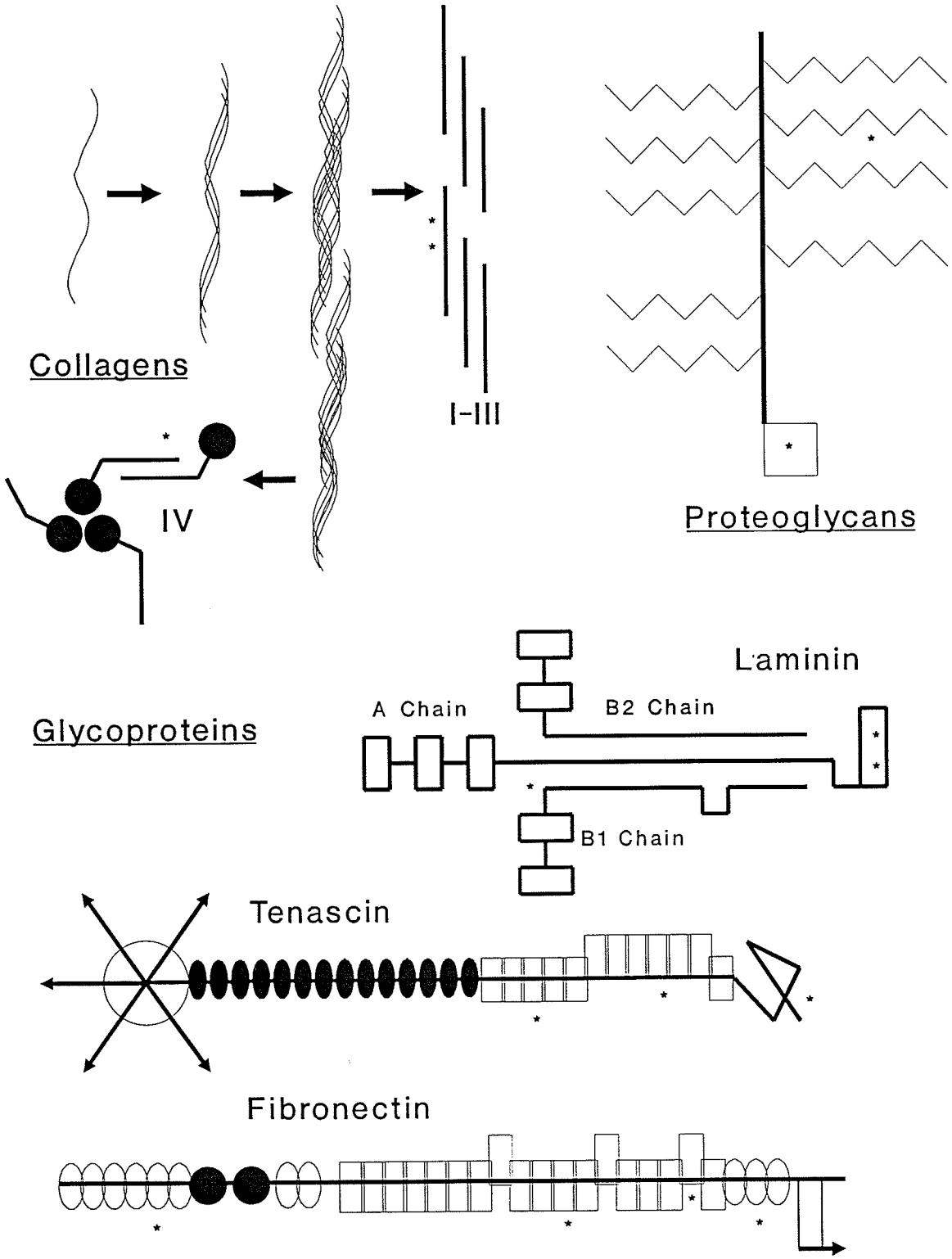
In addition to the cell surface receptors functioning in adhesion, there are several extracellular molecules which contribute to adhesive processes. For purposes of discussion, these molecules may be divided into three broad classes; collagens, glycoproteins and proteoglycans (Figure 2.2). One commonality inherent to these three diverse structures is their ability to associate/aggregate with molecules of their own type. All of them also possess the capacity to interact with other classes of extracellular adhesion molecules. These two properties are crucial to the formation of a multivalent extracellular matrix for interaction with cells.

There are at least fourteen types of collagen (De Sousa et al, 1990). The characteristic feature of collagen is the left-handed 3/10 helix, an atypical (by Ramachandran plot) protein structure stabilized by the presence of glycine and proline (Creighton, 1993). There are four common types of collagen, of which types I-III are fibrillar and type IV is "globular". Types I-III assemble into long fibrils which may be nanometers long. Type IV is composed of a fibrillar collagen domain and a globular domain. Both domains autoaggregate independently, resulting in a meshwork lattice found in basal lamina. The site of collagen binding by adhesion receptors is not known, although the amino acid sequence D-G-E-A has been identified as the target of $\alpha 1 \beta 1$ integrin binding in type I collagen (Staatz et al, 1991). The binding site of heparin sulfates to collagen has also been mapped to a group of basic residues in the amino terminus of type I collagen (San Antonio et al, 1994). Collagens may also interact with extracellular matrix glycoproteins, such as fibronectins, fibrin, and laminins, described below.

The extracellular matrix glycoprotein components include both serum proteins such as fibrinogen, vitronectin and fibronectin, and lamellar proteins such as tenascins and laminins. These molecules are characterized by the presence of multiple domains for

Figure 2.2. Selected Extracellular Matrix Components

The three groupings of extracellular matrix components are depicted (not to scale). Each has several sites for interaction with cellular receptors, identified with small stars. Nascent collagen chains assemble into left-handed triple-helices, which then coassemble to form fibrils. The formation of fibrils through autoassociation is important in the ultrastructure of types I-III collagen, and also plays a role in the assembly of the type IV collagen lattice. Proteoglycans are depicted with an extended protein backbone and carbohydrate substitution along the length of the molecule. The substitution may be extensive, involving dozens of sites, as in aggrecan, or univalent, involving only one site,as in decorin. Additionally, some proteoglycans possess a lectin type domain, as is depicted by the open box. Three glycoproteins are also depicted; laminin, tenascin and fibronectin. The entire laminin molecule, which forms a cross as assessed by EM, is depicted at comparatively reduced scale. Globular domains are depicted as boxes, while fibrillar domains are depicted as lines. The three chains (A,B1, B2) actually form a coiled-coil along the length of the cross. The other two glycoproteins, fibronectin and tenascin, have been depicted in a manner which stresses their modularity. Tenascin is composed of fibronectin type III repeats (open vertical rectangles), epidermal growth factor repeats (filled vertical ovals) and a terminal fibrinogen-like knob. Alternatively spliced domains are depicted as elevated above the horizontal. One of six similar "arms" of tenascin is depicted, the other five are represented by arrows. Fibronectin is composed of type I (vertical open ovals), type II (filled circles) and type III (vertical open rectangles) repeats. Alternatively spliced (from mRNA transcripts) regions are depicted as elevated above the horizontal. One of two similar arms of a fibronectin molecule, connected by two disulfide bonds, is depicted. The arrow represents the second arm.interaction with either cellular receptors or other extracellular matrix components.


Collagens
I-III
IV
Proteoglycans
Laminin
A Chain
B2 Chain
B1 Chain
Glycoproteins
Tenascin
Fibronectin

Several of these molecules are depicted in Figure 2.2.

Laminin and related molecules such as merosin, entactin, kalinin, and nicein are trimeric A-B1-B2 complexes (Ae/Ak/Am, B1e/B1s, B2e/B2k) of total approximately 800kDa. These molecules, which are evolutionarily almost as old as collagens (Shenk and Steele, 1993), possess at least five characterized sites for cell binding. Three of these sites serve as ligands for five different integrin heterodimers (Gehlsen et al, 1989, Hall et al, 1990, Laurie and Laurie, 1994). (Actually, seven integrin heterodimers are known to bind laminin, however, the site of interaction of two of these heterodimers is not yet known)(Sonnenberg, 1993). The third laminin cell binding site, partially characterized, interacts with a cell surface carbohydrate binding protein (ß1-4 galactosy-transferase) (Evans et al, 1994), and a fourth site is available to bind HSPG (cell bound or extracellular)(David, 1993). It is clear, at least in the case of α3ß1 integrin, that different laminin subtypes may be bound with differing affinities (Sonnenberg, 1993). If this observation is extended to include laminin receptors in general, then specific, regional variations in laminin composition may influence the capacity of differing cell types to adhere. Laminin also possesses a site for the binding of type IV collagen, and these two macromolecules are found closely associated in the basal lamina of many tissues. Nontrimeric forms of laminin have also been observed, such as truncated B1 chains, and lone B1k chains. These molecules are expressed in skin, and appear to play a role both in keratinocyte anchorage and differentiation (Sonnenberg, 1993).

Fibronectin is commonly found as a matrix or lamellar protein, but is also present in human serum at approximately 300-400µg/ml (Yamada et al, 1992). Fibronectin, like laminin, possesses many regions for interaction with cellular receptors or other matrix components. Fibronectin is a homodimeric, covalently cysteine-linked, approximately 440 kDa, elongate protein composed of three different types of repeating, globular domains, based upon amino acid sequence. These domains are termed type I, II, and III repeats.

The fibronectin dimers which may assemble into a fibrillar form via N-terminal domain associations. This process is dependant upon cell surface integrin α5ß1 and at least one other uncharacterized structure (Yamada et al, 1992, Schwarzbauer, 1993). Additional associations are made possible by specific sites present along different regions of the fibronectin glycoprotein. Fibronectin possesses two distinct binding sites for heparin sulfates, two distinct sites for fibrin binding, one collagen binding domain, and two distinct sites which are recognized by at least five different integrin heterodimers. The integrin binding sites occur within the "FN-type III" amino acid sequence repeats. These repeats have been crystallographically determined to resemble immunoglobulin-type CH2 domains (Constant region, Heavy Chain, Second Domain)(Dickinson et al, 1994) in tertiary structure. These type III repeats also appear in other glycoprotein matrix components, such as vitronectin and tenascin, in some members of the immunoglobulin superfamily of receptors, and in some type II cytokine receptors as well (Miyake et al, 1992). Fibronectin mRNA may be variably spliced to generate different subtypes of fibronectin, ten of which have been observed (Kornblihtt et al, 1985). The second identified integrin binding site, (specific for α4ß1), is one such region (Humphries et al, 1986). Alternative mRNA splicing represents a mechanism by which fibronectin expressed in the serum or in different tissues can be "customized" with respect to function.

The cell and matrix binding regions have been classified by proteolytic digestion of intact fibronectin to yield protease-resistant fragments, as well as through the use of monoclonal antibodies specific to the active regions on these fragments (Pierschbacher et al, 1981) This capacity to isolate different modules of fibronectin has been useful in elucidating functional relationships between regions. For example, the initially identified integrin binding site in fibronectin contains an R-G-D amino acid sequence which represents the minimum amino acid sequence requirement for integrin-mediated adhesion. However, two of the Type III domains immediately N-terminal to this region act in a

synergistic manner to dramatically strengthen observed adhesion (Yamada et al, 1992). Deletion of these domains will negatively influence the strength of integrin:RGD binding. The minimum R-G-D sequence has recently been demonstrated to occupy a turn between adjacent ß-strands within a type III repeat (Dickinson, 1994). This region is analogous to the V region of immunoglobulin variable domains. The original identification of this R-G-D sequence has proved useful in identifying sites of RGD-inhibited interaction of integrins with tenascin and vitronectin. Both of these proteins also contain R-G-D amino acid sequences in approximately similar positions within fibronectin type III repeats (Yamada et al., 1992).

Fibronectin may also bind secreted proteins, such as α1 microglobulin (Falkenberg et al, 1994) and tumour necrosis factor-α (Hershkovitz et al, 1994). The effects of binding the immunomodulatory α1 microglobulin upon cellular attachment to fibronectin are unknown, but association with TNF-α increases the capacity for cells to bind fibronectin through a protein-tyrosine kinase requiring mechanism (Hershkovitz et al, 1994).

A second serum protein important in the formation of platelet aggregates is fibrinogen. Fibrinogen is a hexameric, glycoprotein of nascent Mr 450,000 Da which possesses central covalent (disulfide) linkages. Fibrinogen is activated by proteolytic cleavage to form fibrin, which self associates into large fibril at wound sites. In addition to sites bound by vitronectin and fibronectin, and the autoassociation sequences unmasked after the proteolytic transition from fibrinogen to fibrin, each fibrinogen molecule may be recognized by four different integrins at four discrete sites. At least one of these sites is unmasked by reducing agents in the central disulfide "knot" of the fibrinogen molecule (Sun et al, 1992).

The tenascins (tenascin-C, X and R) are lamellar proteins (like laminin), are hexameric (like fibrinogen), and are most closely related to fibronectins in domain organization. Tenascins are hexameric molecules composed largely of type III fibronectin

repeats, and are present in fetal, malignant and healing tissues (Matsumoto et al, 1994). Tenascins also possess an RGD sequence within the third type III repeat of each monomer which has been identified as an integrin binding site. A second adhesion site exists in a distal, fibrinogen-like "knot" structure (Joshi et al, 1994). These sites are independent of a third adhesion site bound by annexin II, within a type III repeat, in a calcium dependant manner (Chung and Erickson, 1993). The structure of three flanking type III sequences has been analyzed by X-ray crystallography to determine the interactions and relationships of adjacent domains of tenascin. These domains appear to closely resemble IGSF proteins in organization. (Leahy et al, 1992). Serum proteins other than fibronectin and fibrinogen also appear important in cellular adhesion processes. In response to injury, serum proteins are deposited upon the damaged site, forming a quickly-assembled matrix which cells may attach to. In vitro, these proteins may also be deposited on plastic surfaces from serum used in culture. Vitronectin (Hayman et al, 1985), originally classified as serum S (spreading) factor by virtue of its activity upon adherent cell lines in vitro, is one such protein. Vitronectin is a 65-75 kDa protein which contains a cell attachment site recognized by no less than three different integrins. Vitronectin also possesses a fibrinogen binding site and a heparin sulfate binding site. A fourth important serum protein, which may also be found as part of the extracellular matrix is thrombospondin. In addition to a site recognized by an integrin (and possessing an RGD sequence, presumably in an FN type III domain) (Lawler et al, 1988), thrombospondin is also recognized by HSPGs, the CD36/gp88/gpIV receptor and assembling fibrin fibrils.

The last major class of extracellular matrix macromolecules are the proteoglycans. Proteoglycans are typified by the presence of long, unbranched chains of disaccharide repeats. These carbohydrates usually contain an amino sugar derivative of galactose or glucose as one of the members of the repeat. The identity of these structures is conferred either by unique second sugars, by specific modifications on the amino sugar, or both. For

example, hyaluronan is composed of repeats of glucuronic acid and N-acetyl glucosamine, while heparin sulfate is composed of repeats of glucuronic acid and N-acetyl glucosamine-4-sulfate. A further difference in these carbohydrates is that hyaluronan is found as an isolated saccharide macromolecule (while this technically makes hyaluronan not a proteoglycan, its capacity to associate with protein components is sufficient to allow it to be generally grouped in this category) , while heparin sulfate is found attached to a proteoglycan core protein. Heparin sulfate can be bound to core protein by link proteins, which are extracellular matrix proteins capable of binding both hyaluronan and core proteins. An important feature of these molecules is the described highly repetitive carbohydrate motif. This motif promotes a high valency of interaction of proteoglycans with receptors or other extracellular matrix proteins, which provides a means to increase the avidity of potentially low affinity interactions. Additionally, some soluble proteoglycans possess lectin-like domains (Ruohslahti et al, 1992) and could interact with cell surface components in this manner.

These molecules exhibit a very high hydration index, and, as previously described, may also bind certain cytokines, including basic fibroblast growth factor and transforming growth factor ß1. These properties of proteoglycans allow these macromolecules to act multifactorially upon cells encountering them, influencing cellular recruitment, residency and responses (Aviezer et al, 1994).

It may be noted that many components of the extracellular matrix may also be cell-associated (eg., thrombospondin, fibronectin, proteoglycan) and may mediate adhesion between adjacent cells by "bridging." In this case, neither cell actually requires counter-receptors for the other. Intimate contact may instead be maintained through common cellular interactions with cell-bound extracellular matrix components, such as cell-assembled fibronectin fibrils (Hauzenberger and Sundqvist, 1993, Wilkins et al, 1993).

The extracellular matrix may therefore be viewed as a composite lattice of the three

discussed classes of different macromolecules, each of which interacts with other matrix components, with cellular receptors, and possibly even with cytokines (at least with respect to proteoglycans and fibronectin). The reactions of a cell in response to this milieu will depend not only upon the capacity of a cell to interact, or not, with each individual matrix component, but also upon receptivity to a given signal, cellular interpretations of these interactions, and the final summation of the varied intracellular signalling systems affected.

2.2 Adhesion Receptors Identified on Lymphoid Cells

Many adhesion molecules have been identified on the surface of lymphocytes since the presumptive characterization of CD4 as an adhesion molecule (Table 2.1). Notably, the only group of adhesion molecules from which a member has not (yet) been identified on lymphocytes are the cadherins (Hynes and Lander, 1992).

The sequencing of CD4 and subsequent electron micrography demonstrated the existence of immunoglobulin-fold containing molecules which do not contain paired, hydrophobically-apposed subunits. This was confirmed upon analysis of CD4 by X-ray crystallography (Wang et al, 1990). The CD4 molecule was originally identified as a T subset marker, and subsequently as a coreceptor (along with the T cell receptor) for antigen associated major histocompatibility class II molecules. The CD4 molecule is a highly specific adhesion molecule which functions in the context of T cell help. The CD4 molecule is required as a necessary costimulus for cellular activation during antigenic challenge of the TCR. Thus CD4 also provided evidence that an adhesion molecule could transmit signals into the cell.

It was clear that other early adhesive events characterized in lymphocyte systems, such as the auto-aggregation of certain lymphoid cell lines, and the rosetting of T cells with sheep red blood cells, were not mediated by an antigen specific complex, and were not

Table 2.1 legend: This table lists adhesion receptor identified on lymphocytes which have characterized ligands. This table, adapted from Hynes, 1992 and Shimizu and Shaw, 1990, incorporates information gathered from the literature as well as from within the results section. Receptor: refers to the identified receptor on the lymphocyte surface, while brackets indicate alternative names. The ligand/counter receptor column refers to ligands which have been demonstrated to be bound by the receptors when they are expressed on lymphocytes, or have been demonstrated to be bound by receptors expressed in nonlymphoid cell types [in square brackets].

Table 2.1

ADHESION SYSTEMS CHARACTERIZED IN LYMPHOCYTES

Receptor:	**Ligand/Counter-receptor:**
IGSF	
CD2(LFA-2)	LFA-3
CD4	MHC-II
CD8	MHC-I
ICAM-1	αLß2 integrin (LFA-1)
ICAM-2	αLß2 integrin (LFA-1) αMß2 integrin (Mac-1)
ICAM-3	αLß2 integrin (LFA-1)
LFA-3	CD-2
Integrin	
α1ß1	(Collagen, Laminin)
α2ß1	Collagen, (Laminin, α3ß1)
α3ß1	Laminin, (Collagen,α2ß1, Fibronectin)
α4ß1	Fibronectin, VCAM, Madcam(weak)
α5ß1(FNR)	Fibronectin
α6ß1	Laminin
αLß2(LFA-1)	ICAMs - 1, 2, 3
αMß2(Mac-1)	(ICAM - 2, Fibrinogen, C3b)
αVß3(VNR)	Fibronectin, Vitronectin, Laminin (Fibrinogen, von Willebrand factor, Thrombospondin, Bone Sialoprotein, Osteopondin
α4ß7(LPAM)	Madcam, Fibronectin, VCAM
αEß7	Madcam
Other families	
CD44 (Hermes, pgp-95)	Hyaluronan, Laminin Collagen, Fibronectin
Syndecan I	Collagen,Fibronectin
L-Selectin (Lec-cam)	SlX-Ag, GlyCam, gp-95

TCR or CD4 specific (Dustin et al, 1987).

Three adhesion molecules were originally identified as contributing to lymphocyte adherence processes, and were termed LFA (lymphocyte function associated) antigens 1, 2 and 3. LFA-2 and LFA-3 were determined to be receptor:ligand cognates for each other, and are now known as CD2 and CD58. The strong cell:cell adhesion during antigen presentation was determined to be mediated by LFA-1, a heterodimer of CD18 and CD11a, and its cognate, ICAM-1(CD54) (Haskard et al, 1986, Springer, 1990). LFA-1 is an integrin of the ß2 subfamily, and is also known as αLß2; ICAM-1 is a member of the immunoglobulin superfamily (Staunton et al, 1988). In addition to T cell help, the LFA-1/ICAM-1 interaction was implicated in the autoaggregation of certain T cell culture lines as well as the transient adhesion required in cytolysis of target cells by cytotoxic T cells. ICAM-1 is inducible on endothelium in response to inflammatory cytokines such as tumor necrosis factor, which provides a means for initial attachment to endothelium prior to the extravasation process (Dustin and Springer, 1991, Harlan and Kovach, 1991). The inability of ICAM-1 specific antisera to block certain LFA-1 dependant adhesion processes led to the subsequent discovery of ICAM-2, a further immunoglobulin type cognate ligand for LFA-1, on the surface of some lymphoid cell lines and endothelium. ICAM-1 and ICAM-2 appear regulated at the level of expression, and can be upregulated in different cell types in response to cytokines. A third ligand for LFA-1(αLß2) has recently been discovered, ICAM-3. ICAM-3 is highly homologous with ICAM-1, and shares some common determinants important in LFA-1 binding (Landes et al, 1994). Unlike ICAMs 1 and 2, which may be found on nonhematopoeitic cells, ICAM-3 is found specifically on the surface of leukocytes.

Other receptors have been identified through studies on the capacity of lymphocytes to "home", or traffick specifically to certain tissues. The MEl-14 antigen, which corresponds to L-selectin, is required for homing of lymphocytes to lymph nodes (Lasky

and Rosen, 1992). CD44 has also been suggested to be a homing receptor and an activation marker on certain lymphocyte subsets (Berg et al, 1989). Lymphocyte CD44 has been demonstrated to be a receptor for fibronectin, collagen, laminin and hyaluronan, and a further unidentified lymph node component. While the capacity for lymphoid CD44 to bind fibronectin, collagen and laminin is directly related to chondroitin sulfate covalent modification (Jalkonen and Jalkonen, 1993), hyaluronan binding is mediated by the peptide portion of CD44. The nature of the putative ligand(s) involved in lymphoid trafficking, and attachment to lymph node via CD44 remains unknown.

A third homing receptor, more recently characterized, is LPAM (Lymphocyte:Peyer's Patch Adhesion Molecule). LPAM appears to be required for homing of lymphoid subpopulations to Peyer's patch gut associated lymphoid tissues (Erle et el, 1991). LPAM has been shown to be an integrin, now classified as α4ß7, and to bind to fibronectin, MAdCAM, and VCAM. A very recently discovered homing receptor is αEß7, which has been demonstrated to be enriched in intra-epithelial compartments (Pinola et al, 1994, Strauch et al, 1994).

Integrins other than those observed in homing studies, ie., LPAM (α4ß7) and αEß7, have also been identified on the surface of lymphocytes. The expression of two activation antigens, VLA-1 and VLA-2 (VLA, very late activation) is highly elevated approximately four weeks after stimulation of peripheral T cell populations (Hemler et al, 1983, 1984, Hemler, 1990b). The antigens are detectable on less than 5 to 10 percent of the naive population of cells, but are present on virtually 100 percent after activation. The VLA antigens were discovered to be α/ß heterodimeric complexes, with VLA-1 and VLA-2 sharing a common VLA-ß subunit based upon coprecipitating bands. The use of antisera to this common ß subunit in immunoprecipitation studies suggested the existence of more than the 2 α subunits already characterized. The function of this group of heterodimeric

molecules on lymphocytes was initially unknown.

Insight came when the VLA-ß subunit was discovered to be antigenically identical to the ß subunit of the fibroblast fibronectin receptor (Brown and Juliano, 1987, Takada et al, 1987). The α chain which complexed with this common ß subunit was distinct from the characterized VLA-1 and VLA-2 α subunits, and it was also found to be expressed on activated T cells and thymocytes (Cardarelli and Pierschbacher, 1987, 1988, Hemler et al, 1987). Just as in fibroblasts, this molecule could be demonstrated to be a fibronectin receptor on lymphocytes (Cardarelli and Pierschbacher, 1987, Bernardi et al, 1987). These three antigens are now characterized as integrins α1ß1, α2ß1 and α5ß1, and have subsequently been identified on a great variety of different cell types (Hemler, 1990b). The α2ß1 integrin was determined to be the receptor for collagen on platelets (Wayner et al, 1987) and subsequently on lymphoid cells, but it has also been demonstrated to bind laminin in other cell types (Elices and Hemler, 1989). Of the subsequent VLA antigens (ie. ß1 integrin containing complexes) characterized on lymphocyte surfaces (VLA-3, VLA-4 and VLA-6), only VLA-4, or α4ß1, was originally identified specifically on lymphoid cells (Hemler et al, 1988). This antigen was relatively highly expressed on lymphocytes in the absence of mitogenic stimulation. The α4ß1 integrin demonstrated the capacity to function as a receptor for fibronectin. However, proteolytic studies revealed that α4ß1 recognized a site (III-CS) distinct from that recognized by α5ß1 (CBD) (Wayner et al, 1991, Garcia-Pardo et al, 1990). These observations provided the initial suggestion that more than one integrin might simultaneously bind to a single ligand. In addition to fibronectin, α4ß1 recognizes the immunoglobulin superfamily cell surface receptor VCAM-1 (Osborn et al, 1990, 1994). VCAM-1 is present on the surface of cytokine-activated endothelium, and may play a role in the extravastion of α4ß1 bearing mononuclear cells (Shimizu et al, 1990b). Originally thought to be a lymphoid specific marker, α4ß1 has subsequently been

found on other types of leukocytes, as well as on muscle, neural tissue and some transformed and malignant cell lines (Haugan et al, 1992). The two further ß1 integrins expressed on lymphocytes, VLA-3(α3ß1) and VLA-6(α6ß1), were initially identified on fibroblast cells and platelets, respectively (Takada et al, 1988, Sonnenberg et al, 1988). Both antigens were subsequently observed on the surface of lymphocytes (Hemler, 1990b, Sanchez-Madrid et al, 1986).

The original nomenclature (Very Late Activation Antigen of Lymphocytes) used to identify the integrins discovered on lymphocytes is perhaps somewhat inaccurate, as illustrated through several examples. VLA-4 (α4ß1) expression is relatively high on unstimulated lymphocytes, and does not dramatically increase upon lymphoid activation (Klingemann et al, 1992, Hemler, 1990b). VLA-3 (α3ß1) does not increase, and may even decrease upon lymphocyte activation (Hemler 1990). These antigens are not specific to lymphocytes (Dustin and Springer, 1991). Additionally, the relative expression of all the VLA antigens, with the exception of VLA-4, is higher on many nonlymphoid tissues than lymphoid tissues (Hemler et al, 1983). Conversely, terming these complexes "activation" antigens was completely appropriate for a different reason. Many of the ß1 integrins ("ß1 integrins" is the preferred nomenclature over VLA antigens) on lymphocytes were later determined to stimulate lymphocytes upon integrin-ligand interaction, acting in a comitogenic manner (Matsuyama et al, 1989, Nojima et al, 1990.).

Although the VLA antigens are not unique to lymphocytes, it is evident that many of the adhesion receptors described on lymphocytes are members of the integrin family, and include members of the ß1, ß2, ß3 and ß7 subfamilies.

2.3 Molecular Nature of Integrins

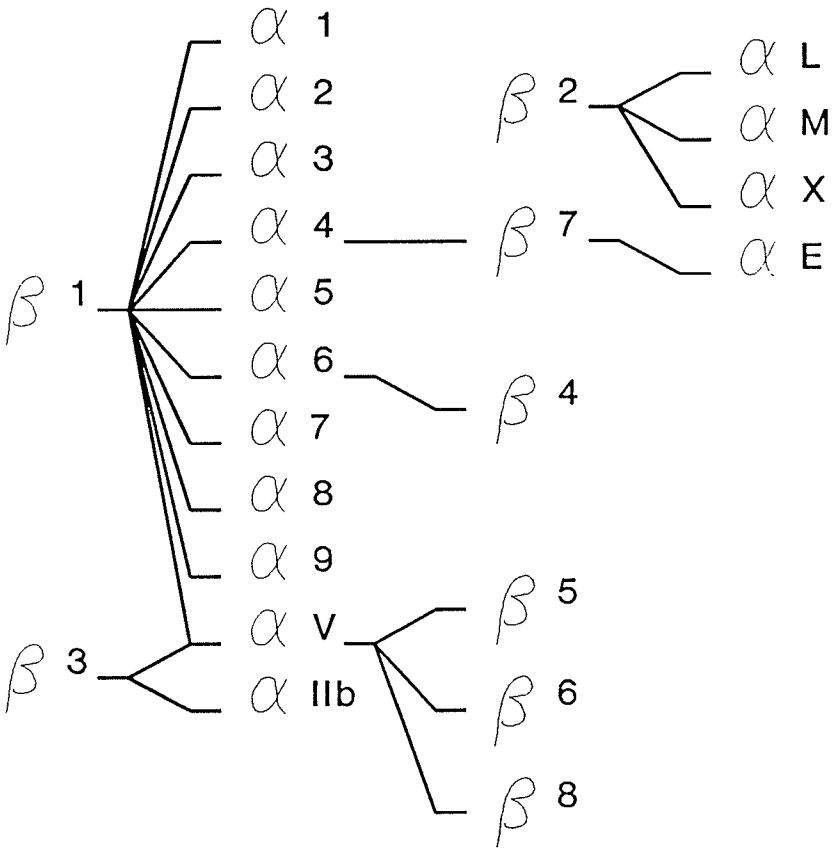
Integrins have always been observed to be surface expressed as αß heterodimers composed of a single α and ß subunit, each of which is a type I transmembrane

glycoprotein. A summary of known α/ß combinations is presented in Figure 2.3. It appears that the expression of integrin α chain is required for the targeting of a pool of ß integrins retained within the endoplasmic reticulum (Cheresh, 1991), which may explain the requirement for dimerization. Neither subunit alone appears to contain the necessary information for surface expression, although they traverse the rough ER as monomers. In support of this theory of integrin dimerization, platelet integrin αIIbß3 may be dissociated on the surface of platelets through treatment with EDTA (certain integrin complexes require divalent cations in order to remain associated). The treatment with EDTA also disrupts the canalicular system of the platelets. Immunogold staining of the platelets reveals that the few intact dimers which remain and the free αIIb subunits segregate to different vacuolar compartments. Free ß3 chains are not observed (Gachet et al, 1993).

The specific α/ß combination is one determinant in the ligand binding specificity of the integrin complex. Other factors are also capable of influencing a specific αß integrin complex's ligand specificities. Integrins depend upon divalent cation binding in order to function (Elices et al, 1991, Gailit and Ruoslahti, 1988, Ignatius et al, 1988, Loftus et al, 1990). In certain integrin heterodimers, the type of cations available will influence the ligand binding specificity of the integrin heterodimer. The αVß3 integrin is a receptor for vitronectin, fibronectin and thrombospondin, but in the presence of Mn^{+2}, will serve as a receptor for laminin (Kramer et al, 1990). Presumably, the binding of a cation to the conserved EF-hand domains will influence the conformation of the protein as a function of its ionic radius (Smith and Cheresh, 1990). The binding of divalent cations is thought to be largely mediated by the α integrin subunits, which display three or four EF-hand type loop consensus sequences (D-x-D-x-D-G-x-x-D), while the ß subunits have only one potentially similar region (Hu et al, 1992). The ligand binding site on both the αIIb and αV integrin subunits has been mapped to this region by photocrosslinking of labelled peptide

Figure 2.3. Known Integrin Heterodimers

The known pairs of integrin α and ß subunits are depicted as connected by bold lines. Only a fraction (21) of the theoretically possible (8 x 15) α/ß heterodimers have been observed. It is postulated that this is due to preferential associations and conformational hindrances which preclude the formation of many integrin dimers.



Known Integrin Heterodimers

15 α subunits

8 β subunits

Assemble into 21 identified heterodimers

ligands (Smith and Cheresh, 1988, DeSousa et al, 1990), thus supporting the observation that the type of ions available might influence the specificity of receptor-ligand interaction. These divalent ion binding consensus sequences are derived from small, cation-binding proteins such as calmodulin, and have been used very successfully to model cation binding domains (Tuckwell et al, 1993).

In addition to the specific α/ß combination and the divalent cations available, it appears that there might exist certain cell-specific (perhaps infrastructural) factors which influence integrin function. Evidence for this stems from observations that a specific integrin αß heterodimer may display a restricted ligand range in some cell types when compared to others (Carter et al, 1990, Kirchhoffer et al, 1990b, Elices and Hemler 1989), even when the appropriate integrins are expressed in the different cell types from the same cDNA construct (Chan and Hemler, 1993).

Integrin α subunits

A commonality among the α integrin subunits, in addition to the described EF-hand sequences, is the existence of short cytoplasmic domains of 25-50 amino acids (Hynes, 1992). These short cytoplasmic domains are highly divergent within a species (between different α subunits, ie., α2 vs α5), but conserved between species (ie., α2 murine vs α2 human)(Hemler 1990, Hynes 1992). These data appear to indicate a highly specialized role attributable to the different α subunits. Different roles for cytoplasmic domains have been elegantly demonstrated via the construction of cytoplasmic domain chimeric constructs bearing common extracellular domains. These chimeras were observed to mediate differential cellular reactions in response to interactions with common extracellular ligands (Chan et al, 1992a).

Integrin α subunits may be broadly classified into two groups, irrespective of the ß subunits they associate with. This method of characterization is borne out by evolutionary

homologies (Figure 2.4a) (Hynes, 1992). The first subgroup of α subunits (α- IIb, V, 3, 5, 6, 7, 8) are post-translationally cleaved (Figure 2.4a)(Hemler et al, 1990a). The cleavage commonly occurs towards the cytoplasmic domain, yielding a C-terminal transmembrane fragment of 25-35kDa which remains linked to the N terminal glycoprotein (125-135kDa) through a disulfide bond. The protease which performs the cleavage is unknown, however, it appears to be ER-resident. It is not known if cleavage is a prerequisite for function in all integrins; it does not appear to be in the case of αVß3 (Cheresh, 1991).

About half of this subgroup of integrin α chains (α- IIb, V, 5) may bind ligand via an RGD sequence. The presence of peptides containing this RGD sequence can inhibit attachment of these integrins to their ligand. No common binding motif has been identified for the non-RGD sensitive α chains. Several of the cleaved α subunits, α3, 6 and IIb, may have alternately spliced cytoplasmic sequences (Tamura et al 1991, Hogervorst et al 1990, Bray et al, 1990). The experiments of Chan et al, (1992a) using chimeric integrin constructs, suggested that differential effects could be elicited by these different cytoplasmic domains in response to (common) ligand binding by the extracellular portion of the integrin.

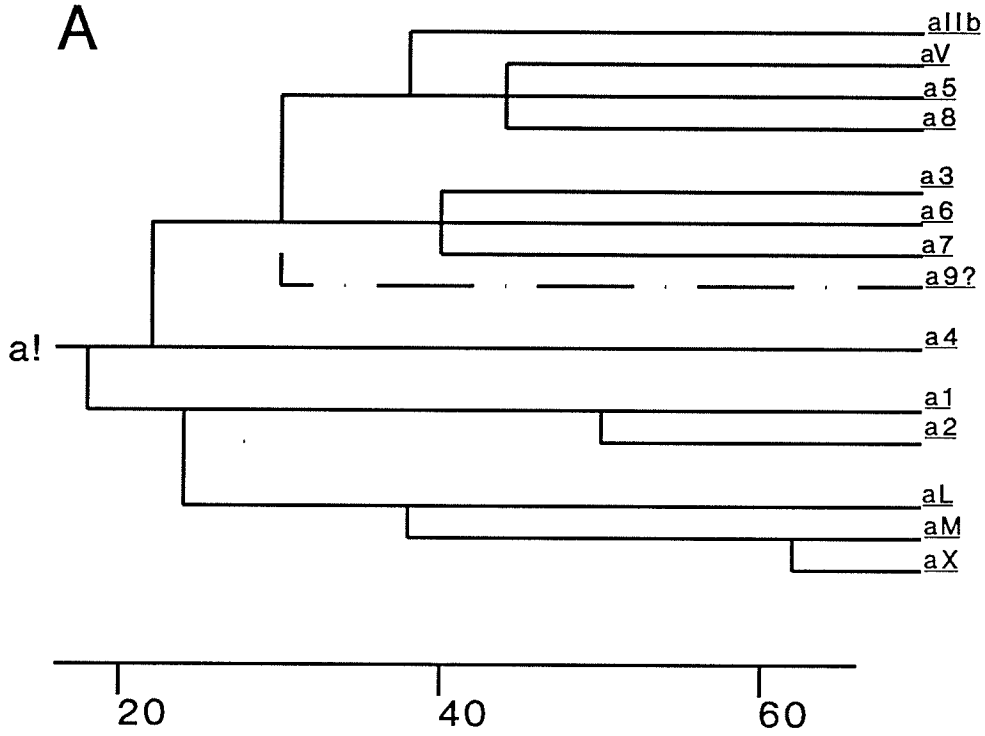
The second group of integrin α chains is comprised of uncleaved subunits. Among this group (α- 1, 2, L, M, X, E), all identified subunits have been demonstrated to possess only 3 divalent ion binding sites and an additional "I", or inserted domain (Takada et al, 1988, Hemler, 1990b, Hynes et al, 1992). The function of this inserted domain has not been confirmed; it may be involved in interaction with ligands. The "I" domain is highly homologous with sequences found in von Willebrand factor and cartilage matrix protein, both of which are known collagen binding proteins (Hemler, 1990b). The homology suggests that the "I" domain in α2 and α1 integrins may also function to bind collagen. However, since the I domain is present in four other α subunits, only two of which have

Figure 2.4. Inter-relationship of α integrin structures

A. Identity tree of integrin α subunits, perhaps derived from some common ancestor integrin α!, depicted along the median. The integrin subunits above the median are all proteolytically cleaved, while the integrin subunits below the median all possess an inserted, or "I" domain, and only three cation-binding sites. The α4 integrin is depicted along the median, possesses no I domain, three putative cations sites, and is proteolytically cleaved in a manner different from other integrins. (Adapted from Hynes, 1992)

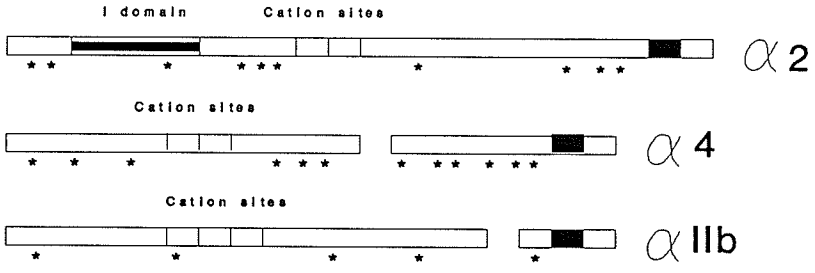
B. Linear-schematic depiction of integrin subunits, displaying transmembrane domain (filled bar), proteolytic sites (spaces), cation binding domains (vertical lines), inserted domain (partially filled region), and sites of glycosylation (stars) (Adapted from Hemler et al, 1991).

A



Approximate % Identity

B



Schematic of α- Integrin Structure

been demonstrated to bind collagen (Wayner et al, 1989, Wright et al, 1993), it is not clear that the role of the I domain is to interact directly with collagen. The I domain has been demonstrated to be either regulatory, or directly involved in the binding of noncollagenous ligands by αMß2 (Diamond and Springer, 1993b).

The α4 integrin is anomalous with respect to the above two convenient groupings. The α4 integrin may be, but is not always, post-translationally cleaved approximately halfway between the carboxyl and amino termini (Figure 2.4b) (Hemler et al 1990a). The cleavage yields disulfide bond linked 70kDa and 80kDa fragments, in contrast to the cleavage characteristic of the first group of α subunits. This cleavage does not appear to effect ligand binding or specificity of α4ß1 heterodimers (Hemler et al, 1990a). The α4 integrin retains only three divalent cation binding sites, very much like the I domain containing α subunits, yet lacks an I domain. The α4 integrin does have a divergent sequence with an extra pair of cysteine bonds in this approximate area. The function of this unique sequence is not understood.

Integrin ß subunits

The ß integrins appear closely related, sharing approximately 40% amino acid identity. The topography of the ß integrins (Mr approximately 130kDa) has been characterized more clearly than that of the α subunits, largely because the ß integrins maintain protease resistant domains, and the α integrins (especially in isolation) are highly protease sensitive.

The ß integrins, with the exception of the highly divergent ß4, may be classified as having at least 5 identifiable "domains" (Figure 2.5). These putative domains are based upon studies by Calvete et al (1989, 1991a, 1991b, 1992a, 1992b), using classical biochemical methods to digest and purify the abundant αIIbß3 integrin from platelets. Automated sequencing and antigenicity were utilized as identification criteria. The five

putatively identified domains of the integrin, from amino to carboxyl terminus, are:

[1] N-Terminal Domain

[2] Ligand Binding Domain

[3] Cysteine Rich Domain (Protease Resistant Domain)

[4] Transmembrane Domain

[5] Cytoplasmic Domain

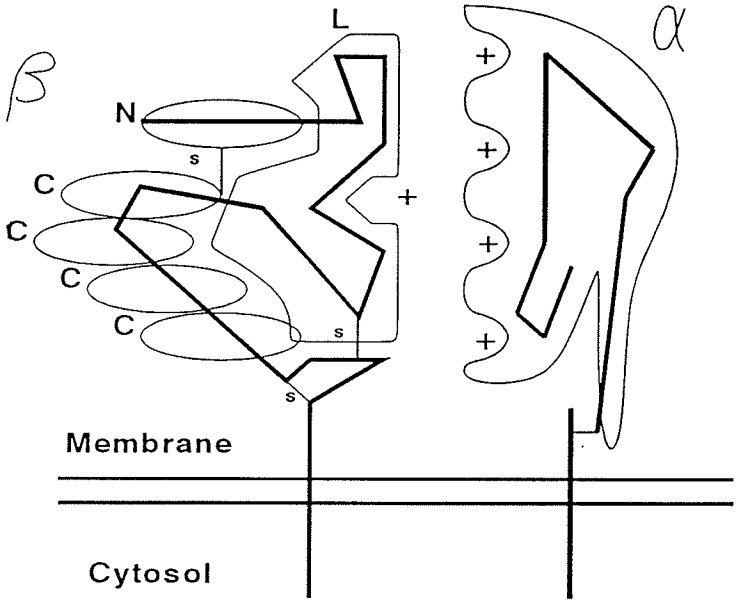
These domains, which will be discussed in greater detail, are summarized in Figure 2.5. The cytoplasmic domain is short in the case of all ß integrins but ß4 (which will be described separately), comprising approximately 50 amino acids. The cytoplasmic domain has been identified on the basis of antigenic recognition, and has been demonstrated to be differentially spliced in ß1 and ß3 integrins (van Kuppevelt et al 1989, Altruda et al 1990). The transmembrane domain has been identified based upon existing algorithms predicting membrane spanning regions. The immediate extracellular portion of the integrin is the cysteine-rich region. As a general observation, all ß integrins share a common cysteine with each of 56 cysteine residues aligning in register (ß7 and ß8 have only 54 cysteine residues aligning in register). The disulfide bonding pattern has been elucidated by biochemical means for the ß3 integrin, and it has been suggested that all integrins may share a common disulfide pattern. This is supported by the studies of Chothia and Lesk (1986), who demonstrated that homologous proteins sharing approximately 40% amino acid identity (as any ß, save ß4, will share with ß3) will have a root mean of squares deviation of only 1 angstrom for atoms along the peptide backbone.

The cysteine rich region is composed of a loop formed by two cysteine residues followed by four repeated sequences of 30-50 amino acids including 8 cysteine residues. The most carboxyl terminal (transmembrane domain proximal) of these four repeats is interrupted by short peptide sequence which includes a further inserted 3 cysteine residues. The number of cysteines present within this linear 250 amino acid (approximately) stretch

2.5. Schematic Diagram of Integrin Structure

An αß integrin heterodimer is depicted approximately as observed by electron microscopy, with domain locations in regions as suggested by Calvete (1994). The α integrin subunit should actually be somewhat larger than the ß, and is shown with a membrane proximal cleavage, a disulfide tether to the membrane bound fragment, and four divalent cation binding sites (+). The ligand binding region is located centrally, between the subunits. The ß subunit domain structure, as assessed by proteolytic digestion of the ß3 integrin, is depicted in greater detail. The N terminal domain (N) is covalently attached to the first cysteine rich-element (C) by a disulfide bond. The protease-sensitive ligand binding domain (L) is located after the N terminal domain, as depicted by the bold tracing, which signifies the domain order with respect to the known amino acid sequence of ß3 integrin. The ligand binding domain is covalently connected to the first cysteine rich element along the peptide backbone, and to the last cysteine rich element via a disulfide bond. Collectively, the four cysteine rich elements comprise the cysteine-rich domain. The ß integrin crosses the membrane via the transmembrane domain, and terminates after a short cytoplasmic segment (in the case of all ß integrins except ß4).

Schematic Diagram of Integrin Structure



is 35. There are 7 further cysteine residues which are included at irregular intervals within the cysteine rich region, yielding a total of 42 cysteine residues involved in the membrane-proximal cysteine rich domain. All but two of these residues are involved in intradomain disulfide-bonding (ie. within the cysteine rich domain), which may explain this regions stability and resistance to proteases. This characteristic has resulted in the phrase "protease resistant core" to apply to this region (Calvete et al, 1991b). Of importance to integrin ultrastructure, four of the cysteines of β3 (which are conserved in all β integrins but β8) are involved in interdomain disulfide-bonding. One cysteine, located N-terminal within the cys-rich element (Cys-430) is disulfide-bonded to the N-terminal domain (described below). Additionally, the C-terminal cysteine (Cys-640) is engaged in an interdomain disulfide bond to a cysteine (Cys 409) present within the ligand binding domain .

The ligand binding domain comprises approximately 300 amino acids, but contains only 7 cysteine residues. Six of these seven residues are involved in intradomain disulfide bonds, while the seventh binds to a cysteine 640, described above. The region from approximately amino acid residue 100 to residue 200 appears to be involved in ligand binding. Photocrosslinking studies have demonstrated small peptides covalently bonding to lysine-126 of ß3 integrin. A specific mutation at aspartate-119 of ß3 integrins prevents ligand binding. Similar mutations in ß2 and ß1 integrin can similarly block attachment, providing strong evidence for the involvement of this region as a ß-specific ligand binding site. This site is also among the first attacked by protease in the well-characterized αIIbß3 integrin complex (Calvete et al, 1991). This, in turn, suggests a high thermal mobility associated with this region (Fontana et al, 1986). While not proven, a high thermal mobility would correlate with the observation that integrin-mediated cellular adhesion is temperature dependant. For example, it has long been know that while CR1 (a complement factor 3 receptor) can bind iC3b at both 4°C and 37°C, CR3, which is an integrin receptor

for complement factor 3 (αMß2) will only function at 37°C (Wright and Detmers, 1988).

The final ß integrin domain identified is the N-terminal domain, which is also enriched in cysteine residues. It is composed of about 50 amino acids and contains 7 cysteine residues. Six of these residues are involved in intradomain disulfide bonding. The final cysteine is disulfide bonded to the N-terminal cysteine rich domain. Thus, after proteolytic digestion, under nonreducing conditions, the N-terminal and cysteine rich domains copreciptitate.

As the fine structure of ß integrins is not well understood (Calvete 1994, Nermut et al, 1988), the N-terminal domain may indeed be part of the cysteine-rich domain; the entire ß subunit appears as a contiguous globule when assessed by electron microscopy (Carrell et al, 1985, Nermut et al, 1988). Digestion with protease will destroy the ligand binding domain in a stepwise manner (Calvete et al, 1989), but will leave a protease resistant "core" (Calvete et al, 1989, Hemler et al, 1984). The addition of reducing agents and subsequent microsequencing can separate this resistant domain into the "N-terminal" domain and a "cysteine-rich" domain (Calvete et al, 1989).

The unique ß4 integrin, which may also be alternatively spliced (Tamura et al, 1990) also expresses a large (approximately 1019 amino acid) cytoplasmic domain, and retains only 48 disulfide bonds which align in register with other integrins (Hogervorst et al, 1991). Only a single α integrin (α6) appears capable of forming a complex with ß4 (Hemler et al, 1989), and this structure localizes to hemidesmosomes (Wayner et al, 1991). A unique feature of this integrin complex is its capacity to readily and strongly bind to a component of the cytoskeleton. The α6ß4 complex specifically (Sonnenberg et al, 1989) binds to intermediate filaments. Other integrin complexes have been implicated in weaker interactions with the actin skeleton, largely by strong fluorescent colocalization within adhesion plaques (Burridge et al, 1989). The cytoskeletal linker proteins, vinculin

(Marchisio et al, 1988) and talin (Singer et al, 1988), have similarly been implicated in colocalization studies, and under permissive chromatographic conditions, a weak interaction between the cytoplasmic ß1 integrin domain and talin can be observed (Horwitz et al, 1987). The cytoplasmic domains of both ß1 and ß3 integrins can be demonstrated to interact with α-actinin, an actin crosslinking protein (Otey and Burridge, 1990). Therefore, it appears that integrins can act to integrate the extracellular environment with the cellular cytoskeleton, but a detailed understanding of this process is currently lacking.

Since adhesion plaques are rich in protein-phosphates (Maher et al, 1991, Kanner et al, 1990), integrins have been extensively analysed to determine if they are phosphorylated (Tapley et al, 1989, Chatila et al, 1989, Dahl and Grabel, 1989, Buyon et al 1990, Horvath et al, 1990, Hibbs et al, 1991, Valmu et al, 1991). While integrins bear sites for both tyrosine and serine/threonine phophorylation, and while these sites may be shown to be direct kinase substrates (Horvath et al, 1990), the role of phosphorylation of cytoplasmic domains in integrin mediated adhesion is not generally clear. Increased phosphorylation is often associated with both increases and decreases in adhesive competence for different integrin heterodimers.

Glycosylation of Integrins

All α and ß integrin subunits sequenced have putative glycosylation sites, and while all have been classified as glycoproteins, relatively little is known about their glycoprotein content. The number of glycosylation sites varies widely from integrin to integrin (5 to 12 N-glycosylation sites in ß integrins, and 4-24 sites in α integrins) (Argraves et al, 1987, Sonnenberg, 1993), and these sites are rarely conserved (see Figure 2.3b for an indication). In the case of ß1 integrin, there are 14 potential glycosylation sites. The number of these available site which actually contain polysaccharide moieties is unknown, but different carbohydrate structures are apparently expressed, including high mannose, bi, tri

and tetra antennary structures (Kawano et al, 1993). Increased fucosylation and decreased sialylation of antennary structures been associated with a decrease in ß1 integrin mediated adhesions to laminin and fibronectin. ß3 integrins which bear only high mannose chains are competent to bind ligands (Cheresh, 1991), therefore, complex oligosaccharides may play a role in down-regulation of integrin function. Such complexes could then potentially be activated by factors displacing the regulatory saccharides, such as cell surface glycosyl-transferases, "competing" oligosaccharide moieties on other cell surface components, or lectin-like extracellular proteins.

There are at least two examples supportive of this proposition. Modification of membrane ganglioside structure or composition has been demonstrated to modify integrin affinity for ligands (Miyake and Hakimori 1993, Conforti et al 1990, Zheng et al 1992). The interaction of P selectin with sialyl Lewis X carbohydrate determinants on soluble αLß2 integrin will increase its binding to ICAM-1, providing evidence for intermolecular interactions (Gahmberg, 1992). These observations suggest that intermolecular or intramolecular interaction with carbohydrates could play a role in the regulation of integrin interaction with ligand.

2.4 Integrin Communications with the Cell

Integrins transmit information into the cell

The redundancy of ligands recognized by integrins and variations in the cytoplasmic domain indicated that integrins might perform functions other than the simple attachment of lymphocytes to specified extracellular ligands. Rather, the variety of cytoplasmic domains among integrins binding to different ligands suggested that the integrins may serve to signal the cell as to the nature of its current extracellular milieu.

There is excellent evidence to indicate that this is so. Early data was derived from

observations that lymphocyte interactions with collagen *in vitro* could influence both antibody (Kemshead and Askonas, 1978) and cytokine (Ofosu-Appiah et al, 1989a, 1989b, Sundqvist et al, 1993) production. Further evidence is supplied by the observation that lymphocytes on ECM secrete tumour necrosis factor α (Hynes, 1992). The integrin αVß3 was demonstrated to be required for IL-4 production by some murine intraepithelial T cell clones (Roberts et al, 1991). Therefore, it appears that interactions with the extracellular matrix, possibly mediated by integrins, could influence lymphocyte activities. The interaction of lymphocyte integrins with fibronectin, laminin, collagen, ICAM-1 or VCAM-1 can act as a comitogen with CD3 or CD2 specific mitogenic antibodies (Matsuyama et al, 1989, Shimizu et al, 1990, van Kooyk et al, 1991). Additionally, lymphocytes on collagen have been observed to spontaneously proliferate (Ofosu-Appiah et al, 1989b). Extracellular matrix components may also augment suboptimal cellular response to antigen (Chan et al, 1991a). However, the manner by which the cells receive signals from integrin interaction with ligand is not completely understood. Ligation of integrin complexes on the surface of lymphoid cells has been demonstrated to cause an increase in cytoplasmic calcium (Schwartz et al, 1992), and may result in the GTP-loading of Ras protein and the phosphorylation of 50kDa cytoplasmic proteins (Kapren et al, 1993). Whether these signals are required for proliferation of T cells or to synergize with other stimuli to induce interleukin-two production is not known. However, the interaction of lymphocytes with fibronectin has been demonstrated to induce nuclear transcription factor AP-1, which is required for interleukin-two production (Yamada et al, 1991). Integrin mediated signalling is known to effect mRNA accumulation in other hematopoietic cells. The interaction of monocytes with fibronectin induces transcription of genes which code for inflammatory mediators (Werb et al, 1989).

Signalling mechanisms within platelets have been more extensively studied, and provides clear examples of integrin-mediated signalling. Collagen binding, dependant

upon the α2ß1 integrin complex, may initiate phospholipase C activity, cytoplasmic alkalization, and an increase in cytoplasmic calcium concentration (Sims et al, 1991, Shattil and Brugge, 1991). Tyrosine kinase activity has been associated with the subsequent occupancy of a second platelet surface integrin, αIIbß3 (Shattil and Brugge, 1991).

Many of the signalling mechanisms observed in platelets have been subsequently observed in other cell types. Tyrosine kinase activity has also been observed in both carcinoma and fibroblastoid cell lines in response to clustering of integrin by antibody and ligand, respectively (Schwartz et al, 1991). The alkalization of the platelet cytoplasm has been observed within fibroblast, endothelial and lymphocyte cells upon attachment to fibronectin (Curtis et al, 1992). The effect of this alkalization is incompletely understood, however, anchorage-independent cell lines have been characterized as typically exhibiting elevated cytoplasmic pH (Schwartz et al, 1990). Further, cytoplasmic alkalization mediated by integrin specific monoclonal antibodies has been to observed to "rescue" cells which would otherwise be expected to become apoptotic (Bates et al, 1994, Frisch and Francis, 1994). The loss of integrin mediated adhesion is considered to be a primary step in the "terminal differentiation" of epidermal keratinocytes (Adams and Watt, 1990).

A lymphocyte specific phenomenon, and perhaps specific to the α4ß1 receptor, is the observation that some antibodies to integrin complexes may induce homo- and heterotypic lymphocyte adhesion. This adhesion is not mediated by the ligated integrin receptors themselves (Pitzalis et al, 1989, Campanero et al, 1990, Bednarczyk and McIntyre, 1990, Shen et al, 1991). It has been suggested that antibody-induced integrin clustering may cause coclustering of distinct secondary intercellular adhesion receptors, thereby suggesting that integrin specific antibodies may induce adhesion as a consequence of integrin clustering (Bauer et al, 1992, Shattil and Brugge, 1991). An alternative possibility exists, however. Since it has been observed that integrins may transmit signals into the cells, it is possible that the antibody ligation results in a transmitted signal which

results in the upregulation of other, distinct receptors. Evidence for this possibility is derived from several varied observations. Not all integrin-specific antibodies induce homo- and heterotypic aggregation, thus indicating that specific regions of the integrin(s) act as triggers (Bednarczyk et al, 1993, Shen et al, 1991). A precedent for integrin mediated signalling to other receptors has been set by observations that ligation of one neutrophil integrin complex with an inhibitory peptide down-regulates a secondary integrin structure (van Strijp et al, 1993). Additionally, in melanoma cells, anti-α4 specific monoclonal antibodies have been demonstrated to interfere with adhesion mediated by cellular chondroitin sulfate proteoglycans to nonintegrin adhesion sites on fibronectin (Iida et al, 1992). Therefore, it is possible that these antibodies facilitate integrin signalling resulting in the modification of secondary adhesion systems.

Cells may regulate the activity of integrins

In contrast to the capacity of integrins to transmit signals into the cell, sometimes termed "outside-in signalling" it appears that integrins may also respond to cellular cues. Many cell types appear to possess the capacity to regulate the activity of their integrins. Integrin mediated adhesion must occasionally be downregulated to facilitate the migration of cells, otherwise they would remain firmly attached in one place (in the absence of an increasing gradient of ligand). Further, it is apparent that during mitosis as well as during some differentiation processes, cells become nonadherent (Neugebauer and Reichard, 1991, Adams and Watt, 1989, 1990). The factors which govern the down-regulation of integrin activity are not well understood. However, these processes, sometimes called "inside-out signalling", appear to occur only in those cells which possess a physiological requirement for integrin regulation.

In contrast to the majority of cell types, in which integrins will bind ligand as a constitutive function, leukocyte and platelet integrins present themselves as functionally

"dormant". Although expressed on the surface of these cell types (abundantly in the case of platelets: one integrin every 80nm of linear plasma membrane, based on average platelet size and integrin complement), no ligand binding is basally apparent. The activation of these complexes from their dormant form is also termed "inside-out signalling", although the precise mode of regulation is not understood.

A variety of agonists can activate platelet integrin αIIbß3 mediated adhesion, including adenosine diphosphate nucleotides, collagen (via the α2ß1 receptor), epinephrine, phorbol esters and thrombin. Importantly, the adhesion competent receptor can be antigenically distinguished from the dormant receptor by monoclonal antibody PAC-1 (Kouns et al, 1990). PAC-1 specifically recognizes the activated form of αIIbß3, designating the appearance of a new epitope upon platelet activation. This suggests either receptor conformation changes, or existing epitopes are unmasked through other means. Evidence for the former was provided by fluorescence resonance energy transfer (RET) experiments, in which the RET efficiency between two nonactivation-dependant epitopes was demonstrated to increase upon platelet activation (Sims et al, 1991). In support of the platelet investigations, lymphocyte studies on the lymphocyte specific αLß2 complex yielded similar results. The activation of lymphocytes with phorbol myristate acetate (PMA, a phorbol ester which is a diacyl glycerol analog), can induce ß2 integrin-mediated binding to ICAM-1 (Shimizu et al, 1990). The activation of the αLß2 integrin complex can be antigenically detected by the presence of the Mab 24 (Dransfield et al, 1992). Thus, two integrin systems (the αIIb/ß3 and αLß2 integrin complexes) provide evidence that the activation of dormant integrins appears to require a conformational change. It is noteworthy that these two integrin complexes are among the most distantly related among the integrins (cf. Figure 2.3a for α-subunit specific evolutionary differences).

In monocytes, PMA has also been demonstrated to induce mobilization of integrin αMß2 and αXß2 to the cell surface from intracellular stores, as well as inducing clustering

of αMß2 already present on the cell surface (Wright and Detmers, 1988). Therefore, in addition to putative conformational changes, integrin expression and lateral organization within the plane of the membrane may be influenced as a result of cellular activation. Certainly, platelet integrins are known to patch upon activation by agonists (Shattil and Brugge, 1990), yet the lymphocyte integrin αLß2 apparently patches even in an inactive state (van Kooyk et al, 1994). It is possible that patching performs different functions with respect to the expressing cell and expressed integrin, or alternately, patching may be a required, but not sufficient, step in the activation of dormant integrin.

It is not clear if this "receptor patching" promotes association with cytoskeletal elements, but ß1 integrin patching in focal adhesions clearly colocalizes with the terminus of cytoplasmic stress fibrils (Burridge et al, 1988, Marchisio et al, 1988), and ß2 integrin may be observed to patch in the cell:cell junction in isolated cell:cell conjugates (Maher et al, 1985). Further, fibronectin may act as an opsonin under certain conditions, indirectly implicating cytoskeletal associations with regions of integrin-dependant adhesion (Wright and Silverstein, 1982, Wright et al, 1984, Bullock and Wright, 1987, Wright and Detmers, 1988), as these associations are required for phagocytosis. These observations clearly indicate that leukocyte integrins may be regulated via cytoplasmic (cytoskeletal and cytosolic) factors by the cell.

The implication of unidentified, cell-specific factors is available from several sources. For example, cDNA constructs, when expressed in different cell lines, possess different ligand-binding activities. Further, the activity of constructs may be upmodulated by deletion or mutation of cytoplasmic sequences, particularly a GFFK amino acid sequence common to all α subunits (O'Toole et al 1991, 1994). Thus it appears that the cytoplasmic integrin sequences may play a role in the negative regulation by the cell, and that deletion of this sequence results in a upregulated functional receptor. This may, in part, explain why integrin constructs lacking both cytoplasmic and transmembrane domains

maintain function (Dana et al, 1991, Larson et al, 1991). Further evidence for integrin down regulation involving sequences C-terminal to the GFFK sequence is supplied by experiments involving chimeric constructs. In these experiments, an integrin α subunit extracellular domain (α4 or α2) may have its activity influenced by a cytoplasmic domain derived from a different integrin (Chan et al 1992b). These chimeras were identical in cytoplasmic amino acid composition up to the GFFK sequence, thus providing strong evidence that post-GFFK cytoplasmic sequence may also modulate integrin function. It is not clear how these sequences do this although it is possible that they bind cytosolic proteins and mask access to the GFFK sequence which might be required for activation and/or stabilization of adhesion. This GFFK sequence may also be a site of interaction with α-actinin (Otey and Burridge, 1992).

There is some evidence to suggest that integrins may be regulated from extracellular sources as well. The ICAM-1 binding activity of a purified, soluble form of αLß2 may be increased by treatment with purified P-selectin (Gahmberg et al, 1992). Since αLß2 is itself rich in Sialyl Lewis X antigen, (the ligand of P-selectin), interactions external to the cell might also influence integrin adhesion. In support of this, activated endothelium rapidly elevates expression of P-selectin, as well as a cell surface ligand for αLß2, ICAM-1, from internal stores. This suggests a model in which the adhesion of leukocytes to the endothelium can be activated in response to local inflammatory stimuli (Dustin and Springer, 1991).

A short peptide derived from the terminal fragment of the IGSF molecule ICAM-2 has been demonstrated to bind to and activate LFA-1 binding to several ligands (Li et al, 1993). Similarly, a peptide derived from a snake venom, echistatin, has been demonstrated to bind to the αMß2 integrin and to not only activate adhesion, but to expand the range of ligands which αMß2 may bind (Wright et al, 1993). It is therefore possible that a range of extracellular compounds may influence integrin mediated adhesion.

Within the plane of the membrane, at least two molecules have been identified which associate with integrins, and could conceivably play some role in their regulation. These molecules are CD9 (Letarte et al, 1993) and Integrin-associated protein (Zhou and Brown, 1994). CD9 is a calcium port which associates with G proteins, but is not present in all cell lines which express integrins. CD9 is only present in pre-B cells, monocytes and platelets. Conversely, integrin-associated protein is expressed on a variety of cell types, even those lacking integrins, such as erythrocytes. While strong links between these molecules and integrins may be displayed in specific cell types, it is not yet clear whether these molecules directly regulate integrins, whether they activate (and/or are activated by) common pathways, or both. For example, monoclonal antibodies directed to CD9 can induce homotypic aggregation of pre-B cell lines, not unlike some integrin-specific antibodies (Letarte et al, 1993). Further investigation in these areas will be required to determine whether different cell types may use different integrin-regulating proteins.

While the ß1 group of integrins on lymphocytes is non-functional upon lymphocyte isolation from the periphery (Hemler, 1990), it is clear that other integrins present on the surface of lymphocytes (ß2 integrins) are activatable, and that a variety of means may elicit their activation. Preliminary evidence, concurrent with these studies, demonstrated that PMA could activate lymphoid attachment to fibronectin (Danilov and Juliano, 1989). Subsequently, other means have been reported to activate ß1 integrins, including the use of "activating" monoclonal antibodies. These antibodies, of which at least three have been reported (Kovach et al, 1992, van-de Wiele-van Kemenade 1992, Chan and Hemler, 1993), are able to bind to and activate dormant human ß1 integrins. The subsequent mapping of ß1-specific activation sites was attempted through the creation of cross-species chimeras (Shih et al 1993,, Takada and Puzon, 1993). These studies have identified at least two general regions on the ß1 integrin subunit which contribute to adhesion. One domain appears to be within 100 amino acids of the membrane, while a second appears to

be within a conserved region of the ligand binding domain. However, these studies complement the studies performed within the current investigation (Section 4.3 and 4.4). Therefore, it is most appropriate that they be described in greater detail, with relevant comparison, during the discussion.

In reflection of the information known at the onset of theses studies (references of 1989 or earlier), principle focus was dedicated initially to the identification of which integrin receptors could be expressed on lymphocytes and which were actually utilized to attach to different components of the extracellular matrix. As the current studies, and the field of integrin research in general evolved, it became evident that these structures were also regulated functionally. Thus, the regulation of function was also priorized for investigation.

3.0 MATERIALS AND METHODS

3.1 Reagents

3.11 General Reagents

All reagents were obtained from Sigma Chemical Co., St Louis MO, unless otherwise specified. Each initial company citation includes the location; all further citations should be considered the same location unless otherwise noted. acetic acid (glacial) (Fisher Scientific, Winnipeg, Canada), acrylamide, ammonium persulfate, ammonium sulfate, bacto-tryptone (DIFCO, Detroit MI), bacto-yeast extract (DIFCO), bromo-chloro-indoyl phosphate, bovine serum albumin (fraction V), calcium chloride, carbenicillin, chloroform, collagen type I (Sigma or prepared as per Kemshead and Askonas, 1978), collagen type II (Collagen Corp, Palo Alto, CA), concanavalin A, cycloheximide (CHI), cysteine, 2-deoxyglucose, dimethyl formamide, dimethyl pimidelate, dimethyl sulfoxide, disodium phosphate, ECL blot development system (Amersham, Oakville ON), ethanol 95% (Fisher), ethylene-diamine-tetracetic acid (EDTA), fetal bovine serum (FBS) (Gibco/BRL, Burlington ON), fibronectin (Sigma, or Telios Pharmaceuticals, LaJolla, CA), fibronectin 15kDa fragment (Telios), fibronectin 40kDa fragment (Telios), fibronectin 120kDa fragment (Telios), Ficoll-hypaque, glucose, glycerol, glycine, Hank's balanced salt solution (Gibco/BRL), hydrochloric acid (Fisher), isoamyl alcohol (Fisher), isopyro-thio-galactoside, laminin (Sigma or Telios), lysozyme, magnesium chloride, manganese chloride, methanol, nitro blue tetrazolium, nonidet P-40 (NP40), papain-Sepharose 4B bead conjugate, phenol, phorbol myristate acetate (PMA), potassium acetate, protein A-Sepharose 4B bead conjugate, protein G-Sepharose 4B conjugate, RPMI 1640 (Gibco/BRL), ribonuclease, Sephadex G-10 (Pharmacia Fine Chemicals, Uppsala, Sweden), sodium acetate, sodium azide, sodium bicarbonate, sodium borate sodium

carbonate, sodium chloride, sodium citrate, sodium chromate-isotope 51 (Amersham) sodium dodecyl sulfate, sodium hydroxide, sodium iodide-isotope 125 (Amersham), staurosporine, sulfuric acid (Fisher), tetracyclin, trisma base, tris-HCl, trypsin, urea, vitronectin (Telios), water (purified water obtained from a Barnstead Nanopure System, R > 16MΩ in all cases).

3.12 Antibodies

Antibody-recognizing conjugates used in these studies include alkaline phosphatase-conjugated goat anti-mouse (Sigma), horse radish peroxidase-conjugated goat anti-mouse (Sigma), fluorescein isothiocyanate-conjugated goat anti-mouse Fab' (Cedar Lane Laboratories, Hornby, ON), and avidin-horse radish peroxidase conjugate (Sigma). Nonconjugated Monoclonal antibodies and their sources are summarized in Table 3.1.

3.13 Plastics and Synthetics

Flat well 96 well plates for adherence assay (76-232-05), tissue culture (76-003-05), or ELISA (76-331-05) and V-bottom 96 well plates (76-023-05) were obtained from Flow Laboratories, Inc (Mclean VA). Flat welled 24 well plates (3047), Falcon Integrid 150mm plates, and 88mm petri plates were obtained from Becton Dickinson (Lincoln Park, NJ). Nitrocellulose (45μm pore) in sheets (Biorad) or precut 82mm cirles (Amersham) was utilized for all immunoblot studies. Semidry transblot-matrix paper was ordered from Whatman (Maidstone, England). Working labware included 15ml conical (2095) and 50ml(2070) conical tubes, and 5ml snapcap tubes (2054)(Becton Dickinson). 1.5ml eppendorf-type tubes (Brinkmann, Lincoln Park, NJ) were used for all nucleic acid manipulations, and microculture of bacteria. Tissue culture was performed using $25cm^2$ canted neck or $75mm^2$ straight neck flasks (Corning Labwear, Corning, NY).

Table 3.1 legend: Antibody refers to the monoclonal designation. All antibodies are monoclonals if raised in mouse (m) or rat (r). Rabbit (rb) derived antibody is polyclonal. Specificity refers to the antigen recognized. A slash indicates the recognition of two distinct antigens. Sources lists those commercial suppliers, collaborators and benefactors who have supplied antibodies for portions of these investigations. Produced antibodies were developed and characterized locally. "ATCC/Produced" antibodies were derived from hybridomas acquisitioned from ATCC stocks.

TABLE 3.1 ANTIBODIES USED IN THESE STUDIES

Antibody	Species	Specificity	Source
3S3	m	$\beta 1$	Produced
4B4	m	$\beta 1$	Becton Dickinson Immunochemicals
6F4	m	$\beta 1$	Produced
12E7	m	CD45	Produced
13B9	m	$\beta 1$	Produced
21C8	m	$\beta 1$	Produced
44H6	m	$\alpha 4$	Dr M Letarte, [Quackenbush and Letarte, 1985]
A-1A5	m	$\beta 1$	Dr M Hemler, [Hemler et al, 1983]
A16G6	m	$\beta 1$	Produced
B3B11	m	$\beta 1$	Produced
B44	m	$\beta 1$	Produced
hFN	rb	FN	Gibco/BRL
hFNR	rb	$\alpha 5/\beta 1$	Telios
hVNR	rb	$\alpha V/\beta 3$	Telios
J143	m	$\alpha 3$	Dr L Old, [Fradet et al, 1984]
JB1	m	$\beta 1$	Produced
JB1A	m	$\beta 1$	Produced
JBS2	m	$\alpha 2$	Produced
JBS5	m	$\alpha 5$	Produced
LM609	m	$\alpha V\beta 3$	Dr D Cheresh, [Cheresh et al, 1989]
mAb13	r	$\beta 1$	Dr K Yamada, [Akiyama et al, 1989]
OKT3	m	CD3	ATCC/Produced
OKT4	m	CD4	ATCC/Produced
OKT8	m	CD8	ATCC/Produced
P1E5	m	$\alpha 2$	Drs W Carter & E Wayner [Wayner et al, 1988], or Telios
P1B5	m	$\alpha 3$	Drs W Carter & E Wayner, or Telios
P1D6	m	$\alpha 5$	Drs W Carter & E Wayner, or Telios
P4G9	m	$\alpha 4$	Drs W Carter & E Wayner, or Telios
P4H9	m	$\beta 2$	Telios
TCRVδ1	m	Vδ framework	Dr M Brenner, [Band et al, 1987]
TCRVγ9	m	Vγ9	T cell Sciences, Boston, MA
TCR$\alpha\beta$	m	any $\alpha\beta$ TCR	T cell Sciences
TS1/18	m	$\beta 2$	ATCC/Produced
TS2/7	m	$\alpha 1$	T cell Sciences
VNR147	m	αV	Telios
WT31	m	$\alpha\beta$ TCR	T cell Sciences

3.2 Tissue Culture Techniques

3.21 Isolation of Lymphocytes from Peripheral Blood

Blood obtained from volunteers by venipuncture was diluted with an equal volume of saline (0.15M NaCl). Diluted Blood, up to 25ml, was carefully layered on 12.5ml Ficoll Hypaque in 50ml conical tubes, and spun at 1500 rpm in an IEC Centra 7 centrifuge (400g) for 15 minutes at ambient temperature. Peripheral blood mononuclear cell populations remained buoyant on the Ficoll Hypaque and were removed carefully by pipette. Cells were washed 3 times under the same centrifugation conditions with Hank's Balanced Salt Solution. Cells were subsequently plated on Falcon Integrid plates precoated with RPMI 1640 supplemented with 25% FBS. Cells were suspended in a volume of the 25% FBS solution equal to the original blood volume obtained by venipuncture. To insure that the plates were not overloaded with cells, which would interfere with the adhesion-dependant depletion of monocyte populations, no more than 15ml of suspension was aliquotted onto each plate. After three hours at 37°C, nonadherent cells were removed by pipetting and repeated gentle washing. Suspensions were washed as previously described, and used in further purifications or immediately cultured.

3.22 Purification of T Lymphocytes

Nonadherent populations were allowed to rosette with 2-aminoethylisothiouronium bromide hydrobromide treated Sheep red blood cells (Unique Ventures Ltd, Teulon MB) (Pelligrino et al, 1975). Cell populations were layered on Ficoll Hypaque as described in 3.21, and centrifuged at 400g. B cell populations remained buoyant on the Ficoll Hypaque, while rosetted T cell populations were pelleted. Pellets were gently washed once in Hank's Balance Salt Solution, and subsequently pelleted in a 15ml conical tube. Cells

were placed on ice, and erythrocytes lysed by approximately one minute exposure to 0.1M NH_4Cl. Lysis was monitored visually by colour change (darkening), and assessed microscopically after cells were washed a further three times in Hank's Balanced Salt Solution.

3.23 Cell Culture

The lymphoblastoid cell lines CEM (American type tissue Culture Collection, ATCC, Rockville, MD), CESS(ATCC), CTLL-2(ATCC), HSB2(ATCC), IM-9 (ATCC), JR2B10, JR2D3 (both kindly supplied by Dr R Warrington, Dept of Immunology, University of Manitoba), Jurkat(ATCC), JY (Dr L Pilarski, Dept of Immunology, University of Alberta), LBRM-3(ATCC), Molt 3(ATCC), Molt 4(ATCC), Namalawa(ATCC), Ramos(ATCC), RPMI 8226(ATCC), RPMI 8866 (American Biologicals Corporation, No longer in operation) U266 (Dr L Qualtiere, University of Saskatchewan), as well as generated hybridomas, were cultured in RPMI 1640 supplemented with 10% FBS. The cell line Daudi (ATCC) was cultured in RPMI 1640 supplemented with 20% FBS. PBL, purified T and purified B cell isolates were cultured in HB 102 Defined Serum-Free Media (Hanna Biologics, Almeda CA).

3.24 Modulation of Adherence Potential

Phorbol myristate acetate (PMA) was solubilized in dimethyl sulfoxide (20µg/ml). Cells were treated with PMA at concentrations as described in individual sections, typically 20-50ng/ml final concentration. OKT3 antibody was used to stimulate cells at 1 µg/ml. Staurosporine (to 1mM from DMSO), EDTA (to 0.5% wt/vol from water), Concanavalin A (to 1:1000 from RPMI 1640), Sodium azide (to 1mM from ddH2O), 2-Deoxyglucose (to 10mM from RPMI 1640), Cycloheximide(to 5µg/ml from DMSO), were used at final listed

concentrations from stock solutions (in brackets) as indicated. Isolated T cell, B cell and peripheral mononuclear cell populations were also activated by culture on monoclonal antibody coated flasks. Activating monoclonal antibody OKT3 (T cells and bulk peripheral populations) or polyclonal anti-μ chain antisera were suspended in HBSS at a concentration of 2.5μg/ml. The sterile solution was pipetted into 25cm^2 flasks, and incubated for 2 hours to allow saturation of protein binding sites on the surface of the tissue culture flask. Unbound antibody was removed by washing the flask in three times with HBSS. Cell suspensions were then added to the flasks for culture.

3.25 Flow Cytometry

Cells were suspended at 0.5-1 x 10^7 cells/ml in PBS, 1%BSA, 0.1% Sodium azide (PBSAz). 100μl per well was aliquoted and spun down in V96 plates (ICN/Flow Laboratories, Mississauga ON, CAN). Cells were resuspended in PBSAz and antibody added to a final concentration of 1μg/ml. Cells were incubated for 60 minutes at 4°C,and subsequently washed three times in PBSAz. 50μl of FITC conjugated goat anti-mouse antibody at a 1:40 dilution of commercial stock was added to the cell pellets, and the pellets resuspended. Samples were incubated for a further 30' at 4°C, and washed a further 4x in PBSAz. Cells were subsequently fixed in PBS, 1% paraformaldehyde (Sigma) and analyzed by flow cytometry.

3.3 Adhesion Assays

3.31 Protein-coating F96 well plates

Appropriate concentrations of protein were suspended in carbonate coupling buffer (0.2M sodium bicarbonate + 0.2M sodium carbonate, water, 1:1:2 by volume, pH 9.9).

Standard concentrations of fibronectin and laminin used, when not otherwise stated, were 5 and 10μg/ml, respectively. The standard concentration of collagen used was 25 μg/ml in the case of type II collagen, or 20μl/ml in the case of the type I rat tail collagen prepared as previously described (Kemshead and Askonas, 1978). Aliquots of 100μl of protein suspension were aliquoted into flat-well (F96), nontissue-culture plates and incubated for 4 hours at room temperature. Plates were either used immediately or stored for up to 5 days at 4°C. Prior to use, coupling buffer was decanted, and wells washed once with RPMI 1640, 3% BSA. Plates were then incubated with 100μl of the same solution for 1 hour at 37°C to block residual, nonspecific protein binding sites.

3.32 Adhesion Assay

Lymphoblastoid cell lines were suspended at 10^6/ml in adhesion assay buffer (RPMI 1640, 1% BSA). Lymphocytes were suspended at twice this concentration due to their smaller size, and subsequent smaller surface area covered; The concentration of cells used for the adhesion assay reflects the number of cells required to coat the bottom of the well while allowing each cell optimal access to the surface. Aliquots of 100μl of cell suspension were placed in coated, blocked, microtitre-plate wells, and allowed to settle and adhere for 60 minutes. Plates were then gently inverted on adsorbent pads to drain the wells, and plates washed a further two to three times to remove nonadherent cells with a handheld multichannel pipettor. Adherent cells were stained by the addition of 50μl of crystal violet dye solution (0.5% crystal violet dye, 20% methanol, 80%H_20, wt/v/v). Excess dye was removed by repeated washes in cold water. Plates were allowed to dry, and cell bound dye was quantitated by solubilization in methanol and absorbance quantitated at 600nm (Wilkins et al, 1991).

3.33 Radiolabelling Cells for Adhesion Assay

An alternative method of quantitation involved the use of radioactive sodium chromate ($Na_2{}^{51}CrO_4$) as a radiolabel (Amersham, Oakville ON). Cells were suspended at 10^8/ml, and mixed with an equal volume of sodium chromate, resulting in a labelling ratio of approximately 0.37Bq/cell (One Bq = 1 disintegration per second). Cells were incubated at 37°C for 60 minutes, and subsequently washed three times in RPMI 1640, 1% BSA to remove unincorporated chromate.

The adhesion assay was performed essentially as described in 3.23, following appropriate radioactive safety procedures. Adherent cells were lysed in a 1% Sodium dodecyl sulfate, ddH_2O solution, and γ emission of the lysate quantitated in a gammacounter. Results are expressed as a percentage of the original quantity of radioactivity incorporated into a 100µl lymphocyte aliquot.

3.34 Perturbation of Adhesion

Prior to aliquoting on plates (as per 3.32), cells were treated with either inhibitors of metabolism or with antibodies (concentrations described in Section 3.24). Cell suspensions were incubated for 20 minutes prior to assay at 37°C in a 5% CO_2 atmosphere. Control groups had appropriate diluent added in the case of metabolic inhibitors (diluents described in Section 3.24), or irrelevant but species-matched antibody in the case of antibody-induced modification of adherence. As often as possible, the control monoclonal was also of the same subclass as the tested monoclonal. In none of the cases did the diluent, or the irrelevant antibody, significantly modify the attachment of cells as compared to secondary control groups to which no additional reagents (diluent or control monoclonal antibody) were added.

3.4 Polyacrylamide Gel Electrophoresis Techniques

3.41 Polyacrylamide Gel Electrophoresis

All gel electrophoresis was performed on either a Biorad Protean II or a Hoeffer Instruments Minigel 200 apparatus. Jurkat cell lysates at a concentration of 5 x 10^7/ml were used, with 50µl being run per lane on the Protean II system, and 25µl per lane being run on the minigel system. Minigels were run at 20 mAmps per gel for 2 hours, while large gels were run at 30 mAmps per gel for 6-7 hours. All gels were cooled with a circulating water refrigeration system. All samples were loaded in either reducing or nonreducing Laemmli electrophoresis buffer (2% SDS, 10% glycerol, 100mM dithiothreitol, 60mM Tris, 0.001% Bromophenol blue, pH 6.8) (Laemmli, 1970), and run on various percent acrylamide gels as specified in the results.

3.42 Radioimmunoprecipitation

Cells were suspended at 2 x 10^7/ml in PBS. Aliquots of 0.5ml were labelled with 5µl commercial $Na^{125}I$ solution (1.85Bq/cell) via three sequential one minute incubations in which 166IU lactoperoxidase and 0.006% peroxide were added to the reaction mixture. The reaction was stopped by transfer of labelling solution to 4.5ml PBSA (PBS, 0.1% BSA) on ice. Suspensions were shaken for 20 minutes on ice, and labelled cells were then layered on 2ml FBS and spun at 500 rpm for three minutes to eliminate debris. Cell pellets were washed twice, and subsequently lysed in 200µl of PBS, 1% Nonidet P40, 10mM PMSF. To a 50µl aliquot, 10µg of specific antibody was added. After 1 hour, rabbit anti-mouse antisera was added, and the resulting immune complexes precipitated by 100µl heat-killed, formalin-treated *S Aureus* Cowan Strain I(SAC)(Sigma). SAC pellets were washed 5 times in NET (0.05% NP40, 50mM Tris, 5mM EDTA, 150mM NaCl, pH 7.4)

and precipitated antigen solubilized by boiling in Laemmli buffer (Harlow and Lane, 1988).

3.43 Sequential Preclearing Assays

As a means to determine antigenic identity, cell lysates were sequentially and serially treated with test antibody and immunoprecipitated. This provided a depletion of specific antigen. Different monoclonals, typically specific for ß1 integrin or CD45 (control antigen), were subsequently used to probe these depleted lysates to assess whether antigenic reactivity remained after immunodepletion.

3.44 Western Blot

All transfers were performed on an LKB Multiphor II semi-dry transfer apparatus, all gels transferred for 90 minutes at .8 mAmps per cm^2 (As per the manufacturer's instructions). To set up transfer, six sheets of novablot transfer paper saturated with transblotting buffer (TB, 48mM Tris, 39mM glycine, .037% sodium dodecyl sulfate, 20% methanol) were placed on the cathode, followed by a sheet of prewetted nitrocellulose, the gel to be transferred, and a further 6 sheets of novablot transfer paper saturated with TB. Transfer was performed as described and nitrocellulose blots were blocked overnight in TBSA-T (25mM Tris, 150mM NaCl, 5% wt/v BSA, 0.5% v/v Tween-20, 0.1% sodium azide) at 4°C. The principle antibody was added to 3ml blocking solution per lane (4µg/ml) and agitated for 90 minutes. Nitrocellulose strips were washed 3 times with TS-A (0.2M Tris, 0.1M NaCl, pH 7.5). Secondary alkaline phosphatase conjugated reagent was added in TS-A, 1:2500 of commercial reagent. After a 30 minute incubation with agitation, nitrocellulose strips were washed a further 4 times with TS-A. Strips were then washed once in TS-C (.1M Tris, .1M NaCl, 50mM $MgCl_2$, pH 9.5), and substrate added (10 ml TS-C, 3.3mg nitroblue tetrazolium, 1.7mg 5-bromo-4-chloro-3-indolyl phosphate

tablets). This method is based upon that of Harlow and Lane (1988), however increaed salt concentrations have been used in the wash buffers to optimize specific signal strength.

3.5 Production of Ascites

3.51 Inoculation of Mice and Harvest of Ascites

Balb/c mice were injected ip with 0.5ml pristane to primed for ascites production. Approximately 10-14 days later, mice were injected ip with 0.5ml PBS-washed hybridoma cells suspended in sterile PBS at 10^5-10^6/ml. Mice were monitored daily for the accumulation of ascitic fluid. Approximately 10-14 days after injection with hybridomas, mice were sacrificed and ascitic fluid collected. Pooled ascites was allowed to clot for 1 hour at 37°C, and debris pelleted by centrifugation. Clear ascitic fluid was removed by pipette and sterilized by filtration for future manipulations (Harlow and Lane, 1988).

3.52 Purification of Antibody

Antibody was isolated through serial ammonium sulfate precipitation. Initially, a 30% saturated solution of ammonium sulfate was generated. The retained supernatant was increased to 50% ammonium sulfate, and incubated overnight at 4°C. The precipitate was pelleted and resuspended in PBS in one tenth the initial volume. The protein solution was dialyzed overnight against PBS, and subsequently adjusted to 3.3M NaCl through the addition of NaCl crystals. The concentration of sodium borate was adjusted to 0.1 M through addition one tenth volume of 1M sodium borate stock solution. This high salt solution was applied to a protein A sepharose 4B column, 4 times serially. Flow rate was dictated by gravity. The column was then washed with 40 or more (if required) volumes of

50mM sodium borate, 3M NaCl, and 40 (or more) volumes of 10mM sodium borate, 3M NaCl. Column-bound immunoglobulin was eluted by the addition of 0.2M glycine, pH 3.5, and eluted fractions neutralized by the addition of 5%(vol) 1M tris, 0.15M NaCl, pH 8.0. Antibody containing fractions were assessed spectrophotometrically at 280nm and by 10% reducing PAGE. Antibody-containing fractions were pooled and dialyzed extensively against PBS, then filter sterilized. Antibody was quantitated spectrophotometrically at 280nm using an extinction coefficient of 1.35 $ODmg^{-1}ml$ (Harlow and Lane, 1988). Alternatively, antibodies were quantitated by Biorad protein assay. The assay was performed as per the method supplied (Biorad). Protein concentration standard curves were generated using monoclonal antibody solutions of known concentration. The assay is based upon the method originally described by Bradford (1976).

3.6 Enzyme Linked Immunosorbent Assays

3.61 Isotyping of Monoclonal Antibodies

Plates were coated with ascites diluted 1:1000 in carbonate coupling buffer, 100µl per well, as described for fibronectin (3.31). Coated plates were then washed 3times with 150µl TBST (100mM Tris, 150mM NaCl, 0.1% Tween). Specific murine isotype-specific goat antisera reagents were then added in TBST at 1:1000, 100µl, for 30 minutes. Plates were again washed 3times in TBST. Alkaline phosphatase conjugated rabbit antisera to goat imunoglobulin (Sigma) was then added to each well, 100µl, for 30 minutes. Plates were washed 4x with 150µl TBST, and substrate (1mg/ml of o-nitrophenyl-ß-d-galactopyranoside in 10mM diethanolamine, .5mM $MgCl_2$, pH 9.5) was added to the wells. Absorbance was read at 540nm.

3.62 ß1 Integrin Antigen Assay

Affinity column-purified integrin was allowed to coat F96 wells at 10µg/ml in CCB. Test antibodies (10µg/ml) in TBST were allowed to bind to integrin-coated wells for 1 hour. Plates were then washed 3times with 150µl TBST, and 100µl of alkaline phosphatase conjugated rabbit-anti-mouse commercial antisera was added at 1:1000 in TBST for 30 minutes. Plates were washed a further 4x prior to the addition of substrate and development and quantitiation as described above (Section 3.61).

3.63 Whole Cell ELISA

Jurkat cells were washed three times and suspended at 10^7 /ml in PBSA (PBS, 1%BSA). 50 µl of cell suspension were aliquotted into each well on a V96 plate and biotinylated monoclonal antibody added (5µl) to yield a final concentration of 1 µg/ml. Cells were incubated for 1 hour, then washed 3times with PBSA, 150 µl/wash. A 50µl aliquot of horse radish peroxidase conjugated avidin, at 1:1000 dilution of commercial stock, was then added and incubated for 30 minutes. Cells were then washed 4x with PBSA, and 100µl substrate (400µg o-phenylene diamine, in 50.6% 0.1M sodium citrate(vol), 49.4% 0.2M sodium phosphate(vol), pH 5.5, 0.03% peroxide) added directly during resuspension of cell pellets. Reaction was stopped by the addition of equal volume of 10M H_2SO_4, and quantitated spectrophotometrically at 492nm.

3.7 Modification of Monoclonal Antibody

3.71 Iodination

Two iodobeads (Pierce) were washed 3times with PBS, dried, and placed in a 5ml snapcap tube containing 200µl of PBS and 18.5MBq ^{125}I, as per the manufacturer's

instructions (Amersham). The beads were preincubated for 15 minutes, and 200µl of antibody at 1mg/ml was subsequently added. The antibody/iodobead mixture was allowed to react for 5 minutes, after which time the supernatant was removed to stop the reaction, and loaded on a 5ml Sephadex G10 column. Iodinated antibody was eluted in the void fraction, two to three fractions ahead of the free radioactive salts.

3.72 Biotinylation

Antibody to be labelled was buffer exchanged to 50mM sodium bicarbonate, pH 8.5, using a Centricon 30 microconcentrator (Amicon Canada, Oakville ON). Final antibody concentration was 10mg/ml. N-Succinyl LC biotin was added directly to the mixture to a final concentration of 400µg/ml, and the reaction was allowed to proceed for 30 minutes. Unreacted biotin was eliminated by buffer exchange in a Centricon-30 centrifuge column. Biotinylated antibody was adjusted to a final concentration of 0.5-1.0mg/ml in PBS containing 0.1% sodium azide, and stored at 4°C.

3.73 Papain Digestion

Papain coupled to Sepharose 4B beads was washed 5x in PBS. Aliquots of 80µl of 1.2mg/ml monoclonal were added to 3.2mg beads (dry weight), supplemented with 10µl 1M sodium acetate, 5µl 1M cysteine, and 1.5µl EDTA. Antibody suspension was incubated overnight (Harlow and Lane, 1988). Fc fragments were recovered with rabbit-anti-mouse Fc and precipitated with protein A beads. Purity of Fab monomers was assessed by 10% reducing PAGE.

3.74 Monoclonal Footprinting of B3B11

Dimethylpimydelate was used to couple B3B11 to protein-G Sepharose beads as

described (Harlow and Lane, 1990). Jurkat cell lysates were prepared as described (Section 3.41), and 200µl were incubated with 20µl of B3B11-beads overnight with shaking at 4°C. Beads were washed six times with NET buffer (Section 3.41), sedimented by microcentrifugation, and liquid removed. Digestion was performed essentially as described by Sheshberadan and Payne (1988); the beads were resuspended in 30µl of stock trypsin solution (1mg/ml in NET), and digestion was allowed to proceed for ten minutes. Beads were washed a further four times, and bead-bound antigen was subsequently solubilized in nonreducing Laemmli buffer. Antigenic identity of integrin fragments solubilized was confirmed by gel electrophoresis and subsequent western blot.

3.8 Immunocompetitive Assays

3.81 Radiocompetitive Assay

Jurkat cells, at a concentration of 10^7/ml, were aliquoted into F96 wells in 50µl volumes. The aliquots were diluted to 100µl through the addition of radioiodinated antibody (25µl, to a final concentration of 0.5-1.0µg/ml) and varying concentrations of "competing" or test antibodies (25µl, to final concentrations 0.1-50µg/ml). The samples were incubated at 4°C for 4 hours. Cells were then washed 5x in PBSAz buffer, and bound radioactivity determined by γ emission. Inhibition of binding was calculated as a function of decrease in radioactivity, assessed by automated gamma-counting (LKB compugamma), as compared to control groups with no competing antibody. The assistance of Jacqueline Stupack during the development of this assay, as well as for providing access to rapid γ-counting, is greatly appreciated.

3.82 Blocking of Biotinylated Monoclonal Binding

Jurkat cells were suspended at a concentration of $5x10^6$/ml. Aliquots of 50µl of cells were transferred to V96 plates, and incubated with increasing concentrations of competing antibody (0.05, 1, or 20µg/ml) for 1 hour at 4°C. Cells were then washed twice with 150µl PBSA, and biotinylated antibody added in a 50µl volume at a final concentration of 2µg/ml. Antibody was allowed to react for 30 minutes at 4°C. Cells were then washed three times with 150µl PBSA, prior to addition of a 1:1000 dilution of horseradish peroxidase-conjugated avidin (200µg/ml commercial stock). Samples were incubated for 30 minutes, and washed with 150µl PBSA four times. Samples were then developed as per the whole cell ELISA, (Section 3.63). Inhibition was determined as the decrease in absorbance from control (noncompetitive) values after spontaneous color development (no biotinylated antibody added) was subtracted.

3.8 Isolation of Plasmid DNA and Bacterial Transformation

3.81 Amplification and Purification of Plasmid

The B3B11 reactive bacterial clone (JM109/DE3 line, provided by C Shen) was subcloned through three rounds, and selected on the basis of tetracyclin and carbenicillin resistance and immunoreactivity of lysates with B3B11. A final clone was selected and amplified by overnight growth in the presence of carbenicillin, 100µg/ml. Cells were pelleted and resuspended in 1/15 original culture volume (typically 100µl aliquots) of 50mM glucose, 10mM EDTA, 25mM Tris pH 8.0 on ice, and then incubated for 5 minutes at room temperature. Freshly made 0.2N NaOH, 1%SDS was added at twice the volume (200µl), mixed by gentle inversion, and incubated for 5 minutes on ice. A 150µl aliquot of

potassium acetate solution, (60% 5M $KCH_3CH_2O_2$, 11.5% glacial acetic acid, 28.5% H_2O) 150µl was added, the solution was mixed by inversion, and then incubated for a further 5 minutes on ice. The mixture was then centrifuged at 16000g for 5 minutes, and the supernatant retained and transferred. Ribonuclease was added to a final concentration of 20µg/ml, and incubated at 37°C for 30 minutes. Plasmid DNA was isolated from protein by the addition of "ICP" solution (chloroform 48%, iso-amyl alcohol 2%, phenol 50%) in equal volume. Upper phase was retained and DNA present was precipitated by addition of absolute ethanol at twice the retained volume. Precipitated DNA was collected by centrifugation at 16,000g for 15 minutes, and allowed to dry thoroughly. DNA was resuspended in 20µl TE (10mM Tris/1mM EDTA, pH 7.4), and used in further manipulations (Methods supplied by Novagen, Inc. within Novatope System Manual and the PET System Manual).

3.82 Transformation and Screening of Competent BL21 Cells

Isolated DNA in TE buffer was added to commercial competent BL21 (Novagen) cells and incubated on ice for 30 minutes. Cells were then transferred to a 42°C water bath for 90 seconds, and subsequently LB (carbenicillin, 100µg/ml) was added. Cells were allowed to recover for 1 hour at 37°C, and were then plated on carbenicillin-containing LB-agar plates. Positive colonies were assessed for B3B11 reactivity by immunoscreening. Nitrocellulose lifts were taken, and cells lysed in a chloroform chamber. Lifts were then laid on 6M urea soaked Whatman 3M chromatography paper to denature protein. Lifts were washed three times in TBST, and allowed to block in TBST/1% collagen for thirty minutes. Probe antibody was added at 2µg/ml (final concentration) and incubated in TBST for 30 minutes. Lifts were washed three times with TBST and the secondary developing

reagent, alkaline phosphatase-conjugated rabbit anti-mouse antisera, was added in fresh TBST at a final concentration of 1:2500. After a thirty minute incubation, filters were washed a further four times in TBST, and developed as per western blot. A clone designated FPG10.B3B11 resulted from the third round of subcloning.

3.83 Induction, Analysis and Purification of Fusion Protein

Clones from frozen stocks were grown overnight in 4ml LB broth in the presence of 200µg/ml carbenicillin. A 1ml aliquot was pelleted, washed once in LB, and resuspended in 0.5ml of fresh media. A 100µl fraction of this was then transferred into a 50ml conical tube containing 25ml of LB/200µg/ml carbenicillin. The culture was incubated for 5-6 hours, or until a 100µl aliquot displayed an OD greater than 0.6 at 540nm, (using media alone as a blank). The culture was pelleted and resuspend in fresh media (LB) containing carbenicillin. Iso-propyl-thio-galactoside was added to a final concentration of 1mM. After 90 minutes, cells were collected by centrifugation and suspended in 5ml TBSE(50mM Tris, 200mM NaCl, 1mM EDTA). Lysozyme was added to a final concentration of 1mg/ml. After 30 minutes at room temperature, an equal volume of 2M urea, 0.5% triton X-100 was added; the suspension was then agitated for an hour at 37°C, centrifuged for 15 minutes at 16000g, and the pellet retained. The pellet was resuspended in 5ml of 2M urea, 0.5% triton X-100, again shaken as described and centrifuged to obtain the pellet. The extraction was repeated a third time. The fusion protein remained insoluble in inclusion bodies, and was solubilized in 8M urea, 2% triton X-100. Purity was assessed by SDS-PAGE with Coomassie brilliant blue staining, and urea removed by dialysis against PBS. A single, major band was apparent at 36kDa, although several faintly staining, (contaminating) bands were also often visible.

SECTION 4.0 RESULTS

SECTION 4.1 Lymphoid Populations Attach to Fibronectin

4.11 Interaction of Jurkat cells with ECM

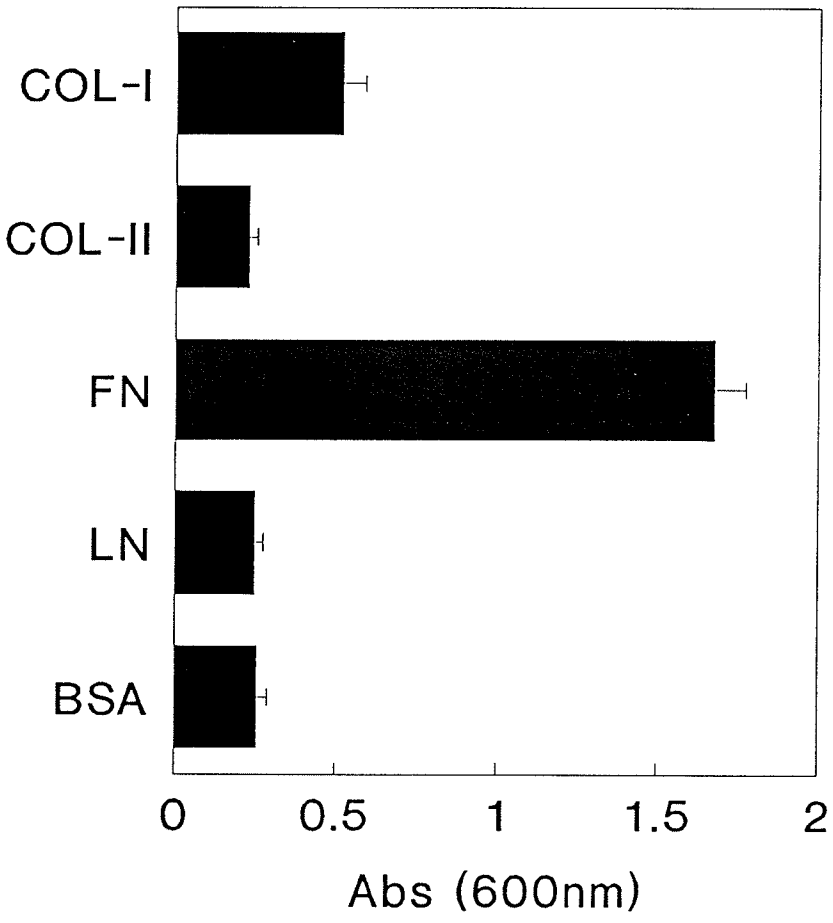
The T leukemic culture line Jurkat was used in a preliminary investigation into the capacity of lymphocytes to interact with extracellular matrix. The Jurkat cell line was selected on the basis of previous data generated within the laboratory (J Wilkins and W Offusu-Appiah, unpublished) which suggested that under some circumstances, the extracellular matrix components collagen and fibronectin could elicit cytokine secretion from Jurkat cells. Further, during these studies, morphological examinations of the Jurkat cells by light microscopy suggested that the Jurkat cells might be acquiring a capacity to adhere to these substrates. Jurkat cells were therefore assessed for attachment to the extracellular matrix components collagen, fibronectin and laminin by an *in vitro* adherence assay.

Jurkat cells were observed to attach strongly to surfaces coated with fibronectin, corresponding to an absorbance of 1.679 (Figure 4.1). Jurkat cells did not appear to attach specifically to murine laminin or to bovine type II collagen, as the observed ODs of approximately 0.229 and 0.249 correlated approximately with the OD observed for attachment to BSA, 0.256. Attachment to type I collagen was suggested by reproducible binding of Jurkat cells which corresponded to an OD of 0.520.

To more fully investigate the interaction of Jurkat cells with fibronectin, and to demonstrate concentration-dependant specificity, fibronectin was serially diluted during the microtitre-well coating process. Jurkat cells were then assessed for attachment to wells coated with varying fibronectin concentrations. Jurkat cells were observed to attach to fibronectin coated surfaces as a function of fibronectin concentration: adhesion was

Figure 4.1 Quantitation of Jurkat Cell Attachment to ECM

Jurkat cells (10^6/ml) were allowed to adhere with microtitre wells coated with various extracellular matrix components; COL-I, type I collagen derived from rat tail, COL-II, type two bovine collagen, FN, human plasma fibronectin, LN murine laminin, BSA, bovine serum albumin. Nonadherent cells were removed by gentle washing. Adherent cells were quantitated by crystal violet dye binding, and absorbance read at 600nm. Mean and Error of triplicates from a representative experiment are shown.


Jurkat Adherence to ECM
COL-I
COL-II
FN
LN
BSA
0
0.5
1
1.5
2
Abs (600nm)

maximal at a concentration of 5 µg/ml or greater (Figure 4.2A). This observation suggested that the interaction of the Jurkat cells with fibronectin was a specific process. Titrations of type II collagen and laminin were also performed, however, no adherence was observed, even when coating concentrations in excess of 100µg protein/ml were used. The type I collagen was not titrated as it was coated via distinct means, utilizing 5µl of the acidic collagen preparation and 1µl of a 3M NaCl solution. The mixture was combined within the well and immediately mixed and spread to cover the well bottom with a sterile pipette tip. Attachment did not increase with increases in collagen applied to wells, and it was not feasible to introduce smaller quantities of the collagen stock. Dilutions of the collagen preparation did not coat plates efficiently, and a loss in cellular adherence was observed.

As a second means to investigate specificity of the Jurkat:fibronectin interaction, microtitre wells coated with fibronectin were treated with nonimmune rabbit antisera, or antisera raised against human fibronectin. Since lymphocytes are also known to express surface-bound forms of fibronectin (Arencibia and Sundqvist, 1989) which could (in theory) autoassociate with immobilized plate-bound fibronectin (Woods et al., 1988), a control group of Jurkat cells were also treated with anti-fibronectin antisera.

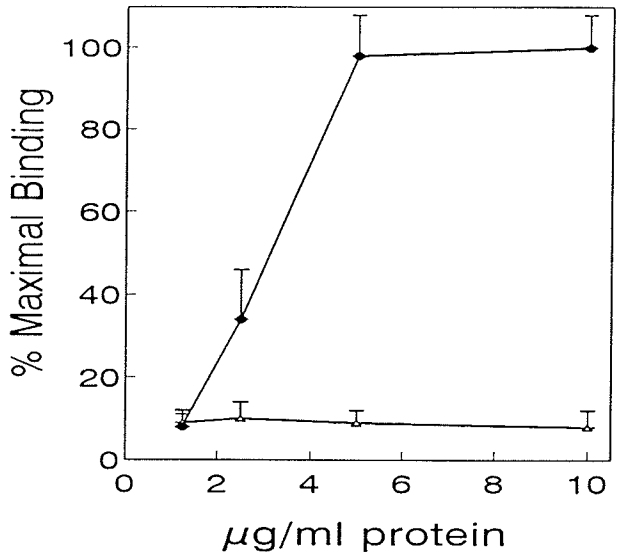
Jurkat cells were not significantly influenced by treatment with antisera to fibronectin, but were not able to attach to fibronectin coated microtitre wells which had been treated with anti-fibronectin antisera (Figure 4.2B). These results suggest that Jurkat cells interact directly and specifically with sites present on substrate fibronectin, and that any putative source of cell bound fibronectin does not appear to play a role in the observed attachment.

To determine the temporal requirements of the observed attachment, the adherence assay kinetics were examined. The attachment of Jurkat cells to extracellular matrix coated surfaces was a rapid process, requiring less than ten minutes (Figure 4.3). By visual assessment, this appeared to correlate with the time required for the cells to sediment from

Figure 4.2 Specificity of Interaction with Fibronectin

A. Fibronectin (square) or BSA (triangle) was diluted in carbonate coating buffer during the microtitre well coating process. Jurkat cells were then assessed for attachment to wells coated with various concentrations of protein. Mean and Error of triplicates from a representative experiment are shown. **B** Commercial rabbit antisera to human fibronectin (HFN) or nonimmune rabbit sera (NIRS) was used to pretreat Jurkat cells or microtitre wells coated with fibronectin prior to adherence assay. Both cells and wells were washed to remove unbound antibody prior to assay. Mean and Error of one of two similar experiments is shown.

A. Titration of Fibronectin



B. Effect of Anti-FN on Adherence

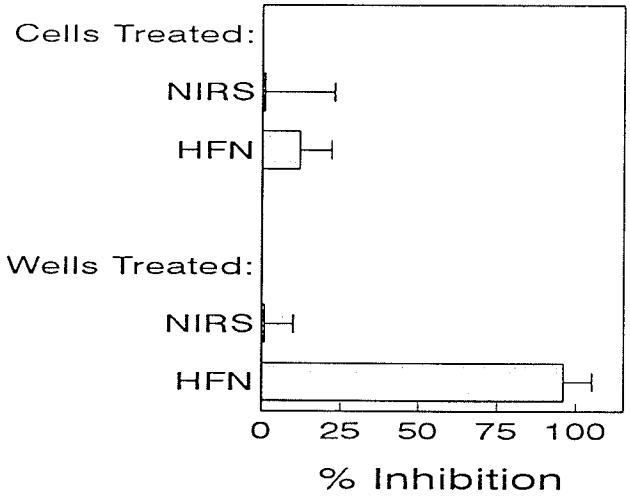
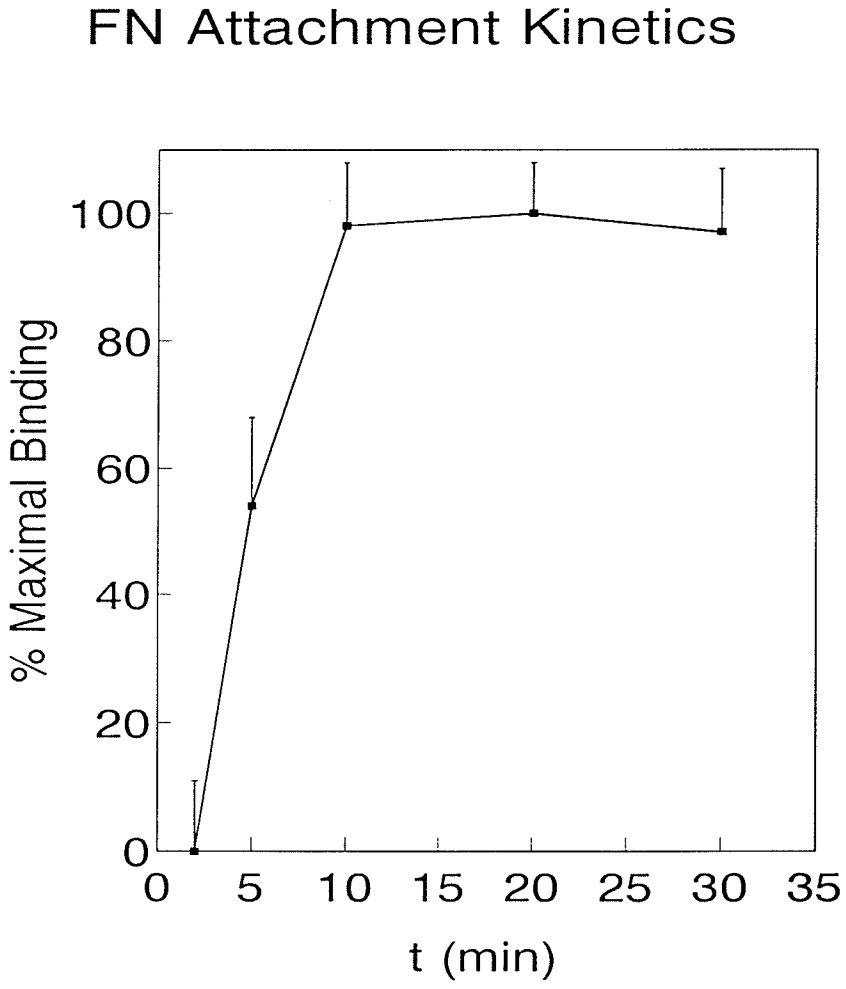


Figure 4.3 Kinetics of Jurkat Attachment to Fibronectin

The specific binding (Abs FN - Abs BSA) of Jurkat cells was assessed as a function of time. Cells were allowed to adhere for different periods, as shown, prior to washing to remove nonadherent cells. Mean and Error for each triplicate time point is depicted. The maximal adherence in this experiment was 0.980 Abs Units.


FN Attachment Kinetics
% Maximal Binding
100
80
60
40
20
0
0
5
10
15
20
25
30
35
t (min)

the assay buffer suspension onto the protein-coated bottom of the microtitre wells.

4.12 Assessment of ECM binding in other cell lines

As described, the Jurkat cell line was used in initial studies due to the suggestion of adherence capacity provided by previous investigations. In order to determine if this was a common property of lymphoid cells, a variety of lymphoblastoid cell lines and isolated lymphocyte peripheral blood lymphocyte (PBL) populations were assessed for their capacity to attach to fibronectin. Jurkat proved to exhibit among the highest binding potentials on fibronectin among the lymphoid cell lines (Table 4.1), although the attachment of Jurkat cells was lower than non-lymphoid cell lines such as K-562 (erythroleukemia) or A-375 (carcinoma). Jurkat cells which were grown on a serum-free media (HB102), did not differ significantly from Jurkat cells grown in serum-supplemented media. Two B cell lines examined also had high adherence potential, JR2B10 and RPMI 8226. These cell lines were perhaps slightly less adherent than the Jurkat cell line (Abs 1.1 to 1.3). The two murine T lines (CTLL2 and LBRM) did not adhere strongly to fibronectin. It is not likely that this is due to species-specific differences in recognition (Erle et al, 1994b), as murine cells can adhere strongly to human fibronectin. These two lines may represent a less adherent lymphoid phenotype, analogous to the human HSB2 cells. Other lymphoid lines varied between 0.340 and 0.750 OD in specific attachment. Thus, a wide variety of cellular phenotypes were observed in culture lines with respect to attachment to fibronectin. The long term culture of these lines does not appear to overtly favour the acquisition of a particular adhesive propensity with respect to fibronectin.

Isolated PBL also attached to fibronectin weakly (OD 0.015), but activation by different means (Concanavalin A, (Con A) (200ng/ml) + Phorbol Myristate Acetate (PMA) (20ng/ml) for 72h, or Interleukin 2 (IL2)(10 units/ml) + allogeneic cell line RPMI 8866 or

Table 4.1 Attachment of Lymphoid cells to Fibronectin

Legend: Lymphocytes: PBL, peripheral blood lymphocytes, Purified T cells, SRBC rosetted lymphocytes, Tonsillar Lc, lymphocytes isolated from tonsil tissue, SFL, lymphocytes purified from clinical synovial aspirates of active rheumatoid disease (n=2), Leukemic B cells, clinical leukemic cells (B lineage) isolated from peripheral blood (n=3), LAK, lymphokine activated killer cells stimulated with B cell lines as indicated (MLR) with IL2, or with IL2 alone (n=3).

Lineage: indicates the lineage of the cell lines assessed for adherence, T, T cell line, B, B cell line, E, Erythroleukemia, M, melanoma, C, lung carcinoma, H, Histiocytic leukemia.

Specific Attachment: Abs FN - Abs BSA. Only Jurkat, JR2B10 and the non-lymphoid cell lines were observed to attach to collagen.

Experiments were performed separately, results shown are representative for each cell type. As all cell lines were not directly tested within the same assay, the results cannot be compared absolutely and are best interpreted in general terms. A guideline might be [**+/-** (<0.200), **+** (0.200-0.400), **++** (0.400-1.000) and **+++** (>1.000)].

TABLE 4.1 ATTACHMENT OF LYMPHOID POPULATIONS TO FIBRONECTIN

Cell type	Lineage	Species	Specific Attachment (O.D.)
CELL LINES:			
Jurkat	T	Human	1.336±.136
JurkatSFM	T	Human	1.289±.112
HSB2	T	Human	0.175±.357
Molt3	T	Human	0.750±.090
CEM	T	Human	0.670±.158
SKW	T	Human	0.554±.127
LBRM	T	Murine	0.273±.043
CTLL2	T	Murine	0.241±.041
JR2B10	B	Human	1.121±.090
NC10	B	Human	0.686±.224
CESS	B	Human	0.340±.224
RPMI 8866	B	Human	0.460±.045
K-562	E	Human	1.421±.136
A-375	M	Human	1.672±.088
A-549	C	Human	1.889±.206
U-937	H	Human	1.119±.077
LYMPHOCYTES:			
PBL		Human	0.015±.021
PBL, PMA		Human	0.052±.019
PBL, Con A		Human	0.006±.020
PBL, PMA/Con A		Human	0.343±.061
Purified T cells		Human	0.043±.025
Tonsillar Lc		Human	0.081±.010
SFL		Human	0.291±.044
Leukemic B		Human	0.032±.013
LAK(IL2+8866)		Human	0.211±.041
LAK(IL2+JR2B10)		Human	0.235±.037
LAK(IL2 Only)		Human	0.041±.035

JR2B10, 120h) was able to significantly increase the observed level of attachment. The activation of PBL with concanavalin A increased their adherence potential upon all matrix components tested, as well as to control BSA-coated wells. Therefore, no increase in specific attachment was observed in the Con A-stimulated populations. However, in the PMA + Con A stimulated population, the increase in fibronectin attachment was much greater than the increase observed BSA attachment, thus the **specific** attachment did significantly increase (calculated as [Abs FN - Abs BSA]).

Lymphocytes were also stimulated through a second activation protocol. PBL were stimulated with interleukin 2 and allogeneic irradiated B cell lines to generate lymphokine activated killer cells. These cells were kindly generated and generously provided by Dr. L. Selin. The lymphokine activated killer (LAK) cells demonstrated an increase in attachment to fibronectin when activated by interleukin 2 and an allogeneic cell line (JR2B10 and RPMI 8866), but not when activated by interleukin 2 alone. In conjunction with the lectin/PMA data, these observations suggested that [1] lymphocyte adherence to fibronectin can be augmented artificially *in vitro*, and [2] adherence induction requires specific, perhaps even multiple, activational signals. Further, it is clear that the Jurkat cell line initially selected for study is not unique in its capacity to attach to fibronectin.

4.13 Preliminary Characterization of Jurkat binding to ECM

Studies were conducted to determine the nature of the receptor on Jurkat cells which mediated binding to fibronectin. Attachment was examined through a variety of biochemical means. Jurkat cells were preincubated with increasing concentrations of trypsin (up to 1350 units/ml for 30 minutes) prior to adhesion assay to determine if the adhesion structures utilized were sensitive to protease. Trypsin was removed by washing prior to assay to preclude the possibility of digestion of substrate. Trypsin was determined to inhibit attachment of Jurkat cells to fibronectin in a dose dependant manner, indicating

that a surface-expressed protein was required in the observed attachment (Figure 4.4A).

The temperature sensitivity of the observed binding was assessed by performing the adherence assay at a variety of temperatures. The binding of Jurkat cells to fibronectin was found to be a temperature dependant process; Jurkat cells attached to fibronectin strongly at 37°C or 22°C, but attached quite weakly to fibronectin at 4°C (Figure 4.4B). Thus, adherence to fibronectin appeared dependant upon temperature.

The role of divalent cations in the observed attachment was also examined. Jurkat cells required the presence of divalent cations in order to attach to fibronectin-coated surfaces. The addition of EDTA, a chelator of divalent cations, was capable of totally abrogating the attachment of Jurkat cells to fibronectin (Figure 4.4C). Jurkat cells attached strongly to fibronectin (Abs > 1.35) in the presence of calcium (500μM), and the calcium could be replaced with magnesium (500μM) without compromising the observed strength of adhesion.

These experiments strongly suggested that the adhesion structure mediating Jurkat attachment to fibronectin was an integrin. The receptor was digestion-sensitive, temperature dependant, and required divalent cations, but not specifically calcium, for function. These results were sufficient to exclude "typical" members of the IGSF family, the selectins, and cell surface proteoglycans as immediate candidate receptors. However, the results were consistent with the use of members of the integrin family by Jurkat cells to attach to fibronectin, a possibility supported by other studies in which ß1 integrins were observed on the surface of T cell populations (Carderelli and Pierschbacher, 1987) and facilitated their attachment to fibronectin (Carderelli and Pierschbacher, 1988, Klingemann and Dedhar, 1989).

Jurkat cells were therefore tested for the expression of ß1 integrin using monoclonal antibody A-1A5, a monoclonal antibody that initially defined the ß1 integrins (Hemler et al

Figure 4.4 Preliminary Characterization of Jurkat Adherence

A. The effect of varying concentrations of trypsin on Jurkat attachment to fibronectin was assessed. Cells were preincubated with trypsin at varying concentrations, and washed prior to assay. Mean and Error of triplicate specific absorbance determinations are shown. **B.** The effect of incubation at different temperatures during adherence to fibronectin was assessed. Jurkat cells were preincubated at the various temperatures for 15 minutes prior to adhesion assay. The assay was then performed at the various temperatures, and adherent cells quantitated. Mean and error of triplicate determinations from a representative experiment are shown. **C.** Jurkat cells were washed in saline, 2mM EDTA and resuspended in phosphate buffered saline (pH 7.4) containing 2mM EDTA), saline containing 500μM calcium chloride ($CaCl_2$), or saline containing 500μM magnesium chloride ($MgCl_2$). The adherence assay was performed in these solutions, and cellular adherence quantified. Mean and errors from triplicate determinations of a representative experiment are shown. Each experiment was performed two (or more) times, with similar results.

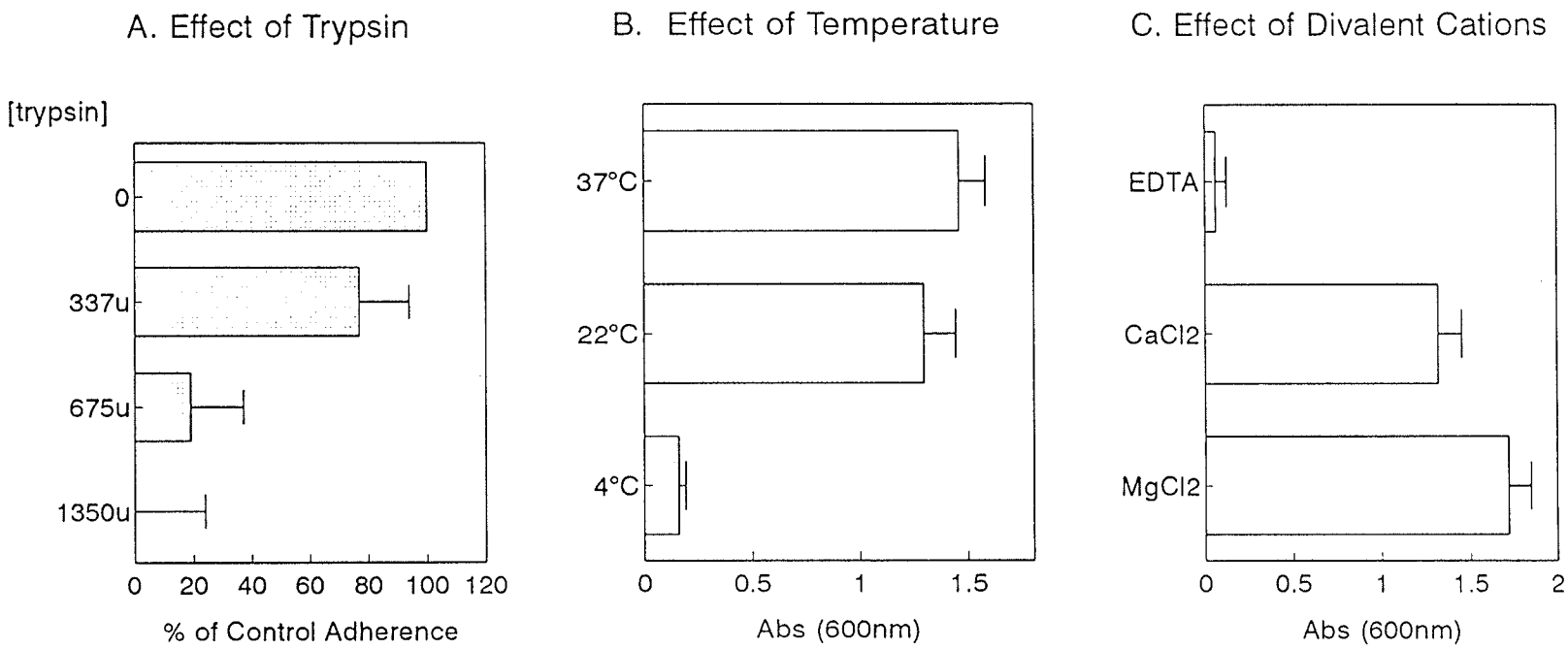
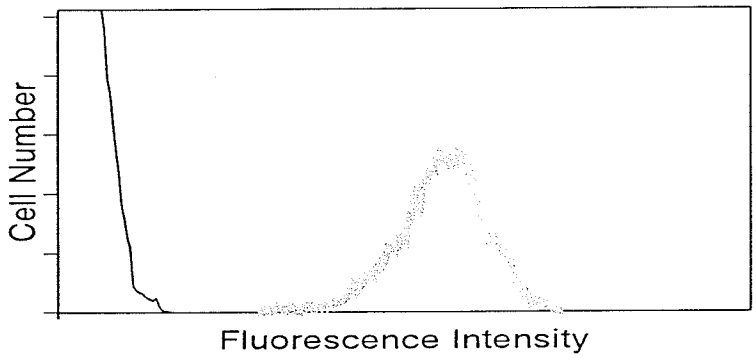

A. Effect of Trypsin
[trypsin]
0
337u
675u
1350u
0 20 40 60 80 100 120
% of Control Adherence
B. Effect of Temperature
37°C
22°C
4°C
0 0.5 1 1.5
Abs (600nm)
C. Effect of Divalent Cations
EDTA
CaCl2
MgCl2
0 0.5 1 1.5 2
Abs (600nm)

Figure 4.5 Characterization of ß1 Integrin on Jurkat cells

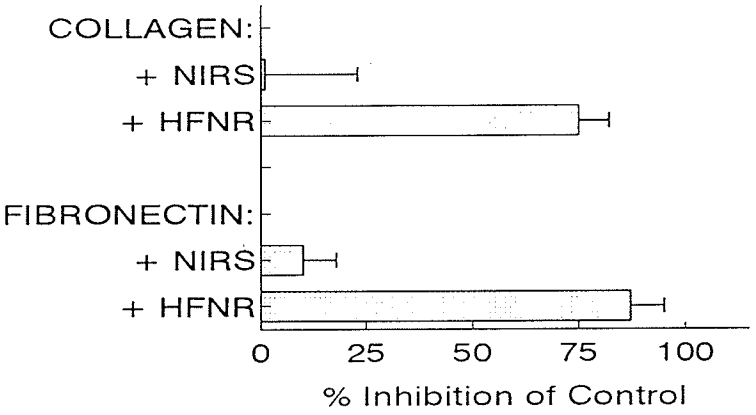
A. FACS. Jurkat cells were labelled with monoclonal antibody A-1A5 to assess expression of ß1, and primary antibody binding was detected with goat anti-mouse FITC-conjugated FAb'. A-1A5 labelled populations (bold line) and nul-labelled (thin line) populations are depicted. a representative experiment is shown. The Y axis is cell number (scale 256), the X axis is relative fluorescence. 10,000 cells were analyzed. **B.** Jurkat cells were pretreated with nonimmune rabbit serum (NIRS) or antisera specific for ß1 and α5 integrin (HFNR), and assessed for attachment to fibronectin or type I collagen. Results are expressed as inhibition of untreated Jurkat cell attachment to either matrix component. Mean and error of triplicates from a representative experiment is shown. Both experiments were performed three or more times.

Expression of ß1 integrin on Jurkat



99.9 % Positive for ß1
Mean Fluorescence Intensity: 132.75

Effect of Antisera to ß1 Integrin on Adherence



1983). FACS analysis (Figure 4.5A) demonstrated the expression of integrin complexes containing ß1 with unidentified α integrin subunits. It was possible that integrins other than ß1 might mediate adhesion to fibronectin. However, the Jurkat cells utilized in these studies do not express ß3 integrins (Shen et al, 1991) and express negligible levels of ß2 integrin (J Wilkins, unpublished observations).

In order to directly test whether Jurkat cells were actually using the ß1 integrin complexes present to attach to fibronectin and collagen, the adhesion assay was performed in the presence of polyclonal antisera specific for ß1 and the α5 integrin subunits, anti-HFNR. This commercial antiserum was capable of strongly inhibiting Jurkat cell attachment to either collagen (>75%) or fibronectin (>80%) (Figure 4.5B). The presence of a nonimmune rabbit serum had no effect. Therefore, it appeared that the Jurkat cells were employing ß1 integrins to bind to collagen and fibronectin.

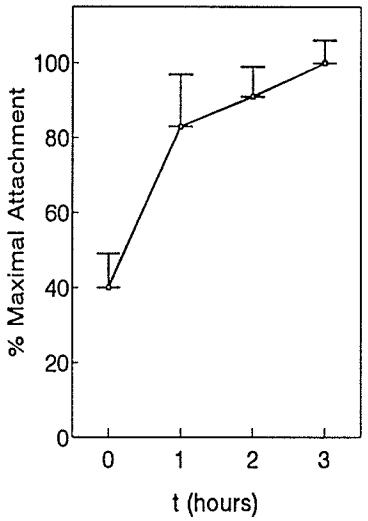
4.14 Cellular activation promotes lymphoid adherence

The accumulated evidence suggested that Jurkat cells were attaching to extracellular matrix by members of the ß1 family of integrins. Integrin αLß2 (LFA-1) had been reported in the literature to exist in a dormant state on the cell surface, and to become activated in response to PMA (Chatila et al, 1989). PMA had also been previously demonstrated to activate a member of a third integrin subfamily (ß3) integrins on platelets (Coller, 1986). Therefore, the possible effect of PMA on Jurkat cell attachment to fibronectin was examined. Jurkat cells were preincubated in the presence of PMA for varying periods, and then assessed for attachment to collagen and fibronectin coated microtitre wells. The PMA-stimulated populations of Jurkat cells demonstrated a stronger adherence to fibronectin than unstimulated populations; adherence was observed to approximately double (Figure 4.6A).

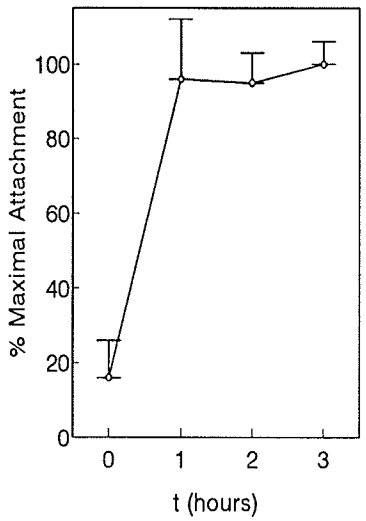
Figure 4.6 Effect of PMA on Jurkat Attachment to ECM

Jurkat cells were treated with the phorbol ester PMA (20ng/ml) for various periods as shown, or untreated (0 time point), and assessed for attachment to fibronectin (**A**) and collagen (**B**) as shown. The treatment periods include the time required to complete the adhesion assay. Mean and Errors of a representative experiment are shown. Jurkat cells were also studied over extended time periods (**C**),(fibronectin shown), to attempt to determine the persistence of enhanced attachment induced by PMA on Jurkat cells.

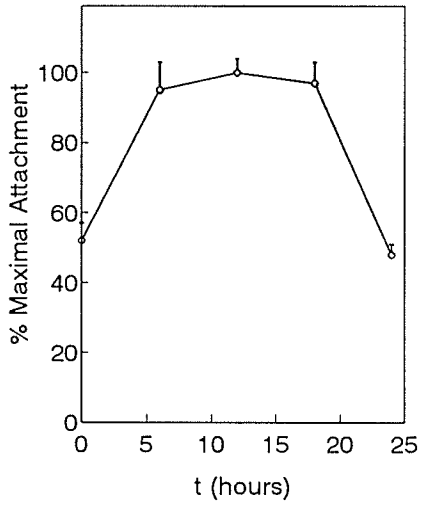
A. Fibronectin



B. Collagen



C. Long-term PMA Kinetics



The observed increase in attachment to collagen was more dramatic. Collagen attachment, as assessed by dye binding, increased by a factor of approximately five (Figure 4.6B). The time points for these results include the one hour required for the adherence assay (PMA was always allowed to remain in the cell suspension for the entire experiment). Therefore, the binding was induced immediately upon addition of PMA (no preincubation was necessary), and was sustained for the length of the assay. In long- term kinetic investigations on fibronectin, the PMA-induced attachment was found to persist for at least 18 hours, but to decrease to basal levels by 24 hours (Figure 4.6C).

These data were consistent with the induction of dormant ß1 integrin on the surface of Jurkat cells. However, it remained possible that PMA might mobilize internal stores of sequestered integrin to the cell surface, as had been reported in ß2 integrin systems using myeloid cell lines (Wright and Detmers, 1988) Alternatively, PMA might induce the (rapid) translation of novel adhesion structures.

In order to test the latter of these two possibilities, Jurkat cells were preincubated with cycloheximide (CHI), a selective inhibitor of protein translation. Cells which had been CHI-treated for one hour were then treated with PMA and tested for their capacity to attach to fibronectin. The CHI-treated cells remained capable of increased attachment to fibronectin following treatment with PMA (Figure 4.7).

In order to address the possibility that Jurkat cells could be mobilizing internal stores of adhesion structures, Jurkat cells were analyzed by flow cytometry before and after treatment with PMA. Jurkat cells did not modify their levels of expression of ß1 integrin (Table 4.2). Since several different α integrin subunits might complex with ß1 integrin, it remained possible that PMA elicited a turnover of ß1 complexes, modifying the α subunit expression patterns while maintaining an apparently stable level of ß1 expression. Therefore, Jurkat cells were coassessed for α integrin expression in PMA stimulated and

Figure 4.7 Effect of Cycloheximide on PMA activation of Jurkat Cells

Jurkat cells were pretreated with cycloheximide (CHI) (10μg/ml) for 1 hour, or untreated. Treated cells and untreated controls were then PMA stimulated(20ng/ml) for 1 hour and assessed for attachment to fibronectin. Nonactivated controls are shown for comparison. Results are the mean of four separate experiments.

Effect of CHI on Adherence

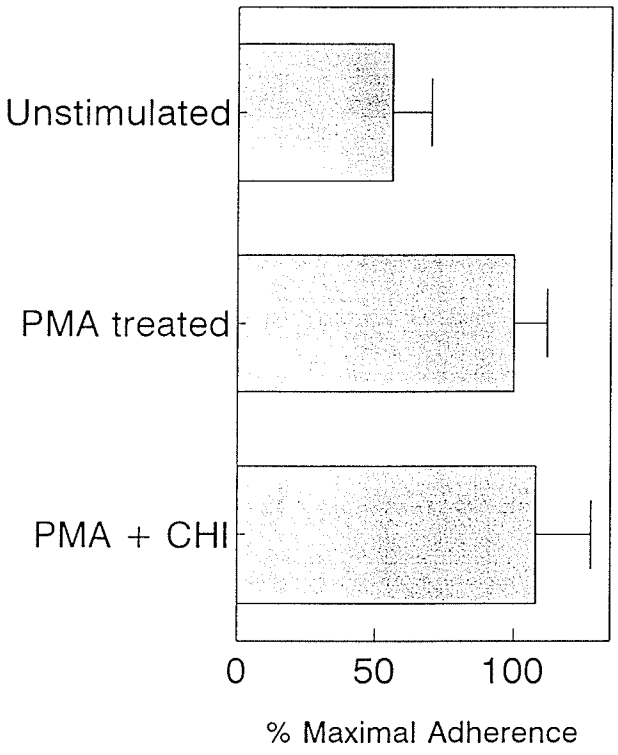


Table 4.2 Integrin Expression on Activated Jurkat Cells

Antigen	Monoclonal	- PMA		+ PMA	
		MFI	% Positive	MFI	% Positive
α1	TS2/7	*	*	*	*
α2	P1H5	101	99	101	99
α3	J143	53	22	51	28
α4	P4G9	108	98	107	98
α5	P1D6	83	97	80	98
ß1	A-1A5	134	99	136	99
CD3	OKT3	137	99	106	98

Legend: Cell populations assessed by flow cytometry were either stimulated (+ PMA) (PMA, 20ng/ml) or treated with diluent only (- PMA).

MFI represents the mean fluorescence index of the positive population.

% Positive are the fraction of cells staining within the gated region, established at the 0.95 confidence interval for nonspecifically labelled (only FITC-conjugated rabbit anti-mouse) populations. The α1 specific antibody TS2/7 did not specifically stain the Jurkat cells by this assessment, signified with a (*).

control populations. Similar to results observed with ß1, the α integrin complement did not appear to change in response to PMA (Table 4.2).

At least one further possibility remained. It was possible that PMA-activated Jurkat cells utilized novel, uncharacterized receptors to increase adherence to fibronectin and collagen. In order to test this directly, PMA-stimulated Jurkat cells were treated with the polyclonal anti-fibronectin receptor (anti- α5+ß1) antisera and assessed for adherence (Figure 4.8). The anti-integrin serum was capable of inhibiting PMA-stimulated Jurkat cell attachment to either collagen or fibronectin, indicating that ß1 integrins remained the adhesion structure used on the PMA-activated cells.

The capacity of PMA to induce attachment was assessed in other lymphoid populations. PMA did not influence all populations equally. The cell line JR2B10 appeared similar to Jurkat, and was capable of increased attachment to fibronectin and collagen in response to PMA (Figure 4.9A, B). In comparison, the HSB2 cell line was capable of attaching to fibronectin, though not collagen. Activation with PMA enhanced the fibronectin adherence in this cell line, though no collagen adherence was induced. Peripheral blood lymphocyte populations were minimally influenced by PMA, increasing attachment to collagen by a greater margin than fibronectin, but less than 0.2 OD units overall. No PMA-specific effect was observed in the Namalawa or RPMI 8226 cell lines. In the case of the Namalawa cells, no specific adherence to either fibronectin or collagen was observed (with or without PMA treatment). In contrast, RPMI 8226 were strongly adherent (Abs > 1.3), however, this adherence was not influenced by treatment of the cells with PMA. Thus, lymphoid cell populations were observed to display a heterogenous response to PMA.

The relatively low adherence observed within the PBL population could have resulted from a lesser expression of required adhesion receptors (ie., ß1 integrins). To

Figure 4.8 Effect of Antisera to ß1 on PMA-activated Jurkat

Jurkat cells were activated with PMA (20ng/ml) for 1 hour and subsequently treated with nonimmune rabbit sera (NIRS) or antisera to the human ß1 and α5 integrin subunits (HFNR)(Human Fibronectin Receptor)(1:100 titre). Cells were then assessed for attachment to fibronectin and type I collagen. Data is expressed as a percentage inhibition of untreated controls. Mean and error of triplicate determinations from a representative experiment are shown.

Inhibition of Stimulated Jurkat

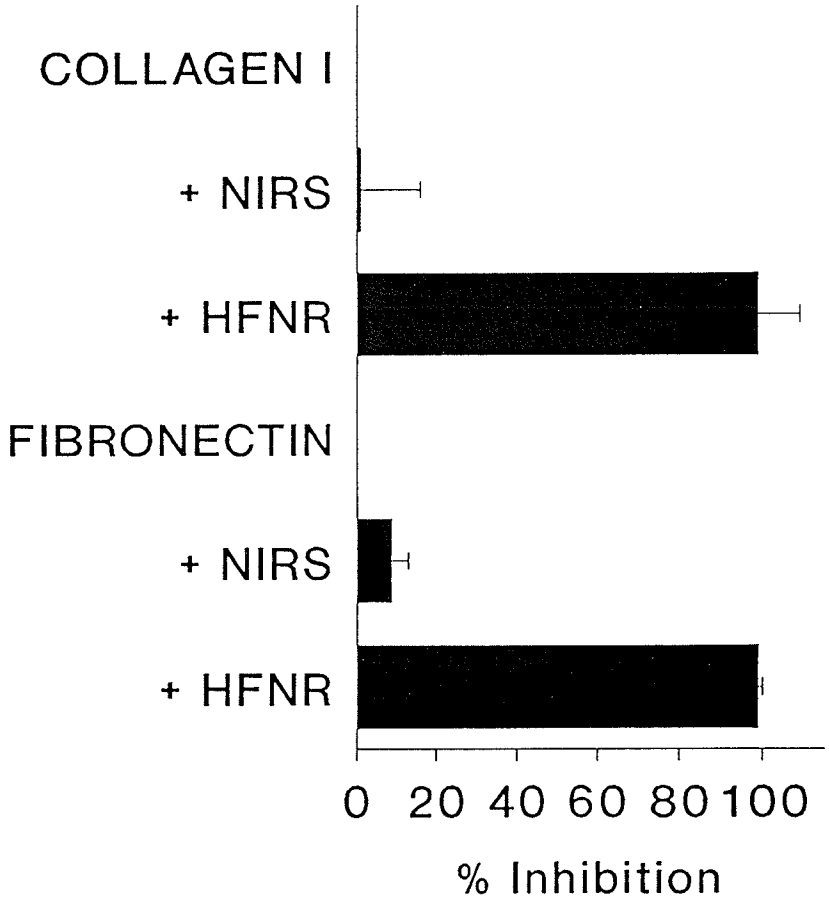
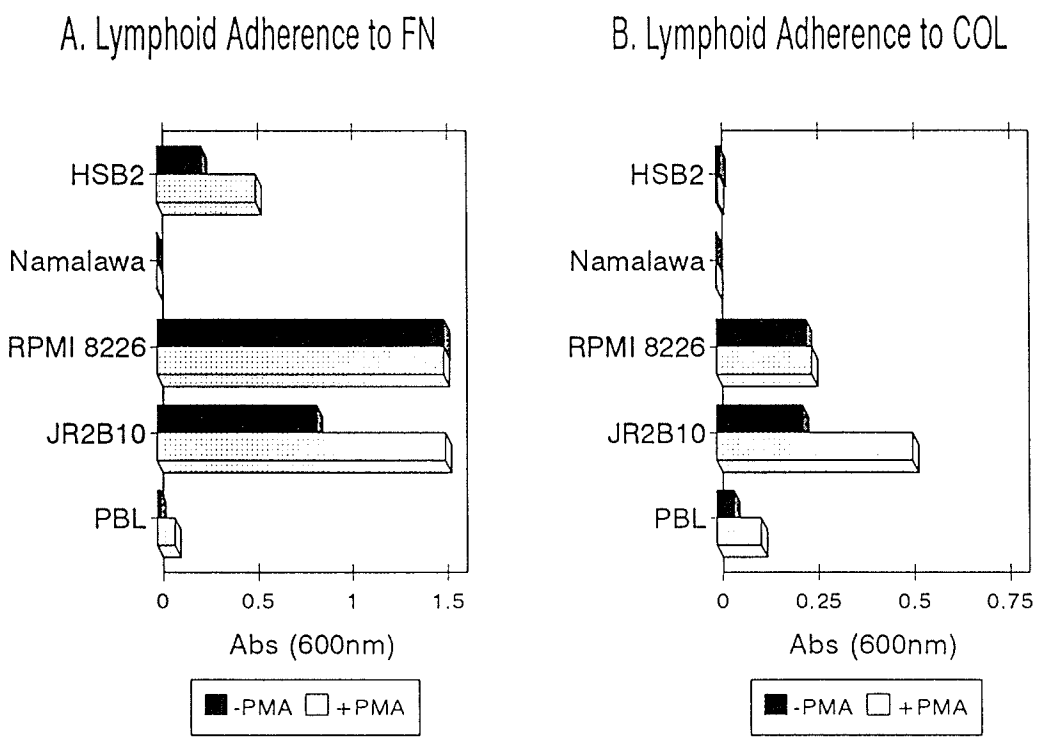


Figure 4.9 Effect of PMA on Representative Lymphoid Cell lines The effect of incubation with PMA (20ng/ml) (light fill) upon the constitutive adhesive potential (solid fill) of several lymphoid cell types was examined on fibronectin (**A**) and collagen (**B**). Representative results for these cell types are depicted; cells were assessed separately. Errors for each of the experiments depicted are less than 11%.


A. Lymphoid Adherence to FN
HSB2
Namalawa
RPMI 8226
JR2B10
PBL
0
0.5
1
1.5
Abs (600nm)
-PMA
+PMA
B. Lymphoid Adherence to COL
HSB2
Namalawa
RPMI 8226
JR2B10
PBL
0
0.25
0.5
0.75
Abs (600nm)
-PMA
+PMA

investigate this possibility, isolated PBL populations were tested for their expression of ß1 integrin. Over 90% percent of lymphocytes assessed were positive for ß1 expression. However, in contrast to the Jurkat cells, a bimodal distribution was observed, with approximately 40% of the cells expressing higher levels of ß1 (These populations are usually termed $CD29^{Hi)}$(Hemler et al, 1987). Since ß1 integrin is equivalent to the CD29, for purposes of simplicity the ß1 designation will be maintained (thus $ß1^{Hi}$), with the remainder expressing low levels of ß1 ($CD29^{Lo}$, or $ß1^{Lo}$) (Figure 4.10). The result of the flow cytometric assessment was identical with other reports (Hemler et al 1983, 1987a,1987b, 1990b). In these reports, it was demonstrated that mitogen stimulation could increase expression of ß1 integrin. Increased ß1 expression was also reported to be a memory cell marker, identifying a population of lymphocytes previously activated (by definition) (Sanders et al, 1988). Thus, two sources of evidence suggested that activation of lymphocytes could result in increases in the mean expression of ß1 integrin. The previously observed increase in attachment within certain cultured, purified lymphocyte populations (Table 4.1) (Con A/PMA stimulated, LAK/allogeneic activated) might reasonably relate to unobserved increases in integrin expression level. Therefore, lymphocyte populations were isolated for purposes of activation, and to subsequently monitor adherence potential changes and levels of ß1 integrin expression.

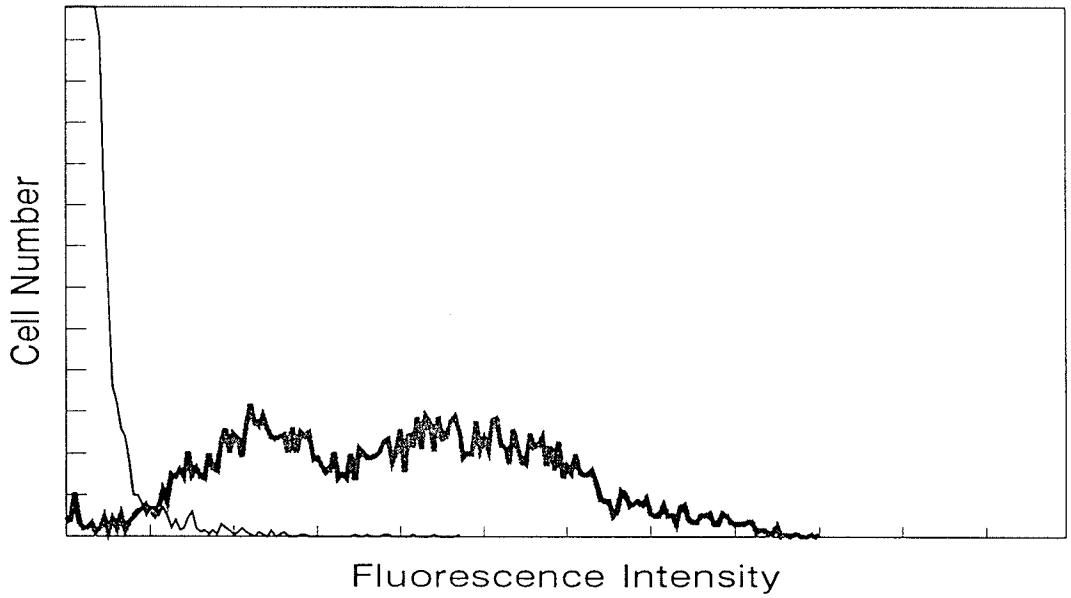
One identified problem with continuing to use concanavalin A as a mitogen, however, was the inherent "sticky" quality that this lectin possessed. Concanavalin A treated PBL exhibited increased attachment BSA as well as to fibronectin, collagen, and other cells. While specific adherence to matrix components was observed, the background adherence increased, and the cells were troublesome to handle with respect to washing and resuspending cell pellets.

Therefore, an alternative mitogen was tested. Monoclonal OKT3 is a mitogenic

Figure 4.10 FACS Analysis of PBLs for ß1 Integrin Expression

PBLs were labelled with monoclonal antibody A-1A5 to assess expression of ß1, and primary antibody binding was detected with goat anti-mouse FITC-conjugated FAb'. A-1A5 labelled populations (bold line) and nul-labelled (thin line) populations are depicted. A representative experiment is shown. The Y axis is cell number (scale to 256)(10,000 cells assessed), the X axis is relative fluorescence intensity (arbitrary units).

Expression of ß1 integrin on PBL



antibody which specifically activates T cells through interaction with CD3, part of the T cell receptor complex. This mitogen was felt to be advantageous because it activated an identified subpopulation of cells through an identified receptor. Further, this mitogen did not render the stimulated cells nonspecifically adherent to glycoproteins, cell binding of lectins such as concanavalin or phytohemagglutinin might.

Purified peripheral blood lymphocytes were divided into four different groups for stimulation. The first group was untreated, the second group was stimulated with PMA, the third group was stimulated with plastic-immobilized OKT3, and the last group was stimulated with both OKT3 and PMA. The adherence properties of the four groups were monitored after a 72 hour incubation at 37°C. The 72 hour period was selected as it had been described in the literature to result in an increase in ß1 integrin expression (Hemler et al, 1990).

The group stimulated with both PMA and OKT3 exhibited a strongly adherent phenotype on both fibronectin and collagen coated surfaces (Figure 4.11). The groups stimulated with either PMA or OKT3 alone demonstrated comparatively weak specific attachment to the matrix components tested (OD < 0.4), yet still exhibited an adhesion potential between 2.5 and 16 fold higher than the untreated peripheral blood lymphocyte population.

The populations of lymphocytes were monitored by flow cytometry for expression of ß1 during the activation procedure. As previously determined, approximately 40% (45.6 ± 5.8%, over three experiments) of freshly isolated lymphocytes express $ß1^{Hi}$. The OKT3 stimulated populations of T lymphocytes demonstrated a gradual increase in the expression of ß1 with time, such that by 48 hours, >80% of lymphocytes were $ß1^{Hi}$, and by 72 hours virtually the entire lymphocyte population was $ß1^{Hi}$.(Figure 4.12A). The PMA stimulated group of T lymphocytes demonstrated a slight (63.8% ± 16.9%), but not

Figure 4.11 Effect of 72h Activation on PBL Adherence

Purified PBL were pooled into four groups which were stimulated with either PMA(20ng/ml), the anti-CD3 antibody OKT3 immobilized on plastic (see methods), both stimuli, or were left untreated. After 72 hours, the cell concentrations were standardized and the populations were assessed for attachment to fibronectin (solid bar) and type I (hatched bar) and II (open bar) collagen. The specific absorbance is shown (Attachment to BSA has been subtracted) from a representative experiment. The standard error is less than 0.10 Abs units in all cases.

Adherence of Stimulated PBL

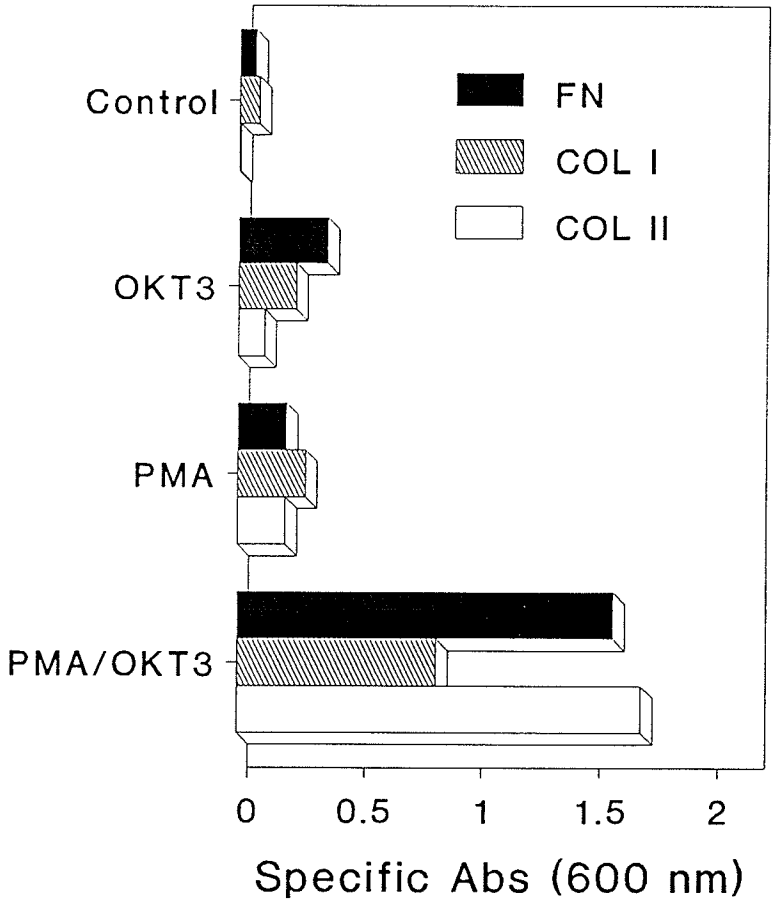
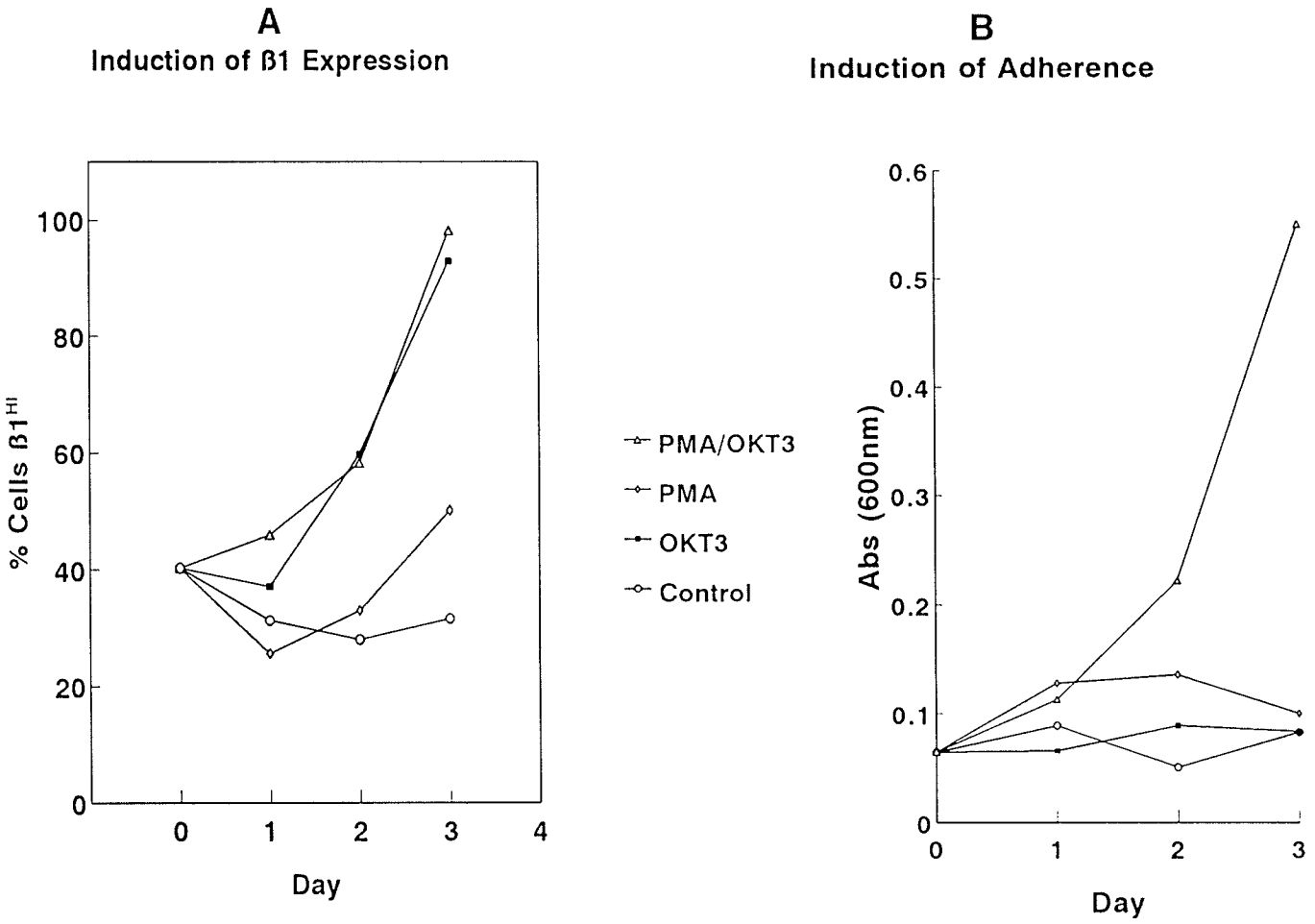


Figure 4.12 Kinetics of Acquisition: ß1 Expression and Adherence

Purified PBL were pooled into four groups and stimulated with PMA(20ng/ml), the anti-CD3 antibody OKT3 immobilized on plastic (see methods), both stimuli, or left untreated (controls). At 24 hour intervals, the populations were assessed for expression of ß1 integrin by FACS and adherence to components of the extracellular matrix (type I collagen is shown). **A.** The percentage of cells in each population expressing $ß1^{Hi}$ were assessed. Cells were labelled with antibody A-1A5. A representative experiment is shown. **B.** The attachment of different PBL populations to collagen was assessed at the various time points. (The standard error is less than 0.05 Abs units in each case). One representative experiment of three is shown.


A
Induction of ß1 Expression
% Cells ß1HI
100
80
60
40
20
0
0
1
2
3
4
Day
PMA/OKT3
PMA
OKT3
Control
B
Induction of Adherence
Abs (600nm)
0.6
0.5
0.4
0.3
0.2
0.1
0
0
1
2
3
Day

significant, increase in the percentage of cells expressing ß1^{Hi}, while the nonstimulated lymphocyte population was unchanged (48.0 ± 18.5%).

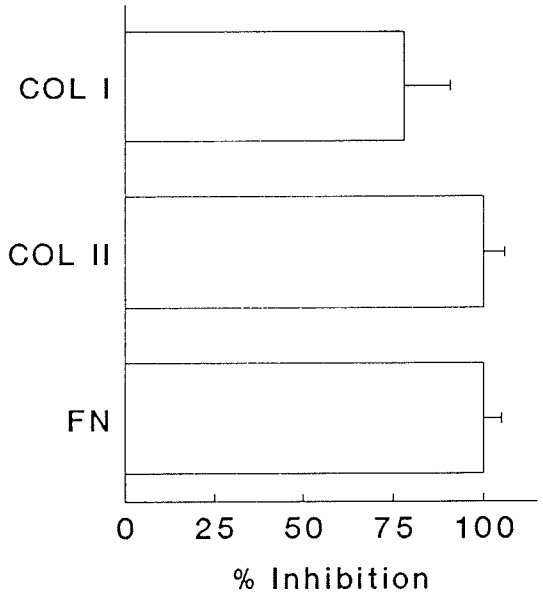
Although high levels of expression of ß1 were detected on virtually all OKT3 stimulated cells at 72 hours, only those cells costimulated with PMA were strongly adherent (OD > 0.5) (Figure 4.12B). This result appeared analogous to, but more dramatic than, the activation of Jurkat cells by PMA. These observations appeared to indicate that a high level of expression of ß1 was, of itself, not sufficient to confer adhesive potential upon a lymphocyte.

To confirm whether the ß1 integrin complex was in fact involved in activated PBL attachment to extracellular matrix, peripheral blood lymphocytes stimulated with PMA and OKT3 for 72 hours were pretreated with anti-ß1 specific antisera. Nonimmune rabbit serum, which has previously been demonstrated not to influence Jurkat cell attachment to collagen or fibronectin, was added to the control group. The attachment of the activated T lymphocytes to either collagen or fibronectin was strongly inhibited by pretreatment with the α5ß1 specific antisera (Figure 4.13) as compared to the group treated with nonimmune rabbit sera. Thus, ß1 integrin-containing complexes could be directly implicated in the process of activated T lymphocyte attachment to matrix. However, the identity of the specific heterodimers mediating adhesion was not known. Previously, α5ß1 had been reported to be a fibronectin receptor on thymocytes (Carderelli and Pierschbacher, 1988), while no lymphoid collagen receptor had yet been reported (Arencibia and Sundqvist, 1989).

Figure 4.13 The effect of anti-ß1 antisera on OKT3/PMA activated PBL

Purified PBL were stimulated with plastic-immobilized OKT3 and PMA (20ng/ml) continuously for 72 hours. Cells were then treated with either nonimmune rabbit sera (NIRS) or (ß1 + α5) specific antisera (HNFR). The effect of the ß1-complex-blocking antisera is depicted as inhibition relative to nonimmune sera treated controls. The mean and error of one of two similar experiments is shown.

Effect of anti-ß1 on PBL Attachment



SECTION 4.2 Expression & Utilization of Lymphoid ß1 Integrins

4.21 Blocking Attachment Requiring Specific α-Integrins

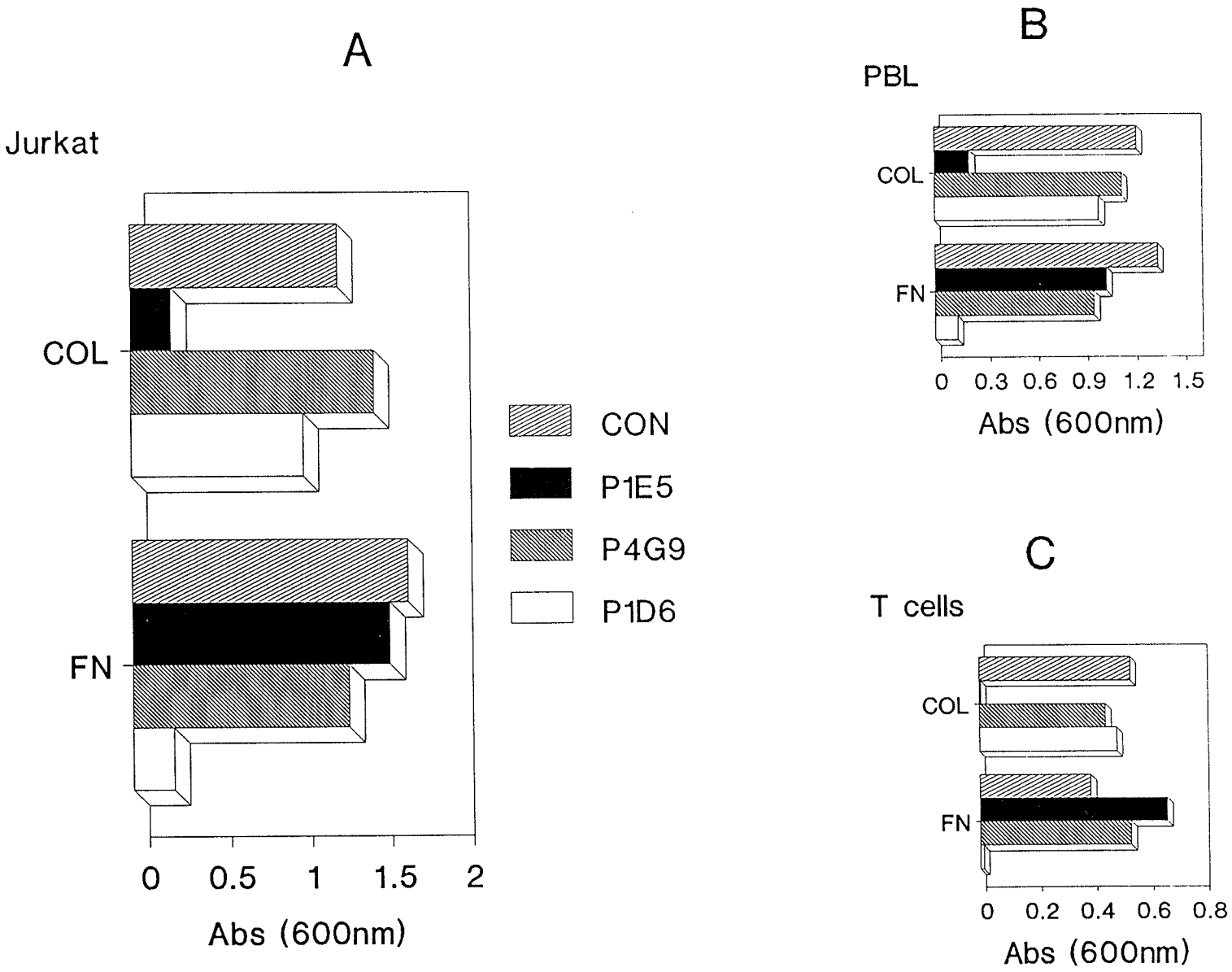
Experiments within the previous section had determined that ß1 integrin-containing complexes were involved in mediating lymphoid attachment to collagen and fibronectin. However, these experiments did not identify which specific α subunits were actually complexed with the common ß1 chain. In order to determine this, monoclonal antibodies, which had a demonstrated capacity to neutralize attachment mediated by specific α integrin-containing complexes, were utilized in attempts to block Jurkat adhesion to collagen and fibronectin. Jurkat cells were preincubated with different monoclonal antibodies specific for individual α integrins, and subsequently assessed for attachment to surfaces coated with either collagen or fibronectin (Figure 4.14A).

Monoclonal antibody P1D6, which is directed against the α5 integrin subunit, strongly inhibited Jurkat attachment to fibronectin. The α5ß1 integrin is the so called "classical" fibronectin receptor first identified on fibroblasts (Argraves et al, 1987). Antibody P4G9, directed against α4 integrin, yielded slight, though consistent, inhibition. Other monoclonals tested, including a control monoclonal antibody specific for the αß form of the T cell receptor (WT31) had no effect upon Jurkat cell attachment to fibronectin. Conversely, neither P1D6 or P4G9 influenced attachment to collagen coated microtitre wells. The inclusion of monoclonal antibody P1E5 (specific for the α2 integrin subunit) by contrast, inhibited attachment efficiently (Figure 4.14A). These observations suggest that, at least with respect to strength of adhesion observed, α2ß1 integrin is the primary collagen

receptor utilized by Jurkat cells, and that α5ß1 integrin is the predominant fibronectin receptor.

Figure 4.14 Characterization of Lymphocyte Attachment

A. PMA stimulated Jurkat cells were preincubated with α-integrin specific antibodies P1E5 (α2-reactive), P1B5 (α3-reactive), P4G9 (α4-reactive) or P1D6 (α5-reactive), and allowed to attach to surfaces coated with either collagen or fibronectin. Similar blocking studies were performed on activated mixed PBL populations (**B**) as well as purified (sheep red blood cell-rosetted) T cell populations (**C**).


A
Jurkat
COL
FN
0
0.5
1
1.5
2
Abs (600nm)
CON
P1E5
P4G9
P1D6
B
PBL
COL
FN
0
0.3
0.6
0.9
1.2
1.5
Abs (600nm)
C
T cells
COL
FN
0
0.2
0.4
0.6
0.8
Abs (600nm)

Similar results were obtained when OKT3/PMA stimulated populations of peripheral blood lymphocytes (Figure 4.14B) or OKT3/PMA stimulated rosette-purified T lymphocytes (Figure 4.14C) were assessed in the blocking assay. Monoclonal antibody P1E5 was observed to block attachment to collagen, and P1D6 was found to block attachment to fibronectin, implicating α2ß1 and α5ß1 as the principle collagen and fibronectin receptors, respectively. In both of these populations, as with the Jurkat cells, the monoclonal antibody specific for α4 had little observable effect on adhesion.

In contrast to this result, previous reports had suggested that two sites on fibronectin possessed integrin binding activity (Humphries et al, 1986), and one such site was identified (Garcia-Pardo et al, 1990, Guan and Hynes, 1990) to be bound specifically by α4ß1 integrin on lymphocytes.

Since Jurkat cells did express α4 and ß1 integrins, it was possible that the putatively formed α4ß1 fibronectin receptor was nonfunctional, or that it was subordinate in the presence of a dominant α5ß1 complex, as had been observed with α3ß1 integrins (Plantefabber and Hynes, 1989). The binding site of α4ß1 on fibronectin is within the CS1 region, occurring at an alternatively spliced site termed the type III connecting segment (IIICS). This site is distinct from the R-G-D amino acid containing "Cell Binding Domain" (CBD), which is the site of α5ß1 integrin interaction. To assist in investigations into cellular binding to these independant sites, chymotryptic digestion of fibronectin can be effected to yield [1] a 40kDa fragment containing the IIICS region, and [2] a 120kDa fragment containing the CBD region. Jurkat cells were capable of attachment to both the 40kDa and 120kDa proteolytic fragments of fibronectin (Figure 4.15). Therefore, it appears that Jurkat cells may attach to regions of fibronectin specific to either the α4ß1 or α5ß1 receptors. The relative level of attachment to the 120kDa fragment (OD 1.2-1.3 range) was much higher than the attachment to the 40kDa fragment (OD 0.350 to 0.450), suggesting that in the Jurkat cell line, greater contributions to cellular adherence are

mediated by the α5ß1 receptor (Figure 4.15). However, why the low level of adherence observed during adhesion studies using the 40kDa fragment was not previously observed in the presence of α5-specific inhibitory antibody (Figure 4.14A) is not clear. Explanations may possibly relate to cellular signalling (resulting in α4-integrin downregulation) transmitted by the blocked α5 complex, or a putative subordinate role for α4ß1 in the presence of α5ß1 ligated by either fibronectin or monoclonal antibody.

4.22 Adhesion Receptors of Cytolytic T Cells

The OKT3/PMA stimulated lymphocytes represented a relatively heterogenous population of stimulated cells, but were largely TCR-αß, CD4+ T cells (approximately 60-70%). Similar patterns of integrin utilization were observed on these cells as well as the TCR-αß, CD4+ Jurkat cell line. This surface phenotype is associated predominantly with "helper" T cells (Wraitch, 1987). Concurrent investigations into characteristics of αß and γδ T cells, performed by Dr L Selin, offered the opportunity to study cytolytic T cell populations. These cells were generated via an activational protocol (interleukin 2 + allogeneic cell line, Wilkins et al, 1992) to expand the cell populations of interest, which were subsequently further purified by cell-sorting.

In order to increase the sensitivity of measured adherence in the adherence assay, cells were prelabelled with ^{51}Cr during these studies. Adherent cells were then quantitated by γ emission rather than dye binding.

A small percentage of cells from both αß and γδ TCR bearing "bulk" populations (FACS-sorted, but uncloned) were capable of adhering to fibronectin, but not collagen, coated microtitre wells (Figure 4.16). Treatment with PMA induced increased the

Figure 4.15 Attachment of Jurkat Cells to Fragments of Fibronectin

Microtitre wells were coated with either native fibronectin (FN)(10μg/ml) , a 120 kDa chymotryptic fragment containing the CBD target site of α5ß1 integrin (10μg/ml), or a 40kDa chymotryptic fragment containing the α4 integrin binding site (40μg/ml). Jurkat cells were assessed for attachment to these fragments. The mean and error values for a representative experiment are shown.

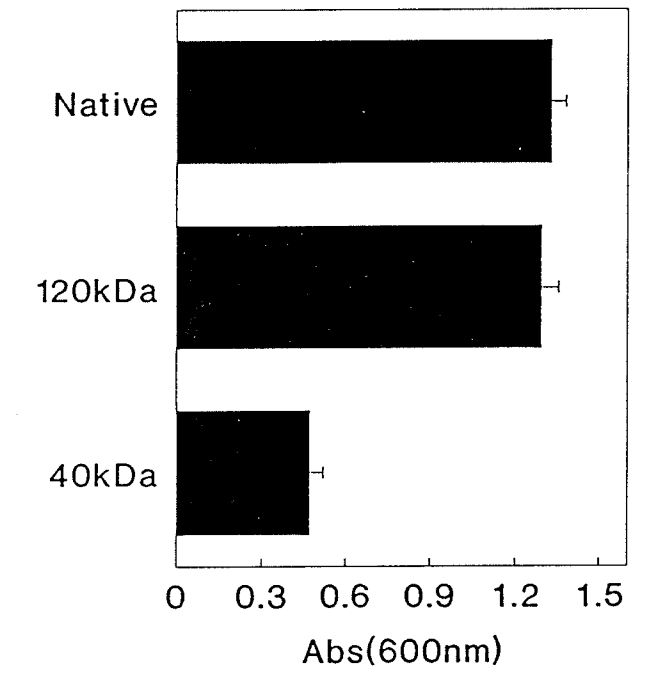
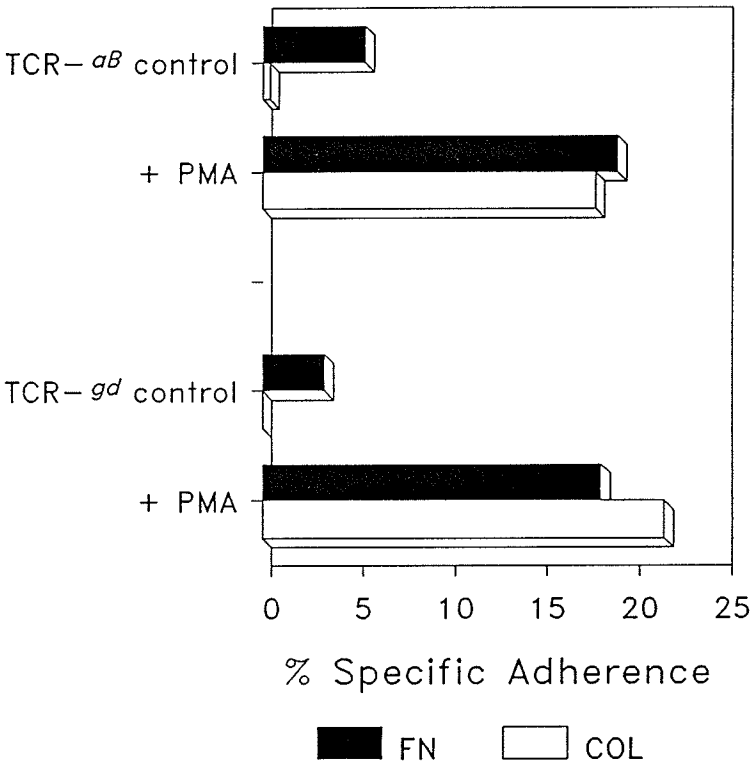

Jurkat adhesion to FN fragments
Native
120kDa
40kDa
0
0.3
0.6
0.9
1.2
1.5
Abs(600nm)

Figure 4.16 Attachment of αß and γδ T cells to Fibronectin and Collagen

FACS-sorted αß (aB) and γδ (gd) populations were assessed for attachment to fibronectin and collagen in the presence (+ pma) or absence (control) of PMA stimulation (50ng/ml). A representative experiment is shown; the standard error was less than 3.6% in each case.

Adherence of Bulk T Populations



proportion of cells which attached to fibronectin in both the αß and γδ populations, and induced a similar proportion of cells to adhere to collagen coated surfaces (Figure 4.16).

The γδ populations were subsequently cloned (Wilkins et al, 1992); the bulk populations was determined to be homogeneously positive for ß1 integrin (Table 4.3), with varying positive expression of other integrins including ß2, but not ß3 integrins (FACS kindly performed by Kosala Sivananthan).

Three of the γδ derived T cell clones, designated as clones #1, #6 and #11, were examined in greater detail. Analysis by flow cytometry demonstrated the presence of integrin α subunits on the surface of clones. The clones were virtually 100% positive for, and expressed high levels of, α4 and α5 chains integrin chains. Clones were also virtually 100% positive for α2, although the expression level was lower than that of α4/α5 (Figure 4.17). Variable expression of α1 was also observed on the clones. The high expression of α2 and α5 contrasts with the lower expression observed on the bulk γδ population. All of these clones also expressed CD8 (Figure 4.17), a classical marker of cytotoxic T cells, which also contrasts with the bulk population, in which only 20% expressed this marker (Table 4.3).

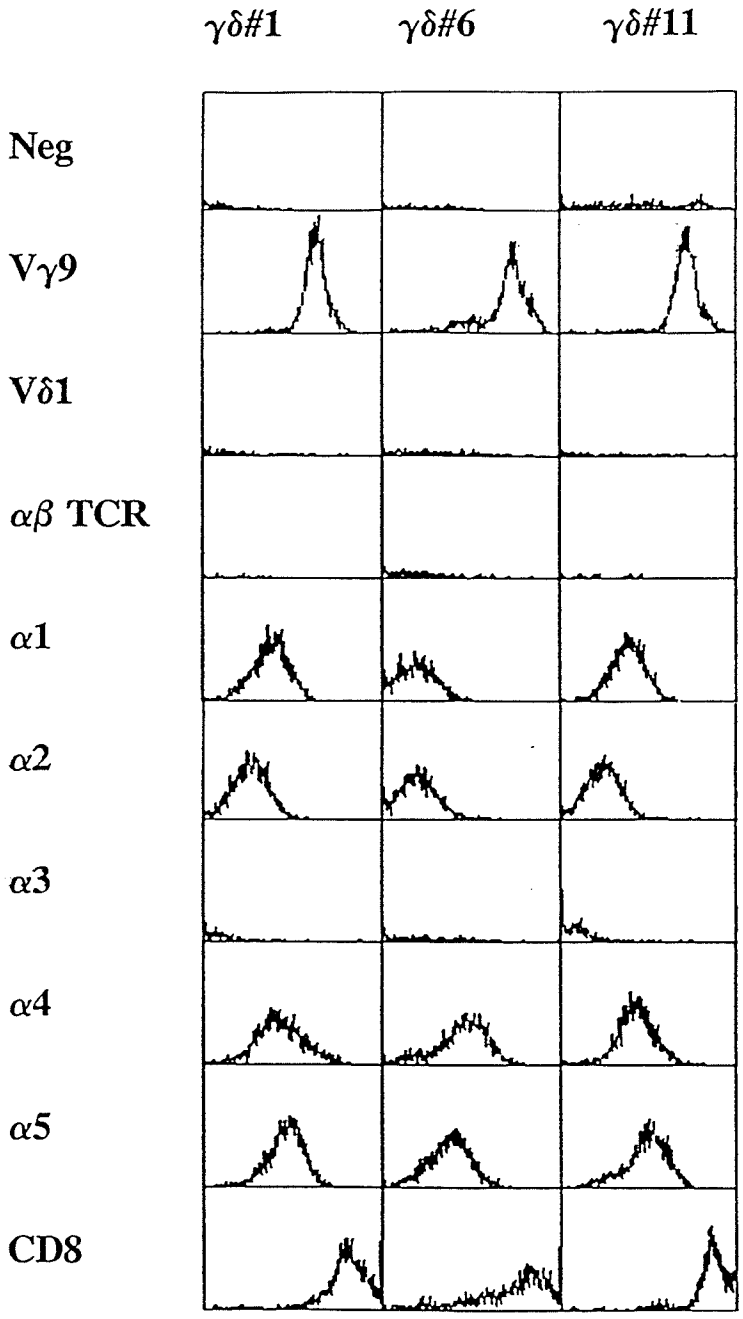
Table 4.3 Analysis of γδ bulk population by flow cytometry

Marker	Antibody	% Cells Positive	MFI
Cell Markers:			
CD2	OKT11	94.2	154.9
CD3	OKT3	91.8	114.9
CD4	OKT4	1.9*	110.2
CD8	OKT8	15.1	120.5
TCRδ1	TCRδ1	91.5	144.2
TCRγ9	TCRγ9	75.8	147.6
TCRαß	TCRαß	6.7	93.4
Integrins:			
ß1	JB1A	95.7	117.3
α1	TS2/7	13.5	91.7
α2	P1E6	17.1	89.8
α3	P1B5	2.3*	99.1
α4	44H6	97.3	123.7
α5	JBS5	28.9	88.4
ß2	TS1/18	90.5	121.2
αVß3	RαVNR	3.1*	105.1

LEGEND: Marker/Antibody identifies the recognized cell surface antigen and the name of the clone which secretes the specific antibody. The αVß3 antibody is polyclonal. % Cells positive are the percentage of cells staining positive for each marker, MFI is the mean fluorescence intensity of the stained populations. An asterisk(*) indicates a negative result with respect to labelled cells gated in a 95% confidence interval.

Figure 4.17 Flow Cytometric Analysis of γδ T cell clones

Clones which were derived from bulk γδ T cell populations (Wilkins et al, 1992) were assessed for expression of surface markers. Cells were assessed with antibodies as described (Table 4.3) for TCR γ9, δ1, αß(framework), CD8, and the ß1 associated integrins α1 (VLA-1), α2 (VLA-2), α3 (VLA-3), α4 (VLA-4) and α5 (VLA-5). The negative control (Neg) cells were stained only with the secondary antisera, ie., FITC-conjugated rabbit antisera to mouse immunoglobulin. As per FACS studies, the "y" axis represents cell number (scaled to 256 cells), while the "x" axis represents relative fluorescence intensity over a scale of three logarithms (base 10).


γδ#1
γδ#6
γδ#11
Neg
Vγ9
Vδ1
αβ TCR
α1
α2
α3
α4
α5
CD8

These cell populations (clones, bulk) were subsequently analyzed with blocking antibodies to determine adhesion structures utilized for adherence. Adhesion of the bulk γδ populations to fibronectin appeared to require ß1 integrin, as the inclusion of monoclonal antibody JB1A, which blocks adhesion mediated by ß1 integrin containing complexes, was able to significantly inhibit attachment to fibronectin (Table 4.4). The addition of α-integrin specific monoclonals was also effective at inhibiting bulk γδ cell attachment to fibronectin, however, no antibody tested had any observable effect upon attachment to collagen. The addition of either α4- or α5-specific monoclonals (P4G9, JBS5) could inhibit attachment to fibronectin, suggesting that both adhesion structures were required to mediate adhesion, or alternatively, that blocking one structure could down-regulate the other. The α2 specific monoclonal P1E5 had no effect upon observed attachment to collagen. Therefore, it appears that unlike previously characterized lymphocyte populations, the bulk γδ T cell populations did not use α2ß1 integrin as their primary collagen receptor. Since ß1 integrin antibodies were similarly ineffective (Table 4.3), it appears that the bulk population may employ non-ß1-integrin, though PMA-activated, collagen receptors.

The γδ clones also exhibited a low level of attachment to fibronectin, but increased attachment could be induced by treatment with PMA. As observed in the bulk populations, adhesion of the clones was blocked by the inclusion of monoclonal antibodies specific either to α4 or α5 (or ß1) integrins (Table 4.4). Surprisingly, and in contrast to the results obtained with the bulk populations, the presence of α2 specific monoclonal P1E5 (or anti-ß1, JB1A) antibody was sufficient to block the attachment of

Table 4.4 The role of ß1 Integrins in γδ T cell Adherence

Clone	Antibodies Identified as Inhibitory	
	Collagen	Fibronectin
αß	NF*	P4G9(89±13), JBS5(66±18)
γδ	NF	JBS5(45±23)
γδ–#1	P1E5(100)	P4G9(100), JBS5(100)
γδ-#6	P1E5(100)	P4G9(100), JBS5(100)
γδ-#11	P1E5(84±11)	P4G9(100), JBS5(93±7)

MAb Specificities:

P1E5: α2 integrin
P4G9: α4 integrin
JBS5: α5 integrin

Legend.

The bulk (FACS-sorted, but uncloned) αß and γδ TCR populations were assessed for attachment to collagen and fibronectin in the presence of previously described, adhesion inhibiting monoclonal antibodies. Those monoclonal antibodies determined to be effective at inhibiting observed attachment (approximately 25%-30% specific adherence observed on collagen or fibronectin), and the mean percentage of observed inhibition (in brackets), are shown.

* NF: No antibody tested was found to be inhibitory to attachment.

°(): The mean percentage inhibition of two blocking studies is shown for α specific monoclonals. The ß1 specific monoclonal JB1A was effective at blocking the γδ clones (>90%), partially effective at blocking unsorted γδ populations (78±20%), and ineffective at blocking the unsorted αß cytolytic populations.

each clone to collagen. The putatively increased role of α2ß1 in collagen adherence of these clones may in part relate to the observed higher level of α2ß1 expression (Figure 4.17).

These results indicated that individual clones could express different adhesion characteristics than the bulk populations from which they were derived. Whether these deviations were due to outgrowth of subpopulations, or were due to tissue culture adaptation of the cell lines during cloning is not clear. However, the specific choice of adhesion molecule expression and utilization during cloning may not be standardized. Preliminary data obtained using a second set of clones (such as 3PL7C), demonstrated a markedly divergent repertoire of α integrins compared to either the parent population (Table 4.5) or the other clones (cf Figure 4.17). This different repertoire correlated with an unusually strong capacity to attach to collagen in the absence of PMA stimulation. However, further investigations with these divergent clones were not possible due to their "loss in passage."

Collectively, the results in the Jurkat cell line, the OKT3 activated T lymphocyte population (principally TCRαß, CD4+), and the cytolytic γδ bulk populations and γδ clones demonstrated that the α4ß1 and α5ß1 integrins were important T lymphocyte adhesion receptors for fibronectin. Further, most of these lymphoid cell populations also supported a role for α2ß1 as a principle receptor for collagen, with the notable exception of the bulk γδ cells.

Table 4.5 Collagen-adherent γδ T cell clones

Clones:	3LP7C	10LP3D	Bulk
Specific Adherence:*			
Collagen	0.833±.035	0.529±.044	0.010±.029
Fibronectin	0.075±.012	0.048±.011	0.166±.038
Integrin Expression:**			
α2 integrin	48.1	40.0	17.1
α4 integrin	69.8	74.1	97.3
α5 integrin	21.8	8.7	88.4

LEGEND:

* Specific Adherence is expressed as units of absorbance at 600nm, ± the standard error of triplicate determinations.

** Integrin Expression results are obtained from flow cytometric analysis, and represent the percentage of the population gating positive within a 95% confidence interval.

4.24 Attachment of B Lymphocyte Cell Culture Lines to Fibronectin

The focus of experiments up to this point had been principally upon T lymphocytes. To determine if B cells maintained a similar capacity to attach to extracellular matrix, a panel of B cell lines (Table 4.6) was screened for adherence to fibronectin immobilized on plastic surfaces. Six of ten B cell lines examined demonstrated specific adherence to fibronectin above an OD of 0.200 (Figure 4.18). Adherence to fibronectin is apparently not a property common to all B cell lines, and among those lines which did adhere, considerable variation was observed in the relative strength of the adherence. There appears to be no correlation between the source of the original isolate of a tumour cell line (Table 4.6) and the capacity to adhere to fibronectin (Figure 4.18). The lines JR2B10 and JR2D3 are subclones from the same bulk parental population but display contrastingly high and low adherence. Therefore, the strength of adherence appears to be more dependent upon some property within a clonal population than upon the original source of the isolate, in agreement with described observations using the γδ T cell clones.

Preliminary studies using rabbit antisera to the α5ß1 complex indicated that B cell binding to fibronectin required ß1 integrin heterodimers. To identify which ß1-containing heterodimers might be contributing to the observed adherence, the 6 cell lines demonstrating significant specific adherence to fibronectin (OD > 0.200) were examined using the monoclonal antibodies known to block the binding of α4ß1 (P4G9) and α5ß1 (JBS5) to fibronectin.

Table 4.6 B CELL CULTURE LINES EXAMINED

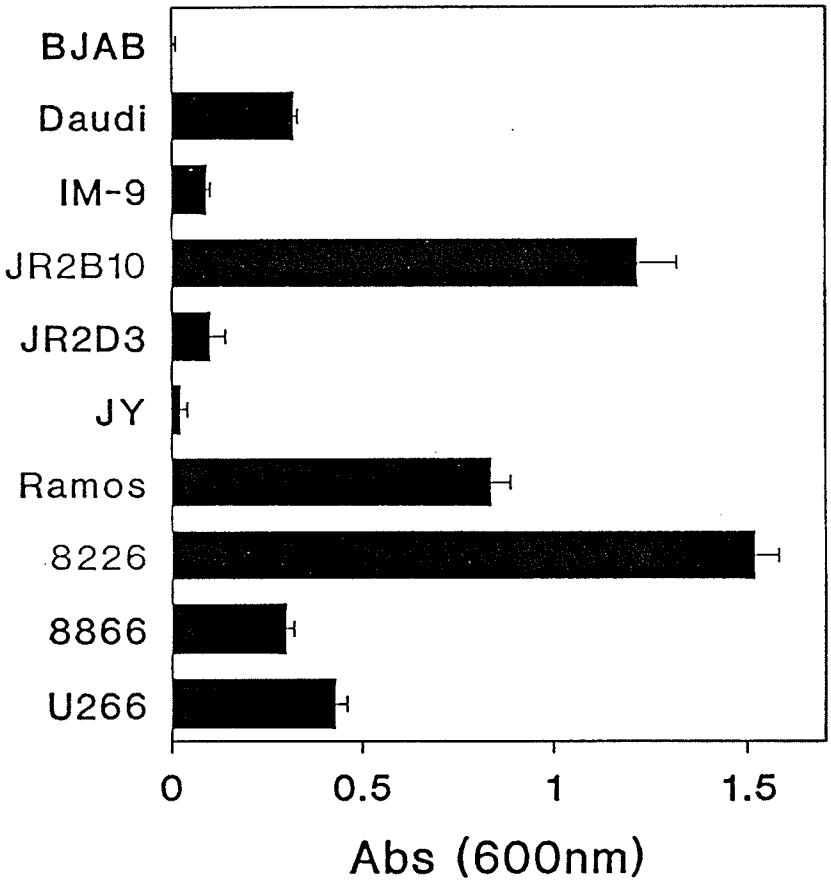
Line	EBV status	Cell Type	Surface Ig	Isolate source
BJAB	-	BL	IgG	Tumour Biopsy
Daudi	+	B	IgM	Tumour Biopsy
IM-9	+	M	IgG^s	Bone Marrow
JR2B10	*	IT	-	Peripheral Blood
JR2D3	+	IT	IgG	Peripheral Blood
JY	+	IT	IgG	Peripheral Blood
Ramos	-	B	IgM	Tumour Biopsy
RPMI 8226	-	M	λ^s	Peripheral Blood
RPMI 8866	+	ML	IgG^s	Peripheral Blood
U266	-	M	IgE^s	Peripheral Blood

Legend:
EBV status: +, positive, -, negative, * EBV immortalized, EBV genome subsequently lost.
Cell type: B, Burkitt's lymphoma, BL, Burkitt's like, M, Myeloma, ML, myelocytic leukemia, IT, in vitro EBV-transformed.
Surface Ig: ^{s} denotes secreted, λ indicates lambda light chain only.

Figure 4.18 Adherence of B cell lines to Fibronectin

A panel of B cell lines were assessed for attachment to microtitre wells coated with fibronectin. Representative specific absorbance (Abs FN - Abs BSA) for each cell line shown. The adherence to BSA (nonspecific) was < 0.1 O.D. units in all cases.

Adherence of B cell lines to Fibronectin



The pattern of cellular fibronectin receptor utilization varied among the cell lines which were examined. Daudi, Ramos, RPMI 8866 and U266 appeared to exclusively bind fibronectin with α4 integrins, as indicated by the sensitivity of the adherence to monoclonal antibody P4G9 (Figure 4.19A). Therefore, these results supported the use of α4 containing integrin complexes as primary fibronectin receptors on these cell lines. The inclusion of α5 specific monoclonal antibody (JBS5) did not influence these cell line's attachment to fibronectin.

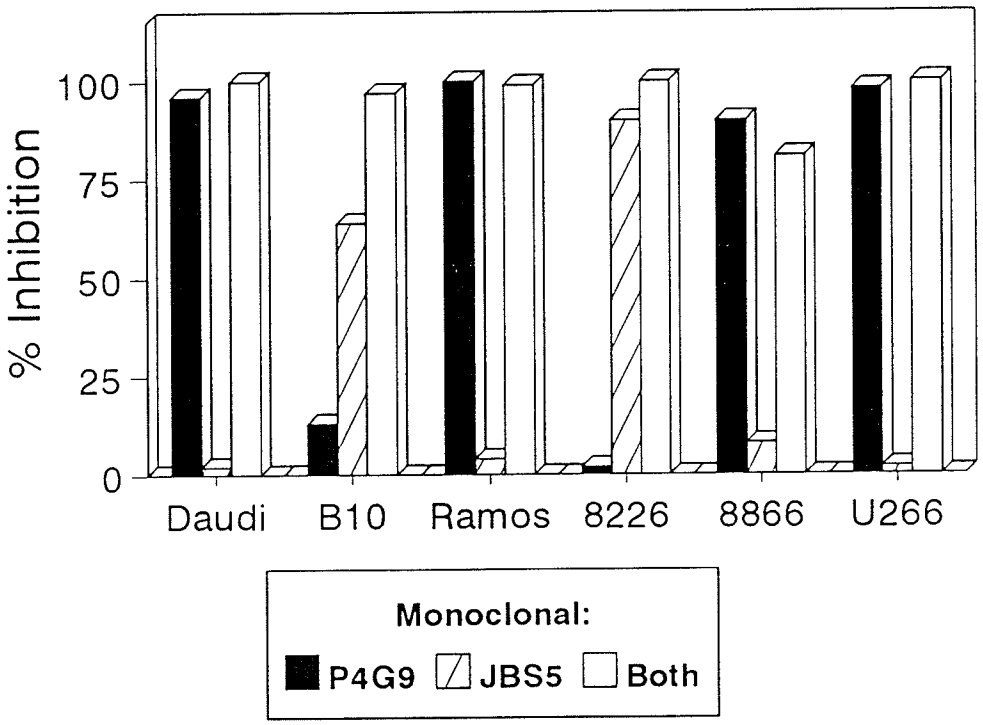
In contrast, RPMI 8226 appeared to primarily use α5ß1, as indicated by the inhibition of fibronectin adherence by monoclonal JBS5. The cell line JR2B10 also appeared to primarily use α5ß1 for fibronectin adherence, although slight inhibition (13%) could be achieved with P4G9 (anti-α4) alone. Complete inhibition of JR2B10 attachment to fibronectin required the combined use of both P4G9 and JBS-5, indicating that both α4 and α5 containing integrins were utilized, and that either heterodimer could function independently of the other.

To confirm the role of ß1 integrin in the adherence process, the mAbs 4B4 and JB1A, specific for ß1, were used to attempt to block B cell attachment to fibronectin. Both monoclonal antibodies were efficient at inhibiting attachment of Daudi, JR2B10, Ramos, RPMI 8226 and U266 cell lines, implying that ß1 was the heterodimer-partner involved in the adherence processes of these cell lines (Figure 4.19B). RPMI 8866 cells were inhibited to a much lesser degree by the anti-ß1 monoclonals, and JB1A appeared less efficient (not statistically significant) than 4B4. This is noteworthy as α4 integrin is known to associate with both ß1 and ß7 integrin subunits, and suggests that non-ß1 integrins might mediate attachment to fibronectin in some cell lines. (Note: the association of α4 with ß7 on our stock RPMI 8866 cells was later confirmed by Erle et al, 1994b).

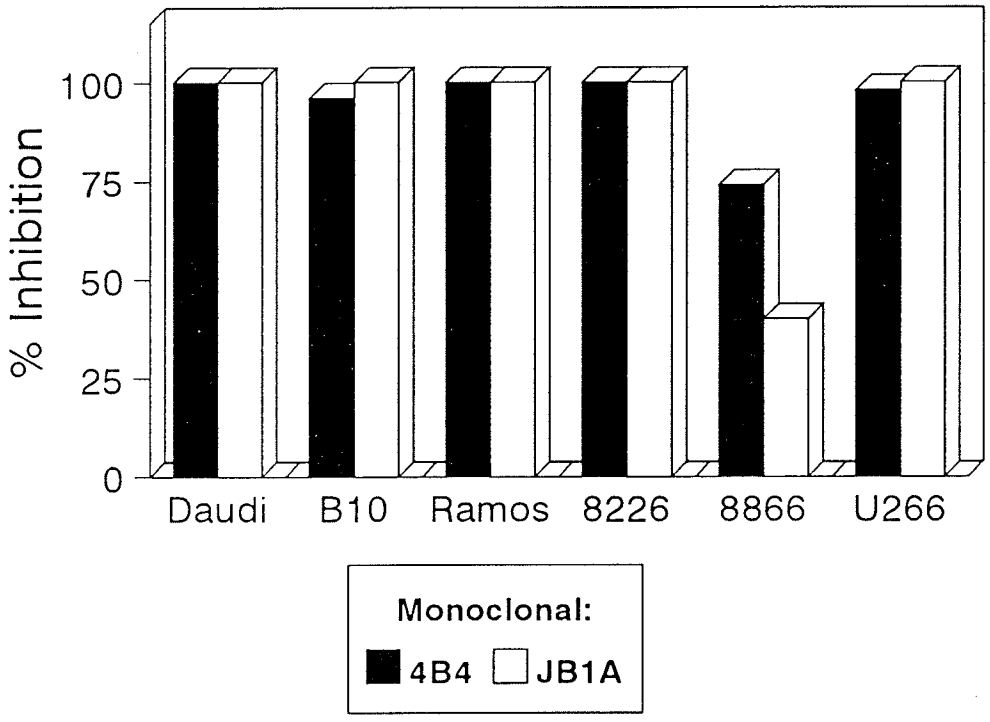
Figure 4.19 Characterization of B cell Attachment to Fibronectin

Cell lines demonstrating adherence above 0.200 O.D. units were assessed for binding structures utilized in attachment to fibronectin. Cells were pretreated with α specific antisera (**A**) to the α4 integrin (P4G9), the α5 integrin (JBS5), or both, and then assayed for attachment to fibronectin. To determine contribution of the ß subunit, two monoclonal antibodies directed against the ß1 subunit (**B**) were similarly used to pretreat Jurkat cells prior to adherence assay. Data shown is the mean of three to five experiments. The standard error is less than 10% in all cases but that of ß1-specific monoclonals blocking the attachment of RPMI 8866 cells (SE - 4B4 = ±23%, SE - JB1A = ±19%), exhibited high variability.

A


% Inhibition
100
75
50
25
0
Daudi
B10
Ramos
8226
8866
U266
Monoclonal:
P4G9
JBS5
Both

B

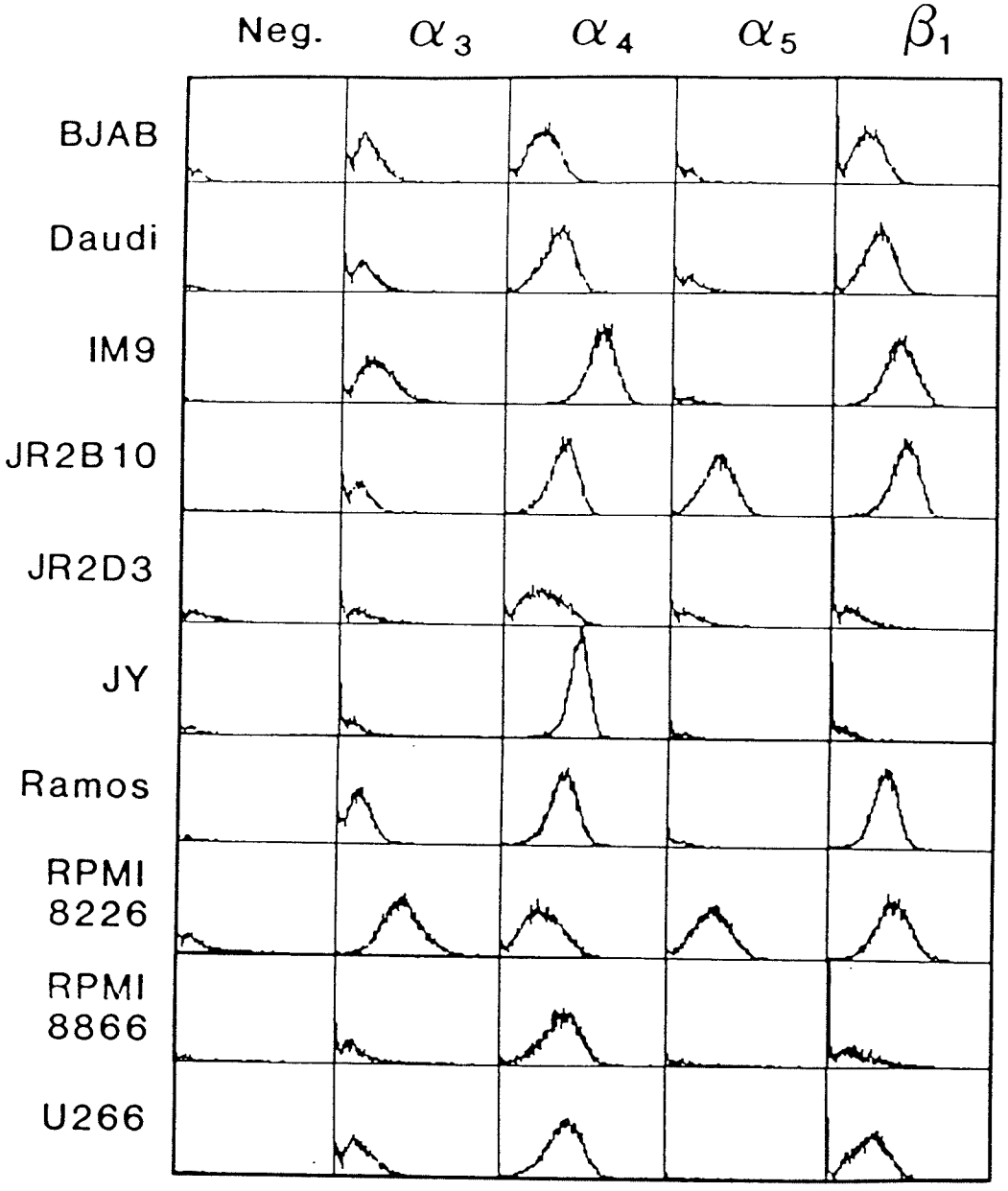

% Inhibition
100
75
50
25
0
Daudi
B10
Ramos
8226
8866
U266
Monoclonal:
4B4
JB1A

Overall, however, the predominant fibronectin adhesion structures characterized on B cell lines, similar to T cell populations, appeared to be the α4ß1 and α5ß1 complexes. To determine whether the observed variation in utilization (α4ß1 vs α5ß1) might be attributed to differential expression patterns, the B cell lines were analyzed for α integrin expression by flow cytometry (Figure 4.20). All lines examined expressed α4 on virtually 100% of the cells, with the exception of RPMI 8226 and JR2D3 cell lines, which expressed it to a lesser degree. Of those cell lines which displayed little or no specific adherence to fibronectin, BJAB cells displayed a very weak expression of the ß1 subunit as compared to the α4 subunit, and this ratio appeared even lower in the JR2D3 cell line. Whether this contributes to the poor adherence potential of these cells is unclear. At least one cell line of moderate (OD 0.400) adherence potential, RPMI 8866, also displayed this pattern. The nonadherent JY cell line appeared to express the α4 subunit in the complete absence of ß1 subunit. This pattern of reduced ß1 expression was not reflected in the nonadherent IM-9 line, which seemed to express abundant α4ß1, based upon the fluorescence intensity of α4 and ß1 subunits. Despite this apparently adequate receptor expression, the failure of IM-9 to adhere supported previous suggestions that the regulation of adherence may occur at a level other than expression.

Within the group of adherent lines, Daudi, Ramos, U-266 and to a much lesser extent RPMI 8866, expressed the subunits necessary to form α4ß1, which was in agreement with the observed adherence inhibition results. RPMI 8226 and JR2B10 expressed the necessary subunits for both α4ß1 and α5ß1 integrins. In addition, RPMI 8226 also expressed high levels of α3 (CD49c), which inferred α3ß1 expression, as α3 is not known to form heterodimers with any ß subunit except ß1. However, the results of the

Figure 4.20 Expression of ß1 integrins on B cell lines

Cell lines were stained for the expression of ß1 (VLA-ß, mAb JB1A), α3 (VLA-3, mAb P1B5), α4 (VLA-4, mAb P4G9) and α5 (VLA-5, mAb JBS5), and analyzed by flow cytometry. The negative control (Neg) was labelled only with the secondary reagent (FITC-conjugated antisera to murine immunoglobulin). The "y" axis represents cell number (scaled to 512 cells), while the "x" axis represents the relative fluorescence intensity over three logarithms (base 10).


Neg.
α_3
α_4
α_5
β_1
BJAB
Daudi
IM9
JR2B10
JR2D3
JY
Ramos
RPMI 8226
RPMI 8866
U266

adherence inhibition data did not implicate α3ß1, a demonstrated fibronectin receptor in certain nonlymphoid cell lines, as a significant binding structure for RPMI 8226 adherence to fibronectin.

It is apparent that if both α4ß1 and α5ß1 were present on the surface of a B cell line, then α5ß1 tended to play the dominant role in adherence, even if the expression of α4 exceeded α5, as it did in JR2B10 cells. The utilization of α5ß1 as a fibronectin receptor corresponded with the two lines which displayed the highest adherence potentials on fibronectin. The level of expression of α4ß1, in itself, was not a good indication of a cell lines' potential to adhere to fibronectin, as demonstrated by the contrast in α4/ß1 expression and fibronectin adherence in the IM-9 and Ramos cell lines.

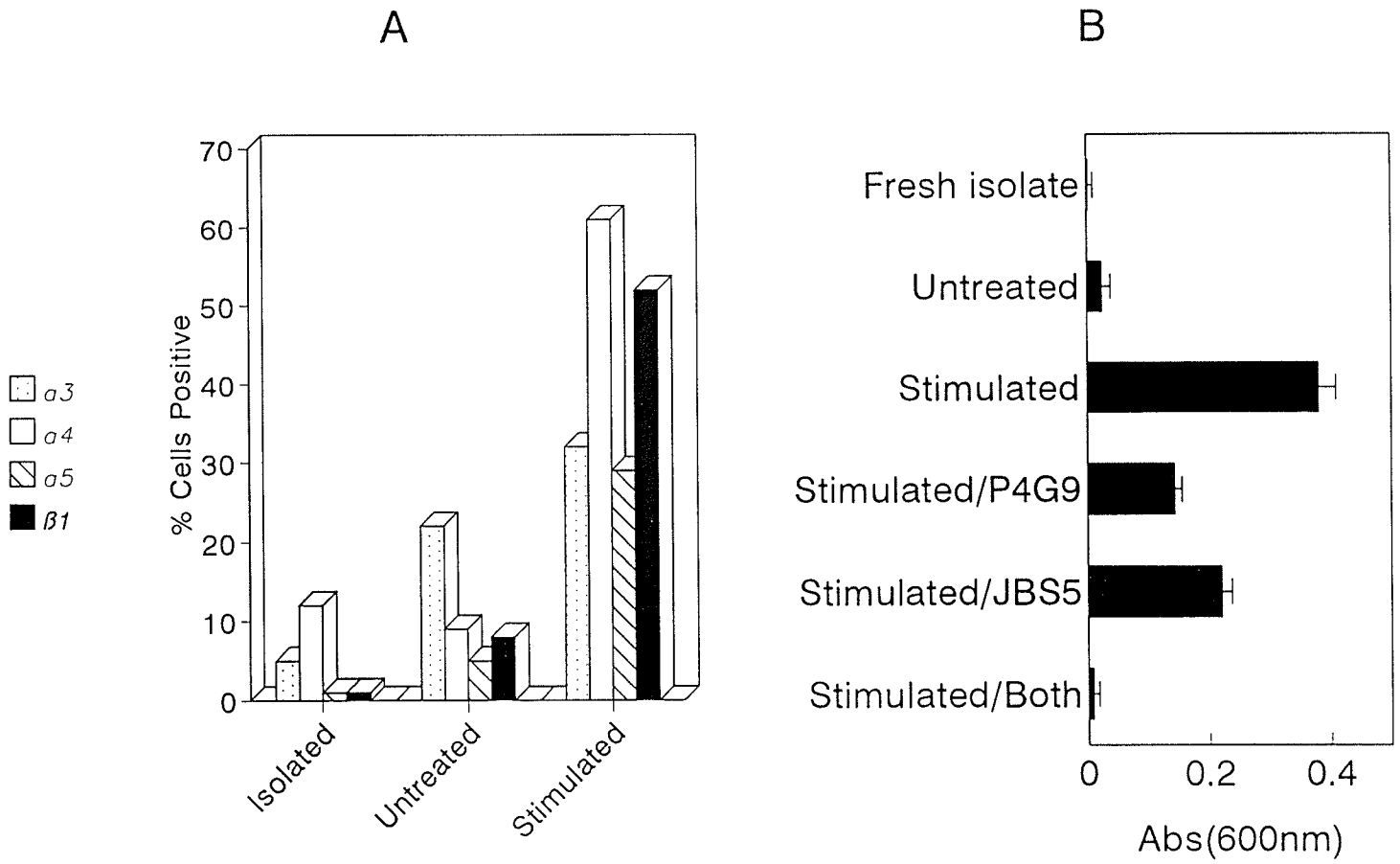
The data generated with the B cell culture lines indicated that B cells might express and utilize α4ß1 and α5ß1. Therefore, purified tonsillar B cells (kindly provided by W Krulicki) were assessed for adherence to fibronectin. No inherent adherence potential for fibronectin was observed, and upon examination of B cells by flow cytometry, ß1 (marker CD29) could not be detected (Figure 4.21A). When cells were stimulated using PMA and anti-μ chain polyclonal sera, they acquired the capacity to adhere to fibronectin (Figure 4.21B). Analysis of stimulated B cells by flow cytometry indicated that 52% of the B population now expressed ß1 (CD29), and in addition α3(marker CD49c), α4(marker CD49d), and α5(marker CD49e) levels were all elevated.

The largest fold increase in integrin α subunit expression was observed in α5(CD49e), but the greatest overall expression was that of α4(CD49d). The α4(CD49d) subunit had been observed to be expressed basally on the surface of a subpopulation of freshly isolated B cells, apparently in the absence of ß1, but likely associated with ß7. To identify which ß1-containing complexes contributed to the adherence of the stimulated B cells to fibronectin, the cells were examined using the α-specific blocking monoclonal

antibodies described. Together, monoclonals P4G9(anti-α4) and JBS-5(anti-α5) were able to virtually abrogate B cell attachment to fibronectin (Figure 4.21B). Individually, P4G9 (anti-α4) and JBS-5 (anti-α5) could inhibit adherence to FN by 65 ± 4% and 45 ± 5% respectively (calculated as the mean ± error of two separate experiments). It therefore appeared that activated normal B cells utilize the α5ß1 integrin complex, as well as α4 integrins (Garcia-Pardo et al, 1990) to adhere to fibronectin.

Figure 4.21 Expression and Use of Integrins by Tonsillar B cells

(**A**). Purified tonsillar B cells were assessed by flow cytometry for expression of integrin fibronectin receptor subunits α3 (CD49c), α4 (CD49d) α5 (CD49e) and ß1 (CD29) either upon isolation (Isolated), after a 72 hour incubation with no stimulus (**Untreated**), or after a 72 hour stimulation with immobilized anti-μ-chain (see methods) and PMA (20ng/ml) (**Stimulated**). (**B**). The same three groups were assessed for attachment to fibronectin. In the adherent "Stimulated" treatment group, the effect of monoclonal antibodies directed against α4 (P4G9) or α5 (JBS5) were assessed for effect upon adherence. One of two similar experiments is shown.


A
B
α3
α4
α5
β1
% Cells Positive
70
60
50
40
30
20
10
0
Isolated
Untreated
Stimulated
Fresh isolate
Untreated
Stimulated
Stimulated/P4G9
Stimulated/JBS5
Stimulated/Both
0
0.2
0.4
Abs(600nm)

4.25 Alternative Adhesion Structures Utilized by B cells

The JY cell line, as well as a significant fraction of isolated tonsillar B cells, were negative for ß1 integrin expression. The previous experiments had implicated ß1-containing integrin complexes as important structures for lymphoid adhesion to collagen and fibronectin. These results implied that ß1 integrin-negative cells must employ alternate means to interact with extracellular matrix. Alternatively, it was possible that ß1 integrin lacking cells possessed no capacity to interact with fibronectin or collagen. Since PMA had demonstrated a capacity to activate "dormant" adhesion potential within lymphocytes, the JY cell line (employed as a ß1 negative lymphocyte model) was treated with PMA to investigate its potential capacity to interact with extracellular matrix.

Untreated JY cells did not display significant adherence to fibronectin, collagen, laminin or vitronectin (Figure 4.22). Following exposure to PMA, a dramatic increase in binding to fibronectin and vitronectin was observed, while attachment to collagen was uninfluenced.

A slight increase in adherence to laminin was also observed (Figure 4.22). Laminin adherence has previously been shown to have specific cation requirements, specifically Mn^{+2}, in other cell types (Kramer et al 1990). The attachment to laminin was further investigated in the presence of different divalent cations (Figure 4.23A). Laminin attachment by JY cells could be maintained or possibly marginally increased by the addition of Mg^{+2}, or maximally increased by the addition of Mn^{+2}.

The observed binding to laminin could be fully inhibited by the addition of LM609, a monoclonal antibody specific for the integrin αvß3 complex (Figure 4.23B). Similarly, the attachment to vitronectin coated surfaces was also blocked by LM609 (Figure 4.23C).

Adherence to fibronectin appeared more complex than that observed with the other two ligands (Figure 4.24). Both LM609 and a polyclonal anti-VNR partially

Figure 4.22 JY cell attachment to selected ECM components

JY cells were assessed for adherence to collagen (COL), fibronectin (FN), laminin (LN) and vitronectin (VN). Open bars represent untreated JY cell attachment to ECM, filled bars represent JY cell adherence after treatment with PMA. The standard error was less than 5% in all cases.

Attachment of JY cells to ECM

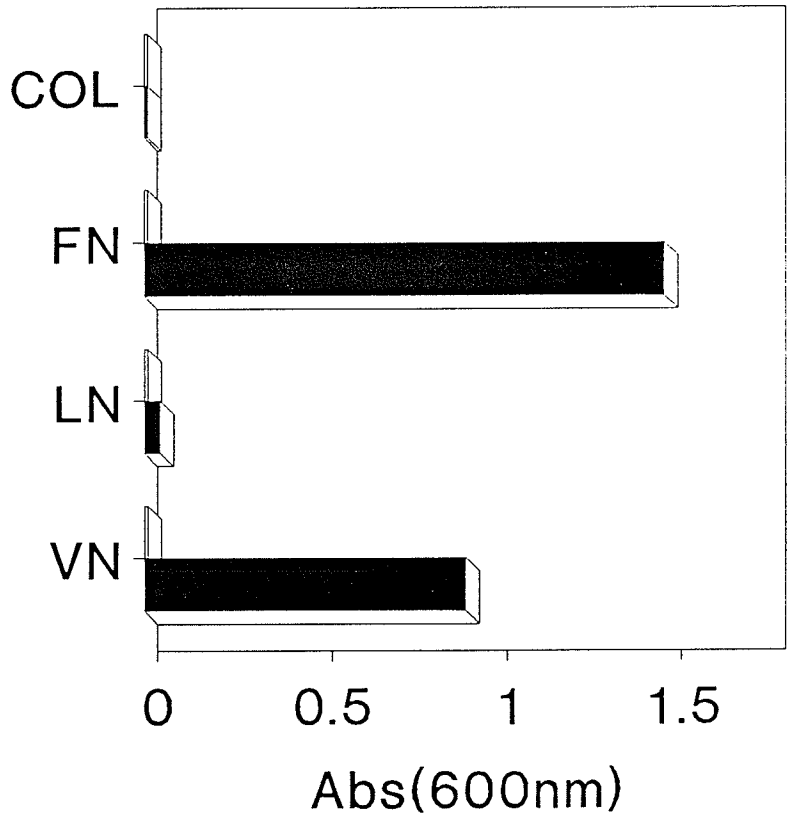
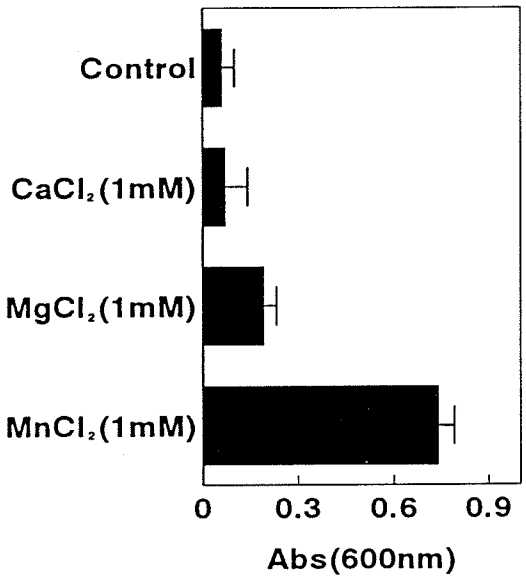


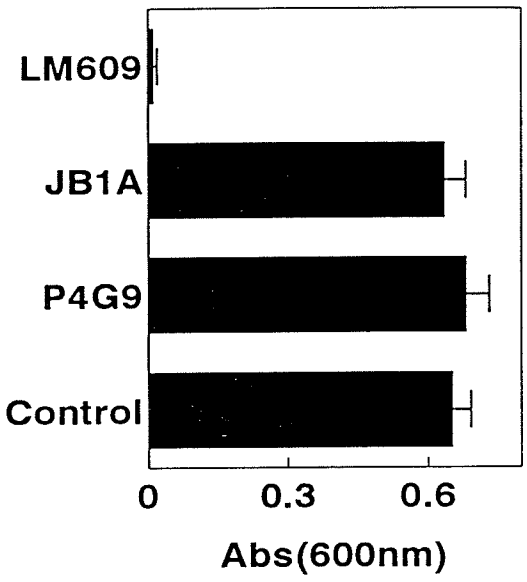
Figure 4.23 Characterization of JY attachment to LN and VN

(**A**) PMA stimulated JY cells were suspended in saline containing similar concentrations of different divalent cations (Ca^{+2}, Mg^{+2}, Mn^{+2}) to examine the effect upon adherence to laminin. (**B**) PMA stimulated JY cells in the presence of Mn^{+2} were preincubated in the presence of monoclonal antibodies to ß1 (JB1A), α4 (P4G9), and αVß3 (LM609) and assessed for attachment to laminin. One of two similar experiments is shown. (**C**) The effect of monoclonal antibodies to specific integrin complexes was assessed upon PMA-stimulated JY cell adherence to vitronectin. Antibodies utilized included LM609 (anti-αVß3), IIb/IIIa (anti-αIIb/ß3), JB1A (anti-ß1), polyclonal antisera to the vitronectin receptor (antiVNR), or control antibody (anti-α5, JBS5).

A


Control
CaCl₂(1mM)
MgCl₂(1mM)
MnCl₂(1mM)
0
0.3
0.6
0.9
Abs(600nm)

B


LM609
JB1A
P4G9
Control
0
0.3
0.6
Abs(600nm)

C

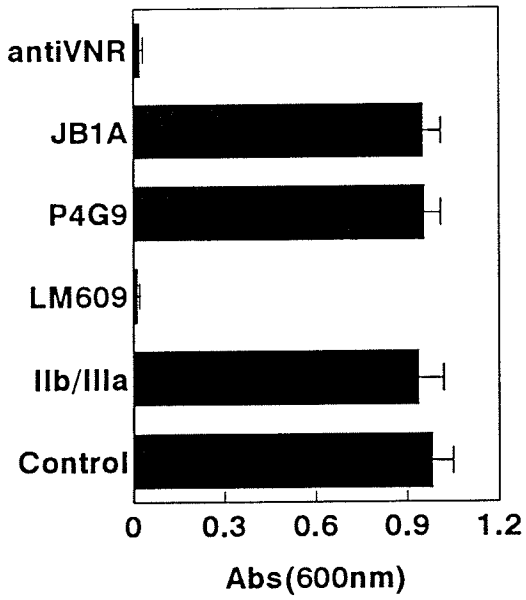
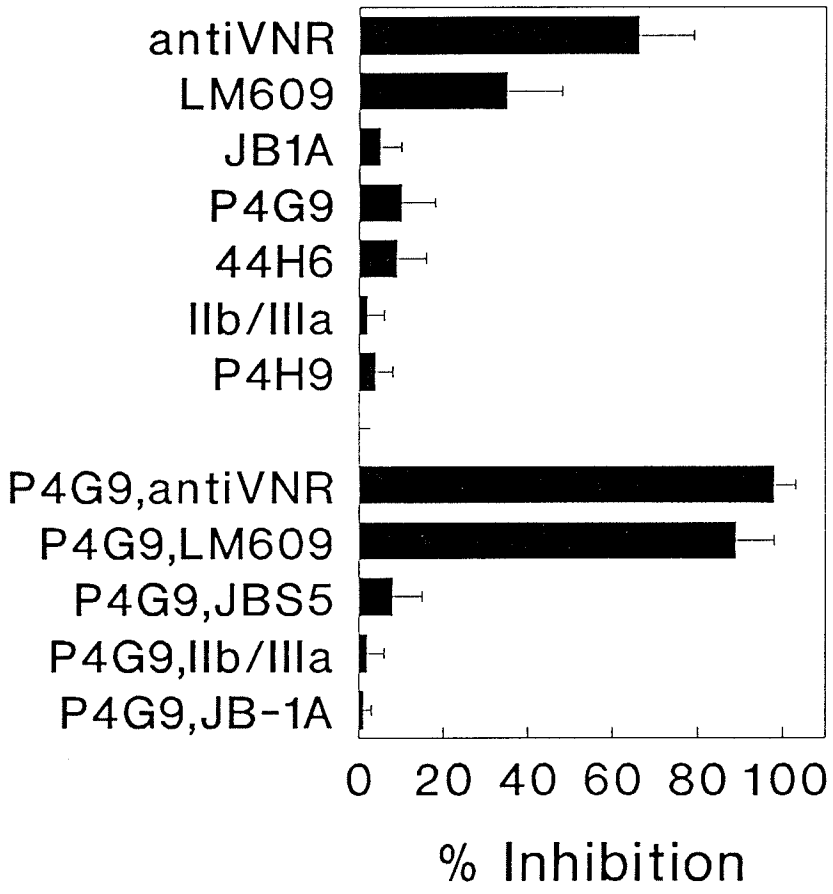

antiVNR
JB1A
P4G9
LM609
IIb/IIIa
Control
0
0.3
0.6
0.9
1.2
Abs(600nm)

Figure 4.24 Characterization of JY cell attachment to FN

JY cells were treated with monoclonal antibodies directed against the α4 integrin (P4G9 or 44H6), αIIb/ß3 integrin(IIb/IIIa), αvß3 integrin (LM 609 and polyclonal rabbit antiVNR), and ß1 (JB1A) and ß2 (P4H9) integrins, alone and in selected combinations. The mean and standard error of three experiments are expressed as inhibition of mock-treated (monoclonal JBS5, anti-α5 integrin) controls.

Monoclonal:


antiVNR
LM609
JB1A
P4G9
44H6
IIb/IIIa
P4H9
P4G9,antiVNR
P4G9,LM609
P4G9,JBS5
P4G9,IIb/IIIa
P4G9,JB-1A
0 20 40 60 80 100
% Inhibition

inhibited adherence to fibronectin. No other single antibody, including those directed against α4 integrin (P4G9, 44H6), caused significant inhibition of attachment. However, when antibodies P4G9 and 44H6 were used in combination with either LM609 or the polyclonal anti-VNR, complete inhibition of JY cell attachment to fibronectin was observed (Figure 4.24). No other combination of antibodies elicited a similar effect. Therefore, it appeared that α4 containing integrin complexes were contributing to the adherence of JY cells to fibronectin.

In no experiment did ß1 directed monoclonals inhibit the attachment of JY cells to extracellular matrix, indicating that PMA stimulation was not inducing adherence through mobilization of internal ß1 stores. Further evidence confirming this was supplied by flow cytometric analysis of JY cells before and after PMA stimulation (Figure 4.25). Comparison of the expression levels of αvß3 and α4 on control and PMA treated JY cells showed no significant difference between the two populations (Figure 4.25). ß1 integrin was not detected on untreated or PMA stimulated JY cells. It did not appear that the changes in JY binding activity associated with PMA stimulation are related to either the upregulation of existing αVß3 or α4 integrin levels, or the induction of sequestered ß1 integrin.

Analysis of radioimmunoprecipitates of surface-labelled JY cells, using LM609 or the αv specific monoclonal antibody, VNR147 revealed the presence of molecular species of 110kDa and 125kDa corresponding to the predicted molecular weight of the ß3 and the αv chains respectively (under reducing conditions)(Figure 4.26, lane G). The α4 specific antibody P4G9 precipitated specific species possibly corresponding to the intact α4 chain-160kDa, an α4 fragment-80kDa and the putative ß chain-110kDa (lane E). Antibody A-1A5 to the ß1 chain did not reveal any reactive material in the JY cells (lane D) but it did precipitate multiple species from the ß1 expressing Jurkat cells (lane B).

Figure 4.25 Effect of PMA on JY cell expression of Integrin

The expression of αVβ3, α4 and β1 integrins on the surface of PMA stimulated and untreated Jurkat cells was evaluated by flow cytometry, labelling with monoclonal antibodies LM609, P4G9, and JB1A, respectively. Controls were only labeled with the secondary reagent (FITC-conjugated rabbit antisera to murine immunoglobulin). The "y" axis indicates cell number (scale 512 cells), while the "x" axis indicates relative fluorescence intensity over three logarithms (base 10). One of two similar experiments is shown.

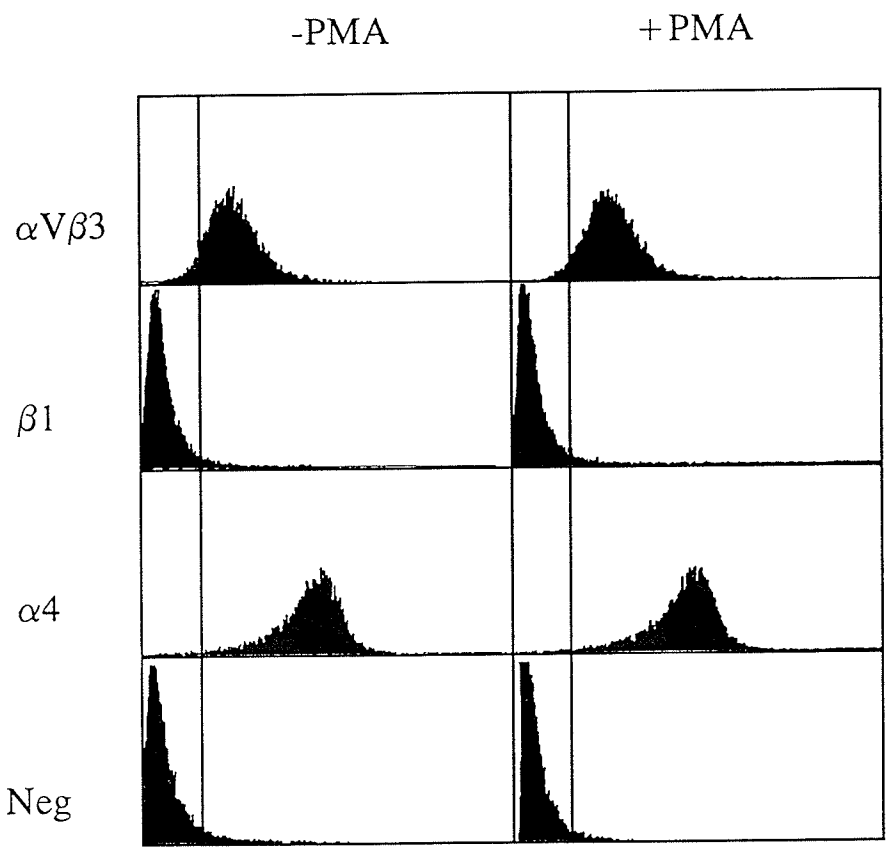
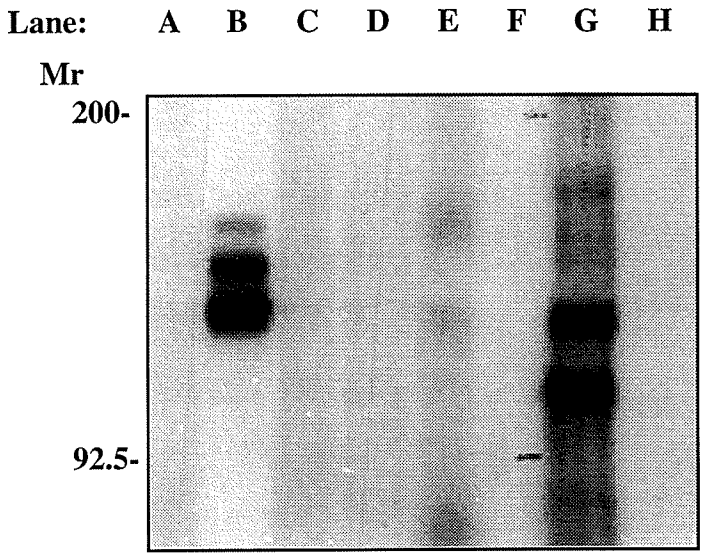

-PMA
+PMA
αVβ3
β1
α4
Neg

Figure 4.26 Immunoprecipitation of JY Integrins

Reducing SDS page analysis of radioimmunoprecipitates of ^{125}I labelled JY and Jurkat cell lines. Lysate source and precipitating antibody (with specificity) are listed as follows; Lane A: JY cells, no Ab, Lane B: Jurkat, A-1A5 (anti-ß1), Lane C: JY, JB1 (anti-ß1), Lane D: JY + A-1A5 (anti-ß1), Lane E: JY + P4G9 (anti-α4), Lane F: Molecular Weight Standards (not radiolabelled, not visible), Lane G: JY + VNR147 (anti-αV), Lane H: Jurkat cells + VNR147 (anti-αV).

Immunoprecipitation of Integrins from JY Cells



JY cell binding to proteolytic fragments of fibronectin was examined in an effort to more clearly define the putative αvß3 and the α4 integrin interactions. The 120 kDa chymotryptic fragment of fibronectin contains the RGD dependent cell binding region of the molecule. The JY cells bound to this fragment as well as to a smaller 15 kDa fragment which contains the cell binding domain in isolation of any other RGD containing regions (Figure 4.27A). The binding to both fragments was inhibited by RGD containing peptides but not by RGE containing control peptides (Figure 4.27A). The RGE peptides serve as a good control as they replace aspartate residues with glutamate residues, thereby preserving a carboxylic acid at the aspartic acid site, preserving the peptide's zwitterionic properties (and no net charge), yet functionally modifying the important aspartic acid site by the "addition" of a carbon spacer on the chemical group containing the carboxylic acid moiety. The peptides should be otherwise nearly identical. Monoclonal LM609 also inhibited binding to either fragment (Figure 4.27B). In contrast, other antibodies, including those directed against α4, did not affect adherence.

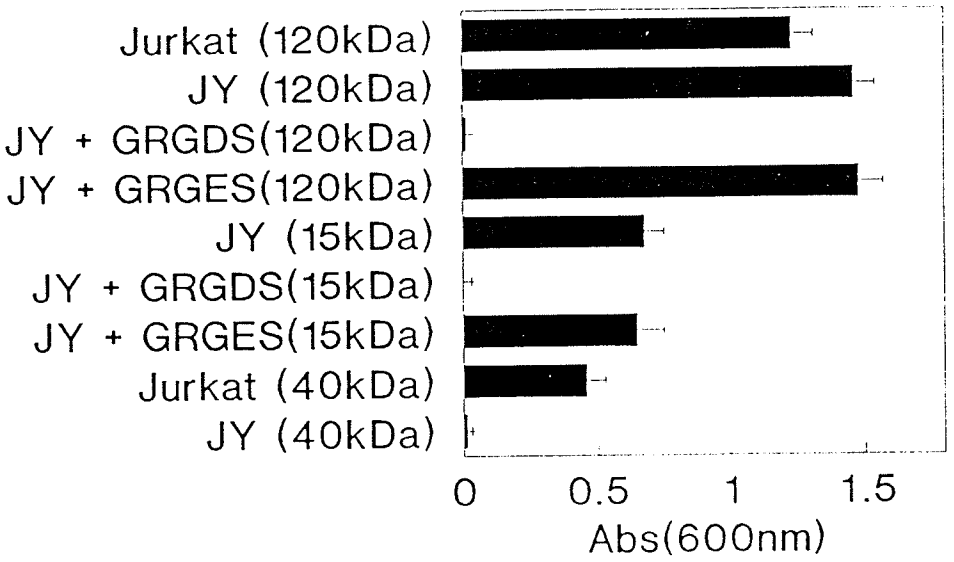
Recent reports indicated that the α4ß7 complex mediated attachment to the CS-1 region of fibronectin (Ruegg et al, 1992, Erle et al 1994b). However, efforts to demonstrate JY binding to a 40 kDa chymotryptic fragment of fibronectin which contains the CS-1 region (but lacks the CBD), could demonstrate no consistent attachment (Figure 4.27A). In contrast the α4ß1 mediated binding of Jurkat cells to this fragment was readily demonstrable (Figure 4.27A), thus indicating that the fragments were functional in the binding assay. Therefore, a direct role for the α4 integrin complex on JY cells (Demonstrated to be integrin α4ß7; Chan et al 1992a) could not be demonstrated in these experiments.

In order to determine if αvß3 utilization was a unique property of the JY cell line, several B cell lines were stimulated with PMA and assessed for their ability to adhere to the

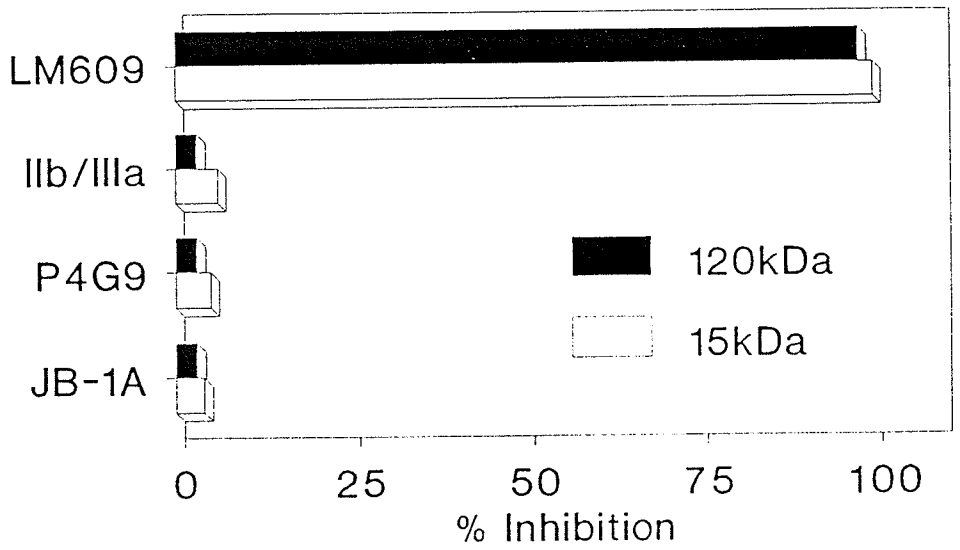
Figure 4.27 Assessment of Fibronectin sites bound by JY cells

The attachment of PMA stimulated JY cells or Jurkat cells to fragments of fibronectin containing the αV/α5 integrin binding site (120kDa and 15kDa), or the α4 binding site (40kDa) was determined (**A**). One of three separate experiments is shown. The effect of inhibitory (GRGDS) or control (GRGES) peptides (150μg/ml) upon the observed attachment to 120kDa and 15kDa fragments was also assessed. One of two similar experiments is shown. (**B**) The attachment to the 120kDA and 15kDa fragments was assessed in the presence on monoclonal antibodies directed against αVß3 (LM609), αIIb/ß3 (IIb/IIIa), α4 (P4G9) and ß1 integrin (JB1A).

A


Jurkat (120kDa)
JY (120kDa)
JY + GRGDS(120kDa)
JY + GRGES(120kDa)
JY (15kDa)
JY + GRGDS(15kDa)
JY + GRGES(15kDa)
Jurkat (40kDa)
JY (40kDa)
0
0.5
1
1.5
Abs(600nm)

B


LM609
IIb/IIIa
P4G9
JB-1A
120kDa
15kDa
0
25
50
75
100
% Inhibition

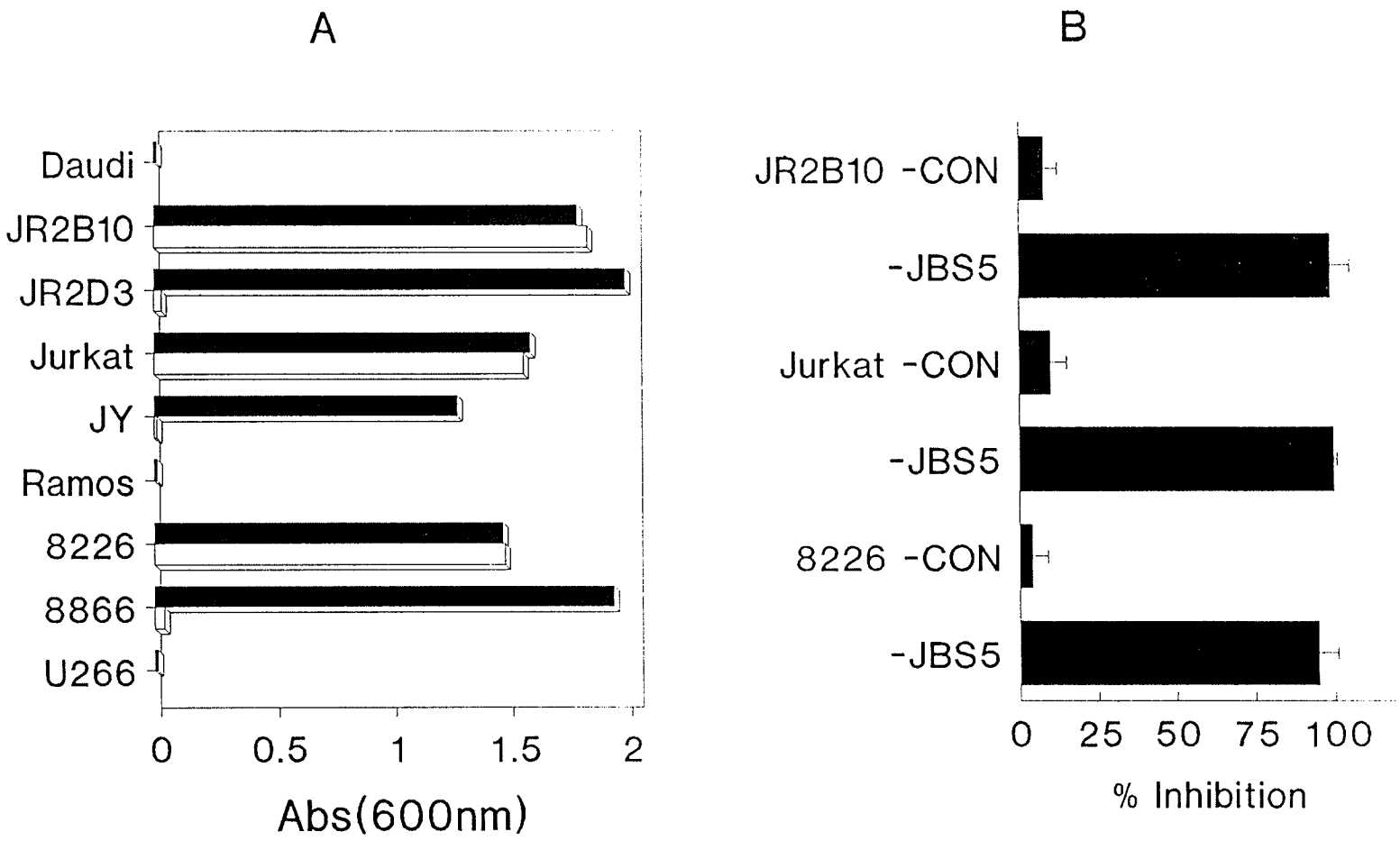
120kDa fragment of fibronectin. As expected, all lines were not adherent. Ramos had previously been classified as an α4ß1 using cell line, and could not therefore attach to the 120kDa fragment (which requires CBD-specific receptors)(Figure 4.28). Those lines which were adherent were then examined for sensitivity of their adherence to inhibition with LM609 (anti-αvß3). The adherence of JY, JR2D3, and RPMI 8866 attachment to the 120kDa fragment could be inhibited specifically and completely by this antibody (Figure 4.28A). These results indicate that B cell lines other than JY may also employ αvß3 as an adhesion structure for fibronectin. All of the lines observed to utilize this receptor were observed to be negative or very weak for ß1 integrin expression (Figure 4.20). Those cell lines which could attach to 120kDa-FN in the presence of LM609 (Jurkat, JR2B10, RPMI 8226), could be blocked by the inclusion of α5 specific antibody JBS5 (4.28B). Therefore, lymphocytes may express at least two different receptors capable of attaching to the CBD containing fibronectin fragment.

4.26 B cell Adhesion to other Matrix components

When binding assays were performed on collagen, only two B cell lines demonstrated appreciable attachment. RPMI 8226 attached to collagen weakly and in a manner independant of PMA (Figure 4.09). In contrast, JR2B10 cells attached to collagen weakly, but this binding was increased by the addition of PMA (Figure 4.09). JR2B10, but not RPMI 8226, cell binding to collagen could be blocked by the inclusion of antibodies specific to either the α2 or ß1 integrin chain (Figure 4.29A). Therefore, only one B cell line demonstrated a capacity to adhere to collagen via ß1 integrins. Purified tonsillar B cell populations were not observed to adhere to collagen in any assays performed (Data not shown).

Figure 4.28 Attachment of Lymphoid Cells to a 120kDa fragment of FN

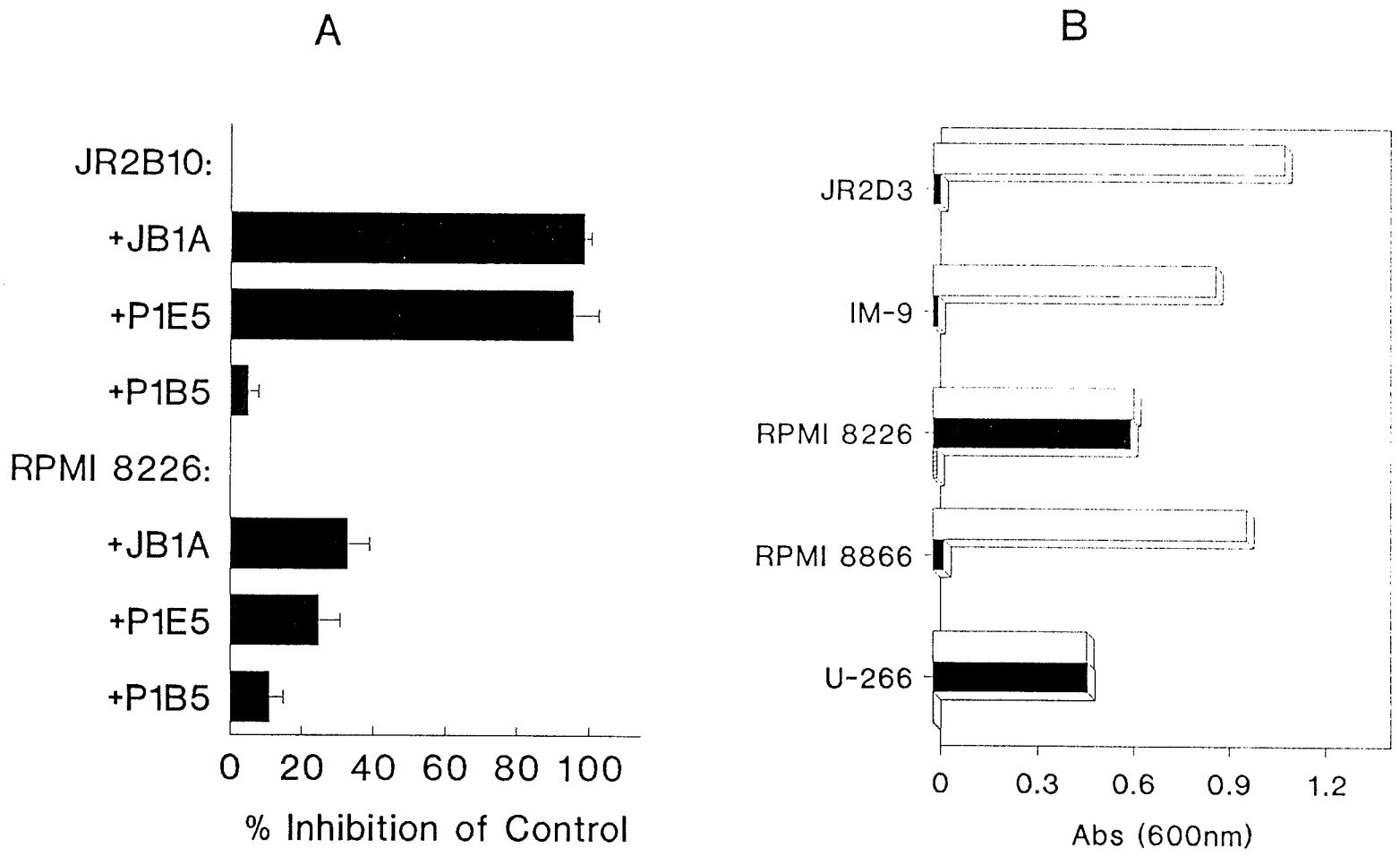
(**A**) A panel of lymphoid cell lines was stimulated with PMA and assessed for attachment to the 120kDa fragment of fibronectin containing the binding site of αV and α5 containing integrins (filled bars). In those cases where attachment was observed, the effects of the anti αVß3 monoclonal LM609 (anti-αVß3) on adhesion were assessed (open bars). (**B**) In those lines adherent to the 120kDa fragment where no inhibition was observed with LM609 (JR2B10, Jurkat, RPMI 8226), the effect of incubation with the anti-α5 monoclonal JBS5 was assessed. Cells were either incubated with a control antibody (LM609)(CON), or with a monoclonal which blocks α5 integrin mediated adhesion (JBS5).


A
Daudi
JR2B10
JR2D3
Jurkat
JY
Ramos
8226
8866
U266
0
0.5
1
1.5
2
Abs(600nm)
B
JR2B10 -CON
-JBS5
Jurkat -CON
-JBS5
8226 -CON
-JBS5
0
25
50
75
100
% Inhibition

However, when the panel of B cell lines previously introduced was assessed for attachment to vitronectin coated surfaces, it was determined that the JR2D3, and the RPMI 8866 cell lines, like the JY cell line, could attach strongly after PMA activation in a manner dependant upon the αVß3 integrin (Figure 4.29B). The cell lines RPMI 8226 and U-266 also attached to vitronectin (Figure 4.29B), but were not affected by the inclusion of monoclonal antibody specific for the αVß3 heterodimer. The binding of these cells could be blocked, however, by the inclusion of polyclonal αvß3 antisera (Figure 4.29B), indicating that either the αV or ß3 subunits might be involved in attachment. The efficacy of the αVß3-directed polyclonal antisera, in contrast to the monoclonal against αVß3, suggests two possibilities. Firstly the αV or ß3 (or both) integrin subunits may be involved in the observed attachment to vitronectin, but may be complexed with alternative integrin subunits (rather than each other). For example, αV integrin may complex with ß1, ß3, ß5, ß6, and ß8. Alternatively, αV and ß3 may complex together in such a way that binding is unaffected, yet the LM609 epitope is modified or eliminated. Although αVß3 can be identified on the surface of RPMI 8226 cells by FACS (37% positive), this does not preclude it appearing in a second, unrecognized form due to post-translational modification.

Figure 4.29 B cell line attachment to Collagen and Vitronectin

A. Collagen attachment: The B cell lines RPMI 8226 and JR2B10, which had both demonstrated a modest capacity to attach to collagen (Figure 4.09), were assessed for attachment to collagen in the presence of α2 and α3 specific inhibitory monoclonal antibodies. **B.** Vitronectin Attachment: The B cell lines were assessed for attachment to vitronectin (open bars) (only adherent lines shown). Adherent lines were subsequently treated with monoclonal LM609, known to block αVß3 (vitronectin receptor) mediated adhesion (filled bars). Where no inhibition with LM609 was observed (RPMI 8226, U266 cell lines), polyclonal antiVNR sera was assessed for effect upon binding (hatched bars). Each experiment was performed twice, with similar results.


A
JR2B10:
+JB1A
+P1E5
+P1B5
RPMI 8226:
+JB1A
+P1E5
+P1B5
0 20 40 60 80 100
% Inhibition of Control
B
JR2D3
IM-9
RPMI 8226
RPMI 8866
U-266
0 0.3 0.6 0.9 1.2
Abs (600nm)

4.3 The Regulation Of Adhesion At The Molecular Level

4.31 Production and Characterization of Monoclonal Antibodies

Biochemical Characterization of Monoclonals

A proven method of probing functional regions of receptors is through the use of site-specific probes, such as monoclonal antibodies (Waldmann, 1989, Davies et al 1990). In order to facilitate such a study of the ß1 integrins, a panel of monoclonal antibody producing hybridomas was generated utilizing immunizing antigen (ß1 complex) derived from Jurkat cell lysates. Alternatively, intact Jurkat cells were used as immunogen.

Five fusions of splenic B cells from immunized mice with NS1 cells were performed (Dr J Wilkins). The initial culture and screening of these hybridomas was performed by Shen Caixia. Further, the biochemical and antigenic characterization of the hybridomas was performed completely by Shen Caixia in the initial two fusions, and her contribution was significant in all further fusions. A summary of those antibodies derived from the fusions used in these studies is shown in Table 4.7, including a list of characterization procedures utilized to identify antigenic specificity.

The monoclonal antibodies JB1, JB1A, B44 and 3S3 were cloned and characterized by C Shen. FACS reactivity of an α2-integrin transfectant, supplied by Dr B M C Chan (University of Western Ontario), was useful in the assignment of α2 integrin specificity to JBS2.

The biochemical characterization of A16G6, B3B11, 13B9, 21C8, and 6F4 was shared (C Shen and D Stupack). Initially, it was demonstrated that the ß1-specific monoclonal antibody A-1A5 (Hemler et al, 1983) could preclear material reactive with these five monoclonal antibodies from Jurkat cell lysates (C Shen). Subsequently, it was

Table 4.7 Characterization of Monoclonal Antibodies

Monoclonal	Isotype			Characterization	Reactivity	
	Fusion		Specificity		EIA	WB
JB1	(1)	IgG2A	ß1	RI, EA, FC, Ft	+	-
JB1A	(2)	IgG1	ß1	RI, EA, FC, Ft	+	+
A16G6	(3)	IgG2A	ß1	RI, EA, FC, Ft	+	N
B3B11	(3)	IgG1	ß1	RI, EA, FC, Ft	+	+
21C8	(3)	IgG1	ß1	ID, EA, FC, Ft	+	-
13B9	(3)	IgG1	ß1	RI, EA, FC	+	N
6F4	(3)	IgG1	ß1	RI, EA, FC, Ft	+	-
3S3	(4)	IgG1	ß1	ID, EA, FC, Ft	+	-
B44	(5)	IgG1	ß1	IW, EA, FC	+	+
N29	(5)	IgG	ß1	EA, FC, Ft	+	+
JBS2	(2)	IgG1	α2	ID, FCL, Ft	+	-

Legend:

Monoclonal: Hybridoma designation. Fusion: Refers to the batch fusion the monoclonal producing hybridomas were cloned from. Isotype: "IgG" refers to an undetermined isotype which may be bound by protein A or protein G coupled to Sepharose-4B, and is composed of 25/50 kDa chains when assessed by 15% reducing polyacrylamide gel electrophoresis. All other isotypes confirmed by ELISA.

Specificity: The antigenic reactivity assigned to the monoclonal, ie., ß1 integrin, α2 integrin. Characterization: Refers to the means used to confirm antigenic reactivity of the monoclonal antibody. ID, immunodepletion of reactivity by known antibody, RI, reciprocal immunodepletion of cell lysates by monoclonal vs known antibody, IW, specific reactivity with purified antigen in western blot, EA, reactivity in antigen or cell based ELISAs, FC, flow cytometric expression on known ß1 positive cell lines, negative on known ß1 negative cell lines, Ft, positive on transfected cell lines expressing specific antigen, negative on others. Reactivity: Refers to the reactivity of the antibody in immunoassays. All antibodies are functional by flow cytometry. EIA: Enzyme-linked immonoassay using cell or purified antigen. WB: western blot, N indicates reactivity only on non-reduced blots.

demonstrated that A16G6 (also called JB1B)(Figure 4.30) as well as B3B11, 13B9 and 6F4 could preclear A-1A5-reactive material from Jurkat lysates. Therefore these five antibodies were assigned ß1 reactivity based upon their capacity to react with the same material in a Jurkat cell lysate as a monoclonal of defined ß1 specificity.

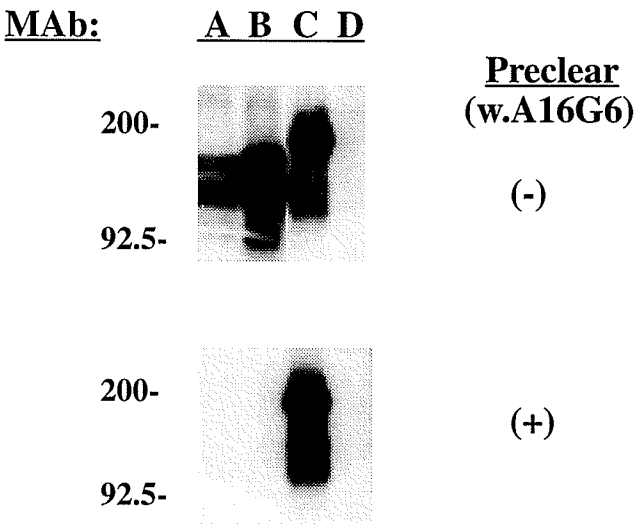
The monoclonal antibody 21C8 could not be demonstrated to completely remove all A-1A5 reactive material, and thus could not be assigned ß1 reactivity by this method. However, several observations suggested that this monoclonal bound ß1 integrins. Monoclonal 21C8 was reactive with immunoaffinity column-purified ß1 integrin (prepared by C. Shen) in ELISA (Figure 4.31A). Monoclonal 21C8 immunoprecipitated material with the same molecular weight as other ß1 integrin specific monoclonals. Additionally, 21C8 was reactive with Chinese Hamster Ovary (CHO) cells transfected with human α5 and ß1 integrins (transfectants prepared by N Hunt), but not control CHO cells expressing only α5 or endogenous hamster integrins (Figure 4.31B). Monoclonal N29 was cloned by limiting dilution (D Stupack), and antigenic specificity of the hybridoma was assessed by modification of integrin function (see below) and, similar to 21C8, reactivity with the described CHO transfectants and purified ß1 integrin in ELISA.

Functional Characterization of Monoclonals

After biochemical characterization and the assignment of specificity, the monoclonal antibodies were assessed for their capacity to modify integrin function. Jurkat cells were treated with specific monoclonal antibodies, (10μg/ml) and assessed for their attachment to fibronectin and collagen coated surfaces (Figure 4.32A). Three different functional classes of monoclonal antibodies were observed in these studies. The first class of monoclonal antibody observed was typified by JB1 and was functionally inert, ie., neutral with respect to effect on integrin mediated attachment to extracellular matrix. The addition of these ß1

Figure 4.30 Immunodepletion with A16G6 removes A-1A5 reactivity

Surface-iodinated Jurkat cells were solubilized. Jurkat-derived lysates were then either depleted of test antigen (Ab1 +) or not treated (Ab1 -). Depletion of test antigen was accomplished by serial treatment of Jurkat lysates with monoclonal A16G6(JB1B) (Note that A16G6 and JB1B are synonymous). After each addition of A16G6(JB1B), antibody was immunoprecipitated with protein A to remove bound antigen complex. Lysates were considered depleted when A16G6 (JB1B) was unable to precipitate any further antigen. Monoclonal antibodies A16G6(JB1B), A-1A5, B3B11 and CD45 all demonstrate activity in nondepleted lanes (Ab1 -) Antigen-depleted lysates were probed with A-1A5 (specific for ß1), or anti-CD45 (MAb 12E7). Depletion of lysates with A16G6 removed A-1A5 reactivity, but not CD45 reactivity (Ab1 + lanes). Reciprocal depletion with A-1A5, followed by probing with A16G6, yielded similar results (not shown).

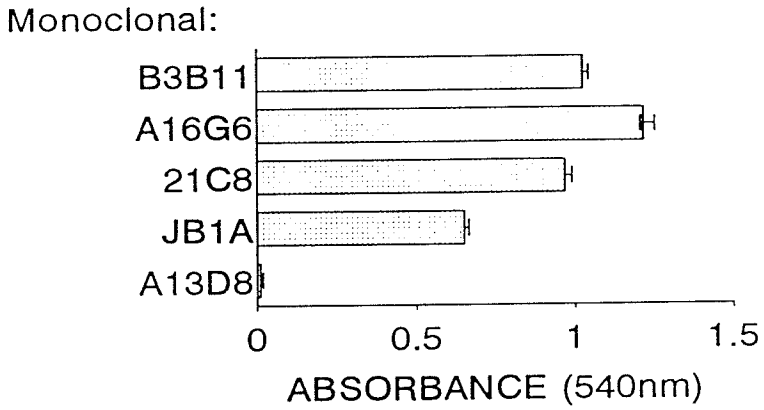


Legend: (A) MAb A-1A5, (B) MAb A16G6,
(C) MAb 12E7 (vs CD45),
(D) No MAb added.

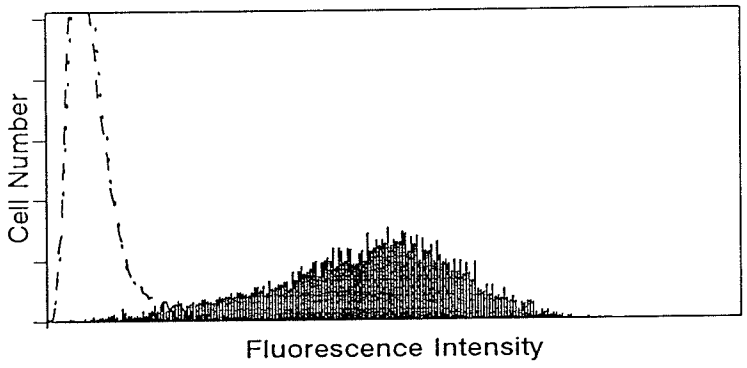
Figure 4.31 Reactivity of 21C8 with purified ß1 and CHO constructs

A. Affinity-purified ß1 was allowed to coat ELISA plates, and the reactivities of 21C8 as well as other ß1-specific (A16G6, B3B11, JB1A) and a non-ß1-specific monoclonal (A13D8), were assessed by ELISA. One of two similar experiments is shown. **B.** Chinese hamster ovary cells (CHO) which had been transfected with either α5 integrin chain(CHOα5), or both α5 and ß1 (CHOα5ß1) chains were assessed for their expression of 21C8 epitope. The negative control (secondary FITC conjugated antisera only) for CHOα5ß1 transfectants is shown. Note that the histogram for the CHOα5 expression of 21C8 is superimposed upon the negative control for CHOα5ß1. The similar negative control for CHOα5, which has been omitted for clarity, is also virtually identical to these two histograms. The scale along the "y" axis is 240 cells (a clipped 256-scale histogram), while the "x" axis represents 3 logarithmic progressions of increasing fluorescence intensity (base 10). (CHO transfectants produced and provided by N Hunt.)

A.



B.



CHO a5/ß1 97% Positive for 21C8
Mean Fluorescence Intensity: 112.1

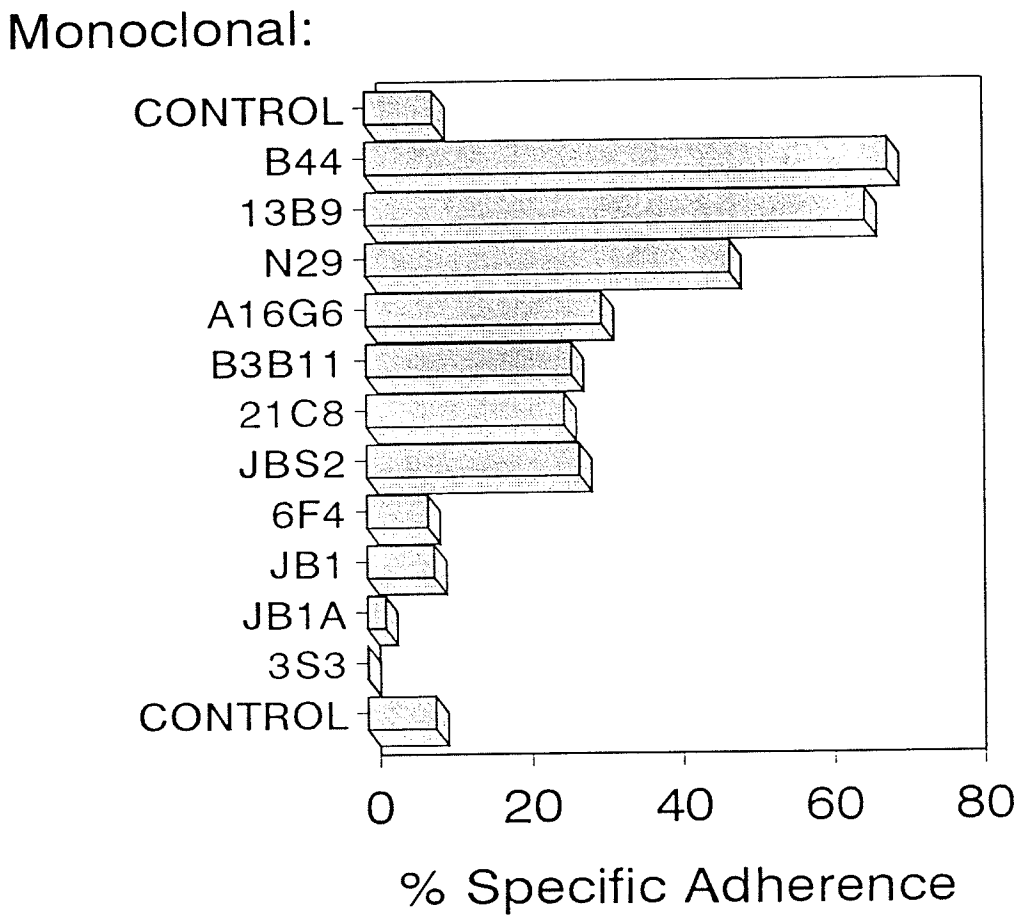
specific antibodies did not modify the observed Jurkat adherence to any extracellular matrix components tested. The second class of antibody observed was typified by monoclonal JB1A, and was inhibitory. Similar to the blocking monoclonal antibodies utilized in section 4.2, the addition of JB1A could inhibit Jurkat adhesion to fibronectin, or collagen in a dose-dependant manner (Figure 4.32B). The third class of antibody observed were adhesion activating antibodies. This class of monoclonal induced Jurkat adhesion to collagen or laminin in a dose-dependant manner (Figure 4.32B, collagen shown. Note: Although Jurkat cells had initially been determined to be nonadherent on laminin-coated surfaces, a later report in the literature ascribed species-specificity to laminin adherence. Thus, Jurkat cells were later determined to adhere to human laminin in a ß1 integrin-dependant manner. However, as the focus of the current studies had been specific to fibronectin and collagen adherence, laminin adherence assays were performed infrequently). Proteolytically (papain) produced Fab fragments of A16G6 were also capable of increasing attachment; therefore it did not appear that monoclonal divalency was a requirement for monoclonal antibody-mediated integrin activation (Figure 4.32B).

The results of these preliminary functional studies, combined with previous data, suggest some characteristics of the native integrin heterodimer. The ability of monoclonal antibodies to activate adhesion suggested that exterior sites on the integrin molecule might be regulatory. Thus, it appeared that adhesion might be regulated by factors external as well as internal to a lymphoid cell. The capacity for antibodies specific to either the ß1 subunit or the α2 subunit to activate attachment to collagen suggested that regulation of adherence was a process which could involve both α and ß integrin subunits. The ability for either α or ß specific monoclonals to block attachment to ligands (as described in section 4.2) supported this possibility. Therefore, further experiments were designed to examine the relationship of α and ß integrins in the regulation of collagen adhesion in the Jurkat model.

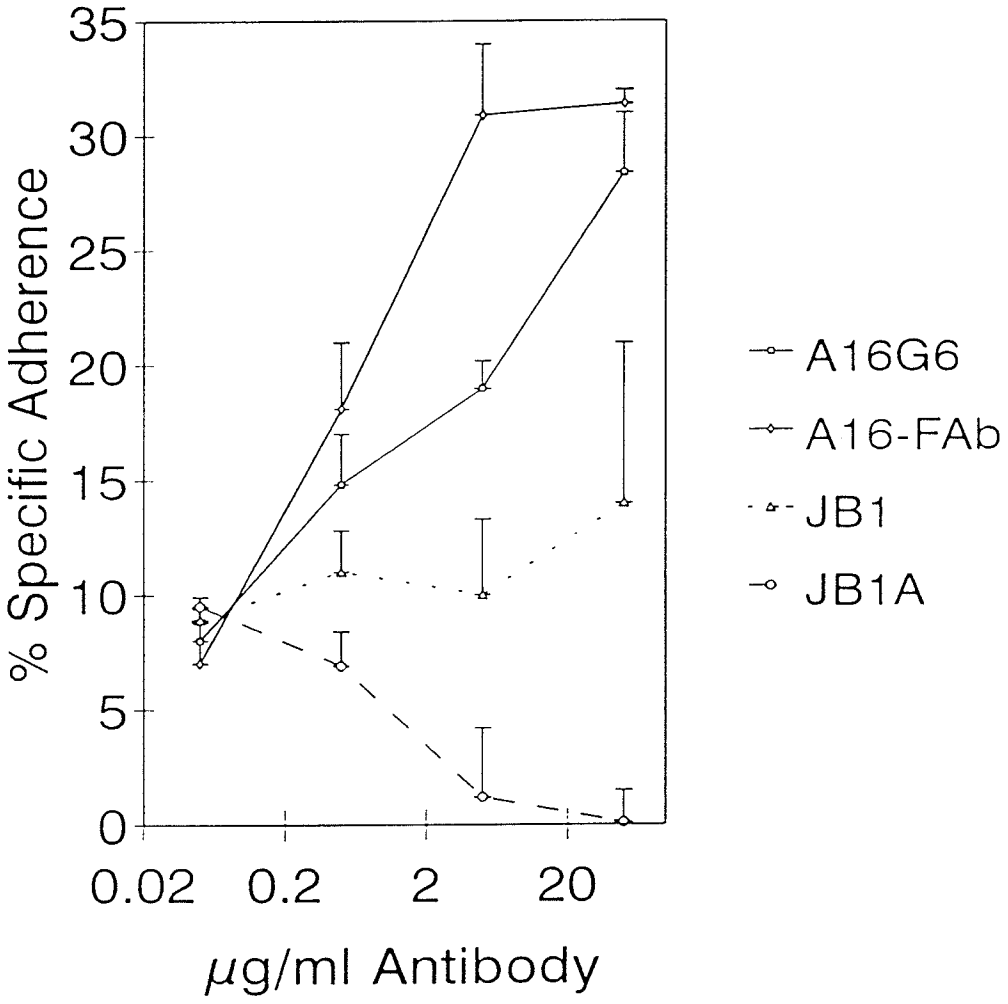
Figure 4.32 Effect of Monoclonals on Jurkat Attachment to Collagen

(**A**) The attachment of Jurkat cells to collagen was examined in the presence of integrin specific monoclonal antibodies (10μg/ml). Jurkat cells were pretreated with antibody for 30 minutes and subsequently allowed to attach to collagen-coated microtitre wells for 1 hour. (**B**) Effect of titration of one antibody observed to exert no significant effect on collagen attachment (JB1), one antibody which blocked adhesion (JB1A) and one antibody which promoted adhesion (A16G6), and an FAb fragment derived from it. A representative experiment is shown. All antibodies depicted in (**A**) were titrated. All appeared to have maximal effect above 5-10μg/ml.

A


Monoclonal:
CONTROL
B44
13B9
N29
A16G6
B3B11
21C8
JBS2
6F4
JB1
JB1A
3S3
CONTROL
0
20
40
60
80
% Specific Adherence

B


35
30
25
20
15
10
5
0
% Specific Adherence
0.02
0.2
2
20
μg/ml Antibody
A16G6
A16-FAb
JB1
JB1A

4.33 Investigations utilizing α2ß1 integrin on Jurkat cells

The adhesion-activating monoclonal antibody JBS2 was not ß1 specific (Table 4.7). This anti-α2 integrin specific monoclonal was unique in the respect that it induced attachment only to collagen; it had no effect upon cellular attachment to other extracellular matrix components such as fibronectin or laminin (Figure 4.33A).

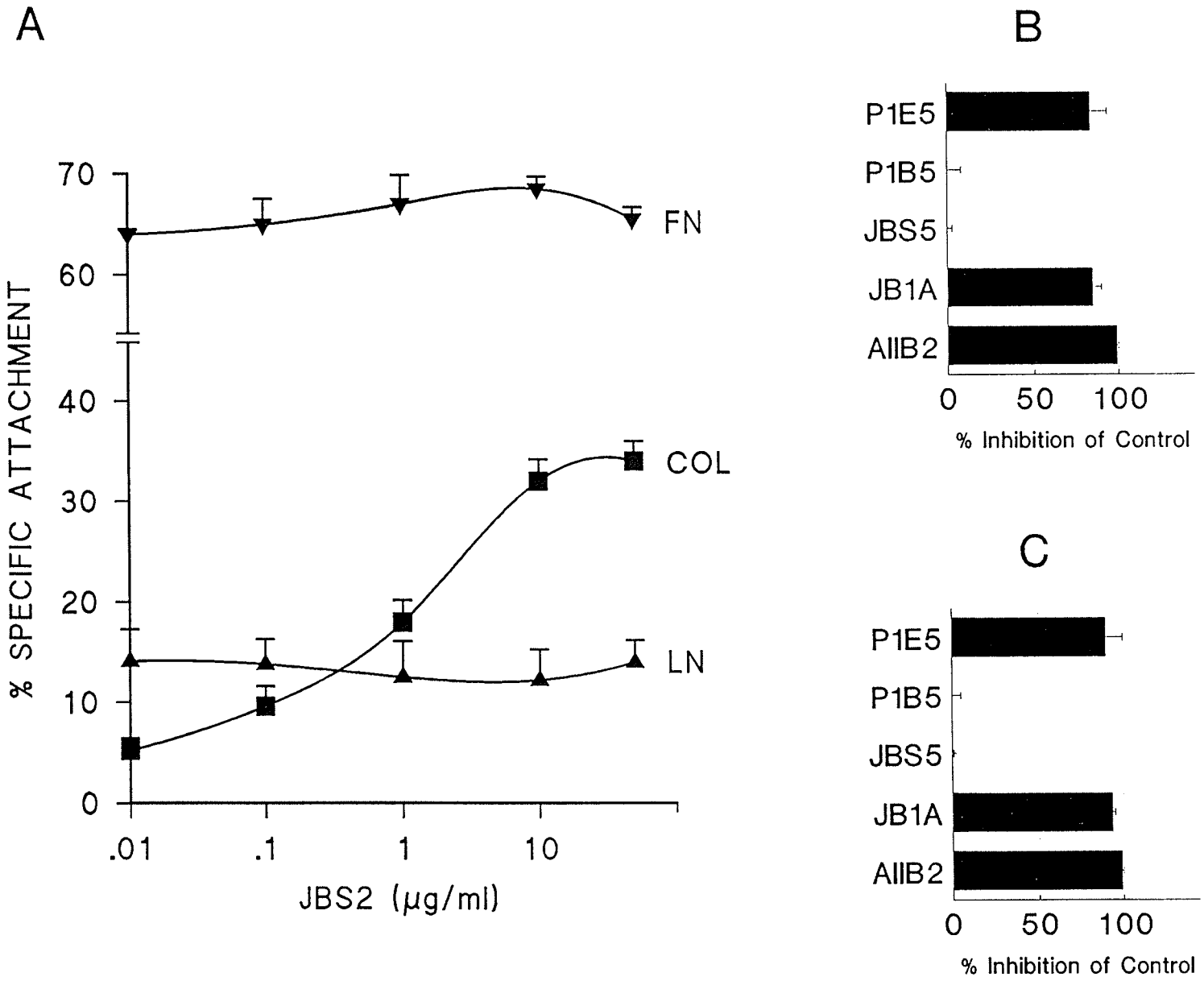
JBS2 induced attachment mediated by the α2ß1 integrin complex, as assessed by the capacity for α2 or ß1 specific inhibitory antibodies to block attachment of Jurkat cells induced by JBS2 (Figure 4.33B). The α2ß1 integrin has previously been demonstrated to be the Jurkat receptor for collagen on PMA activated cells (Figure 4.17A). Induction of adhesion by activating ß1 specific monoclonals similarly activated the α2ß1 receptor (Figure 4.33C), as assessed by blocking studies using specific monoclonal antibodies.

Since all three of these distinct stimuli (α2 specific monoclonal, ß1 specific monoclonal, PMA) activated collagen adherence via the same receptor. It was therefore possible that they might activate the receptor via similar means. To test this possibility, Jurkat cells were pretreated with staursporine, and inhibitor of protein kinase C, or diluent, and then subsequently treated with maximally stimulating concentrations of each of the agents (20μg/ml monoclonals, 50ng/ml PMA). The staurosporine-treated cells were activated to attach to collagen by adhesion-inducing monoclonal antibodies specific to either α2 (JBS2) or ß1 (A16G6) integrins, but were not induced to adhere by PMA (Figure 4.34A). In contrast, all activating agents were capable of inducing diluent-treated cells to attach to collagen. This result demonstrates that the activation of Jurkat cell adherence by PMA, but not by monoclonal antibodies, is sensitive to the inclusion of serine/threonine protein kinases, such as protein kinase C.

While the previous experiment had dissociated between monoclonal and phorbol ester-mediated activation of Jurkat cells, it was still not clear whether monoclonal

Figure 4.33 Activation and Analysis of Jurkat Collagen Adherence

(**A**) Jurkat cells were pretreated with the monoclonal antibody JBS2, specific for α2ß1 integrin, at various concentrations and assessed for effect upon attachment to collagen, fibronectin and laminin. A representative titration is shown. (**B**) Jurkat cells which had been activated to adhere to collagen with MAb JBS2 (20μg/ml) were subsequently treated with monoclonal antibodies P1E5 (anti-α2), P1B5 (anti-α3), JBS5 (anti-α5), JB1A (anti-ß1) or AIIB2 (anti-ß1) to assess which structures were mediating attachment to collagen. One of three similar experiments is shown. (**C**) Jurkat cells which had been activated to adhere to collagen with MAb A16G6 (20μg/ml)(cf. Figure 4.35B) were similarly characterized for collagen adhesion. One of three similar experiments is shown.


A
70
60
40
30
20
10
0
% SPECIFIC ATTACHMENT
FN
COL
LN
.01
.1
1
10
JBS2 (μg/ml)
B
P1E5
P1B5
JBS5
JB1A
AIIB2
0
50
100
% Inhibition of Control
C
P1E5
P1B5
JBS5
JB1A
AIIB2
0
50
100
% Inhibition of Control

antibodies directed against α2 and ß1 activated Jurkat cells via similar or distinct means. To test this, a combinatorial assay was devised in which all reagents were used at saturating concentrations (ie., concentrations at which addition of further reagent caused no further increase in observed specific adherence). Under these circumstances, it was hypothesized that those reagents which activated Jurkat adhesion via different means would exhibit complementation, while no effect would been seen among reagents which activated via similar means. The inclusion of PMA served as a control, as PMA had been previously demonstrated to activate cells in a dissociable manner from the monoclonal antibodies.

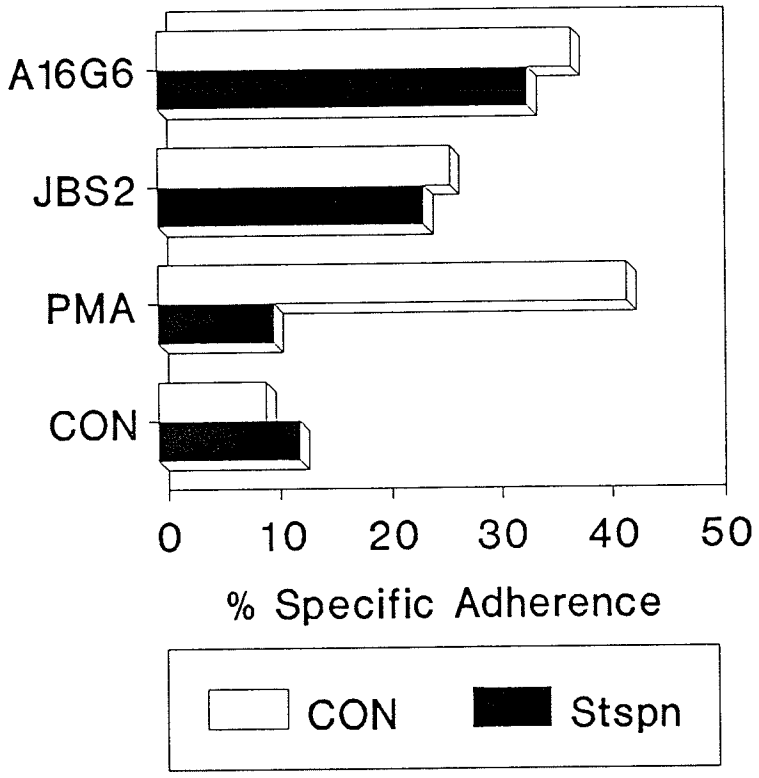
The combination of PMA with antibodies exhibited an additive effect, a result which was predicted by, and integral to, the experimental design (ie. that distinct and dissociable mechanisms should complement each other) (Figure 4.34B). The addition of staurosporine was able to specifically inhibit the additive attachment of Jurkat cells induced by combinations of PMA (with either α2 or ß1 specific monoclonals), to levels comparable to that induced by the monoclonal alone. This experiment suggests that the specific portion of the additive increase in adherence provided by PMA can be selectively inhibited by staurosporine.

The addition of α2- and ß1- specific antibodies was also observed to increase attachment to collagen in a cooperative manner (Figure 4.34B). The additivity was unaffected by the inclusion of staurosporine, therefore, it does not appear that the increase in adherence induced by A16G6/JBS2 results from protein kinase C activation. These results further suggested that the induction of Jurkat cell attachment to collagen mediated by α2 and ß1 specific monoclonals were distinct events. These results suggested a model of lymphoid α2ß1 integrin in which regulatory regions can be activated through the triggering of cytoplasmic signalling pathways (PMA), or through extracellular perturbations of either the α2 (JBS2) or ß1 (A16G6) subunits.

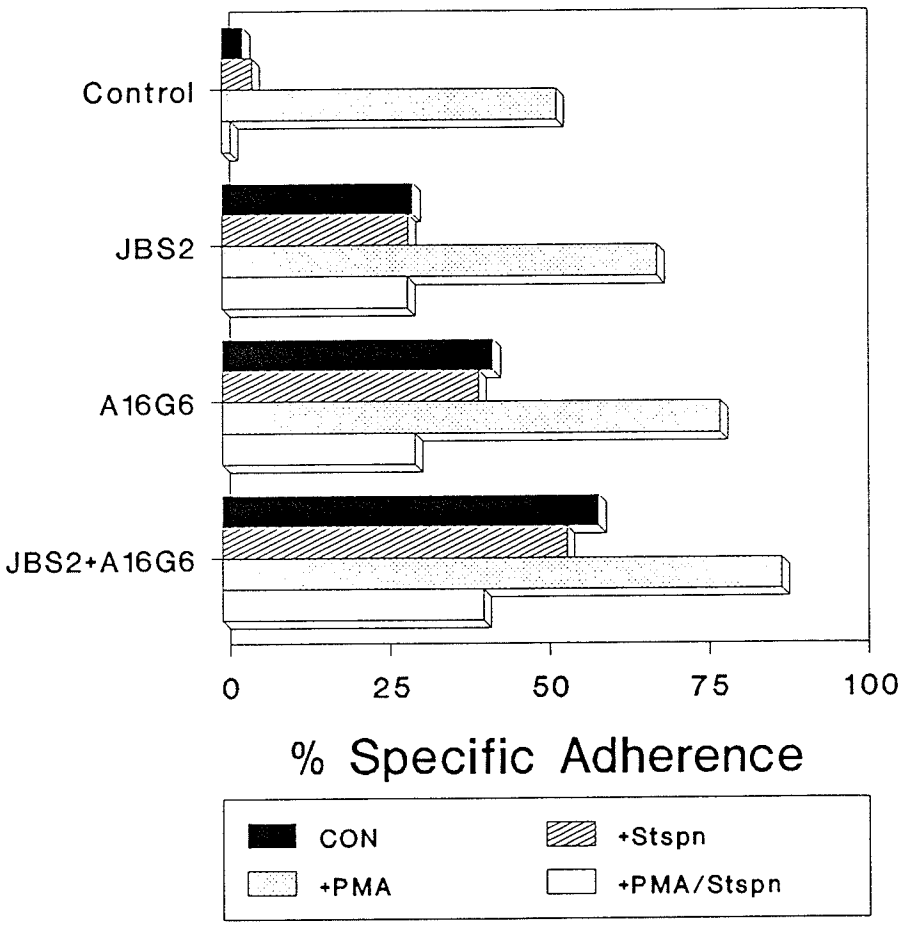
Figure 4.34 Effect of Staurosporine on Induced Collagen Attachment

(**A**) Jurkat cells which had been pretreated with either staurosporine (1μM) or diluent (DMSO) for 15 minutes were treated with PMA, JBS2 or A16G6 to examine the effect of staurosporine on the induction of collagen adherence. A representative experiment is shown (The standard error is <3% in each case). (**B**) Stimulatory antibodies A16G6 and JBS2, at maximally efficacious concentrations (20μg/ml) and PMA (50ng/ml) were used alone and in combinations to examine the induction of adherence in control and staurosporine-treated Jurkat cells. The results shown are the mean of three separate, triplicate determinations.

A


A16G6
JBS2
PMA
CON
0
10
20
30
40
50
% Specific Adherence
CON
Stspn

B


Control
JBS2
A16G6
JBS2+A16G6
0
25
50
75
100
% Specific Adherence
CON
+Stspn
+PMA
+PMA/Stspn

A further examination of the interplay between α2 and ß1 was performed by cotitration of these antibodies. Varying titrations of α2 and ß1 were used to stimulate Jurkat cells prior to adherence assay. A dramatic increase in adherence was observed when low concentrations (1μg/ml) of JBS2 and A16G6 were combined (Figure 4.35). The increase observed was greater (1.5 fold) than that observed when either of these monoclonals was used alone at 10μg/ml. The magnitude of the observed synergism discounted the possibility that the induced attachment might be simply due to a simple additive effect (eg. 1 μg A16G6 + 1μg JBS2 = 2μg/ml activating monoclonal). This is a very interesting observation as it suggests possible mechanisms by which the adhesive potential of a cell might be increased by low concentrations of multiple stimuli.

4.4 Regionalization of Activating Sites on ß1 Integrin

4.41 Competitive Mapping Using ß1 Integrin Antibodies

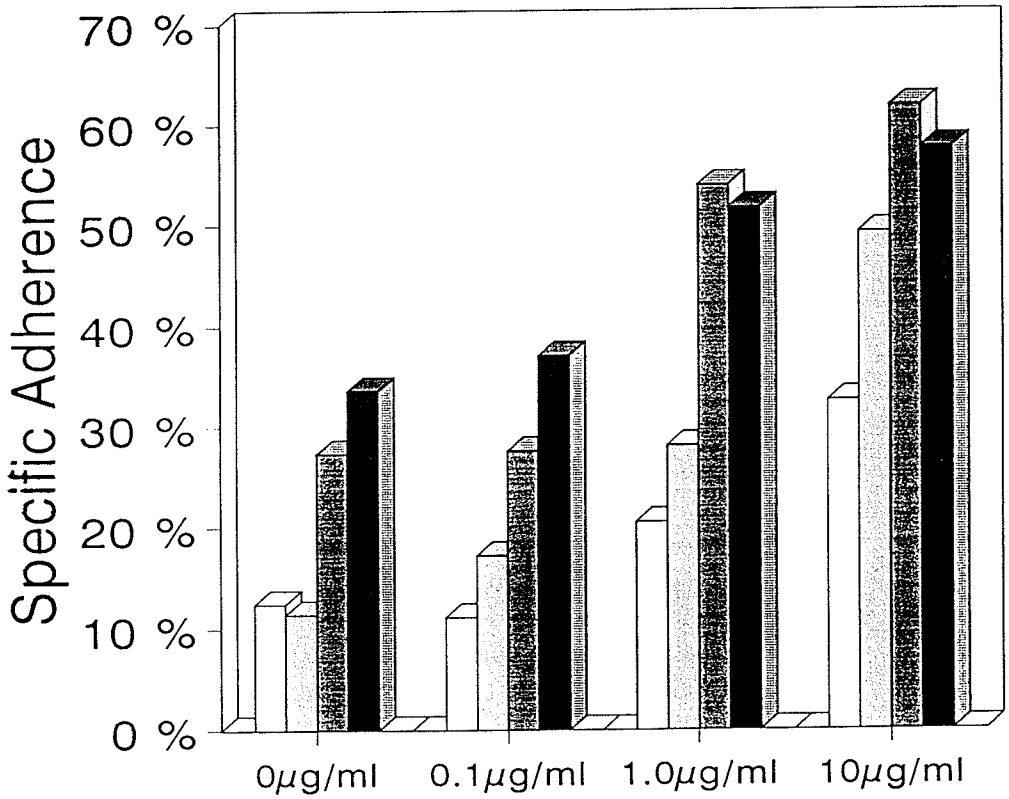
In order to determine if any of the monoclonal antibodies shared specificity for regions of the ß1 molecule, the panel of antibodies was analyzed via competitive assays, in which unlabelled antibody was used to compete with labelled antibody for access to epitopes on cell surface, native, ß1 integrin heterodimers. This approach was selected due to the capacity it offered to group the antibodies into competitive groups. Competitive groups often, but do not always, reflect proximal epitope location on the antigen of interest.

Monoclonal antibodies may compete for binding for two distinct reasons. The first is that the epitopes overlap as a function of the approximately 600-800 square angstrom area

Figure 4.35 Cotitration of JBS2 and A16G6

The adhesion of Jurkat cells to collagen was examined in the presence of varied concentrations of monoclonal antibodies JBS2 and A16G6. The results shown are the mean of two separate, triplicate determinations. The mean % adherence for each bar is displayed in the table below; the standard error is less than 5% in all cases, and was typically less than 3%.

Cotitration of JBS2 and A16G6



[A16G6]	0μg/ml	.1μg/ml	1.0μg/ml	10μg/ml
0μg/ml	12.4 %	11.1 %	20.5 %	32.6 %
.1μg/ml	11.4 %	17.2 %	28.1 %	49.3 %
1.0μg/ml	27.3 %	27.6 %	53.9 %	61.9 %
10μg/ml	33.6 %	37.1 %	51.7 %	57.7 %

[JBS2]

which is the monoclonal "footprint" (binding site, plus occluded areas) (Davies et al, 1990). A second type of competition occurs when the competing antibody induces a conformational change in the target molecule which masks or alters the target epitope of the labelled monoclonal.

Whole cells were used for the ELISA as a source of native integrin, and the competitive cell-ELISA modified after Epstein and Lunney (1985). To study the competitive interactions of the panel of monoclonal antibodies with ß1 integrin, each monoclonal was labelled with biotin. Unbiotinylated, competing antibodies at 20:1, 1:1 and 1:20 ratios were then allowed to preblock Jurkat cells for 1 hour prior to the addition of the biotinylated antibody. Binding of the biotinylated antibody was quantitated by addition of avidin-conjugated horse radish peroxidase, followed by colorimetric development with OPD substrate and quantitation at 492nm. Results were expressed as a percentage of control, (no competing antibody), after subtracting spontaneous color development. The results of a representative biotin-based blocking study are depicted (Figure 4.36A).

Some of the competitive relationships were initially tested by radiocompetitive assay. Three monoclonal antibodies (one activating, one neutral, one inhibitory) were radioiodinated and tested for binding to intact Jurkat cells. Direct competition assays were performed, with the competing and blocking antibodies added simultaneously (Figure 4.36B).

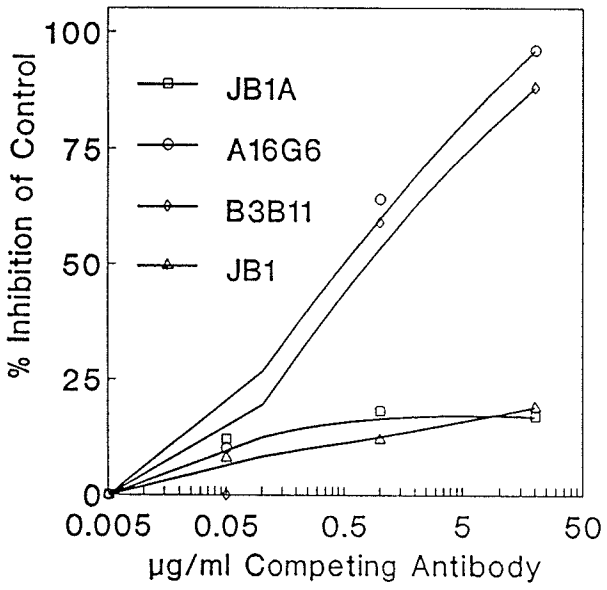
All results obtained in the biotin-based competitive studies were in agreement with radiocompetitive assay. The results of both methods of study are summarized in Table 4.8. The monoclonal antibodies A16G6, B3B11 and 21C8 were found to cocompete with each other. Each of these antibodies was adhesion-inducing in the functional assay. Therefore, these monoclonals appeared to recognize an independent, function-activating region on the ß1 complex. Similarly, the adhesion-inducing monoclonals 13B9 and B44 were also found to co-compete with each other. These monoclonals may represent a

Figure 4.36 Monoclonal Immunocompetitive Assays

(**A**) Jurkat cells were preblocked with varying concentrations of competing antibody, as shown, and then assessed for binding of biotinylated A16G6. Binding was quantitated subsequent addition of horse-radish peroxidase-conjugated avidin, and expressed in comparison to unblocked control cell binding of biotinylated A16G6. Results shown are the mean of three separate experiments. (**B**) Jurkat cells were assessed for binding of radio-iodinated A16G6 (1μg/ml) in the presence of different concentration of competing antibody as shown, and quantitated by γ emission. Results are the mean of two separate experiments and are expressed relative to unblocked binding of radio-iodinated A16G6 to Jurkat.

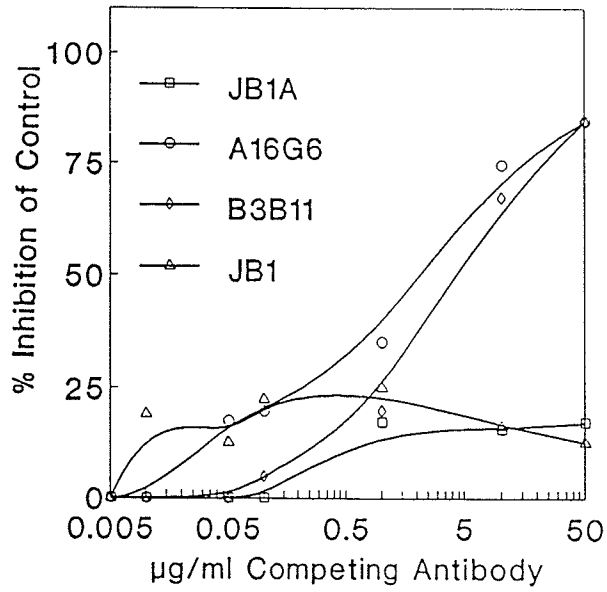
A

A16G6-Biotin



B

A16G6-^{125}I



second unique region on ß1 integrin capable of regulating adhesion.

The monoclonal antibody JB1 was determined to cocompete with monoclonal 6F4 for binding to Jurkat cells; both of these monoclonals are neutral with respect to integrin function. No other antibodies were observed to compete with each other reciprocally, although partial inhibition of B3B11 was observed when 6F4 or JB1A was used as a blocking agent. Partial competition has been suggested to represent adjacency of two epitopes (Wilson, 1988). If so, this would suggest that the JB1A and 6F4 epitopes are within 35 angstroms of the B3B11 epitope. However, the inability of B3B11 to reciprocally partially inhibit either 6F4 or JB1A did not support this possibility. Rather, it suggested that the observed inhibition was due to some conformational effect elicited by 6F4 or JB1A binding.

The accumulated data (Table 4.8) supported the existence of three distinct regions on the ß1 integrin molecule which may contribute to functional activation: The two regions bound by the competitive groups described above, and the epitope recognized by monoclonal N29. If the JBS2 epitope is included, then the α2ß1 integrin complex appears to possess no less than 4 putative regulatory sites positively influencing functional activation of α2ß1 integrin heterodimers.

Table 4.8 Summary of Competetive Assays

Competing Antibody:	Labelled Antibody: ▼ A16G6	B3B11	21C8	N29	B44	13B9	▼ JB1	6F4	▼ JB1A	3S3
A16G6	+	+	+	–	–	–	–	–	–	–
B3B11	+	+	+	–	–	–	–	–	–	–
21C8	+	+	+	–	–	–	–	–	–	–
N29	–	–	–	+	–	–	–	–	–	–
B44	–	–	–	–	+	+	–	–	–	–
13B9	–	–	–	–	+	+	–	–	–	–
JB1	–	–	–	–	–	–	+	+	–	–
6F4	–	±	–	–	–	–	+	+	±	–
JB1A	–	±	–	–	–	–	–	–	+	–
3S3	–	–	–	–	–	–	–	–	–	+
AIIB2	–						–		–	
mAb13	–						–		±	
A-1A5	–						–		–	
A10B4	–						+		–	

Legend:
▼ Indicates antibodies which were iodinated in addition to biotinylation.
(+) indicates effective competition (>80% inhibition) in either assay. All results were similar in both the iodination and biotinylation experiments.
(±) indicates partial competition (40%), as demonstrated in either the iodination or the biotinylation experiments.
(-) indicates no effective competition observed.

4.42 Localization of the B3B11 Epitope

One approach to mapping the location of linear epitopes involved antigenic screening of bacterial expression libraries. Each colony randomly expressed various fragments of an antigen as part of a bacterial fusion protein. This was accomplished by digestion of cDNA encoding the protein of interest, followed by ligation of these DNA fragments into a bacterial expression vector. Limitations inherent to this method included the inability to detect carbohydrate-dependant as well as nonlinear (conformational) epitopes. Thus, this method is not suitable for identification of all epitopes.

To analyze the location of epitopes from the panel specific to β1, plasmid pECE encoding ß1 cDNA was randomly digested with DNase in the presence of manganese. The resulting 50-150bp fragments were adenosine-tailed and ligated into the novatope expression vector. This work was performed by Dr J Wilkins. Competent JM109 DE3 cells were then transformed (by Shen Caixia) with novatope vector expressing random inserts and colonies were screened for the presence of immunoreactive fusion protein. Since the protocol required antibodies with the capacity to react with a linear epitope, B3B11 and JB1A were among those antibodies selected for use in initial screening. Both B3B11 and JB1A were functional in immunoprecipitation, cell and antigen based ELISA, and reducing and non-reducing Western blots. These monoclonals were therefore considered to be good candidate antibodies to probe the ß1 peptide library.

Three colonies were initially characterized (Shen Caixia) to be reactive with antibodies in the initial screening cocktail. Two of these colonies were found to be reactive with JB1A. Subsequent characterization of the JB1A epitope is discussed in section 5, and was performed by C Shen and N Hunt.

A single JM109 colony was found to be reactive with B3B11. This colony was subsequently cloned and amplified. Plasmid DNA was purified and transfected into the

BL21 cell line for protein expression. Transfected cells were immunoscreened and cloned through three rounds of selection. The bacterial clone isolated, designated FPG10.B3B11, was immunoreactive with monoclonal antibody B3B11, but not other ß1 specific antibodies (Figure 4.37A).

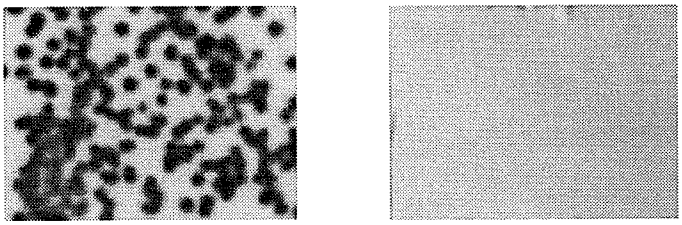
The fusion protein encoded within FPG10.B3B11 is a composite T7 phage G10 capsid-protein and inserted integrin sequence. The protein is predicted to have a molecular weight of approximate 30kD, plus insert. Clone FPG10.B3B11 was subjected to SDS PAGE analysis. A single, strong protein band was evident when fusion protein expression was induced by 1mM isopropyl-ß-D-thio-galacto-pyrannoside, with an apparent molecular weight of approximately 36 kD (Figure 4.37B). When cell lysates were transferred to nitrocellulose and analysed by western blot, a strong signal was obtained at the same relative molecular mass as the induced fusion protein (Figure 4.37C); the fusion protein was not reactive with a second ß1 specific monoclonal, JB1A. Further, B3B11 was not reactive with G10 fusion protein bearing a JB1A-specific insert (4.41C).

Therefore, these data suggested that the insert specific to clone FPG10.B3B11 contained the B3B11 reactive antigen. This antigen was specific to monoclonal B3B11,. When other monoclonals which cocompeted with B3B11 for binding to Jurkat cells (A16G6, 21C8), were used to probe FPG10.B3B11 lysates, no reactivity was observed (Figure 4.38A). Since SDS-PAGE and immunoblot might interfere with conformational epitopes, purified fusion protein was coated onto microtitre wells and assessed for reactivity with B3B11 and cocompeting monoclonals A16G6 and 21C8 (Figure 4.38B).

Figure 4.37 Analysis of BL21/FPG10.B3B11 Reactivity

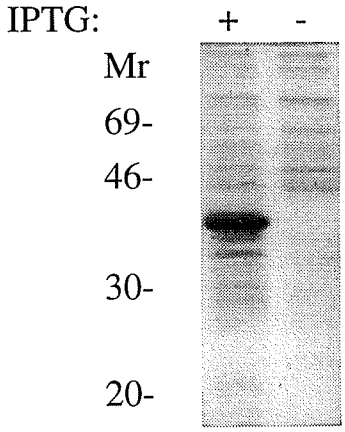
(**A**) Nitrocellulose lifts of colonies of clone BL21/FPG10.B3B11 were assessed for their reactivity. Blots derived from the same plate were treated with ß1 specific antibodies B3B11 or JB1A (control). Antibody binding was assessed colorimetrically. (**B**) Effect of IPTG (1mM) upon the protein production of BL21/FPG10.B3B11 cells was assessed. Treated (lane 1) and non-treated cells (lane 2) were analyzed by 15% reducing SDS-PAGE after 1 hour. (**C**) IPTG treated BL21/FPG10.B3B11 cells or BL21/FPG10.A (specificity control) cells were lysed and subjected to SDS-PAGE on a 15% reducing gel. Gels were subsequently transblotted to nitrocellulose, and reactivity to B3B11, JB1A and OKT3 (control) was assessed.

A.

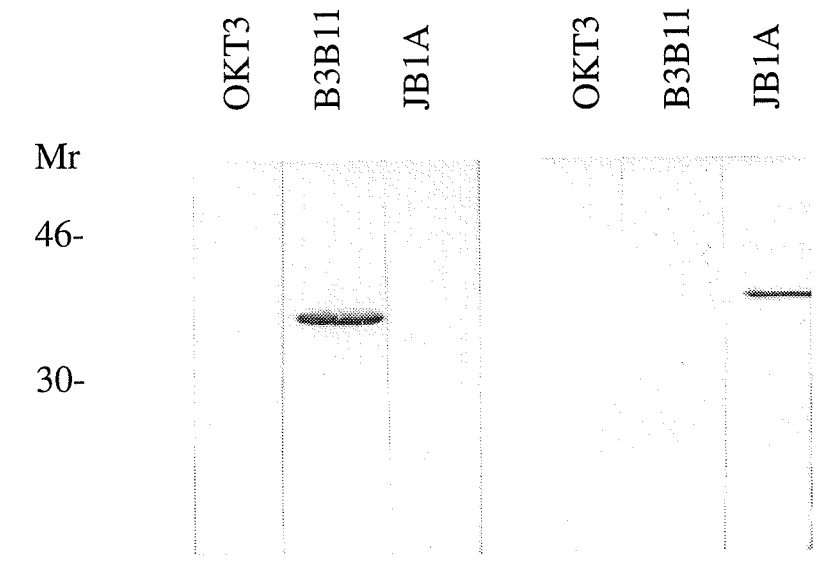


B3B11 JB1A

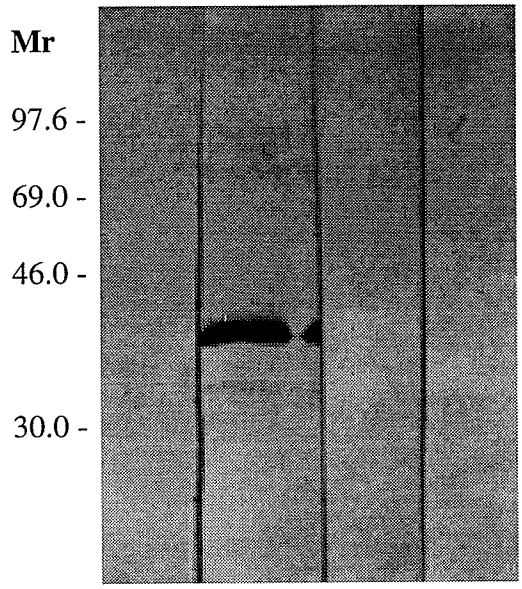
B.



C.



A. Western Blot



Lane: **A** **B** **C** **D**

Legend: **A: A16G6** **B: B3B11**
C: 21C8 **D: 13B9**

B. ELISA

Antigen

	FPG10.B3B11	**FPG10.HSVtag**
Monoclonal		
B3B11	**0.764 +/- .027**	0.013 +/- .001
aHSV	0.009 +/- .001	**1.348 +/- .002**
A16G6	0.013 +/- .005	0.018 +/- .003
21C8	0.099 +/- .018	0.054 +/- .006
13B9	0.020 +/- .002	0.014 +/- .014

Only B3B11 was specifically reactive with FPG10.B3B11 fusion protein. B3B11 was not reactive with control fusion protein G10.tag, which did react specifically with control αHSV monoclonal provided in the novatope kit. Collectively, these data suggest specific reactivity of B3B11 monoclonal with FPG10.B3B11 fusion protein. Further, the inability of B3B11 to react with other G10 fusion proteins suggests that the specificity resides within the inserted sequence.

DNA isolated from B3B11 reactive clones was isolated, and the insert was subsequently sequenced (by Dr Odd Bres and Shen Caixia). The insert was determined to be in frame and to code for a 35 amino acid sequence specific to the human ß1 integrin chain, amino acid residues glutamate-(636) to histidine-(670) (Figure 4.39). This sequence is predicted to be within the protease-resistant core of the integrin, within the most membrane- proximal cysteine-rich repeat motif.

4.3 Tryptic Fragments of ß1 integrin React with A16G6, B3B11 and B44

A proteolytic approach was utilized as a second method to investigate the location of B3B11 epitope relative to a competing (A16G6) and a noncompeting (B44) monoclonal. These antibodies were selected based upon their strong reactivity (ie.sensitivity) in western blot. The experiment was designed such that B3B11 (coupled to Sepharose beads) would be used to [1] purify ß1 integrins, and [2] protect ß1 integrin complexes during proteolytic digestion by trypsin. After proteolysis, and subsequent removal of cleaved peptides, the digest was subjected to SDS-PAGE, transferred to nitrocellulose, and assessed for the presence of products reactive with monoclonals by western blot.

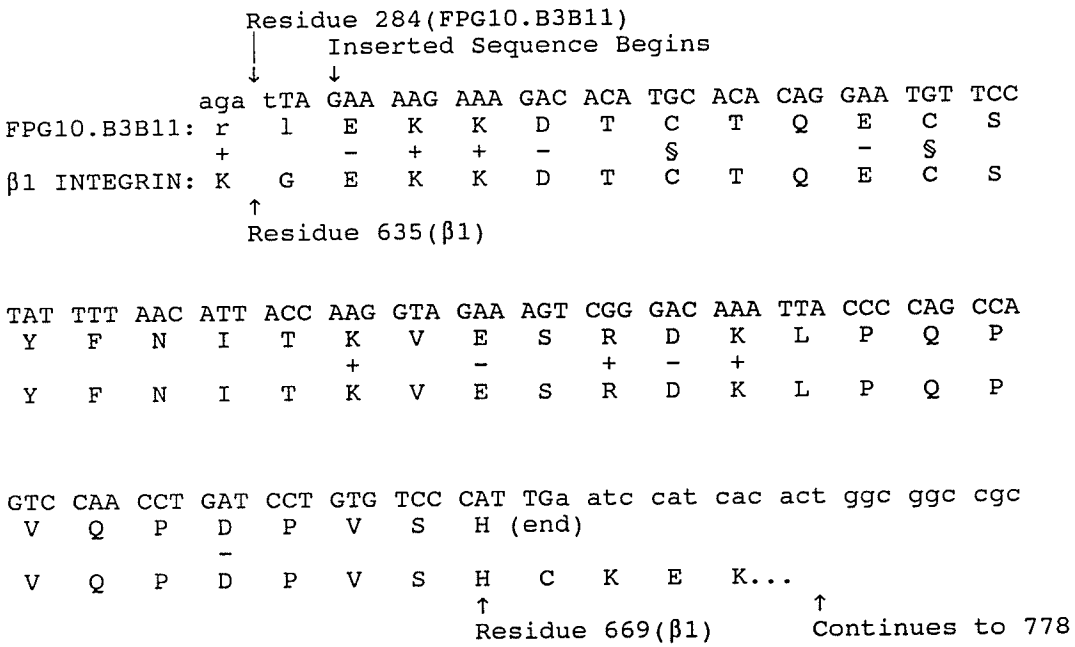
It was predicted that if the A16G6 epitope was located within the same region as B3B11, it should be protected from tryptic attack (Sheshberadaran and Payne, 1988). Therefore, A16G6 should recognize most, if not all, of the B3B11-column derived

Figure 4.39 The B3B11-reactive fusion protein inserted DNA sequence

Plasmid (pTope) DNA encoding FPG10.B3B11 was purified and sequenced by S Caixia and Dr O Bres. The unique sequence of the fusion protein, corresponding to the insert, is shown. Codons are translated in frame. Acidic (-), basic (+), and disulfide bond(§) forming residues are highlighted.

FPG10.B3B11

Insert Sequence (Capitals)



digestion products. In contrast, since monoclonal B44 did not compete with B3B11, it was predicted that the B44 epitope might not be protected by B3B11, and could therefore be susceptible to digestion by trypsin.

An immunoaffinity column was made using monoclonal B3B11, and ß1 integrin was purified by passage of Jurkat cell lysates. After thorough washing of the column, aliquots of column beads (with ß1 still uneluted) were digested with the protease trypsin or diluent-treated (controls). Beads were again extensively washed after digestion to remove all digested fragments not reactive with (ie., bound by) monoclonal B3B11. Aliquots of beads were then boiled in nonreducing Laemmli buffer to release bound antigen digests. The samples were probed for antigenic reactivity by western blot.

Three proteolytic breakdown products, each smaller than the original ß1 integrin, were resolved when the digest was probed with B3B11 (Figure 4.40). The peptides were approximately 110kDa, 80kDa and 55-65 kDa. The size of these fragments, and the lack of any smaller (further digested) proteolytic products is consistent with previous studies using ß3 integrin (Calvete et al 1991a, Du et al, 1993), in which the core of ß integrins was found to be strongly resistant to protease digestion.

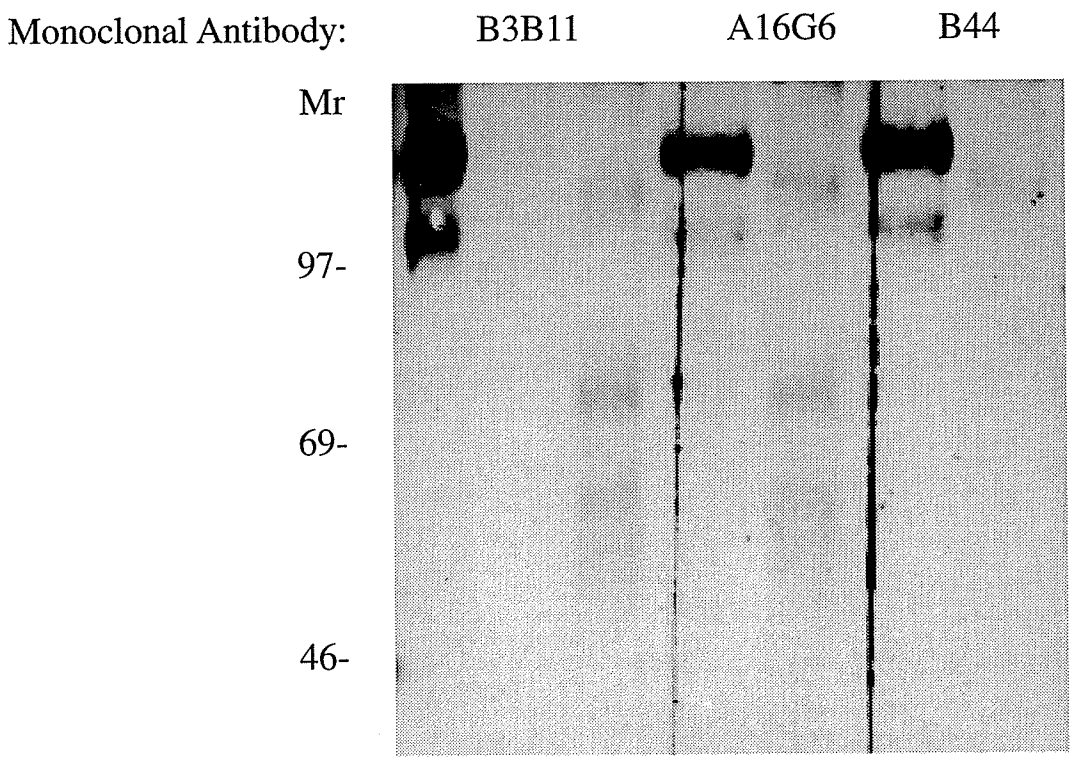
When monoclonal antibody A16G6 was used to probe the B3B11 column-derived digest, three products could be resolved, with molecular weights identical to those resolved by B3B11. Since the digested material was derived from a B3B11 column (and must therefore be B3B11-reactive by definition), it appeared that the cocompeting monoclonals A16G6 and B3B11 share reactivity with each identified tryptic product. The smallest fragment which could be bound by both antibodies had an apparent molecular weight of approximately 55-65kDa under the nonreducing conditions used (Figure 4.40).

The second antibody, monoclonal B44, which had not been observed to compete with B3B11 (or A16G6) for attachment to Jurkat cells was also used to probe the derived digests. In contrast to B3B11 and A16G6, monoclonal antibody B44 could only resolve

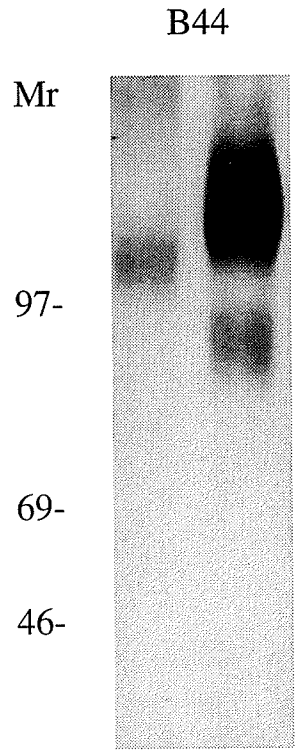
Figure 4.40 Antigenicity of B3B11-reactive tryptic ß1 fragments

(**A**) Jurkat cell lysate (lanes 1, 3-7) was allowed to incubate with protein-G Sepharose-B3B11 beads. After washing, beads and any material bound were subjected to tryptic (1mg/ml) digestion (lanes 2, 3, 5, 7) for 15 minutes at ambient temperature. Washed beads were eluted in Laemmli buffer and eluant analyzed by SDS-PAGE (5-20% gradient gel) and subsequent immunoblot. Reactivity with B3B11 column-eluted material was assessed with monoclonal B3B11 (lanes 1-3), A16G6 (lanes 4, 5) and B44 (lanes 6, 7). Note that no immunoreactivity was observed when the beads were digested and no Jurkat lysate (antigen source) was added (lane 2). (**B**) An enriched fraction (5x) of B3B11 column-eluted tryptic material was analyzed by SDS-PAGE (7.5% gel), immunoblotted and assessed for reactivity with B44.

A.


Monoclonal Antibody:
B3B11
A16G6
B44
Mr
97-
69-
46-
Lane:
1 2 3 4 5 6 7
Jurkat lysate added:
+ - + + + + +
Trypsin digested:
- + + - + - +

B.


B44
Mr
97-
69-
46-
1 2
+ +
+ -

the highest molecular weight digestion product, at approximately 110kDA (Figure 4.40A). Indeed, the lack of reactivity by B44 did not appear to be an artifact of due to gel-loading or reduced (rather than eliminated) antigenicity, as even very heavy loading (5 equivalents) of gels failed to demonstrate any antigenic reactivity (Figure 4.40B) .

These observations suggested that noncompetitively bound sites on the ß1 integrin could be dissociated by proteolysis. Further, the results demonstrate that A16G6 and B3B11 recognize the protease-resistant core of ß1 integrin, an observation consistent with the sequence obtained from the FPG10.B3B11 fusion protein (present within the cysteine-rich repeats) and the immunocompetitive studies. The reactivity of B44 with only the largest digestion product was consistent with loss of B44 epitope as one of the initial cleavage events in a sequential cascade, as has been described for integrin β3 (Calvete et al., 1991a). Thus, these results are suggestive of B44 recognition of a region of ß1 integrin other than final protease-resistant core (55-65 kDa fragment). Since the protease resistant core, under non-reducing conditions, is composed of [1] the cysteine-rich repeats, disulfide-bonded to [2] the N-terminal domain, (Calvete et al 1991a), the B44 epitope appears to reside in the ligand-binding domain (the only other extracellular domain).

Altogether, the mapping (competitive, proteolytic) data supports a model in which there are at least two regions on the ß1-integrin glycoprotein capable of functionally activating attachment mediated by ß1 integrin complexes. The existence of at least two regions is supported by the competitive data and the proteolytic "footprinting". The random fusion protein library, in conjunction with the proteolytic digestion, provide strong evidence that one region is present on the ß1-protease resistant core. The proteolytic data suggests, but does not absolutely prove, that the other region may be present within the protease sensitive ligand binding domain.

5.0 DISCUSSION

5.1 Lymphoid Cells Adhere to Extracellular Matrix Components

5.11 *Lymphoid cells display varied constitutive and activatable potentials for adhesion*

Of the human lymphoid cell culture lines which were tested for adherence, Jurkat cells were observed to possess the greatest capacity to attach to fibronectin, corresponding to OD 1.5 at 600nm under standard adhesion conditions. The phorbol ester PMA, which is an activator of protein kinase C (Brown and Juliano, 1988) was capable of increasing attachment of the Jurkat cells to fibronectin. This effect was maximal under limiting concentrations of fibronectin (2.5µg/ml) (Figure 4.2), approximately doubling adhesion (OD 0.8 to OD 1.8) in some experiments. When Jurkat cells were ^{51}Cr labelled and allowed to attach to wells coated with higher concentrations of fibronectin (5µg/ml), 60-70% of the cells displayed specific adherence. At lower concentrations of fibronectin (2.5µg/ml) , approximately 30-40% of the Jurkat cells adhered, and this was also approximately doubled by PMA (data not shown). The effect of PMA, however, was most evident on collagen attachment, where increases of 5-10 (Figure 4.6, 4.34) fold were common. The effect of PMA was blocked by staurosporine (Figure 4.34), an inhibitor of serine/threonine kinases with highest potency against protein kinase C. Indeed, the attachment induced by PMA was reduced after long term incubation (24 hours)(Figure 4.6) which may correspond with the ability of PMA to down-regulate pKC after long term treatment (Woods and Couchman, 1992). Although it is possible that staurosporine inhibited other, unidentified kinases, the most obvious explanation is that staurosporine inhibited its known primary target, protein kinase C. Of note, PMA does not upregulate adhesion in all lymphoid cells (Figure 4.9, 4.16), nor does staurosporine completely

abrogate adhesion (Figure 4.34).

Although Jurkat cells displayed a high adherence potential relative to other lymphoid cell lines, this level was still less than that of other, non-lymphoid cell lines, such as A-375, A-549, K-562 and U-937 (Table 4.1). It is interesting that these cell lines (with the exception of U-937 (a myeloid line), grow as adherent cultures. In adherent cell culture, integrin-mediated attachment to substratum appears to be required to prevent apoptosis (Meredith et al., 1993). In contrast, all of the lymphoid cell lines grow as suspension cultures. There appears to be no selective reason why these cell lines should retain the capacity to adhere to fibronectin-coated surfaces. This may explain why several of the cell lines, such as BJAB, CEM, and Cess do not appear to possess the capacity to attach specifically to extracellular matrix (fibronectin and collagen) coated surfaces (Table 4.1, Figure 4.19). However, it is clear that many of the lines did maintain the capacity to attach to components of the extracellular matrix, despite the apparent lack of constant utilization. The capacity to interact with fibronectin was varied, with some cell lines interacting weakly (eg., Daudi) and others more strongly (eg., Ramos) as assessed by cells remaining attached following washes of similar stringency (ie., performed on the same microtitre plate). The Daudi/Ramos comparison appears fitting, as both of these cell lines adhere via the same integrin structure ($\alpha 4\beta 1$)(Figure 4.20) in our studies, both express approximately equivalent levels of the integrin subunits as assessed by flow cytometry, and neither significantly increases adhesion in response to PMA (data not shown).

Other cell lines, such as JY, RPMI 8866 and JR2D3 displayed very little, if any, capacity to attach to fibronectin. However, when these cells were activated with PMA, a multifold increase in cellular attachment to fibronectin and vitronectin (10 to 20x) was observed (Figure 4.22, Figure 4.28). This response, which was mediated by integrin $\alpha v\beta 3$, appeared more dramatic than that induced by PMA in Jurkat cells. This is however,

likely a reflection that Jurkat cells do express a constitutive capacity to attach to extracellular matrix components. Indeed, the specific level of attachment after PMA activation, assessed by colorimetric assay, was always higher in the Jurkat cells than the JY cells (comparative data not shown, but see figure 4.27, PMA activated JY is similar to unactivated Jurkat on the 120kDa fragment of fibronectin). Importantly, the level of the appropriate integrins in either of these two cell lines, JY or Jurkat, was not influenced by PMA treatment (Figure 4.25 and Table 4.2). Thus, the effect of PMA was not to increase the number of receptors *per se*, but rather to increase their activity in some manner. For example, the activity of a receptor may be influenced by its mobility in the membrane (Olurundara et al, 1993).

5.12 *Are there physiological correlates to in vitro observations?*

This variety of phenotypes observed among the cell lines with respect to capacity to adhere to fibronectin might be explained in different ways. One manner of explaining the varied adherence potential may relate to the transformed status of many of the cells utilized in these studies. Many cellular transforming factors can influence cellular adhesion both positively and negatively. For example, the viral src gene can directly phosphorylate β1 integrin, and it appears that this prevents localization in adhesion plaques (Johannson et al., 1994), although varied cellular phenotypes are observed in rous sarcoma virus transformed cells (Horvath et al., 1990, Tapley et al., 1989). Oncogenic transfomation of cells can induce the expression of new integrin (Plantefabber et al, 1989, Cheresh et al., 1989 Kramer et al, 1991) and nonintegrin (Benchimol et al., 1987, Peach et al., 1993) adhesion molecules. Thus, the transformed status of many cells will clearly influence their expression of adhesion molecules, and also possibly their capacity to adhere to different ligands.

Alternatively, it is plausible that these cells may maintain certain characteristics that they possessed *in vivo* upon immortalization. Many of the cell types which undergo

dramatic changes in integrin expression upon transformation are nonhematopoietic. Primary cell culture is associated with slow growth, contact inhibition and a requirement for attachment (Meredith et al, 1993) in these cell lines. In contrast, freshly isolated peripheral blood lymphocytes may be activated to undergo a rapid proliferative phase, accompanied by changes in the expression of cell surface adhesion molecules (as seen in Figure 4.12). Activation of the T cells through the T cell receptor by antigen (Chan et al., 1991a), by CD3 or by CD2 (Shimizu et al., 1990c, van Kooyk et al., 1989) can activate cellular adhesion via integrins, and this adhesion appears to be integral to lymphocyte function, as adhesion may augment proliferation (Morimoto et al, 1989, Shimizu et al., 1990b) cytokine secretion (Godfrey et al., 1988, Offosu-Apiah et al, 1989a,b), and possibly even immunoglobulin secretion in B cells (Kemshed and Askonas, 1978). Thus it is possible that, in the most optimistic scenario, many of the lymphoid cell lines may represent cells "frozen" at some stage corresponding to a potential physiological counterpart. The utilization and regulation of specific adhesion receptors may mimick that occuring *in vivo*. If this is true, at the least the characterization of various binding structures on different lymphoid lines may provide clues as to binding structures which may be used physiologically. Indeed, it seems obvious that lymphocytes must use adhesion molecules as a means to traffick, become recruited to local inflammatory sites, or even to provide signalling events to transmit information into the lymphocyte regarding the external milieu. In those situations where the *in vitro* expression of adhesion molecules in lymphocytes has been compared to *in vivo* behaviour, good corellations have been found (Ferguson et al, 1991, Rodrigues et al, 1992 Haman et al, 1994).

Within the current studies, some similarities were observed between the Jurkat cell line and the activated T cells. Jurkat cells are not activated to proliferate or to adhere by the inclusion of OKT3 monoclonal antibody (data not shown), yet did demonstrate a constitutive capacity to attach to the extracellular matrix which was augmented by PMA

treatment. In this respect, they resemble PBLs which have been prestimulated by the monoclonal antibody OKT3 for 72 hours. These PBLs are more adherent than, for example, unstimulated T cells (Figure 4.11), they are not activated by the addition of further OKT3 antibody, and their adhesion potential is augmented by the addition of PMA. Additionally, both OKT3 stimulated PBLs (within the time frame examined) and Jurkat cells proliferate in culture. Thus, Jurkat cells appear to be a reasonable model of proliferative CD4+ T cells for adhesion and regulation experiments. Indeed, the principle receptors determined on Jurkat and T cell populations are the same (Figure 4.14).

The general observation (that isolated lymphocyte populations utilized integrins to attach to the extracellular matrix, and utilized them in a regulated manner) supported the use of lymphoblastoid cell lines to examine aspects of lymphocyte adherence. The common criticism of the use of cell culture lines (that they are no longer "physiologically relevant" due to tissue culture adaptation) appears to be less applicable within our model . Clearly, the characteristics of a tissue culture adapted cell line may vary from their physiological counterparts, and the capacity to adhere could conceivably be one such trait. However, the lymphoid tissue culture lines examined did not demonstrate common adhesion characteristics. No generalizations could be made regarding "culture advantages" of an adherent or nonadherent lymphoid phenotype, nor could any advantage be ascribed to specific adhesion structures. A heterogenous distribution of both overall adhesion potential and structures utilized was observed. Therefore, although it is clear that the tissue culture lines do vary somewhat from their physiological counterparts, they remained useful tools in the study of lymphoid adhesion by providing [1] a consistent, homogenous supply of cells for use in adhesion assays, and [2] a source of lymphoid-derived integrin for use as antigen and for protein analysis.

4.13 *Lymphocyte subpopulations express different adhesion molecules*

Populations of lymphocytes at differing stages of maturity and isolated from different locations may bear different extracellular matrix receptors (Carderelli and Pierschbacher, 1988, Sanders et al, 1989, Murakamie et al, 1990, Hemler, 1990, Erle et al, 1994a,b), and these structures are important in lymphocyte functions (Godfrey et al, 1988, Ferguson et al, 1991, Roberts et al, 1991, Rodrigues et al, 1992, Sundqvist et al, 1993, Galea et al, 1994). The differential expression of integrins has been observed on peripheral blood lymphocyte subpopulations. Memory (and thus, by definition, previously-activated) T lymphocytes possess increased levels of several adhesion molecules, including ß1 integrin (Sanders et al,1989). The stimulation of lymphocyte populations *in vitro* with anti-CD3 monoclonal antibodies has been observed to increase the expression of α4, α5, and ß1 integrins (Kohn et al,1991). Similarly, PBL stimulated with PHA will increase their expression of α1, α2, α4, α5, α6 and ß1 integrins (Hemler, 1990). The purified T lymphocytes which were examined during the current studies displayed a similar response when stimulated with OKT3 (Figure 4.12), as an increase in the expression of ß1 was observed. Of relevance to the previously discussed points, OKT3-stimulated populations, despite elevated expression of ß1 integrin, exhibited a relatively modest increase in adherence potential in the absence of PMA. However, the term "relative" is appropriate in this instance, as OKT3 stimulation still induces a 2.5 to 12 fold increase in the adherence of OKT3 stimulated cells compared to untreated controls. In the presence of PMA, OKT3 stimulated cells became highly adherent, approximately 30 to 40 fold more adherent than untreated controls. This serves to illustrate a major observed paradigm; Regulation of integrin-mediated adhesion can occur at two different levels; receptor expression and receptor function.

The observation that the OKT3-stimulated population were only "modestly" adherent may appear to contradict other reports (Shimizu et al, 1990c, Shimizu and Shaw,

1991) in which it has been demonstrated that OKT3 can activate isolated peripheral blood lymphocyte populations to become adherent. However, these "apparent" differences in observations may be reconciled in several different ways. Principally, the method of patching of the lymphocyte CD3 complex (thus eliciting activation) was different in the compared investigations. In the experiments performed by Shimizu et al, an anti-mouse polyclonal antisera was used to elicit CD3 capping, while in the current investigations, OKT3 was immobilized on the plastic of the tissue culture flask. A major difference this would create is that the OKT3 antibody would therefore be present within the microtitre wells during the adhesion assay in the reported study, but would not be in our own studies. Even if some small quantity was able to leach off the plastic of the culture flask, no secondary reagent would be present as an endogenous crosslinking agent.

A second observation is that in the studies of Shimizu et al (1990b, 1991) the induced adhesion was observed to be transient, decaying after 30 minutes. Within our own studies, the adhesion assay procedure was performed for sixty minutes. Therefore, any transient response might have been overlooked. The PBL in our studies were not assessed until they had been stimulated for 24 hours under the various stimulatory procedures. This might conceivably allow the extinction of transiently-induced adhesion signals.

A final point addresses assay sensitivity and consistency of assays between different laboratories. It is possible that the studies performed within this investigation were less sensitive than those of Shimizu et al (1990b, 1990c). Our wash protocol was determined to be more stringent (ie. forceful) than that of other labs (from personal communication with Dr Shimizu). However, to reiterate, we did observe an increase in adhesion potential of several fold with PMA or anti-CD3 stimulated populations as compared to untreated controls. However, this effect was overshadowed by the dramatic increase in adhesion potential observed when these two stimuli were combined.

5.13 *Integrin receptors may be differentially regulated on the same cell*

A very interesting phenomenon determined during these studies was that on the surface of the same cell, specific integrin receptors for the extracellular matrix could be differentially regulated. Selective usage could help explain the common expression of some integrin subunits on different lymphocyte subpopulations. The RPMI 8866 cell line provides an interesting model for discussion. This cell line expresses very low levels of ß1 integrin, but relatively high levels of α4 (Figure 4.20). The α4 chain has been demonstrated to be associated with ß7 integrin on this cell line (Erle et al, 1994b), and to be constitutively capable of binding to a 40kDa fragment of fibronectin via α4β7 integrin. This data agrees with our own observations that RPMI 8866 cells may attach to fibronectin via α4 containing integrin heterodimers (Figure 4.19A), that β1 integrin plays a minor role in attachment relative to α4 (Figure 4.19B), and that α4 is expressed in excess of β1(Figure 4.20), directly suggesting the presence of a second heterodimer.

This cell line also expresses the αVß3 integrin complex, but in a dormant or functionally down-regulated form. The addition of PMA activates this receptor, which binds the 120kDA fragment of fibronectin containing the RGD-type cell binding domain. This clearly demonstrates that a cell may express different integrins yet maintain them in different states of functional competence. This has clear implications in both processes of lymphocyte recruitment and retention. The α4 integrin complexes are also capable of mediating attachment to IGSF molecules (VCAM and/or MAdCAM)(Springer, 1990a, 1990b, Erle et al., 1994b), a property which may be important in initial recruitment of lymphocytes to trophic sites and/or sites of inflammation. The interaction of α4 with surface expressed VCAM on endothelial cells may induce the expression of proteins and or proteases as well as other activational signals required for subsequent extravasation through the endothelium (Burkly et al, 1991, Romanic and Madri, 1994). The same receptors might allow interactions with fibronectin sufficient to mediate cosignalling for activational

events (Davis et al, 1990, Shimizu et al, 1990a, 1990b, Nojima et al., 1990) as well as facilitating cellular migration (Springer, 1990b). If, in some subpopulation of these activated cells the events elicited the activation of the αVß3 receptor, then the properties of the activated cell would again change. The capacity to interact with fibronectin would be strengthened(by the addition of a second potential receptor), the range of ligands which the cell could interact with would be greatly expanded, and future signalling events might be modified might be modified by engagement of the newly activated receptors, thus altering cellular responses to future stimuli. An example of such an event is the observation that in certain murine intraepithelial lymphocytes, the "signal" from the extracellular cofactor fibronectin, required for IL-4 production, is dependant upon αVß3 receptor occupancy (Godfrey et al et al, 1988, Roberts et al 1991). The expression of dormant integrin in the presence of active integrin not unique to RPMI 8866 cells: similar phenomena were observed with RPMI 8226 cells (α5 active, α4, αV inactive)(Figure 4.19, 4.28), and Jurkat cells on fibronectin (α5 active, α4 inactive)(Figure 4.14).

A second example of apparently differential regulation of integrin complexes on the surface of lymphoid cells is provided by the Jurkat cell line. The Jurkat cell line expresses α2ß1 integrin in a relatively dormant form, while the α5ß1 integrin appears constitutively active, as assessed by cellular adherence patterns of unstimulated Jurkat cells, ie. strongly adherent on fibronectin coated surfaces, weakly adherent on collagen. This phenomenon might be partially explained by the observation that Jurkat cells express a higher number of functional α5ß1 complexes as compared to α2ß1 complexes. It could be argued that some threshold number of integrin receptors need to be engaged to facilitate cellular adhesion, and that only a small subpopulation of a given type of expressed integrin complex is competent to mediate adhesion. Thus it is possible that there are too few "competent" α2ß1 complexes to effectively engage the matrix. It would then follow that the various means of activating the α2ß1 integrin complex demonstrated (PMA, α2-specific, ß1 specific mAbs)

simply increase the proportion of α2ß1 integrins in an adhesion-competent state.

However, this theory appears somewhat unlikely. The combined activational protocols (monoclonal antibodies with PMA) can activate the α2ß1-mediated level of cellular adhesion to collagen to approximately that of fibronectin (ie., approximately 60% of cells attaching)(cf Figure 4.33, 4.35), which suggests that only a very small portion of the expressed Jurkat α5ß1 integrin complexes are actually used to mediate adhesion to fibronectin (no more than the total complement of α2ß1 receptors, assuming comparable binding affinities among integrins **and** that the α2β1 receptors are used 100% efficiently). Additionally (or alternatively) the activity of PMA may be much more highly specific for the induction of collagen adhesion relative to fibronectin adhesion. No reason for such an activity by PMA is immediately apparent; it appears to be a nonspecific activator of integrins of most types (Hynes, 1992). With regard to inside-out signalling activity to upmodulate integrins, the cytoplasmic tails of α2 and α5 have been documented to behave similarly (Chan et al., 1992a). Further, if the PMA is acting "more specifically" on elements of the extracellular domain of α2, it is clear that this activity can still be augmented further by exogenous stimuli, such as monoclonal antibody JBS2 (Figure 4.35).

Therefore, the most obvious supported model is that Jurkat cells differentially regulate their α2 and α5 containing integrin heterodimers. A somewhat similar situation is also observed in platelets, where one integrin is basally active (in this case α2β1) and a second integrin (αIIbβ3) requires activation, which can be facilitated by ligation of the active (α2β1) integrin (Staatz et al, 1989). It is not clear if this type of receptor cross-talk exists in Jurkat cells, the issue was never seriously addressed for a variety of reasons. However, in preliminary experiments, the Jurkat cells seemed to readily attach to collagen surfaces (OD > 0.5) which had been "spiked" with low levels (1ug) of fibronectin.(unpublished observations).

5.2 Identification of Integrin Utilization by Blocking Assay

5.21 *Rationale for using monoclonal antibodies to block adhesion structures*

The blocking assay was the principle means of determining the adhesion structures utilized by the various cell lines to mediate attachment to fibronectin and collagen. The basis of the blocking assay was derived from the assumption that it was possible to sterically inhibit the interaction of integrin with ligand. It was known that integrins interacted directly with ligand, and that the ligand binding site was composed of regions of both α and ß subunits. Therefore, it should theoretically be possible to determine the identity of the αß complex used by indexing the α and ß specific monoclonals capable of eliciting inhibition of adhesion to a given ligand.

Previous reports that certain integrins (αVß3, α5ß1, α2ß1) that had been studied could interact directly with column-immobilized ligand, such as vitronectin, fibronectin and collagen suggests that integrins play a direct, rather than passive (ie., upregulating alternative adhesion structures) role in matrix binding. This observation is central to rationalizations that the blocking assay functions by purely steric means.

5.22 *Signalling, coreceptors, and other complications*

Evidence has accumulated, however, that simple interaction with ligand is not necessarily the entire role played by integrins. A second possibility is that the engagement of integrin complexes at specific sites by monoclonal antibodies may induce signalling events which may modify alternative adhesion systems. A primary observation of integrin mediated signalling was that integrins can act comitogenically to induce lymphocyte proliferation (Matsuyama et al, 1989, Nojima et al, 1990). It appears that in part this is due to the induction of transcription factors which are required for transcription of lymphokine growth factors (Yamada et al, 1991). Rapid signalling events include the induction of

homotypic and heterotypic lymphocyte aggregation in response to low concentrations of antibody (Bednarczyk and McIntyre, 1990). More rapid still than either of these events (measured on a timescale of days and hours, respectively) is the phosphorylation of cytoplasmic proteins, which occurs within minutes of engagement of surface integrins by monoclonal antibodies, particularly α4 (Yoshihisa et al, 1992) and ß1 (Kapron-Bras et al, 1993). Other events include the loading of Ras proteins with GTP and the accumulation of cAMP within the cytoplasm (Kapron-Bras et al, 1993). These signalling events could conceivably influence the function of alternate cellular adhesion systems.

Evidence for such a process occurring is provided by the observation that a monoclonal antibody specific for the integrin α4 subunit is capable of mediating inhibition of attachment of a melanoma cell line to a fragment of fibronectin which lacks the α4 binding region (Iida et al, 1992). Attachment to this region of fibronectin is mediated by a heparin sulfate proteoglycan, possibly syndecan, and can blocked by the addition of competing heparin sulfate. This observation suggests that monoclonal antibody interaction with integrin subunits can directly influence adhesion mediated by alternative structures. A similar observation was made during characterization of the adhesion structures mediating JY cell line adhesion to fibronectin. It was apparent that multiple structures were used to attach to native fibronectin. Attachment to the 120kDa fragment of fibronectin was demonstrated by both antibody-blocking data and the use of synthetic peptides. The data further suggested that α4ß7-mediated attachment to a putative secondary site. This site could not be identified, as the JY cells were not adherent on the 40kDa fragment containing the known α4 integrin binding site. In reflection of the observations by Iida et al, an alternative interpretation is that the α4-specific monoclonal antibody may have contributed to the inhibition of the JY cell attachment to fibronectin by downregulating attachment mediated by alternative receptors, such as heparin sulfate proteoglycans (HSPGs). Attachment to native fibronectin may therefore have been mediated by αVß3 and cell-

surface HSPG, αVß3 and α4ß7, or possibly all three (or more!) receptors.

In specific regard to the latter possibility, it is possible that the α4ß7 and the HSPG moiety may act as coreceptors. Synergy regions within the fibronectin molecule have been previously determined which dramatically improve the attachment of α5ß1 integrin to the RGD domain of fibronectin, yet the mode of action of these sites is not known (Obara et al, 1988). Cell binding to the fragments of fibronectin containing the synergy regions are blocked by α5 or ß1 specific monoclonals, therefore it does not appear that a separate receptor is utilized. However, it is possible that a putative receptor for the synergy region works in concert with α5ß1, and becomes inoperative when the α5ß1 integrin is inoperative (or blocked). In support of this, a third heparin-binding region of fibronectin has been associated with the central region of fibronectin, an area which contains the identified α5ß1 synergy region (David, 1993). This model is in agreement with the α4ß7-mediated attachment: A heparin-binding domain is located approximately the same distance from the α4 binding site as the synergy regions are from the α5ß1 binding site (Yamada, 1992). Thus, an α4ß7-dependant coreceptor (possibly HSPG) may require a functional (unblocked) α4ß7 integrin to facilitate adhesion. Alternatively, the presence of the second interaction may serve to stabilize adhesive interactions.

The entire concept of coreceptors or alternatively "surface cofactors," has not been addressed within the current studies (and is a relatively recent development in integrin studies). It is clearly possible that multiple receptors simultaneously engage the fibronectin and collagen coated surfaces on the microtitre wells, and that it is the summation of these "events" which influences lymphocyte function. However, since the goal of these investigations was identifying those structures principally involved (as assessed by apparent contribution to adhesion) in mediating lymphoid attachment to extracellular matrix proteins; it was rationalized that the blocking studies might allow dissection of this process. At the outset of these studies it was not even clear whether integrins were receptors, per se,

or simple matrix-binding structures.

A second, more elaborate method to investigate contribution to attachment involves reconstitution of adherence by transformation of selected cell lines with cDNA encoding adhesion structures. This method, complementary to the blocking assay, has directly implicated α2ß1 expression in adhesion to collagen (Chan and Hemler, 1993), α5ß1 in adhesion to fibronectin (Schreiner et al, 1989), αVß3 in adhesion to vitronectin (Cheresh, 1991), and αIIbß3 in adhesion to fibrinogen and von Willebrand factor (O'Toole et al, 1991).

Collectively, there is evidence to suggest [1] that integrins mediate signalling (Matsuyama et al, 1989, Nojima et al, 1990, Davis et al, 1990, Yamada et al, 1991, Yoshihisa et al, 1992, Kapron-Bras et al, 1993) [2] that integrin expression is important in cellular attachment to extracellular matrix, and [3] that integrins can bind ligands directly. Additionally, at least one antibody to α4 integrin (P4G9) can inhibit cellular attachment to sites on fibronectin not known to be bound by α4 integrins (Iida et al, 1992). An important consideration is that although many α integrins may complex with the common ß1 subunit, each complex is a distinct receptor with distinctive properties conferred by the α subunit. Therefore, the modes of inhibition observed in the blocking assay could conceivably be distinct and even integrin heterodimer-specific.

In the case of α5ß1 and αVß3, the observations that these integrins interact directly with ligand, confer specific ligand binding into transfectants, (Cheresh, 1991, Schreiner et al, 1989) may bind to discrete identified portions of fibronectin (as small as 105 amino acids)(Pierschbacher et al, 1984), and may be eluted from ligand by peptide inhibitors (RGD peptides) suggests a specific and direct interaction with ligand. The simplest (and perhaps "most likely") model is that monoclonal antibodies specific to these molecules inhibit cellular attachment by steric hinderance to receptor occupancy. Similar observations have been made with respect to the α2ß1 receptor (in regard to collagen attachment)(Chan

and Hemler, 1993), and these observations should, in theory, be extended to considerations involving the α2ß1 integrin on Jurkat cells.

However, a case for direct interactions involving the α4ß1 integrin is decidedly weaker. There is currently no evidence which clearly demonstrates a direct interaction between purified α4 integrin complexes and fibronectin. While fibronectin binding of cell lines expressing only α4ß1 or α4ß7 may be abrogated by specific peptides present within the III-CS domain of fibronectin (LDV, REDV) (Garcia-Pardo et al 1990, Wayner et al 1991, Erle et al 1994b,), it is not clear whether this is due to a competitive interaction or purely via some signalling mechanism. Antibodies against α4 in particular seem highly capable of eliciting signalling events, such as modulation of cellular second messenger levels (Bednarczyk and McIntyre, 1990), phosphorylation of a 105kDa protein (Yoshihisa et al,1992), and induction of lymphocyte aggregation (Bednarczyk et al, 1993, Letarte et al, 1993, Shen et al, 1991). These events may be important in the modulation of a coreceptor, or alternatively, a completely separate receptor system. However, any suggestion of signalling by α4ß1 integrin must address the observation that on α4ß1-bearing lymphoid lines, attachment to fibronectin may be inhibited by either α4 specific or by several different ß1-specific antibodies. Therefore, if α4ß1 does not bind ligand directly, but rather regulates a secondary receptor, then interaction with ß1-specific antisera must also transmit similar signals. This suggests that the blocking of other receptors by these ß1 specific monoclonals (eg., α2β1) must occur concurrent with α4ß1 mediated signalling events, although these processes are unaffected by monoclonal antibodies to α4. Thus, although the α4 integrin-specific blocking data is weakest at directly implicating the α4 integrin receptors as adhesion structures (especially with respect to α4ß7), it is clear that the α4-integrin is integral to lymphoid adhesion to fibronectin.

5.23 *On the validity of using fragments of fibronectin...*

Recently (Sanchez-Apparicio et al, 1994), the α4ß1 integrin has also been demonstrated to be a receptor for the classical cell binding domain (CBD) of fibronectin. This region has been previously shown to be bound by only α5 and αV containing integrins.

This report would appear to undermine some of the experiments which were performed during these investigations. It calls into question the experimental rationale of utilizing different proteolytic fragments of fibronectin to identify receptor use (α4 binds 40kDa, α5 binds CBD) on lymphoid cell lines. However, the reported α4-mediated binding to the CBD, (which has been observed in the Daudi and Ramos cell lines), occurs specifically when these cell lines are stimulated by monoclonal antibody TS2/16 (but not by PMA). TS2/16 is an adhesion-inducing monoclonal antibody which apparently binds to the ß1 integrin ligand-binding domain (Takada and Puzon, 1993) and subsequently induces some conformational change. It is not yet clear if these results are physiologically relevant. If treatment of the cells with monoclonal antibody TS2/16 does mimick a physiological activational signal, then the ramifications are profound; α4ß1 may exist in a variety of activational states, and can bind an increasing number of ligands as a reflection of activational status. A similar model has been previously proposed by Chan and Hemler (1993), to display differential ligand binding capacity of the α2ß1 integrin when expressed in nonlymphoid cell lines. The obvious alternative is that mAb TS2/16 may activate these cell lines in a completely physiologically-irrelevant manner. Further investigations will be necessary to resolve this issue completely. In either event, none of the experiments performed during these investigations (involving fragments of fibronectin) were performed in the presence of adhesion activating monoclonal antibodies, and therefore retain their originally intended validity.

5.24 *Interpretations of integrin expression and utilization*

With the limitations implicit to the interpretations of the blocking studies forwarded, the results of these studies may be discussed with respect to those performed by other investigators. Three important general observations come from these (and other) adhesion structure characterization studies; [1] different lymphocyte populations appear to use common integrin receptors to interact with fibronectin and collagen, although populations vary in their expressed integrin repertoire, [2] expression and apparent utilization of integrin complexes were not always coincident, and [3] integrins expressed on lymphocytes appeared to function in a regulated manner.

The first observation appears to contradict the rationale for studying integrin repertoires on different lymphocyte subpopulations. An original hypothesis, that in order to traffick in distinctive manners, lymphocytes might express different repertoires of adhesion receptors, did not appear valid. The only cell-specific receptors suggested by these investigations were the αVß3 and α4ß7 integrins, which were only observed on EBV+, IgG+ B cell lines (JY, RPMI 8866, and JR2D3)(Table 4.6). However, this observation cannot withstand careful scrutiny. The α4ß7 receptor has been observed on the surface of T cells (Erle et al, 1991, 1994b), and the αVß3 receptor has been observed on the surface Jurkat cell clones (E6) available from ATCC (J Wilkins, unpublished data). With respect to integrin utilization patterns, various lymphocyte populations were observed to utilize α5ß1 or αVß3 to attach to the 120kDa fragment of fibronectin. There is circumstantial evidence to suggest that α4ß1 might also be involved in fibronectin attachment to the 40kDa fragment of fibronectin (HEP-II, III-CS domain), and further that JY α4ß7 might adhere to fibronectin at an uncharacterized site. In light of the previously discussed signalling vs blocking debate, the details of this process remain to be elucidated. A summary of the integrins observed to mediate lymphoid attachment to fibronectin and collagen in these studies is presented in TABLE 5.1.

TABLE 5.1 SUMMARY OF OBSERVED INTEGRIN UTILIZATION

INTEGRIN	KNOWN LIGANDS	LIGANDS OBSERVED	OBSERVED LYMPHOID CELL-TYPE UTILIZATION
α2ß1	COLLAGEN	+	Some T & B LYMPHOID LINES
	LAMININ	-	ACTIVATED BULK $\alpha\beta$ T CELL ISOLATES, T CELL CLONES
α3ß1	COLLAGEN	-	T & B LYMPHOID LINES
	LAMININ	-	BULK T AND B ISOLATES
	FIBRONECTIN	-	T CELL CLONES
α4ß1	FIBRONECTIN	+	PRESENT ON ALL LYMPHOCYTES
	VCAM	-*	EXAMINED, FUNCTIONAL ON MOST.
α5ß1	FIBRONECTIN	+	SOME T & B LYMPHOID LINES
			BULK T AND B ISOLATES
			T CELL CLONES
αVß3	FIBRONECTIN	+	ACTIVATED B CELL LINES
	COLLAGEN	+	(Typically ß1-deficient)
	VITRONECTIN	+	

LEGEND.
Integrin heterodimers observed on lymphoid cells are listed with known ligands. The observed utilization patterns (form the current investigation) are listed in the third row, and the cell types observed to utilize the integrins in the fourth.
* Adhesion to VCAM not assessed within these studies.

5.25 *α5β1 and α4β1 as lymphocyte fibronectin receptors*

The α5ß1 receptor was observed on the surface of both T cells and B cells. The discovery and report of α5ß1 expression and utilization on B cells was novel; it was previously reported specifically that α4 integrins mediated B cell attachment to fibronectin (Garcia-Pardo et al, 1990). On all α5 expressing lines observed (with the exception of the γδ T cell clones, discussed below) the α5 integrin appeared to be the predominant receptor for fibronectin. Blocking α5 integrin with monoclonal antibody was sufficient to strongly inhibit binding of all α5 bearing cell lines, while the effect of α4 blocking reagents on these was very weak. The Jurkat cell line adheres much more strongly to a fragment of fibronectin bearing the α5 site (equivalent to that observed on intact fibronectin) than to the fragment bearing the α4-specific binding site, despite a higher expression of α4 (>α5) integrin on the surface of Jurkat cells.

It appears that the α4ß1 receptor can mediate high adherence potential in a cell line. The Ramos cell line, which is moderately adherent (OD approximately 0.900) expresses only α4ß1 integrin on its surface (Figure 3.20, Garcia-Pardo et al, 1990). This adherence can be inhibited by either α4 or ß1 specific antibodies, implicating α4ß1 integrin as a fibronectin receptor. The Ramos and Jurkat cell lines express comparative levels of α4ß1 though it appears that the α4ß1 performs differently in these two cell lines. This may result from several factors, including the possible expression of α4ß1 integrin-specific adhesion supporting infrastructural components.

Alternatively, it is possible that in the Jurkat cell line (as compared to Ramos), the α4ß1 integrin is directly influenced by the presence of other integrins. It is conceivable that the α4ß1 integrin competes with other integrin structures (such as α2ß1 and α5ß1) for access to common cytoplasmic elements required for adhesion. In support of this, it is clear that α4 chimeras which bear α2 and α5 cytoplasmic domains behave similarly to each

other, but very different than wild type α4. The chimeric constructs (α4/α2 α4/α5) support strong adhesion (tightly immobilized cells) in comparison to cells expressing wild type α4 (highly mobile cells, less adherent) (Chan et al., 1992a).

Early reports lend support for this. The concept of "integrin hierarchy" was first introduced by Plantefabber and Hynes (1989). This concept was originally observed in α5ß1/α3ß1 integrin expressing carcinoma cell lines. In cell lines which expressed only α3ß1, this complex was observed to be the major fibronectin receptor. However, in cell lines co-expressing α5ß1 and α3ß1, the α3ß1 integrin appeared to lose functional status, and the α5ß1 heterodimer became the primary fibronectin receptor. The results of the current investigation are consistent with this report. In none of the lymphoid cell lines studied was α3ß1 observed to be a contributing fibronectin receptor. The results of the current experiments might therefore be considered to expand the putative hierarchy of integrin to α5ß1 > α4ß1 > α3ß1 in lymphoid cell lines.

However, γδ cell clone attachment to fibronectin was inhibited completely by monoclonal antibodies directed against either α4 or α5 integrin. One interpretation of this result is that both of these receptors play a crucial role in the observed attachment to fibronectin. The contribution from each may be required to facilitate a threshold level of interaction necessary for adherence. Alternatively, blocking one receptor might effect disengagement of the second, presumably by some signalling mechanism previously alluded to (Section 5.21).

The observations in γδ cells is contrasted by data obtained with the Jurkat, JR2B10 and RPMI 8226 cell lines, as well as the bulk stimulated B and T cell populations, which maintain adhesion to fibronectin in the presence of an α4-specific blocking monoclonal. It is possible that γδ cells maintain distinct means to regulate the same integrin adhesion receptors relative to other lymphocyte populations.

5.25 *α2β1 is a lymphoid collagen receptor*

The only integrin heterodimer identified which to mediated attachment to collagen was α2ß1, although both α3ß1 and α1ß1 have been observed to be collagen receptors in nonlymphoid cell lines. In those cases where ß1 integrin mediated the attachment to collagen, it was always determined to be mediated by the α2ß1 complex. These results are in agreement with other reports, in which only α2ß1 has been demonstrated to be a collagen receptor in lymphocytes (Goldman et al, 1993). While all populations which expressed α2ß1 were determined to be adherent on collagen coated surfaces, α2β1 could not always be identified as the sole receptor for collagen. The RPMI 8226 and γδ T cell bulk populations, which did express low levels of α2ß1 and were collagen adherent, could not be inhibited with α2 specific monoclonal antibodies. Thus it appears that alternative receptors were utilized as the principle collagen receptors in these cells. Possible alternate receptors identified on lymphoid cells include heparin sulfate proteoglycans such as syndecan (Elenius et al, 1990), the chondroitin-sulfated form of CD44 which binds to the N-terminal region of collagen fibrils (Jalkonen and Jalkonen, 1993) and CD26 (Gorrell et al, 1991).

Among the B cell populations examined, only the RPMI 8226 and JR2B10 cell lines express the α2ß1 collagen receptor, and neither of these B cell lines secretes immunoglobulins (8226 does secrete lone λ chains)(Table 4.6). A previous report had suggested that further study of B cell interaction with collagen was warranted (Kemshed and Askonas, 1978), as gelatin positively influenced IgG secretion *in vitro.*

Interestingly, none of the IgG+ B cell lines (IM-9, JY, JR2-D3, RPMI 8866) examined express α2ß1. Further, when B cells were isolated from tonsil (Figure 4.21) or peripheral blood, no attachment to collagen was observed (unpublished observations). The current investigation does not provide support for α2ß1 integrin as the collagen receptor

which facilitates the observed increase in IgG secretion. There are differences between the studies, however. It is not immediately evident that the B cells were actually adherent to gelatin-coated plastic surfaces in the original report. Further, gelatin will differ from native type I and II collagen in several aspects, possibly offering sites for receptors other that $\alpha 2\beta 1$. It is also noteworthy that populations of B cells which express CD44 (which binds collagen via a chondroitin sulfate moiety) at high levels have been identified to be enriched in IgG-secreting cells (Murakamie et al, 1990).

5.27 *Varied roles for the same structure on different lymphocytes?*

If lymphocytes predominantly utilize similar structures to adhere to common elements of the extracellular matrix, it becomes difficult to account for variations in lymphocyte recruitment/retention, trafficking and adhesion observed in vivo and in vitro (Berg et al, 1989, Hu et al, 1992). The current studies suggest that rather than different structures being utilized on different lymphoid lineages, each lineage appears to vary clonally with respect to adhesion receptor complement. Different $\delta\gamma$ T cell clones express varied levels of specific integrins, even expressing patterns in apparent contradiction to the heterogenous parental population. Thus, different $\gamma\delta$ T cells will express and utilize slightly different combinations of common receptor types, creating an inherent bias in adhesion characteristics of different $\gamma\delta$ T cells.

Similarly, the JR2B10 and JR2D3 populations are derived from the same parental bulk population, but display different integrin repertoires (having only integrin $\alpha 4$ in common), different potentials for adhesion, and different ligand specificities. The observation that these two lines had different "resting" (ie., unstimulated) adhesion potentials is of interest, because it represents a further mode of differentiation between lymphocyte subsets. Despite the fact that for the most part, lymphocytes use common structures (often integrins), to adhere to different components of extracellular matrix, two

lymphocytes which express the same receptors can behave differently according to the activational status of the integrin. For example, the Ramos and IM-9 cell lines both express α4ß1 integrin, but only the Ramos cells bind to fibronectin (Figure 4.18). Therefore, in addition to expressed repertoire, the concept of "functional repertoire" might be appropriate.

Cells which either lack α4ß1 functionality or α4ß1 expression may not be interchangeable concepts. Only if the original recruitment (or stimulus) during a physiological process (such as trafficking), absolutely requires functional α4 might these two different states may be functionally interchangeable. However, if a different receptor (for example, L-selectin) is involved in initial cellular recruitment, subsequent activational events might differentially influence a cell expressing "downregulated α4" as compared to a cell completely lacking α4 expression. This example could apply to many other functionally regulated receptors (including other integrins), on lymphocytes. This particular example is somewhat less applicable to α4 than to other integrin receptors, as the only lymphocytes yet identified which actually lack α4 expression are immature T and B cells (Hemler, 1990).

The regulated function of the integrins expressed on lymphocytes appears to be a feature which is a characteristic of the lymphocytes themselves. As an example, the αVß3 integrin was not known to be functionally regulated prior to its description on certain B cell lines in the course of the current studies. The integrin αLβ2(LFA-1) is a very important integrin in cell:cell contact, and is highly regulated. Yet when cDNA for this integrin is expressed in a nonhematopoietic cell line, the integrin was discovered to by constitutively active (Larson et al., 1991). Similar results are obtained when expression of the highly regulated platelet integrin αIIbß3 integrin is reconstituted in K-562 cells (O'toole et al, 1991). These observations support the contention that hematopoeitic cells carefully regulate their adhesive potential. Integrin regulation is an observed characteristic of

hematopoeitic cells; other cells typically express integrins which are continuously in an active state (except perhaps during cell division, or during certain processes of cellular differentiation, resulting in deactivation of functional integrin (Hodivala and Watt, 1994).

It is not clear how integrins are regulated. Integrins might be basally inactive, and require some cofactor, such as "Integrin Modulatory Factor" (Hermanowski-Vosatka et al, 1992) or "Integrin Associated Protein" (Brown et al, 1990) for activation. It is also possible that integrins are basally active, but may be inactivated by direct interaction with cell-specific constituents (negatively regulated). A variation of this possibility is that certain cell types may modify their integrins (presumably through some posttranslational, ie. covalent, modification) to an inactive status, and that this status is readily reversible. Given [1] that the expression of ß1 integrin on lymphocytes is low in comparison to most tissues, [2] that tissues depend upon integrins constantly to assist in maintenance of general tissue structure, and [3] the fact that integrins probably evolutionarily predate lymphocytes (Hynes 1992), it is perhaps more likely that integrins are negatively regulated in lymphocytes. However, none of the models proposed is absolutely exclusive of the others.

The induction of integrin-mediated adhesion with PMA suggests that the "neutralization" of negatively-regulating factors is a rapid process. Alternatively, nonlymphoid cell lines may constitutively possess cofactors necessary for adhesion, while nonadherent lymphocytes might require rapid induction or activation of these cofactors to elicit integrin-mediated adhesion. Although a number of events which trigger lymphocyte-integrin functional activation have been recorded in the literature, including stimulation by CD2 and CD3 specific monoclonal antibodies (van Kooyk et al, 1989), exposure to antigen (Chan et al, 1991a), and treatment with ionophores or phorbol esters (Brown and Juliano, 1988), the molecular interactions involved in the process of activation have only recently begun to be studied.

5.3 Regions Important in the Regulation of ß1 Integrin Function

5.31 *A novatope approach to identifying regulatory regions of ß1*

Different strategies have been employed to determine which factors are involved in integrin regulation, including probing extracellular regions of ß integrins or ß integrin chimeras with antibodies (Coller, 1986, Altieri et al, 1988, Keizer et al., 1988, van Kooyk et al, 1991,Kovach et al, 1992, van de Wiele-van Kemenade et al, 1992, Kapron-Bras et al, 1993) or with other proteins such as peptides and snake venoms(Parise et al., 1987, Rojiani et al, 1991, Li et al, 1993, Wright et al, 1993). To map the specific regions important in integrin regulation and function, either mutational/chimeric constructs were utilized in conjunction with these characterized probes (Shih et al, 1993, Takada and Puzon 1993), or purified integrin was digested and binding location was determined by microsequencing (Smith and Cheresh, 1988, Calvete et al, 1989-1992, 1994). Additional clues were provided by naturally occurring mutations associated with loss of integrin function, such as leukocyte adhesion deficiency (LAD)(Kishimoto et al, 1989, Arnaout et al, 1990)(β2 integrin disease) or Glanzmann's thrombasthenia (Coller et al, 1991, Chen et al, 1992)(β3 integrin disease). Our strategy was to randomly digest ß1 integrin cDNA with DNAse in order to generate random fragments for insertion into and expression in a fusion-peptide library. This strategy was used successfully by another lab concurrently with these studies to map an activating region of β3 (Du et al., 1993).

The original observation, that certain ß1-specific monoclonal antibodies have the capacity to activate ß1 integrin-mediated attachment to extracellular matrix, provided a means to map the location of putative regulatory regions. This concept was not unique to the current investigation. In addition to the six ß1-specific and one α2-specific activating monoclonal antibodies presented within these studies, three other activating monoclonal antibodies specific for human ß1 have been reported in the literature (A-1A5, TS2/16, 8A2)

(Hemler et al, 1983, van de Wiele-van Kemenade et al, 1992, Kovach et al, 1992, respectively) The mapping of activating epitopes recognized by monoclonal antibodies has been attempted as a means of understanding modes of integrin regulation.

Chimeric and mutant constructs of ß1 and ß3 have allowed the mapping of the activating, membrane-distal site of functional regulation to a region in the ligand binding domain(Takada and Puzon, 1993) and a region adjacent to the membrane (Shih et al, 1993)(Du et al, 1993). The membrane distal site requires sequences from two sections of the ligand binding domain thought to be proximal as a consequence of tertiary structure. These sites occur between amino acid residues (115-141) and (200-271) in ß1 integrin trans-species chimeras. (Note that there are two kind of chimeras; chimeras of two species, ie., mouse/human β1, and chimeras of different integrins within a species, ie., β1/β3 human chimeras). The membrane-proximal region was mapped to the region between the third cysteine-rich repeat and the membrane (493-680 in ß1 integrin)(Shih et al., 1993). These regions were not localized further, and in at least one case (A-1A5, Takada and Puzon, 1993) reactivity of the monoclonal antibody was inconsistent throughout the study. This illustrates a significant disadvantage in the creation of chimeric constructs; it is never clear if a given construct will assemble properly. Glycosylation sites and nuances of folding may vary between species. For example, the mapped human B3B11 epitope is highly homologous in mice, yet B3B11 does not appear to react with mouse cells in flow cytometry (tested once, CTLL2 cells). It is possible that this epitope is available for reactivity in Western blot, but this was not tested. (This observation is made even more interesting by the later findings of H Ni, who could localize the B3B11 epitope to a five amino acid sequence which is absolutely conserved in mice)(H Ni, J Wilkins unpublished data). Since proteins are three-dimensional structures, the modification of one region may modify epitopes which are distant along the linear primary sequence, but are proximal due to tertiary structure. Thus the conformation, known to be important in

integrin activation, may be modified by the construction of chimeras or mutants.

One advantage of the chimeric constructs, however, was that any monoclonal which recognizes cells by either flow cytometry or radioimmunoprecipitation could be utilized in rapid screening. While many might be "lost" or misinterpreted due to the limitations discussed, a large number of monoclonals could be rapidly screened. In contrast, only two antibodies from the panel utilized in these studies (Table 4.7) were positive on the novatope screen (Monoclonal antibodies JB1A and B3B11). The epitope recognized by JB1A (an inhibitory antibody) was further localized by Caixia Shen using the novatope approach and by Nina Hunt with a phage library (Devlin et al, 1992). The JB1A epitope is located in a region linking the ligand binding domain to the N-terminal domain, between amino acids 83 and 90. The apparent distance of this epitope from the B3B11 site is supported by the immunocompetetive data (Table 4.8) which suggests JB1A and the B3B11 epitopes are not proximal to each other. The putative regulatory regions identified during these studies, in addition to that determined for JB1A, are illustrated in Figure 5.1A.

Clearly, the reactivity of different monoclonal antibodies will dictate their usefulness in different approaches to map regulatory regions. For example, monoclonal antibodies which do not perform western blot, or are specific to carbohydrate epitopes, are of limited usefulness for immunoscreening prokaryotic libraries. No reactivity in western blot is listed for any of the literature-described (A-1A5, TS2/16, 8A2) antibodies, which is a possible indication that they do not perform well under these conditions. Monoclonal A-1A5 was not able to blot when tested within our lab (C Shen, D Stupack, J Wilkins, unpublished data).

Among those activating monoclonals produced during the course of these studies, three proved excellent for performing western blot (B3B11, B44, N29), while two were of limited usefulness (A16G6, 13B9, functional in nonreducing conditions). A single

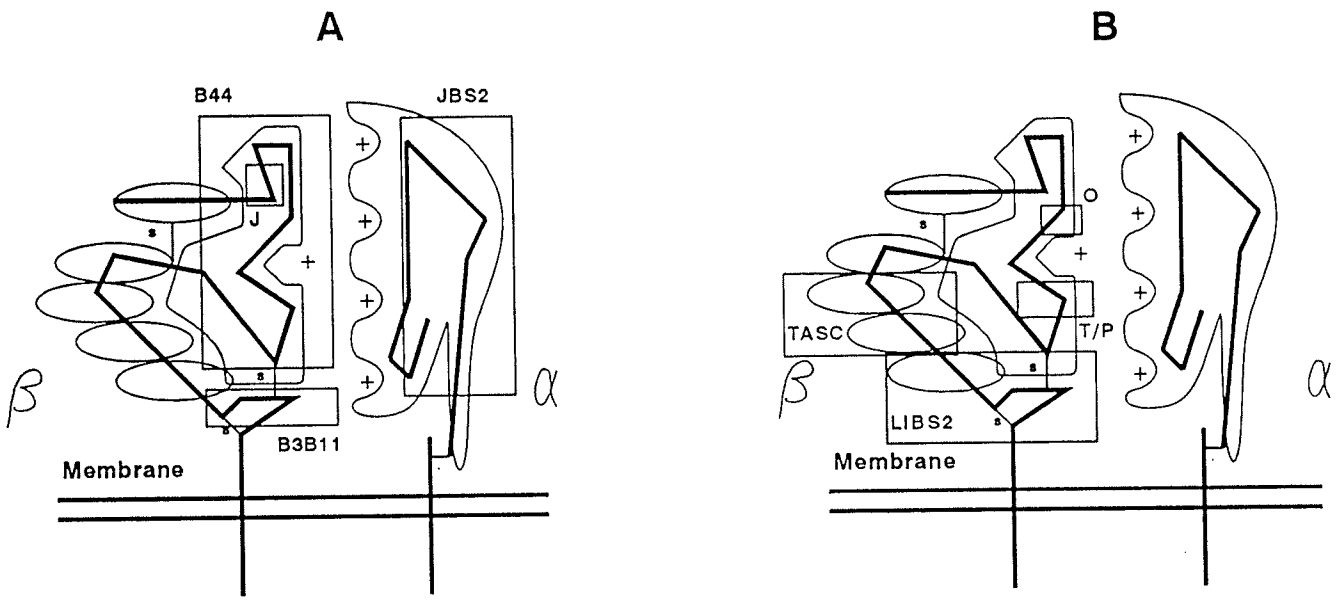
activating monoclonal was never observed to exhibit activity in western blot (21C8). All of the blot-positive antibodies were utilized to screen the pECE-ß1 derived novatope expression library. However, the only activating monoclonal to yield a positive colony in the current generated library is B3B11, although the library was subsequently reprobed (Caixia Shen). With respect to monoclonal antibodies A16G6 and 13B9, which seem dependant upon nonreducing conditions (and therefore probably disulfide bonds), no positives may have been observed due to a requirement for larger cDNA inserts in the fusion peptide (to form the para-conformationally dependant epitope).

This observation has been documented in studies on ß3 integrins. The adhesion - activating monoclonal antibody termed LIBS-2 (which activates the integrin αIIbß3) is positive for reactivity in western blot, but is highly sensitive to reducing conditions. The LIBS-2 monoclonal antibody requires an insert size of at least 602-669(ß3) to facilitate recognition of fusion protein (Du et al, 1993). The monoclonal antibody is not functional against smaller fragments of ß3 (even under nonreducing conditions). Monoclonal antibody A16G6 may recognize an area of β1 similar to that recognized by LIBS-2 in β3. The A16G6 epitope is similarly reduction sensitive, it maps to the homologous region of ß1 integrin indirectly (by its observed competition with B3B11), and both LIBS-2 and A16G6 react with the protease resistant core of their respective β integrins (Figure 4.40 and Du et al, 1993). The identified B3B11 epitope also reacts with the protease resistant core of β1, and has been mapped within a homologous region of ß1 (635-669) to that identified in ß3(602-669)(Du et al, 1993). Altogether, these observations suggest that a reduction-sensitive, function-activating epitope may reside in this region of ß integrins.

The reason for lack of reactivity of the N29 and B44 monoclonal encoded epitopes is not self-evident, as these epitopes are very active in reducing western blots of Jurkat cell lysates. Several explanations may address the lack of reactivity within the novatope system. It is possible that these epitopes are coded for by regions of DNA which are "hot

Figure 5.1 Proposed Regulatory Regions of Integrin Heterodimers

A. The regions suggested to be regulatory during the current study are highlighted on the schematic diagram of integrin, including the membrane proximal region bound by B3B11 (ß1 635-669). The monoclonal B44 has putatively been assigned to the ligand binding domain(ß1 101-373, based on conserved proteolysis of ß3), and the JBS2 epitope has been assigned generally with the α2 subunit. (However, the since JBS2 binds to Jurkat cells in the presence of EDTA, it does not appear that the divalent-cation binding domains contribute to the JBS2 epitope.) The inhibitory JB1A site, identified by C Shen, and N Hunt, is indicated with a J. **B.** Domains suggested to be regulatory in other ß integrin studies include the TASC (ß1-avian 493-602) and LIBS-2 (ß3 602-669) sites discussed in the text, as well as site identified by the chimeric mapping studies of Takada and Puzon (T/P)(ß1 211-219). One other (O)(ß1 120-140, ß3 110-130) inhibitory site intimately involved in the binding of RGD sequences (in RGD-dependant receptors) and possessing the conserved divalent cation binding motif is also boxed (ß110-142).


A
B44
JBS2
J
B3B11
Membrane
β
α
B
TASC
T/P
LIBS2
Membrane
β
α

spots" for random digestion by DNAse. During the creation of DNA fragments to ligate into the expression (pTope) vector, the epitopes recognized by these monoclonals may be commonly destroyed. Alternatively, it is possible that these monoclonals recognize epitopes formed by post-translational mechanisms not present, or not active, within the prokaryotic expression system. Examples of post-translational modification might include methylation of lysine residues, sulfation or phosphorylation of tyrosine residues, or (perhaps most likely), O- or N-linked glycosylation. In support of the final possibility, preliminary investigations using Jurkat-derived integrin lysates suggest that B44 is lost upon the chemical removal of carbohydrate (A Li, J Wilkins, unpublished observations). In combination with the proteolytic data generated in section 4.4, this observation suggests that B44 epitope may reside on a carbohydrate-dependant epitope within the ligand-binding domain of ß1.

The tryptic footprints of B3B11-protected ß1 integrin support the contention that A16G6 recognizes the same region of ß1 as B3B11, while B44 recognizes only the initially produced proteolytic product. It is not clear if the digestion of ß1 with trypsin mimicks exactly the digestion of ß3 (the only ß integrin analyzed extensively by proteolysis and cosequencing) (Calvete, 1994). However, due to the high degree of homology between ß1 and ß3 integrins, it would be expected that they would be digested in approximately the same manner. As a conservative estimate, if one assumes that the largest digestion product (110kDa) does not lack any of the extracellular domain, but rather lacks the transmembrane and cytoplasmic domains, then one could still predict that the B44 epitope be localized to within ß1 102-373. This location would be within the ligand binding domain, and is consistent with investigation employing mutational (Calvete, 1994) and chimeric (Takada and Puzon, 1993) approaches implicating this region in the regulation of adhesion. The study of Takada and Puzon suggests the region 200-271 may be a target site for monoclonal-mediated upregulation. With respect to B44 reactivity being glycosylation

dependant, there exists two N-glycosylation sites in this linear stretch of peptide, N-192 and N-249 (Argraves et al, 1987). O-glycosylation sites remain to be characterized.

5.32 *Several sites are known to regulate β integrin function*

Several different domains within an integrin complex have been reported to be associated with the upregulation of ligand binding. These include the ß integrin ligand-binding domain (including region around residue 130(ß1) and 200-230(ß1) (Takada and Puzon, 1993, Calvete, 1994), the ß integrin membrane-proximal region (602-669 ß3, 493-602 ß1-avian)(Du et al, 1993, Shih et al, 1993), and the ß integrin cytoplasmic domain (O'toole et al, 1994, Laflamme et al, 1994). On α integrins, three additional regions, the "I" domain (not present on all integrins)(Randi and Hogg, 1994, Muchowski et al, 1994, Kamata and Takada, 1994, Diamond et al, 1993), the divalent cation binding regions (Loftus et al, 1990, Smith and Cheresh, 1991b) and the cytoplasmic domain (Chan et al 1991a, O'toole et al 1994) have been associated with the regulation of integrin function. It is possible that more regulatory regions have yet to be identified.

The current study identifies monoclonals which appear to recognize at least two different sites of activation of ß1 integrin. The site recognized by B44 (and therefore perhaps by 13B9, based on competitive data), putatively in the ligand binding domain, and the membrane proximal regulatory region. The latter region has been previously reported in the literature in both the human ß3 integrin (ß3 602-669, identified by mAb LIBS-2) and the avian ß1 integrin (avian ß1 493-602, identified by mAb TASC). The mode of function of this region is not absolutely known, it is not clear how interactions at this site activate adhesion within the ligand binding domain. The binding of monoclonal LIBS2 to αIIbß3 can activate adhesive potential of the ligand binding site, located approximately 12-16nm distant, as assessed by rotary shadowing EM (Du et al, 1993).

The TASC antibody has been suggested to induce integrin attachment to collagen or laminin by causing displacement of the α and ß subunits relative to one another, due to increased access afforded a second monoclonal antibody, called "G". "G" recognizes ß1-specific sequences at the putative αß integrin interface (Shih et al, 1993). Thus it has been postulated that separation, or lateral movement, of the α and ß subunits is a necessary requirement in integrin activation. The B3B11 epitope, which has been mapped to ß1 635-669, and cocompeting monoclonal antibodies A16G6 and 21C8, may conceivably activate ß1-mediated adhesion through similar means.

If adjacency of α and ß membrane-proximal (or transmembrane) sequences can downregulate integrin function, this might explain a requirement for lateral displacement of the subunits to facilitate ligand binding. Further, it might also explain why integrins which lack transmembrane and/or cytoplasmic domains are observed to be constitutively active (O'toole et al, 1994, Hayashi et al, 1990, Marcantonio et al, 1991).

There is good evidence to suggest that the ß1 integrin cytoplasmic domains may modulate integrin function by modulating integrin distribution. The cytoplasmic sequence alone contains the information required for localization within adhesion plaques (LaFlamme et al, 1994), presumably through cytoplasmic interactions with proteins capable of binding the ß1 cytoplasmic domain, such as α-actinin (Otey and Burridge, 1990a, 1990b, Otey et al, 1994), pp58vsrc (Tapley et al, 1989), and talin (Horwitz et al, 1986, Geiger et al, 1992). ß1 or ß3, but not α5 or αIIb cytoplasmic-domain Taq-constructs can successfully compete with and prevent ß1-mediated interactions with cytoplasmic elements, disrupting adhesion. Additionally, ß1 cytoplasmic deletions fail to localize to sites of adhesion, thereby further implicating this region in modulation of cellular adhesion (Laflamme et al, 1994). Further evidence for cytoskeletal interactions are provided by recent observations that cadherin chimeras bearing ß1 cytoplasmic domains will recruit talin to cell:cell tight junctions, a location it is not otherwise located (Geiger et al, 1992). It appears the

cytoplasmic domain of ß1 is important in the regulation of the avidity of interaction of the external domains with ligand. Since focal adhesions are rich sites for protein kinases (Kanner et al, 1989), PMA may influence the stability of these structures. It is possible that protein kinase C may activate a phosophatase within or adjacent to focal adhesions, as cytoplasmic tyrosine-phosphorylated ß1-integrin does not localize to adhesion plaques (Johansson et al, 1994). A polyclonal antisera directed against a phosphorylated form of the ß1 cytoplasmic domain does not stain adhesion plaques, but rather a diffuse distribution throughout the membrane. Antisera directed against nonphosphorylated ß1 stain adhesion plaques strongly (Johansson et al, 1994). Thus, pKc may actually be involved in the stabilization of adhesion plaques by the activation of phosphatases, rather than kinases. It is clear that pKc is also required in the receptor-signalled turnover of adhesion plaques (Hall et al, 1994) to facilitate migration.

5.33 *The α integrin regulatory regions*

The inserted, or I domains of the integrins αM (Diamond et al,1993b), αL Randi and Hogg, 1994) and α2 (Kamata and Takada, 1994) have been implicated in the regulation of ligand-binding specificity and affinity of αß integrin complexes. Additionally, the divalent cation domains been implicated in the regulation of α integrins, by both circumstantial evidence (changes in divalent cation concentrations may change both ligand binding potential and specificity) (reviewed in Hynes, 1992) and one directed study which demonstrated antigenicity and ligand binding capacity as a function of the coordination sphere of a bound ion (Smith and Cheresh 1991b). Additionally, the epitopes recognized by certain activating monoclonal antibodies which have been characterized (NKL-16, anti-αL, van Kooyk et al, 1994, mAb 24,anti-αM, L, -X, Dransfield et al, 1992) are dependant upon divalent cations.

The existence of activating monoclonal reagents specific for α integrin subunits supplies precedent for the existence of JBS2. No α-integrin specific monoclonal has previously been identified which activates a ß1 containing heterodimer (eg., α2-ß1). The JBS2 epitope is maintained in the presence of EDTA, and is therefore does not appear dependant upon divalent cations (unpublished observations). This suggests that the JBS2 epitope may be independent of the divalent cation-binding sites.

The α2 integrin is one of only two ß1 partners (α1 is the other) which possesses an I domain. There is no direct evidence to suggest that JBS2 binds to the I domain, however, this region is known to be regulatory in α2 (Kamato and Takada, 1994) and might represent an initial site to begin future investigations.

The α integrin cytoplasmic domains have been implicated in the regulation of the αß integrin heterodimers, although the precise mechanism is not known. The control of the external domains by internal sequences has been suggested to mediate inside→out signalling. Within these studies, only PMA was used to activate Jurkat adherence in a (presumably) inside→out manner. It is possible that PMA-mediated activation could also lead to protein-binding (or release) of cytoplasmic regions of the α integrin subunit. This model allows integrin complex-specific activation. Discrete integrin heterodimers could be activated by protein interactions with their distinctive cytoplasmic domains.

However, since all α integrins also possess a conserved KxGFFKR amino acid sequence immediately adjacent to the transmembrane domain, one might also observe coordinated activation. The specific KxGFFKR region has been demonstrated to be required for down-regulation of the human αIIb/ß3 integrin in a CHO expression system (O'Toole et al, 1994). Mutants lacking the complete cytoplasmic domain are constitutively active (including secreted forms which lack a transmembrane domain)(Dana et al, 1991, Murata et al, 1991), while regions which terminate after KxGFFKR require activation (ie.,

treatment with PMA) to elicit function. However, it is clear that sequences C-terminal to the KxGFFKR also influence integrin regulation, as αIIb (external, transmembrane)/α5 (cytoplasmic)-chimeras possess constitutive binding activity, while native αIIb integrins are down-regulated. Further, mutation within specific post-GFFK sequences can dramatically influence cellular adhesion (O'Toole et al, 1991, 1994).

5.34 *Interrelationship of Activating Regions*

The activating reagents, in combination with the Jurkat cell line, (which expresses low collagen binding potential) allowed combinatorial studies of the activation of α2ß1 integrin. Jurkat cell attachment to collagen could be induced by treatment with PMA, JBS2, or ß1-specific monoclonal antibodies. The stimuli provided by JBS2, PMA and A16G6 were studied in detail. These reagents were found to cooperatively induce attachment to collagen.

The contribution of PMA could be inhibited with staurosporine, an inhibitor of serine/threonine protein kinases, and especially protein kinase C, in the presence or absence of the stimulating monoclonals. In contrast, activation mediated by the monoclonal antibodies alone was not affected by staurosporine. These results, in conjunction with other reports in the literature (van Kooyk et al, 1989, Shimizu and Shaw, 1991), demonstrate a difference in PMA-dependant and non-PMA dependant activational events.

Neither α2 nor ß1 monoclonal antibody activated events were influenced by staurosporine, yet it appeared that they were distinct events as a cooperative increase in binding was observed when these reagents were used at low (synergistic) or saturating (nonsynergistic, not purely additive) concentrations. A simple model for the monoclonal antibody-mediated induction of adhesion is the stabilization of an external conformation of integrin which is competent to adhere; however, this external adhesive form of the integrin can be further stabilized by the presence of intracellular activating signals supplied by

PMA. Thus, PMA probably influences a different regulatory site than monoclonal antibodies.

PMA activation of protein kinase C will influence many cellular systems. The activation of protein kinase C alters the lipid composition of the inner membrane leaflet as well as influencing the ionic potential of the cytoplasm (Schwartz et al, 1991a,b). Since membrane composition will influence the activity of integrins (Smith et al, 1992, Conforti et al, 1990), it is possible that the modification of membrane composition alone is required for the activation of ß1 integrin. Additionally, activated protein kinase C phosphorylates proteins on serine and threonine residues, many of which are themselves (regulated) protein kinases (Meredith et al, 1993, Woods and Couchman, 1992). This cascade could also result in the activation of integrin-binding proteins or complexes, or integrin-modifying enzymes.

For example, the polymerization of actin may lead to integrin clustering on the surface of Jurkat cells, thus increasing the avidity of interactions, effectively increasing cellular adherence (Otey and Burridge 1990b). Since at least three proteins, α-actinin (Otey et al, 1990a), talin (Horwitz et al, 1986) and the SH2 domain of v-src kinase (Geiger et al, 1994) have been demonstrated to bind to the cytoplasmic domain of ß1 integrin, it is likely that others may as well. It is clear that the cytoplasmic domain of the ß1 integrin appears important in the localization of integrin heterodimers to sites of adhesion. This capacity to localize at adhesion sites is conserved in chimeras bearing only the cytoplasmic domain of the ß1 molecule(Laflamme et al, 1994). Conversely, the recruitment of ß1 integrin to sites of adhesion can also be negatively infuenced by phosphorylation on cytoplasmic tyrosine residues (Johansson et al, 1994).

Thus it is clear that protein kinase C might may activate adhesion through several means. (PMA-activated protein kinse C might influence membrane composition, actin polymerization, α or ß cytoplasmic domain binding proteins, and protein kinase and protein

phosphatase activity.) Each of these activities are independent of external monoclonal antibody binding, yet could conceivably influence cellular adherence in the presence of the monoclonal antibody. For example, binding A16G6 might, as suggested for other monoclonals binding in the membrane proximal region (Shih et al, 1993), effect separation/rotation of the α and ß integrin domains. However, in the absence of supporting internal signals, the monoclonal may never be fully effective; indeed, it is even possible that the cytoplasmic domains may be bound by a cytoplasmic regulation complex in direct conflict with the mode of activation of the monoclonal.

The monoclonal antibodies may activate integrin adherence through a variety of means. As previously mentioned, the simplest possibility is that monoclonal-binding stabilizes a form of ß1 integrin which is more competent to adhere to ligand. However, if the monoclonals function purely through this means, then the data from the competitive assay suggest that several sites on an integrin heterodimer are functional in stabilizing adherence.

Site 1: A16G6, B3B11, 21C8 **Site 2**: N29

Site 3: B44, 13B9 **Site 4**: JBS2, (α2ß1 specific)

Further, the ability of monoclonals to cooperate (JBS2 + A16G6) suggests that discrete conformational/activational states exist within antibody-bound integrin heterodimers. This suggests highly regulated, multiply influenced, integrin- ligand interaction.

An alternative possibility is that the monoclonal antibodies elicit signalling events which result in changes in integrin regulation. In support of this, phosphorylation of a 50kDa protein and GTP loading of ras has been observed following JBS2 and 21C8 binding to Jurkat cells (Kapron-Bras et al, 1993). This evidence suggests a means of modulating integrin adherence with external signals, yet utilizing internal control mechanisms. The monoclonals induce internal signals which result in the upmodulation of

integrin activity. However, if these signals were identical to those induced by PMA, one might not expect additivity in the combinatorial experiments. Indeed, PMA was observed to upregulate the GTP loading of ras in these studies.

Importantly, no direct correlation has been made between a requirement for the integrin-mediated signalling events and the induction of cellular adhesion. Since integrins which have bound ligand, or specific monoclonal antibodies, are known to transmit signals into a cell, these observations may reflect signalling resulting from the integrin-antibody interaction. Since monoclonal antibodies may activate solubilized integrins to bind to immobilized ligand-columns, cellular cytosolic constituents do not appear to be an absolute requirement for monoclonal-induced adhesion (Arroyo et al, 1992, Chan and Hemler, 1993).

Collectively then, the evidence suggests that lymphoid integrins may be negatively regulated by internal cellular factors. Further, it does not appear that the external monoclonal antibodies influence integrin-mediated attachment through the same means as internal signals, despite the observation that the ligation of integrin by monoclonal antibodies may elicit signalling events. Based upon mapping or mutational experiments performed within this investigation and others, it appears that integrins may have several different conformations which lead to activation. In addition to the rotation/separation of the membrane proximal domains putatively assigned to the membrane proximal regulatory region (Shih et al, 1993), and the discussed possible cytoplasmic interactions of both α and ß cytoplasmic domains, it is clear that interactions with other regions of a heterodimer may elicit change.

Since JBS2 and N29 have not been mapped, it is not clear how these monoclonals upregulate adhesion. JBS2 might bind to the I (regulatory) domain of $\alpha 2$ as suggested, thus influencing adhesion. However, JBS2 might alternatively bind to a membrane proximal domain of $\alpha 2$, thus influencing adhesion in a manner similar to that postulated for

TASC (and possibly A16G6, B3B11, 21C8), but with an α specific, rather than ß specific, target site. Since A16G6 and JBS2 induce adhesion cooperatively, the latter scenario suggests that A16G6 cannot optimally separate/rotate the α2ß1 integrin to induce adhesion. Future investigations will be required to determine the role of JBS2 in α2ß1 activation.

Similarly, N29 has not been mapped. It appears to recognize a unique site on the surface of β1 integrin relative to the other activating monoclonal antibodies (Table 4.8). Flow cytometric analysis of the nonadherent IM-9 cells (Figure 4.18) suggests a differential distribution of ß1 integrin-specific activating epitopes (unpublished data). The distribution of the N29 epitope appears similar to that of A16G6 (and B3B11, 21C8) as compared to B44 (and 13B9). The strong reactivity of monoclonal N29 in western blot may prove useful in the future characterization of this epitope.

5.35 *Carbohydrate effectors as physiological activators of integrins*

The rotation or removal of a single carbohydrate group can dramatically affect access to a given epitope (Wilson et al, 1988). It may be that in addition to protein conformational considerations, accessibility of either ligand binding or regulatory regions of integrins may be influenced by carbohydrates. The binding of oligosaccharide moieties on detergent solubilized αLß2 (LFA-1) by recombinant, soluble P-selectin increases affinity for immobilized ICAM-1 (Gahmberg et al, 1992). Thus, a precedent is set for alteration of integrin affinity by interaction with associated carbohydrate. One simple model to explain this phenomenon is that carbohydrate masks an important binding site which is displaced by the binding of carbohydrate by selectin (or putatively, monoclonal antibody). Alternatively, carbohydrate binding by P-selectin may simply be a corequisite for binding of secondary domains, which themselves alter integrin conformation. However, the observation that integrins with aberrant glycosylation patterns are deficient in adhesion suggests a more integral role for carbohydrate (Symington et al, 1989, Akiyama

et al, 1989, Kawano et al, 1993, Kitigawa and Paulson, 1994). In contrast to variant glycosylation, a lack of processing of carbohydrate precursors on integrins does not appear to seriously impact adhesive potential (Cheresh, 1991). This data further suggests that a cell-specific means of controlling integrin function might relate to cellular glycosylation machinery.

The data generated in this investigation suggests and supports a multi-factor regulation of lymphocyte integrin ligand binding activity. The synergy of effect observed between JBS2 and A16G6 suggests potential physiological strategies for upregulation of specific, dormant integrins. Soluble P selectin may be present within serum at a concentration of 1-2µg/ml (Gahmberg et al, 1992); the addition of secondary stimuli at local sites may effect larger increases in adhesion than observed with single stimuli alone (Figure 4.35). The L selectin is also observed to be cleaved (Kahn et al, 1994), but no role has yet been demonstrated for the soluble form. Given that common adhesion receptors are expressed on lymphoid cell types with divergent trafficking patterns, distinct methods of adhesion receptor regulation may be required. The regulation of integrins may be coupled to, or perhaps dependant upon, the expression of other cell-specific cofactors or coreceptors.

5.36 *The end of the beginning...*

The identification of regulatory regions of ß1 integrin are a small first step towards future investigations directed at a molecular understanding of integrin function. An understanding of the nature of putative regulatory factors within the cytoplasm and those potentially present extracellularly should increase our capacity to manipulate integrins and therefore integrin-mediated responses. Obvious targets for ongoing research include the role of carbohydrate, the identification of specific cytoplasmic regulatory elements, dissection of the PMA and nonPMA modes of activation, and downstream consequences of

cell interaction via integrin ligands. The growth of research on integrins has been enormous since 1986. On average, an integrin-concerning paper is now (November, 1994) published every day. However, efforts must again be redoubled if we are to facilitate an understanding of the varied roles of adhesion in development and disease. Through this, we will further our capacity to devise therapies against and manipulate adhesion dependant processes, impacting directly on metastasis, thrombosis/hemostasis, transplant rejection and rheumatic disease.

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