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I am submitting herewith a thesis written by Lokesh Kumar Wuluvarana entitled " Cloning and Evidence of an Alternatively Spliced Isoform of Lymphocyte Function Associated Antigen – One." I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Life Sciences.

Robert E. Hall, Major Professor

We have read this thesis
and recommend its acceptance:

Robert N Moore

Accepted for the Council:

Associate Vice Chancellor and
Dean of The Graduate School

CLONING AND EVIDENCE OF AN ALTERNATIVELY SPLICED ISOFORM OF LYMPHOCYTE FUNCTION ASSOCIATED ANTIGEN - ONE

A Thesis
Presented for the Master of Science
Degree
The University of Tennessee, Knoxville

Lokesh K. Wuluvarana
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DEDICATION

This thesis is dedicated to my parents

Mr. Appaiah Wuluvarana and Mrs. Anusuya Wuluvarana

and brothers

Dr. Hemant Wuluvarana and Mr. Nanda Wuluvarana

who gave me unconditional love and support during the course of my degree.

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ABSTRACT

Lymphocyte associated function - one integrin (LFA-1 - CD18 / CD11a) is an adhesion molecule which is expressed in cells of leukocyte origin. Antibodies against LFA-1 are known to block T cell responses to antigen presenting cells, T cell help to B cells, NK mediated killing, macrophage killing of tumor cells, and leukocyte endothelium adhesion / extravasation. In this study, the presence of an alternatively spliced isoform of LFA-1 (designated as LFA-1 minor) was demonstrated in T cells, B cells and monocytes. A 114 bp deletion was identified at the extracellular region of CD11a (αL), which is very close to the transmembrane domain of LFA-1. Computer analysis showed that the deleted region contains a casein II kinase phosphorylation site. Northern blot analysis indicated the possibility of several other alternatively spliced transcripts of CD11a. Further experiments showed that the expression of the alternatively spliced isoform of CD11a was modulated by the mitogens like Con-A, LPS, and PMA. It is suggested that the alternatively spliced isoform may have different binding affinity and/or signalling properties and may play an important role in the regulation of the function of LFA-1.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

Ca^{2+}	Calcium ions
cAMP	Cyclic adenosine monophosphate
CD	Cluster of differentiation
cDNA	Complementary deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
COOH	Carboxy terminal
CNS	Central nervous system
Con-A	Concanavalin-A
DTT	Dithiothreitol
DEPC	Diethyl pyrocarbonate
ECM	Extracellular membrane
EDTA	Ethylene diaminetetraacetic acid
HCl	Hydrochloric acid
KCl	Potassium chloride
Kda	Kilodaltons
MAb	Monoclonal antibody
Mg^{2+}	Magnesium ions
$MgCl_2$	Magnesium chloride
Mn^{2+}	Manganese ions
Mr	Molecular weight
NaCl	Sodium chloride

NH_2	Amino terminal
PMA	Phorbol myristate acetate
RNase	Ribonuclease
SSC	3M Sodium chloride 0.3M sodium citrate
Tris-HCl	Tris (hydroxymethyl) aminomethane hydrochloride

CHAPTER I

INTRODUCTION

Integrins are cell surface heterodimeric receptors that in the presence of divalent cations promote both cell-matrix and cell-cell interactions. An individual integrin is composed of a single α and a single β subunit, each of which spans the plasma membrane. Thus integrin represents the connection between the extracellular environment and the intracellular compartment (11).

The adhesion of cells to each other or to elements of the extracellular environment is crucial in many aspects of physiology. Cellular growth and differentiation, development, hemostasis, wound repair, and lymphocyte recruitment and extravasation are a few of the important process which require the proper and regulated adhesion of various cell types. The adhesive properties of blood cells are mediated in part by families of cell surface receptors with distinct structural and functional characteristics. These families include the selectins, cadherins, and those receptors with immunoglobulin like domain.

Each integrin β subunit (90-110 KDa) can complex with a distinct set of α subunits (120-180 KDa) creating heterodimers with characteristic binding specificities. Table 1* shows all the known integrins and their ligand(s). The topographies of α and β are similar. The majority of each is extracellular, and N-terminal sequences of both

* All figures and tables may be found in the appendix.

subunits comprise the ligand binding pocket. β-subunits have a number of cysteines which form extensive intramolecular disulfides. α-subunits typically contain 3-4 repeats of a putative divalent cation binding motif and some undergo a post-translational cleavage (45).

Functions of Leukocyte Integrins

Since the early studies of Metchnikoff in 1880s, a large body of data have been accumulated that established a crucial role for circulating polymorphonuclear leukocytes (PMN) in host defense against pyogenic infections. These studies also suggested the contribution of PMN to host defense and tissue destruction. Generating monoclonal antibodies to leukocyte surface antigens and characterizing these antigens led to better understanding of cell adhesion and its significance in the process underlying both beneficial and detrimental roles of PMN. These studies established the critical role of CD11/CD18 leukocyte adhesion molecules in vivo and have generated novel means for controlling PMN-induced tissue injury (43).

Leukocytes continuously circulate in the body screening for the presence of altered cells, infecting microbes and foreign antigens. Lymphocytes enter lymphoid organs through the high endothelial venules and remain there for sometime, reappear in lymphatic vessels and return to the blood. Specific immune recognition is essential for immune responses, and here Class I and Class II transplantation antigens play a central role by interacting with the T-cell receptor complex. But the specific immune recognition is physically weak and therefore, other molecular systems have developed in order to

strengthen and regulate such interactions. But these molecules are also utilized for leukocyte accumulation in various tissues and for several other non-immune leukocyte-target-cell interactions (20). Lymphocyte – extracellular interactions utilize both activation -independent and dependent adhesion receptors. Lymphocyte homing into peripheral nodes is targeted by primary interaction of L-selectin with carbohydrates of the peripheral node HEV addressin; homing to intestinal Peyer's patches may involve by primary adhesion to the mucosal vascular addressin. Trafficking to both these lymph node tissues involves the activation-dependent integrin LFA-1 (8, 9, 44).

The term "leukocyte integrins" is slightly ambiguous and is probably outdated. In the early years, the integrin family was neatly subdivided in subfamilies on basis of β-subunit usage. Thus, the β1 subfamily was identified with VLA proteins, the β2 subfamily was referred to as leukocyte integrins, and the β3 subfamily was known as cytoadhesins. This classification also appeared to mirror the functional involvement of β1 and β3 integrins primarily in cell-extracellular matrix (ECM) interaction, which β2 integrin appeared to participate mostly in cell-cell adhesive function. (11)

Leukocyte specific integrins are called β2 integrins or CD11/CD18 according to the cluster of differentiation antigen nomenclature. Thus molecules have a common β2-chain (CD18), and presently 4 different α-chain have been described (CD11a,b,c,d). CD11a/CD18 (LFA-1) is enriched in lymphocytes, CD11b/CD18 in neutrophils and CD11c/CD18 in monocytes and macrophages. (20).

Recently, a novel β2 integrin was discovered and was designated as CD11d (αd). The expression of CD11d/CD18 was prominent in macrophages in specific red

pulp, lymph node regions and bone marrow. In peripheral blood, the αd expression was limited to a minor subpopulation of $CD8^+$ T-cells, which included small lymphocytes and large granular lymphocytes. (15).

CD11a/CD18 (LFA-1) is expressed on all leukocytes and serves as general adhesion molecules involved in leukocyte homotypic and heterotypic interactions. CD11a/CD18 plays a particularly important role *in vitro* in the proliferation of T cells to antigen and mitogen, in the interaction of cytotoxic T cell and NK cells with their targets cells, in the activation of B cells and their differentiation into immunoglobulin –secreting cells and in leukocyte –endothelium adhesion. CD11a/CD18adhesion functions are in part mediated by CD54, an endogenous ligand with wide cell distribution (10, 35).

All integrins evidently assemble in the endoplasmic reticulum from two polypeptides coded for by genes on separate chromosomes. The CD18 gene is on chromosome 21 and in fact patients with trisomony 21 (Down's Syndrome) express increased levels of the CD11/CD18 integrins and show enhanced adhesion. Such individuals often suffer from repeated infections, which could mean that excessive adhesion may be harmful. The α-chain genes (CD11 a,b,c) are clustered on chromosome 16 (20).

An infrequent recessive autosomal disorder known as leukocyte adhesion deficiency (LAD), which is genetically inherited results from pan-β2 integrin deficiency in leukocyte surface expression. This genetic deficiency has been corrected *in vitro* in cells derived from LAD patient by transfection of a wild-type β2 subunit, thus completely restoring expression and cell adhesive functions to the αLβ2. The typical symptom of

LAD patient are recurrent bacterial and fungal infections affecting mainly the skin, gut and mucous membranes, impaired pus formation, poor wound healing and delayed severance of the umbilical cord. LAD patients exhibit persistent leukocytosis, yet monocytes fail to emigrate to inflammatory sites, as evidenced by necrotic lesions conspicuously devoid of leukocytes (11).

The Leukocyte Function Associated-1 molecule (LFA-1) is involved in cell-cell adhesion. This is a heterodimer consisting of αL (Mr = 180,000) and β2 (Mr = 95,000). The N-terminal sequencing of the α-subunits in the mouse and human has suggested that LFA-1, Mac-1, P150,95 are structurally related. N-terminal sequencing of the α-subunits in the mice and humans has suggested that they are structurally related; however, the cell-surface expression and function of these molecules differs. LFA-1 is expressed on virtually all leukocytes and is involved in a large number of adhesion-dependent phenomena. mAb directed against LFA-1 inhibit antigen-specific T-helper cell function and cytolytic function such as cytotoxic T-lymphocyte mediated killing, antibody-dependent cytotoxicity by granulocytes and natural killer activity. LFA-1 is also involved in antigen-independent interactions that mediate cell localization to sites of inflammation such as leukocyte adhesion to endothelial cells, fibroblasts, epidermal keratinocytes and synovial cells. (29). By the use of mAB, it appears that LFA-1 and ICAM-1 interactions are crucial for the maturation of CD 8^+ T cells (38).

Ligands of LFA-1

CD11a/CD18 binds to the immunoglobulin domain of the ICAM-1 (CD 54) and ICAM-2 (CD102) on the surface of stimulated or unstimulated endothelial cells and leukocytes, to ICAM-3 (CD50) on lymphocytes, and to Landstein-Weiner blood group (ICAM-4) on RBC (4). It was shown that neuronal glycoprotein Telencephalin (TLN) is a cellular ligand for the CD11a/CD18. It was demonstrated that purified TLN-Fc fusion protein mediates leukocyte adhesion dependent on CD11a/CD18, and reciprocally, that purified CD11a/CD18 mediates cell adhesion dependent on TLN. The tissue distribution of TLN is unique among ICAMs, in that only the telencephalic regions (the cerebral neocortex, olfactory cortex, hippocampus, striatum, amygdala, septum, and olfactory bulb) of the CNS express this molecule and it is present on neurons and absent from glial cells (47).

LFA-1 is able to bind selectively to its ligands ICAM-1 and 3, suggesting that LFA-1 can exist in distinct ligand -specific binding states. In the case of ICAM-1, the natural physiological regulators of LFA-1 mediated binding to ICAM-1 are unknown. It has been shown that ICAM-1 plays a role in the activation of LFA-1 binding to ICAM-3. (7).

Structure of Alpha Chain of LFA-1

Characterization of LFA-1 α subunit at the protein and mRNA level was done to reveal its sequence and primary structure. Hydrophobicity analysis showed that the LFA-

1 α-subunit is a typical transmembrane protein with a 25-residue hydrophobic single sequence, an extracellular domain of 1062 residues, a single hydrophobic transmembrane region of 29 residues, and short cytoplasmic tail of 53 residues. It has 12 glycosylation sites (29).

The CD11 polypeptides exhibit similar characteristics. The external domain of the α -chain has 7 internal repeats and 3 characteristics divalent cation consensus binding sites (EF –Hand-like) resembling those found in Ca^{2+} -binding proteins such as calmodulin and parvalbumin (DXXDXXXD). In fact, the α - chain has been shown to bind Ca^{2} and this binding cannot be reversed by Mg^{2+} . This implies that Ca^{2+} and Mg^{2+} have separate binding sites. The extracellular domain also has an inserted domain ('I' domain) consisting of an insertion of 200 amino acids near the amino terminal region of the protein that is not present in the sequenced ECM receptor integrins. This is homologous to the domain of the same size vWF and cartilage matrix protein. Members of I-domains superfamily serve important recognition function in several proteins (2). The I-domain is present in the N-terminal region of the all the sequenced α -subunit of the 3 β2 integrins (αL, αM, αX) and 2 β1 integrins (VLA-1 and VLA-2). It is homologous to the 3 "A" domains of von Willebrand Factor (vWF), a plasma glycoprotein that mediates adhesion of platelets to subendothelial matrix.

The LFA-1 I-domain is expressed in an eukaryotic system as an isolated fragment which retaines a conformation similar to that of the native molecule in that the majority of the tested LFA-1 I –domains mapped to the I-domain. The majority of the LFA-1 I – domain mAbs (13 out 18) block LFA-1 binding to ICAM-1. Interestingly, these 13 mAbs

also block binding to ICAM –3, while two other I – domain antibodies show a selective inhibition of LFA-1 binding of ICAM-3 and not to ICAM-1, suggesting that I –domain is also involved in LFA-1 binding to ICAM-1. The presence of ICAM-1 binding site in the LFA-1 I –domain was shown indirectly by inhibition of the T cell –directed LFA-1/ ICAM-1 adhesion and indirectly in a binding assay. These results indicated that I – domain contains a functionally discrete binding site for ICAM-1 (37). The role of divalent cations Mg^{2+}, Ca^{2+} and Mn^{2+} in LFA-1 binding to ligand intercellular adhesion molecule-1 (ICAM-1) and induction of the divalent cation-dependent epitope recognized by mAb 24 was studied. Manganese strongly promoted both expression of the 24 epitope and T cell binding to ICAM-1 via LFA-1, suggesting that Mn^{2+} is able to directly alter the confirmation of LFA-1 in a manner that favors ligand binding. In contrast Mg^{2+} required removal of Ca^{2+} from T cell LFA-1 with EGTA. This study also suggested the inhibitory effect of Ca^{2+} in the activation LFA-1 (16).

The cytoplasmic domain of α -chain is important in signaling (1). It has 53 amino –acid residues. A conserved GFFKR sequence has been identified in the cytoplasmic domain of CD 11a. It has been found that this region regulates the assembly and adhesiveness of integrin LFA-1. Analysis of the mutants α1090* (has a deletion before the position 1090 i.e. before the GFFKR region), α1095* (has a deletion after the position 1095 i.e. after the GFFKR region) and α ΔGFFKR (has an internal deletion of the sequence GFFKR) were done for cell surface expression of LFA-1. These results suggest that these mutations might affect the stability of the mutant proteins or the efficiency of α and β subunit dimerization. The levels of mature α and β subunits

immunoprecipitated by TS 1/18 mAb from pulse-chased cells expressing mutant α1090*, α1095*, or α △GFFKR were similar and comparable to mature wild type and α and β subunits. However, the levels of the α1090*, α1095* and α△GFFKR precursors precipitated by TS 1/22 mAb from pulse-labeled cells were 7.3-, 4.1-, and 5.5-fold, respectively, higher than the wild-type α-precursor. These results indicate that to express similar levels of LFA-1 heterodimers on the cell surface cells need to synthesize more mutant α -precursor than the wild-type α -precursors.

Truncation of the cytoplasmic domain after the GFFKR sequence (α1095*) did not alter ICAM-1 binding of the transfectants. In contrast, Jurkat-$\beta_{2.7}$ cells that expressed mutant α1090* that was truncated before the GFFKR sequence exhibited more than 4 –fold higher basal control binding than the wild –type transfectants. This result suggests that the GFFKR sequence is an important regulator of LFA-1 adhesiveness. An internal deletion of this sequence (α△GFFKR) also resulted in constitutively high binding of transfectants to ICAM-1, further demonstrating the importance of this highly conserved sequence motif (31).

After ligation of LFA-1 to the counterreceptor, the cell spreading abilities of some of the mutants were studied. It was found that unlike the wild-type LFA-1, all the truncated forms, including those with individual cytoplasmic domain seem to be required for cell spreading. Moreover, although manganese significantly enhanced the adhesion of truncated LFA-1 to ICAM-1, it had no effect on cell spreading (36).

Regulation of Expression of Integrin Receptors

Many mediators (signaling molecules, drugs, or other agents) regulate integrin levels. Changes in protein levels are most commonly shown by FACS analysis, immunoprecipitation, or immunostaining. Parallel changes are also shown at the mRNA level (23).

The best studied peptide growth factor is TGF-β (members of the family of Transforming Growth factor -β). TGF-β increases β1 expression in several cell types at both protein levels. In some cases, the increase in β1 integrin dimers does not occur due to increase in β1 synthesis but due to increase in α and it is suggested that TGF-β enhances the assembly of β1 with its α partner. Other examples of integrin that are increased by TGF-β stimulates include α1, α2, α3, α5, αv, β3, β5, and β6.

Other growth factors and cytokines that regulate integrin levels are PDGF-AB, PDGF-BB, EGF, NGF, acidic FGF, IL-4, IL-1β, IL-6, β-family chemokines (MCP-1, MCP-1α, RANTES), Tumor Necrosis Factor-α (TNF-α), TNF-β, IFN-γ, M-CSF, and GM-CSF. Several signaling molecules and hormones such as retinoic acid, 1, 25-dihydroxyvitamin D3 and corticosterone have also been shown to have effects.

Some of the pharmaceutical agents that regulate integrin levels are phorbol esters, the tyrosine kinase inhibitor genistein, forskolin (which increases cAMP levels), gossypol (a biphenolic agent), thalidomide and dexamethasone (5). Phorbol ester treatment of the U937 cell line leads to the induction of αX/β2 and then downregulation of α4/β1 from the cell surface. It has been demonstrated that during the HL60 and U937 myeloid differentiation, the levels of αX and α4 mRNA are differentially regulated, α4 and α5

mRNA levels are down regulated during the granulocytic differentiation of HL-60 cells, while they are differentially regulated during monocytic pathway and the expression of αX is regulated at the transcriptional level, while that of α4 is mainly controlled by post-transcriptional mechanisms (23).

Mechanical stress also alters integrin levels. Some of the infectious agents that have been found to affect integrin levels include Pneumocystis carinii, HIV and cytomegalovirus.

It has been found that after HIV-1 infection, both chronically infected H9 $CD4^+$ lymphocytes acquired the ability to adhere to some extent to type IV collagen and laminin, but not to type-I collagen. H9 cells chronically infected with two of the 3 HIV-1 stains studied showed approximately sevenfold increase in attachment to fibronectin, while the same cells infected with the human retrovirus HIV-2 did not (51).

A number of integrin promoter regions have been characterized, including α2, α4, α5, αL, αM, αX, αV, αIIb, β1, β2, β3, avian β3, mouse β7. All these promoters lack both TATA and CAAT boxes. Many integrin promoters have binding sites for transcription factors like SP1, Ets, PU-1. Some of them have AP-1 sites (30).

Since the discovery of intervening sequences (introns) in eukaryotic transcription units and the process of their removal by splicing, much has been learned about the general mechanisms involved in this form of RNA processing. Intron removal and splicing must be a precise process. To ensure fidelity in splicing, short consenses sequences are found at intron boundaries.

Alternative splicing of RNA is an important mode of regulation of expression of a protein. The different types of alternative splicing are: single initiation site, multiple poly (A) sites, differential splicing; multiple initiation sites, single or multiple poly (A) sites, differential splicing at the 5' end; single initiation site, single poly (A) site, variable splicing; multiple 5' ends or multiple 3' ends, splicing unaffected.

The major late transcription unit of Adenovirus type 2 (Ad2) was the first characterized example of a complex transcription unit that uses multiple Poly (A) sites and alternative modes of RNA splicing. The complex arrangement of this gene allows for a diverse group of proteins to be produced from a single transcription unit that is 25kb long (27).

Several other eukaryotic genes have now been identified to use alternative polyadenylation sites along with differential patterns of RNA splicing. The genes for rat calcitonin and immunoglobulin heavy chain are spliced in this manner. The alternative mRNA products from these two genes are regulated in development or tissue specifically such that alternative protein products are produced (27). Alternaltive splicing has also been reported in several integrins (6, 13, 22, 28, 33, 46, 48, 53).

In our laboratory, studies have been done to determine the expression of several integrins in tumor cell lines. The presence of a DNA that is homologous to the alpha subunit of LFA-1 was shown. This DNA had approximately 100 base pairs less than that of the alpha subunit of LFA-1. Based on this observation, we hypothesize that there exists an alternatively spliced isoform of LFA-1. This isoform of LFA-1 may play an important role in the regulation of its function in cells of different origin. To further characterize

this molecule, it was cloned and sequenced. These results indicate that this molecule is highly homologous to the alpha subunit of LFA-1. Experiments were done to study the expression of this molecule in cell lines of different origins (T cell, B cell, and monocytes) and also in response to various activators.

CHAPTER II

MATERIALS AND METHODS

Cells and Cell Lines

Molt-4 (human T-cell leukemia), CEM (human T- lymphoblastoid), DHL (human B-cell lymphoma), THP-1 (human monocytic leukemia) cells were maintained in RPMI-1640 tissue culture medium containing 10% fetal bovine serum, 2 mM glutamine, 100 units/ ml penicillin and 100 µl/ ml streptomycin (21).

Cell Activation

All cells were grown at 37 °C. Cells that had total counts upto 10^4 were resuspended in 100 ml of fresh medium and treated with treated separately with mitogens. CEM and Molt-4 cells were treated separately with Concavalin-A (1.0 µg/ ml of culture). DHL-4 and THP-1 cells were treated separately with Lipopolysaccahride (10 µg/ ml of culture). All cells were treated with PMA (50 ng/ ml of culture. The treated cells were incubated at 37 °C for 16-20 hours before RNA isolation.

Preparation of RNA from Mammalian Cells

Mammalian cells were grown in tissue culture medium as described above and were counted using a haemocytometer. A total of 10^8 cells were taken and centrifuged at 2000g for 10 minutes. All solutions were prepared using diethyl pyrocarbonate (DEPC) treated water.

The method followed is described elsewhere (12). A denaturing solution (solution D) of the following composition was prepared: 4M guanidinium thiocyanate, 25 mM sodium citrate , pH 7.0; 0.5% sarcosyl, 0.1M 2-mercaptoethanol. To the pelleted cells, 14 mls of solution D, 0.1 ml of 2 M sodium acetate, (pH 4.0), 1 ml of phenol (water saturated), and 0.2 ml of chloroform-isoamyl alcohol mixture (49:1) were added sequentially, with thorough mixing by inversion after the addition of each reagent. The final suspension was shaken vigorously for 10s and kept on ice 15 minutes. Samples were then centrifuged at 10,000g for 20 min at 4^0C. After centrifugation, RNA was present in the aqueous phase whereas DNA and proteins were present in the interphase and phenol phase. The aqueous phase was transferred to a fresh tube, mixed with 1ml of isopropanol, and then placed at -20^0C for 2-3 hours to precipitate RNA. RNA was collected by centrifugation at 10,000g for 20 minutes. The resulting pellet was dissolved in 0.3 ml of solution D, transferred into a microfuge tube and precipitated with 1 volume of isopropanol at -20 ^{0}C for 16 to 18 hours. After centrifugation at 10,000g for 10 minutes, the pellet was washed 3 times with 80% ethanol, air-dried and resuspended with 10 mM Tris-HCl and stored at -80^0C.

Isolation of mRNA from Total RNA

Messenger RNA was isolated using an mRNA isolation kit (Boehringer Mannheim, Indianpolis, IN). This kit applies an oligo (dT) cellulose column which is an affinity adsorbent which contains chains of thymidylic acid up to 30 nucleotides long covalently attached to celllulose (Whatman CF11) via the terminal 5'-phosphate of the oligonucleotide. Under stable ionic conditions thymidine and adenosine form specifically complementary

base pair structures with the aid of hydrogen bridges. Therefore poly (A) rich mRNA can be separated from other RNA species which do not contain any poly (A) region (eg. rRNA, tRNA). The method followed was as described elsewhere (3, 32).

For 1.7 mg of RNA, 34 mg of oligo (-dT) cellulose was used. This was rehydrated in a 15 ml sterile tube with 4 ml of elution buffer (0.01 M Tris-HCl (pH 7.5), 0.05 % SDS, 1 mM EDTA). The resin was pelleted by centrifugation at 3000 x g for 5 minutes. The wash with elution buffer was repeated twice. The resin was equilibrated twice by washing with binding buffer (0.01 M Tris-HCl (pH 7.5), 0.5 M NaCl, 0.5 % SDS, 1 mM EDTA) and centrifuging with between washes. This pellet was stored in room temperature under 1 ml of binding buffer until the addition of RNA. The NaCl concentration of RNA was adjusted to that of binding buffer by addition of 5M NaCl. The RNA whose NaCl concentration was adjusted, was added to the pelleted resin. The resin was resuspended by inverting several times and then it was incubated for 30-60 minutes with intermittent agitation. The resin was pelleted by centrifugation at 3000 x g for 5 minutes. The supernatant was removed and the resin was resuspended in 5 ml of binding buffer and the pelleteting process was repeated. This wash was repeated twice. The pellet was then resuspended in 500 µl of binding buffer and transferred into Eppendorf tube. This was centrifuged and the supernatant was removed. mRNA was eluted by adding warm elution buffer (preheated at 65 °C). This was centrifuged and the supernatant was tranferred to another tube. The tube with the pellet was eluted again. To the pooled supernatant, 0.1 volume of 3M sodium acetate and 2 volumes of absolute ethanol were added and was incubated at –80 oC overnight. After this the tubes were

centrifuged at 10, 000 rpm for 15 minutes. The mRNA pellets were washed twice with 80 % ethanol (adjusted with DEPC treated water).

Reverse Transcriptase Reaction

The method for the synthesis of cDNA have been described in Hall, R.E. et al., 1996. A 5X stock solution (5x buffer, 0.5µg oligonucleotides/µg of RNA, 1 mM of each dNTPs) for reverse transcriptase reaction was prepared. Four µg of RNA was heated to 65^0C for 5 minutes and kept on ice for 3 minutes. A reaction volume of 20 µl contained 10 µl of the 5X stock solution, 20 units of RNasin/DTT, 4 µg of RNA, 16 to 20 units of avian myeloblastosis virus (AMV) reverse transcriptase enzyme, DEPC treated water to make up to a final volume to 20 µl. The reaction mix was incubated at 42^0C for 90 minutes. Two µg of RNAse was added to the reaction and it was further incubated at 60 minutes at 37^0C.

Polymerase Chain Reaction (PCR)

A master mix was prepared consisting of 50 µl of 10x PCR buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl, 15 mM $MgCl_2$, 0.01% gelatin), 12.5 µl of 50 mM $MgCl_2$, 20 µl 10 mM dNTP'S, 12.5 µl of 100% formamide, volume made up to 250 µl with sterile water. The composition of the 20 µl PCR mix was as follows: 10 µl of master mix, 1.6 µM of each forward and reverse primers, 1 unit of *Taq* DNA polymerase, volume made up to 20 µl with distilled water. This reaction was submitted to 25 PCR cycles on a Perkin –Elmer GeneAmp PCR System 9600 as follows: melting at 94^0C for 1 minute, annealing at 55^0C for 1 minute,

extension at 72^0C. For the studies on expression of α -subunits of LFA-1 major and LFA-1 minor, the following primers were used in PCR:

Forward Primer (Designated LFA-1):

5'----CC CCC AAA GGC CCC AAG ATC C----3'

Reverse Primer (Designated LFA-2):

5'--- C AGA GTC CTT CTC ATG GAG GGG----3'

Forward Primer for LFA-1 major only (Designated LFA-Major):

5'----AAG CAC ATG TAC CAG GTG AGG----3'

Forward Primer for LFA-1 minor only (Designated LFA-Minor):

5'----AAG CAC ATG TAC CAG GAG CCT----3'

Forward Primer for Mac-1:

5'----CCC AGA ACC AAC AAA ACC GAA TTC C----3'

Reverse Primer for Mac-1:

5'----TTC GGC CCC CGG GGG ACC CCC T----3'

Forward Primer for P150, 95:

5'----CCC AGG ACC AGC AAG ACC ACC----3'

Reverse Primer for P150, 95:

5'----GGG ATC ATT TCT CAC TGG GCG GGC----3'

Forward Primer for β2 subunit:

5'----C GGG CAG TAC TGC GAG TGT GAC----3'

Reverse Primer for β2 subunit:

5'----C CTA ACT CTC AGC AAA CTT GGG G----3'

The forward primers were different in these reactions but the reverse primers were the same.

Agarose Gel Analysis

10 µl aliquots of PCR were analyzed on 1.2% agarose gels electrophoresed at 75 V, 25 mA in 40 mM Tris-acetate (pH 8.5), 2 mM EDTA (TAE buffer) followed by staining with 0.5 µg /ml ethidium bromide. *Hae* III restriction endonuclease-digested Phi-X 174 replicative form DNA (BRL) was electrophoresed in parallel lanes and used as a size marker. In RT-PCR experiments, contols for integrity of RNA and lane loading consisted of parallel RNA samples run with β-actin primers.

For gel analysis of RNA, 6-10 µg of RNA was mixed with denaturing dye (1mM EDTA, 50 % formamide, 6.5 % formaldehyde and 0.4 % xylene cyanole/bromophenol blue) was heated at 55^0 C for 5 minutes and then cooled on ice for at least 3 minutes. The RNA samples were then analyzed by electrophoresis on 1.1 % agarose gels in 20 mM 3' –(N morpholino)- propanosulphonic acid (MOPS) pH 8.0). Lanes containing a RNA ladder (BRL) and ribosomal RNA for size reference were stained with ethidium bromide (0.5 µg/ml) and photographed under UV transillumination.

Cloning of PCR Products

Amplified DNA bands were cut out of agarose gels and purified using the procedure from Gene Clean kit (Bio 101, Vista, CA.). DNA was then cloned by using the

pCR 2.1 TA Cloning Kit (Invitrogen, Carlsbad, CA.). For ligation, the ratio of concentration of vector and insert used was 1:3. The ligation reaction mix consisted of the following: 150 ng of DNA fragment, 1 µl of 10X Ligation Buffer (60 mM Tris-HCl, pH 7.5; 60 mM $MgCl_2$; 50 nM NaCl, 1 mg/ml bovine serum albumin; 70 mM β-mercaptoethanol; 1 mM ATP; 20 mM dithiothreitol; 10 mM spermodine), 2 µl pCR 2.1 (Invitrogen) vector (25 ng/µl), 1 µl T4 DNA Ligase (4.0 Weiss units) and sterile water was added to make the volume to 10 µl. This was incubated at 14^0 C for 16 to 18 hours. The PCR product of the 570 second DNA base pair second band DNA band was gel purified and cloned into pCR 2.1 vector. The map of pCR 2.1 TA cloning vector is shown in Figure 2. *Taq* polymerase has a nontemplate-dependent activity which adds a single deoxyadenosine (A) to the 3' ends of PCR products. The linearized vector supplied in this kit has single 3' deoxythymine (T) residues. This allows PCR inserts to ligate efficiently with this vector. Thus PCR products generated with *Taq* polymerase have a high efficiency of cloning in the TA cloning system as the 3' A-overhangs hybridize with the T residues on the vector. Restriction digestion and Southern blot analysis was done to confirm the size of the insert.

After ligation, 2 ml of 0.5 M β-mercaptoethanol was added to a 20 ml polypropylene tube containing 50 µl of one shot competent *E.coli* cells. Two µl of the ligation mix was pipetted into the tube containing the competent cells and was gently stirred by using a pipette tip. This was incubated for 30 minutes on ice. This tube was subjected to heat shock for 45 seconds at 42^0 C. This was then placed on ice for 2 minutes. To the ligation mix, 450 µl of SOC medium (2% Tryptone, 0.5% Yeast Extract, 10 mM NaCl, 2.5 mM KCl,

10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM dextrose) was added. The tube was incubated at 37^0 C for 1 hour at 225 rpm at 37^0 C rotary shaking incubator. Aliquots of transformation mix were spread over LB agar-ampicillin plates, after which 40 µl of 40 mg/ml X-Gal was spread over the plates (40 µl of IPTG was also spread whenever TOP10F' was used). These plates were incubated at 37^0 C for 18 to 20 hours. White colonies were picked and were analyzed for the presence of insert by restriction endonuclease analysis.

DNA Sequencing and Computer Analysis

Recombinant plasmids were purified by alkaline lysis and ethanol precipitation and both strands were sequenced by dideoxy chain termination method using the Sequenase II kit (United States Biochemical, Cleveland, OH). Computer analysis of the nucleotide sequence and amino acid sequence was done using the Software package Genetics Computer Group (Madison, WI). Various searches were made on the available genetic databases.

Southern Blot Analysis of Alpha Subunit of LFA-1

Agarose gels were soaked in denaturing buffer (87.75g of sodium chloride, 20 g of sodium hydroxide, volume made up to 1000 ml) for 25 minutes in the shaker. Gels were then soaked in neutralizing buffer (60.5 g of Tris base, 87.75g of sodium chloride, pH 7.0, using Hydrochloric acid, volume adjusted to 7.0). The gels were blotted onto Biotrans (ICN, Irvine, CA) nylon membrane in 6X SSC for 16-18 hours. The nylon membrane was crosslinked by exposing to UV light in a Stratalinker.

Prehybridization was performed in a solution of 6X SSC, 5X Denhardt's solution, 0.05% sodium pyrophosphate, 100 to 250 μg/μl boiled herring sperm DNA, 0.5% sodium dodecyle sulfate at 65^0C for 16 to 18 hours.

The radiolabelled probe was prepared using the random primer method which is as follows. Twenty five ng of probe DNA in a 1.5 ml Eppendorf tube (the volume should not exceed 11.5 μl) and was heated to 95 to 100^0C for 5 minutes and then kept on ice for 3 minutes. To this, 5 μl of 5X reaction buffer, 2.5 μl 10X decamer solution, 5 μl of [^{32}P]-deoxycytidine triphosphate (-dCTP) or [^{32}P] deoxyadenosine (-dATP) (3000 Ci/mmol, 10 mCi/ml) and distilled water was added to make up the volume to 24 μl. To this mix, 1 μl of Klenow DNA polymerase (1 Units/μl) was added. This was incubated at room temperature for 5 to 6 hours. The volume of the reaction mix was then diluted to 100 μl with sterile water. The reaction mix was passed through a calibrated spin column of G-50. The probe was collected in a 1.5 ml Eppendorf tube. The required amount of probe was heated at 95^0 C for 5 minutes and kept on ice for 3 minutes. The probe was then added to the hybridization bag containing the nylon membrane. The bag was sealed and incubated at 65^0 C for 18 to 20 hours, washed to a stringency of 0.2X SSC at 65 °C. The nylon membrane was then dried and exposed to a film (Kodak, Rochester, NY.).

Densitometer Studies

The autoradiograms were analyzed by video densitometer on a Lynx 4000 Molecular Biology Workstation, (Applied Imaging, Santa Clara, CA, USA). The values of densitometry were standardized by statistical analysis like mean and standard error on mean and these

were represented on bar graphs. Bar graphs with error bars were plotted by using Jandel Scientific software.

Northern Analysis

Lanes containing an RNA ladder (Promega, Madison, WI) and ribosomal RNA for size reference were stained with ethidium bromide (0.5 μg/μl) and photographed with a ruler under ultraviolet (UV) transillumination. Gel lanes for Northern blotting were washed twice (30 minutes each) in DEPC water, once in 10 X SSC, and then transferred (16 to 18 hours, room temperature) onto Biotrans nylon membranes (ICN, Irvine, CA) employing a wick and 10 X SSC. The blots were crosslinked by exposure to UV light in a Stratalinker, baked at 80^0 C for 90 minutes under vacuum, and incubated at 41^0 C for 16 to 18 hours in prehybridization buffer consisting of 10 X SSC, 5X Denhardt's solution, 50 % formamide, 0.5 % SDS, 0.1 % sodium pyrophosphate and 150 μg/ml denatured herring sperm DNA. Hybridization was carried out at 41^0 C for 16 to 18 hours with ^{32}P heat denatured DNA probe (10^6 c.p.m./ml, specific activity > 10^8 c.p.m./ μg DNA). This was followed by washing in 3X SSC and 1X SSC. The blots were then exposed to X-ray film (Kodak Biomax) and developed.

CHAPTER III

RESULTS

Reverse Transcriptase and Polymerase Chain Reaction (RT-PCR)

RT-PCR was done using the RNA of Molt-4 cells to amplify the ∝ subunit of LFA-1. After electrophoresing the PCR products on 1.5% agarose gel, staining with ethidium bromide, it was observed that along with the predicted PCR products of ∝ subunit of LFA-1 (672 base pairs), there was another cDNA that was amplified. By gel analysis, it was determined that the smaller fragment was approximately 550 base pairs (designated as LFA-1 minor) in length. PhiX 174 DNA digested with Hae III restriction enzyme, was used as molecular weight markers. PCR using the primers of β -actin was done to check the integrity of the mRNA.

Southern Blot Analysis of Alpha Subunit of LFA-1

The PCR products were blotted on to a nylon membrane by the procedure described in Methods and Materials. After probing with ^{32}P labelled partial cDNA of α -subunit of LFA-1 major (representing a partial cDNA of the α subunit of LFA-1), it was shown that both hybridized (Figure 3). The PCR amplified β actin did not hybridize, eliminating the possibilty of non-specific hybridization. This showed that the smaller 550 bp band had significant homology with LFA-1 α subunit . This experiment was repeated several times to confirm the results. In Figure 3, the arrow shows the 550 base pair DNA band.

Cloning of PCR Products

Initial attempts to reamplify the smaller band failed possibly because of its very low quantity. A large batch of PCR products prepared with LFA-1 α subunit primers was electrophoresed and the bands were cut out of the gel and then purified and cloned into TA cloning vector 2.1. Plasmid DNA was isolated from this clone and was digested with *EcoRI* and electrophoresed on a 1.5 % agarose gel to visualize the insert.

Figure 4 shows the restriction endonuclease digested reaction of the plasmid DNA electrophoresed on the gel. The arrow in the figure shows the position of the inserted DNA. The size of the inserted DNA conforms to the predicted size of α subunit of LFA-1 major. Lane 2 contains the α -subunit of LFA-1. This shows the size comparison between the two DNA fragments. This gel was blotted on to nylon membrane and hybridized with with α -subunit of LFA-1 cDNA to confirm the cloning experiment. The results indicate that the α -subunit of LFA-1 has been cloned.

DNA Sequencing

The clone representing the 550 bp product (α -subunit of LFA-1 minor) was sequenced by the dideoxy method using Sp-6 and M13 primers. Figure 5 shows the sequencing gel of plasmid DNA of LFA-1 minor clone. It indicates a 114 bp deletion, accounting for the smaller size PCR product. Interestingly, after the deletion the same frame is maintained. This strongly suggests that this could be an alternatively spliced product of LFA-1. The figure also shows the site where the cDNA was inserted into the

clone and also the splice site. The deleted 114 base pairs are shown in the figure. This deletion was in the extracellular region that was very close to the transmembrane domain. There are no published records of characterization of this region of CD11a. Figure 6 shows the complete sequence of the clone of α-subunit of LFA-1 minor. The sequences are the same as that of CD11a except for the 114 base pair deleteion. The position of the deletion is shown by astrix in the figure. Figure 7 shows the comparison of the α - subunits of LFA-1 major and LFA-1 minor. The deleted DNA sequence (114 bp) and protein sequence (38 amino acids) are shown in the figure. The deletion is on the extracellular region, which is close to the transmembrane domain.

Computer Analysis of DNA and Protein Sequences

Computer analysis was done on the sequences of the deleted 114 base pairs using GCG and BLAST software packages. There was significant homology with some of cell adhesion molecules in the databases containing DNA sequences of various organisms.

The nucleotides of the deleted portion of the DNA was translated to amino acid sequence. The amino acid sequences were analyzed for its putative functions using the 'motif' search. This search revealed that the deleted portion had a Casein Kinase II phosphorylation site. The function of this phosphorylation site in the extracellular region is not known.

Northern Blot Analysis

Northern blot analysis was done by using mRNA (Poly A selected) of THP-1 cells. The results are shown in Figure 8. There was a significant hybridization at approximately 5 Kb on the 1.1 % denaturing gel blot. RNA marker ladder and Ribosomal RNA were also electrophoresed on adjacent lanes to serve as size marker. In the past, northern blot analysis was done by Larson et al. (29). Here the CD11a mRNA was shown at 5.5 Kb position, which is higher than the size of 28 s rRNA. Our observations show that the 28 s and CD 11a are at almost the same position on 1.1% agarose gel (denaturing). The size of 28s rRNA is approximately 4.8 Kb. The hybridization at 5.0 Kb is very close to the size of 28s rRNA but there is no hybridization at 18s rRNA region. Also the figure shows the agarose gel of different fractions of RNA. Although equal quantities of samples were loaded, the mRNA samples have lesser amount of 28 s rRNA, which implies that some of rRNA was removed during the process of mRNA isolation. There are 2 interesting observations on this experiment: (i) There is a significant hybridization at approximately 1.0 Kb, which is below the position of 18s rRNA, eliminating the possibility of non-specific hybridization to 28 s and 18s rRNA. (ii) There are additional hybridization signals below the hybridization position at 5 Kb. This suggests the presence of alternatively spliced products.

Studies on the Expression of α -Subunit of LFA-1 Major and LFA-1 Minor

Cell lines of various origins (T-cell, B-cell, monocytes) were cultured with different

mitogens in order to compare modulation of the two transcripts (α subunit of both LFA-1 major and LFA-1 minor). The cell lines used were as follows: CEM (human T lymphoblastoid cell), Molt-4 (human T cell acute lymphoblastic leukemia), DHL (human B lymphoma cell), Reh (human acute lymphocytic pre B cell leukemia), THP-1 (human monocytic cells). After culturing cells with and without mitogen for 16 to 18 hours, RNA was extracted, subjected to RT-PCR, and the products were analyzed by agarose gel electrophoresis followed by blotting on nylon membrane and hybridizing with α subunit of LFA-1 major. Figure 9 shows the results of southern hybridization. The primers that were designed at the splice site of CD11a (described in Materials and Methods), enhanced the amplification of the α subunits of LFA-1 major LFA-1 minor in separate reactions. The autoradiograms were then submitted to densitometric measurement of band intensities. Tables 2 and 3 show the results of densitometry. Five batches of experiments were done to study the expression pattern of the α subunits of LFA-1 major and LFA-1 minor. These readings were later subjected to further statistical analysis (mean and standard error on the mean). These were plotted in bar graphs with error bars.

Figure 10 shows the expression of α -subunit of LFA-1 major in response to various mitogens in CEM, DHL-4, Molt-4, THP-1 cells. In case of CEM cells, there was no significant modulation of expression in response to these mitogens Con-A (T cell activator) and PMA (protein kinase C activator). The error bars show the deviation in results from the mean of different sets of experiments. In case of Molt-4 cells stimulated with PMA, the expression alpha subunit of LFA-1 major is very high but the standard error of the mean is

also high (+/- 5.97 on an average of 7.7). This shows that there is less consistency in the results of these experiments.

Figure 11 shows the graph of the expression of α -subunit of LFA-1 minor in CEM, DHL-4, Molt-4 and THP-1 under the same conditions. The results in Figure 11 indicated no significant modulation but the error bars were high, which implies that there was variation among the various batches of experiments.

These analyses revealed that there was some modulation of expression of the α subunit of LFA-1 major and LFA-1 minor in response to the mitogens but no significant trend in the modulation was established as indicated by the high errors of the mean and in some cases the errors of the mean were very high (Molt-4 cell- Figure 10). By using the densitometry readings, bar graphs were plotted to compare the basal level of expression of α -subunits of LFA-1 major and LFA-1 minor (Figure 12). In case of LFA-1 major, DHL-4 shows very high level of expression, when standardized to that of CEM cells, but the error bars are also very high, which shows that these results are not consistent. Studies on the expression of α -subunit of LFA-1 minor indicate that there may be a higher level of expression in THP-1 (Figure 12). Figure 13 shows the comparison of the expression of α subunit of LFA-1 minor Vs. LFA-1 major. The expression of α -subunit of LFA-1 major were 10 – 60 times higher than that of α - subunit of LFA-1 major. The errors on the mean were very high in some cases. In case of THP-1, the ratio expression of α -subunits of LFA-1 minor Vs. LFA-1 major was lower (approximately 1 : 5.5), when compared to other cell lines and the error on the mean is not high. This shows that there may a higher level of expression of alpha subunit of LFA-1 minor in THP-1 when compared to CEM, DHL-4 and Molt-4.

There have been several observations made based on the densitometric studies. The summary of the important observations are as follows:

1. There is modulation of the expression of α -subunits of LFA-1 major and LFA-1 minor but there was no specific trend observed. The levels of β -actin (control) were the same even under activated conditions. There may be some other factors which affect the expression of α -subunits of LFA-1 major and minor.
2. THP-1 may be expressing higher levels of α -subunit of LFA-1 minor, when compared to other cell lines (CEM, DHL-4 and Molt-4).

CHAPTER IV

DISCUSSION

Leukocyte adhesion is of pivotal importance and the structure of the leukocyte integrins in relation to their function has been extensively studied. (20, 31, 37). LFA-1 is involved in T- helper and cytotoxic T-lymphocyte function. It is also involved in antigen independent interactions that mediate cell localization to sites of inflammation such as leukocyte adhesion to endothelial cells, fibroblasts, epidermal keratinocytes and synovial cells (29). In all these activities, the avidity of LFA-1 for its ligand plays a very crucial role.

The mRNA of CD11a is 5.5kb and is expressed in lymphoid and myeloid cells. The RT-PCR in our experiment was done by using internal primers at a region that spans extracellular, transmembrane and intracellular domains. The RT reaction results in the production of cDNA from RNA and the PCR with the above-mentioned primers, results in the amplification of the region of CD11a that is covered by the primers. In our experiments, we used formamide to increase the specificity of PCR. Formamide increases the stringency of annealing of primers to the template and also removes non-specific annealing. By analysis of PCR on agarose gel along with the molecular markers (ϕx 174 DNA digested with Hae III restriction enzyme), it was found that the PCR amplified fragment of CD 11a has a molecular weight of 673 base pairs. Formation of a second less prominent DNA band was observed and the molecular weight of which was approximately 570 base pairs i.e approximately 100 base pairs less than that of CD 11a

band. On Southern blot analysis, it was confirmed that this second band of DNA was homologous to CD11a.

The 550 base pair RT-PCR product (termed LFA-1 minor) was cloned into the pCP 2.1 vector and sequencing by dideoxy method. The sequence of LFA-1 minor was compared to the published sequence of CD11a (29). We found that 114 base pairs were deleted in this clone and the gene remains in-frame thus suggesting that it arose by alternative splicing with 38 amino acids deleted in the mature protein. This region was in the extracellular domain – very close to the transmembrane domain. The extracellular region contains I-domain, which is the binding site for its ligand Intercellular Adhesion Molecule-1 (ICAM-1) (37); it has binding site for divalent cations, which regulate the function of LFA-1 (16). The deleted 114 base pair region of LFA-1 minor has not been characterized yet.

The genomic structure and exon-intron boundaries of CD11a have not been elucidated. The CD11 genes have been mapped to the p11-p13.1 region of chromosome 16, which has been implicated in chromosomal abnormalities in patients with acute myelomonocytic leukemia. (42, 34). The α-subunits of β_2-subunits integrins are highly homologous to each other. The CD11b (α-subunit of Mac-1) and CD11c (α-subunit of p150,95) have 63% identity at the amino acid level and 68% at the nucleotide level. CD11a displays 35% amino acid identity but is clearly homologous to the other α subunits, CD11a and CD11c. The nucleotide sequence of exons and the intron-exon junctions of CD11b have been elucidated (19) . The exon 27 of CD11b has 114 base pairs and is located in the region that is similar to the deleted portion of α-subunit of LFA-1

minor. Based on this, it is reasonable to speculate that the deleted 114 base pairs of LFA-1 minor is indeed a complete exon and that LFA-1 minor is an alternatively spliced isoform of LFA-1.

Initial attempts to conduct Northern hybridization with total RNA failed because of the low copy number of LFA-1. Therefore mRNA was isolated for Northern blot experiments. Northern blot analysis showed that there was hybridization at approximately 5 Kb region on the blot. Also there was hybridization at approximately 1.0 Kb region. This could presumably be an evidence of an alternatively spliced product of LFA-1. As mentioned earlier, since the second hybridization band is lower than the 18 s rRNA, the chances that the hybridization is non-specific. There are several faint bands below the 5 Kb region. This suggests that there may be some alternative splicing of LFA-1. There is possiblity of some other alternatively spliced isoforms of LFA-1, which are expressed in low quantities but have some specific function.

The deleted region of LFA-1 minor was analyzed using 'Fasta' search in the software from Genetics Computer Group (GCG). Analysis of DNA showed homology to many of cell surface molecules. By using the 'Gap' utility of GCG, attempts were made to map the sequences of deleted region to the known genomic DNA sequences of chromosome 16 in the region p11 to p13.1. No matches were found. Protein analysis using 'Motif' search showed that this region had a Casein II Kinase (CK-2) Phosphorylation site. Casein II Kinase is a protein serine/threonine kinase whose activity is independent of cyclic nucleotides and calcium. CK-2 phosphorylates many different

proteins and is involved in signal transduction. It is also known to phosphorylate extracellular proteins and alter the function of such proteins (18).

A similar observation was reported in Drosophila γ-Aminobutyric acid receptor gene Rdl. Gamma aminobutyric acid (GABA) is the major inhibitory neurotransmitter in both vertebrate and invertebrate nervous system. Extensive alternative splicing of 'a' or 'b' and 'c' or 'd' extracellular region of GABA has been observed. One of the spliced exons (exon 'd') has a putative site for casein II kinase phosphorylation. Phosphorylation sites are typically found in the cytoplasmic region. The importance of a casein II kinase phosphorylation here is unclear (18).

The observation of an *in vivo* isoform of LFA-3 called LFA-3 Δ D2, is notable in this regard. LFA-3 is the major naturally occurring ligand for the T-lymphocyte receptor CD2. Both these molecules are members of Ig superfamily. Two of these isoforms the are transmembrane (TM) form and glycosyl -phophatidylinositol –linked (GPI) form. The TM form contains a membrane spanning domain and short cytoplasmic domain, whereas glycosyl -phosphatidyl -linked (GPI) form is anchored within the membrane. The cDNA sequence for a third isoform of LFA-3 termed LFA-3 Δ D2, was reported in human T cell line Molt-4. This isoform encodes the TM-LFA-3 anchoring region and truncated extracellular region consisting of only the membrane distal domain, domain 1, of the other isoform. The speculation here is that the lack of proximal domain would be expected to have only minimal effect on the function of LFA-3 Δ D2. The structure of LFA-3 Δ D2 places the major functional (distal) domain closer to the cell membrane. This change may allow this isoform to establish a closer and perhaps more effective

region of cell-cell contact which may enhance signal transduction through adhesion-dependent pathways (25).

In the past, alternative splicing of α and β - subunits of integrins have been documented. Alpha-3, α 6, α 7, β1, β2 and β4 have alternative splicing at the cytoplasmic region (53). Alternative splicing in some of them is developmentally regulated. Also it is suggested that this may be important in determining the specificity and affinity of integrin receptors for their ligands (53). The fact that a CK2 phosphorylation site is not available on the extracellular site of LFA-1 minor may have some importance on its activity. CK2 is known to be involved in phosphorylation of extracellular proteins. Also a deletion of 114 base pairs from extracellular region of LFA-1 minor, may render the ligand binding sites closer to the cell membrane. This may result in better cell-cell interaction. It is possible that the expression of LFA-1 minor is developmentally regulated and has varied specificity for its ligands.

Several surface molecules have been known to produce multiple forms by alternative splicing of its RNA. The fibronectin (FN) gene has been become the paradigmatic to illustrate genome evolution by exon shuffling, generation of protein diversity by alternative mRNA splicing, and topological coordination between transcription and splicing (27). FN plays key roles in the adhesive and migratory behavior of cells related to fundamental processes such as embryogenesis, malignancy, hemostasis, wound healing, host defense, and maintenance of tissue integrity. Alternative splicing in 3 sites of the primary transcript gives rise to multiple FN polypeptides (40). This process is type, development and age regulated.

Platelet endothelial cell adhesion molecule –1 (PECAM-1) is a 130 KD member of the Ig gene superfamily that is expressed on human platlets, at the intercellular junctions of resting endothelial cells, and on circulating monocytes, granulocytes, and certain T cell subsets. Experimental evidence shows that the cytoplasmic tail is unusually complex in its genomic organization and lends itself to the production of multiple PECAM-1 isoforms that appear to be generated by alternative splicing (26). Other observations show that alternative splicing generates isoforms of murine intercellular adhesion molecule-1 (24).

It has been shown that the Drosophila integrin PS2 α transcripts undergo developmentally regulated alternative splicing. Two different subunits have been identified (PS1 α and PS2 α), each of which associates with the same β subunit. Immunoprecipitation of these integrins with monoclonal antibodies against the α subunits yielded a variety of peptides, which suggested that there may be a number of different α-subunit isoforms differing in size. Two forms of PS2 α are frequently observed: a canonical © form and a form lacking the 75 nucleotide exon 8 (m8) (6).

Attempts were made to quantitate the expression of LFA-1 minor by ribonuclease protection assay. The α subunit of LFA-1 minor was cloned into pGEM 3fz vector. The purpose of doing this was to synthesize sense and antisense RNA transcripts using the promoters Sp6 and T7, which were available on the vector. Some difficulties were encountered in producing RNA transcripts. This could presumably be due to the presence of many pause sites on the transcripts of LFA-1 minor. Also there are chances that the

insert may have a phage polymerase termination signal (Maxiscript invitro transcription Kits (Ambion) Version 9701).

In order to obtain initial information regarding possible control of expression of LFA-1 minor, a series of cell lines were stimulated with various agents followed by RT-PCR analysis of LFA-1 major and minor transcripts (Figure 9). The cell lines used were: CEM (leukemic T cell), DHL-4 (lymphoma B cell), Molt-4 (leukemia T cell), THP-1 (monocytic leukemia). These cells were treated with various stimulants. Concavalin-A (Con-A) is a lectin that activates T cell. CEM and Molt- 4 cells were treated with Con-A. Bacterial Lipopolysaccahride (LPS) is a polyclonal activator of B cell . DHL-4 and THP-1 cells were activated with LPS. PMA is an activator of protein kinase-C (50). All cells were treated with PMA.

The PMA activated cells adhere to the flask. RNA was isolated from all these cells and RT-PCRs were done. Specific primers were used in PCRs, as indicated in the Materials and Methods section. This gave a better representation of LFA-1 major and LFA-1 minor DNA. The expression of these 2 products were quantitated by densitometer studies on Southern blots. The densitometer readings were taken under the same amounts of light and focus. The important observations are that THP-1 produces higher quantities of LFA-1 minor, when compared to that of other cells tested. CEM and THP-1 produce higher amounts of LFA-1 major, when compared to that of DHL-4 and Molt-4. Activation studies clearly show modulation of expression of LFA-1 major and LFA-1 minor, but these results have not been consistant in many cases (Figure 10-13). The PCRs

with β actin primers show consistent amounts of basal and activated expression. This indicates that the LFA-1 expression is modulated by mitogens.

The diversity of integrin supergene family of receptors has been highlighted by the observations that individual α subunits can pair with multiple β subunits. In addition, several intergin subunits have been shown to be alternatively spliced at the cytoplasmic domain. They include α3, α6, α7, β1, β3, and β4. (53). β1C, an alternatively spliced form of β1 integrin is involved in the inhibition of the cell cycle progression (33).

Some of the alternatively spliced products have been developmentally regulated and are dependent on the cell cycle. From the results of our experiments, it is clear that the reasons for the inconsistent modulation of the expression of LFA-1 minor by the activators like Con-A, LPS and PMA, is because of the differences in the stages of cell cycle among the cell lines used in the different batches of experiments. The relationship between cell adhesion and cell cycle progression have been documented in the past (52, 17). In our experiments, we did not monitor the phase of cell cycle i.e. the cells were harvested depending on the cell count and not on the stage of cell cycle. There is strong possibility that the alternatively spliced form of LFA-1 has a different specificity for its ligands and such interactions may serve some specific function depending on the stage of development. Also the presence of LFA-1 minor may serve some inhibitory function similar to that of alternatively spliced integrins β1c, TGF-β, TNF, INF receptor family, which serves as an inhibitor to cell cycle progression (33). However, it is very critical to determine the function of the missing exon during binding to its ligand.

We have provided some evidence for the presence of an alternatively form of CD 11a. Elucidating the intron-exon boundary will confirm this. Using primers of the consensus intronic sequence can be used in genomic sequencing of chromosome 16 in the region p11 to p13.1, to elucidate the intron-exon boundary. Since CD 11a genes have been mapped to chromosome 16 p11 to p13.1, the complete sequence of this region will enable one to determine the exact location of this exon on the genome (34). As mentioned before, the 114 base pair (or 38 amino acid) deletion is present on the extracellular region, it may affect the binding to its ligand. This may cause the protein to fold in a different fashion and this may serve a different function. This deletion causes the I domain and the cation binding domain to be placed closer to the transmembrane domain and cytoplasmic domain. As a result, this may enhance the interactions between the cytoplasmic domain and the extracellular domain of LFA-1 minor.

The deleted portion of LFA-1 minor has a Casein II Kinase phosphorylation site. Casein II Kinase is known to phosphorylate extracellular proteins. This phosphorylation may be important in regulating its binding affinity for its ligands. The absence of this CK2 phosphorylation may render the α subunit of LFA-1 minor, constitutively active or inactive. The whole process may act as an efficient way of modifying cellular responses to an integrin ligand as a function of differentiation. The fact that there is no obvious trend in the expression of LFA-1 major Vs. LFA-1 minor indicates that this is dependent on the cell cycle. Also the fact that CEM and THP-1 produces higher quantities of LFA-1 minor, indicates that its expression is dependent on cell type, which is similar to that of α 3 and α 6 (46). Developmentally regulated α7 and

Drosophila PS2 α have been proposed to affect receptor function. With this precedent, it may be important to determine the possible role of LFA-1 minor in development and differentiation.

Further studies should include harvesting cells at phases of the cell cycle and studying the expression of LFA-1 minor Vs. LFA-1 major. Expression studies should be done at the RNA level by quantitative RT-PCR and also at the protein level by immunohistochemistry with antibodies against CD 11a. Transfection of cells separately with clones of LFA-1 major and LFA-1 minor and then studying its properties will enable one to determine its avidity to its ligands and also its preference of ligands. With Studies on the expression of these molecules in many more cells of leukocyte lineage will give a better understanding of its expression at different stages of differentiation and development and also its possible role in these stages.

In conclusion, these findings add to the structural complexity and diversity of integrins. These also give rise to novel modes of regulating the function of integrins. These findings may be of therapeutic importance depending on the precise cellular responses evoked by the ligand engagement by LFA-1 minor during differentiation and development.

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APPENDIX

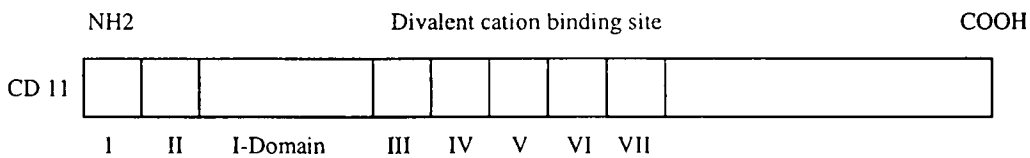


Figure 1: Schematic structure of an alpha chain of beta 2 integrin. The alpha chains are formed by similar repeats (I-VII) including three divalent cation -binding sequences (V-VII). The ~200 amino acid domain is essential for ligand binding, and contains a Mg2+ binding site. The alpha chains are non-covalently linked to a common beta chain, which is unusually rich in Cys residues. (Gahemberg et al. 1997. Eur. j. Biochem. 245: 215-232)

pCR™2.1 The map of the linearized vector, pCR™2.1, is shown below. The sequence of the multiple cloning site is shown with a PCR product inserted by TA Cloning®. The arrow indicates the start of transcription for the T7 RNA polymerase.

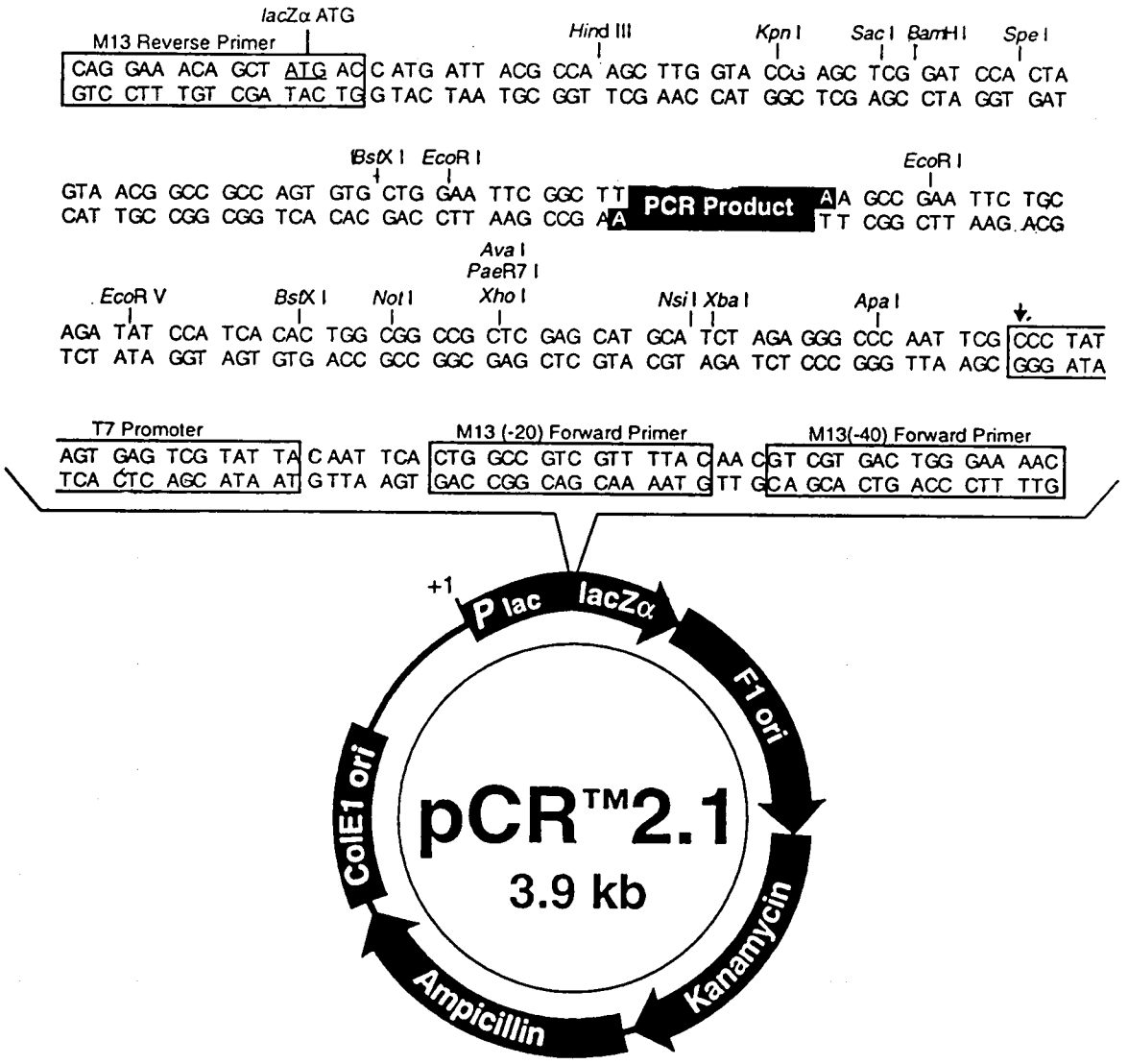


Figure 2: The map of pCR 2.1 TA cloning vector (Invitrogen).The vector has -dT overhangs to facilitate the ligation of PCR products, which have -dA overhangs. Note that the vector is modified at the unique EcoRI site during preparation of pCR 2.1. The inserted PCR product is flanked on each side by EcoRI site. (Invitrogen product catalog, 1997)

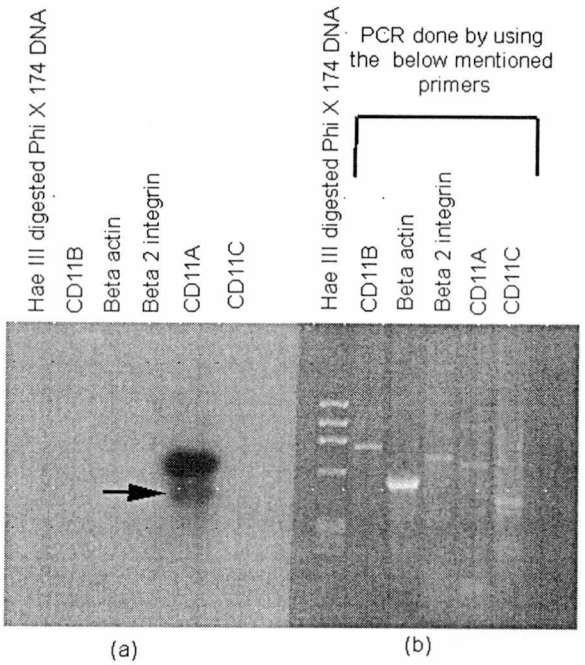


Figure 3: (a) Southern blot analysis of alpha subunit of LFA-1. RNA was isolated from Molt-4 cells. RT-PCR and Southern blot analysis were done by using partial cDNA of alpha subunit LFA-1 as a probe. The presence of an approximately 550 base pair DNA is shown by arrow. Note that the smaller sized DNA is homologous to alpha subunit of LFA-1. (b) RT-PCR was done by using the primers as indicated in the figure. These reactions were electrophoresed on 1.5% agarose gel.

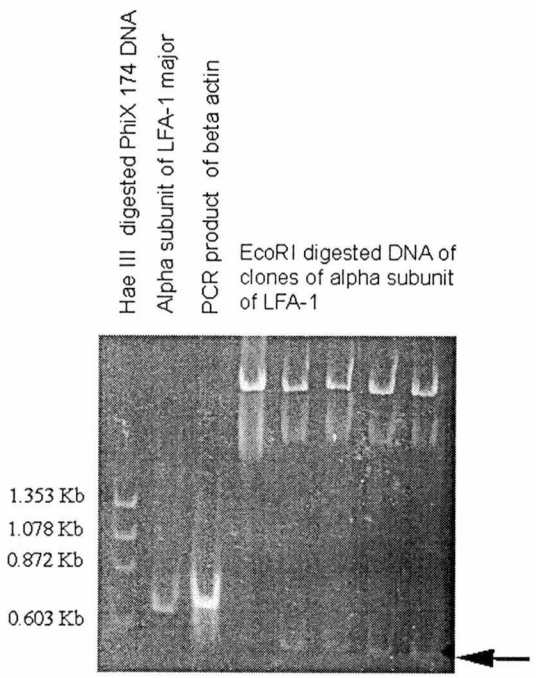


Figure 4: Results of Cloning of Alpha subunit of LFA-1 minor. The alpha subunit of LFA-1 minor was cloned into pCR 2.1 vector. Plasmid DNA was isolated from the clones and was digested with EcoRI restriction endonuclease. These reaction were electrophoresed on 1.2% agarose gel. Hae III digested PhiX 174 DNA was as a molecular marker. The inserts are shown by arrow.

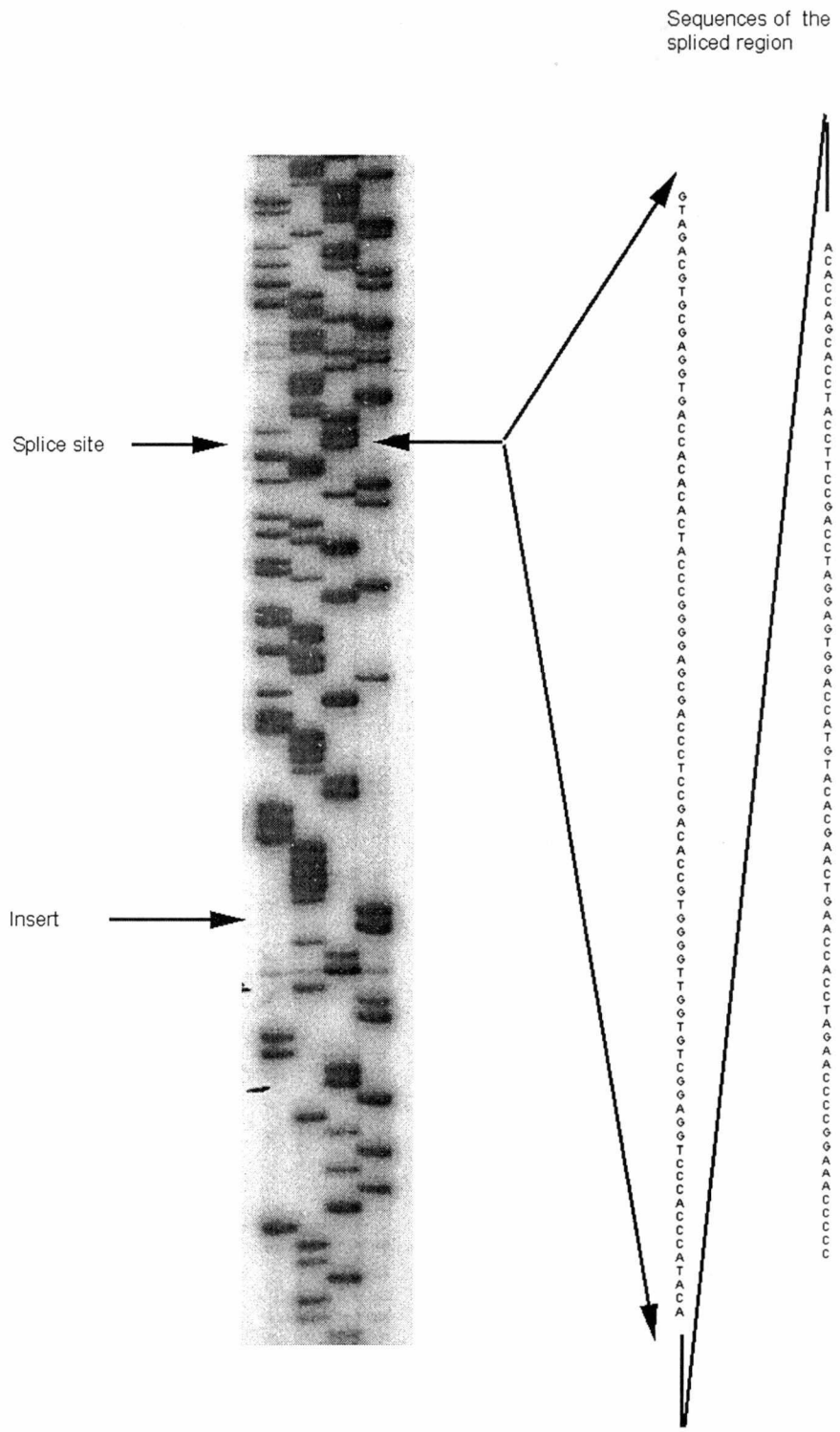


Figure 5: DNA sequencing gel of alpha subunit of LFA-1 minor. The arrows indicate the region in the vector where the DNA fragment was cloned and the region which had the 114 base pair deletion. The deleted sequences are shown.

CC CCC AAA GGC CCC AAG ATC CAC CAA GTC AAG CAC ATG TAC CAG *

GAG CCT CCC GTG CCC TGC CAC TAT GAG GAT CTG GAG AGG CTC CCG

GAT GCA GCT GAG CCT TGT CTC CCC GGA GCC CTG TTC CGC TGC CCT

GTT GTC TTC AGG CAG GAG ATC CTC GTC CAA GTG ATC GGG ACT CTG

GAG CTG GTG GGA GAG ATC GAG GCC TCT TCC ATG TTC AGC CTC TGC

AGC TCC CTC TCC ATC TCC TTC AAC AGC AGC AAG CAT TTC CAC CTC

TAT GGC AGC AAC GCC TCC CTG GCC CAG GTT GTC ATG AAG GTT GAC

GTG GTG TAT GAG AAG CAG ATG CTC TAC CTC TAC GTG CTG AGC GGC

ATC GGG GGG CTG CTG CTG CTG CTG CTC ATT TTC ATA GTG CTG TAC

AAG GTT GGT TTC TTC AAA CGG AAC CTG AAG GAG AAG ATG GAG GCT

GGC AGA GGT GTC CCG AAT GGA ATC CCT GCA GAA GAC TCT GAG CAG

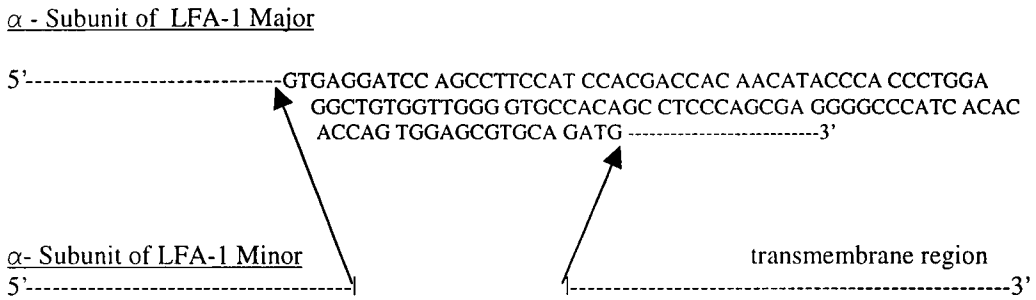
CTG GCA TCT GGG CAA GAG GCT GGG GAT CCC GGC TGC CTG AAG

CCC CTC CAT GAG AAG GAC TCT

Figure 6: DNA Sequence of subunit of LFA-1 Minor. The transmembrane domain is underlined.

(* - The point of 114 deletion when compared to alpha subunit of LFA-1)

Comparison of DNA Sequence of Alpha Subunits of LFA-1 Major and LFA-1 Minor



Comparison of Protein Sequence of Alpha Subunits of LFA-1 Major and LFA-1 Minor

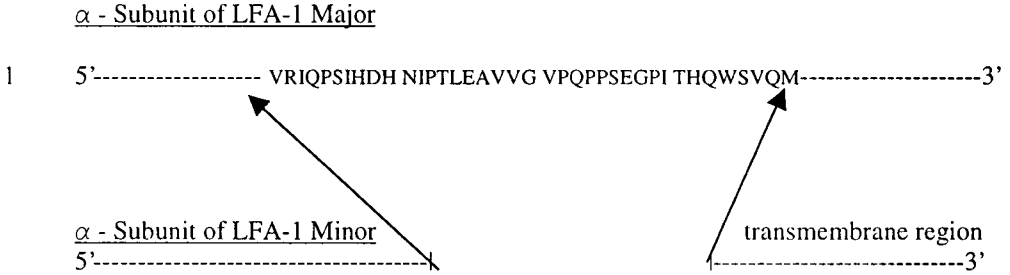


Figure 7: Comparison of DNA and protein sequences of the alpha subunits of LFA-1 major and LFA-1 minor. The deleted DNA and protein sequences in the alpha subunit of LFA-1 minor are shown.

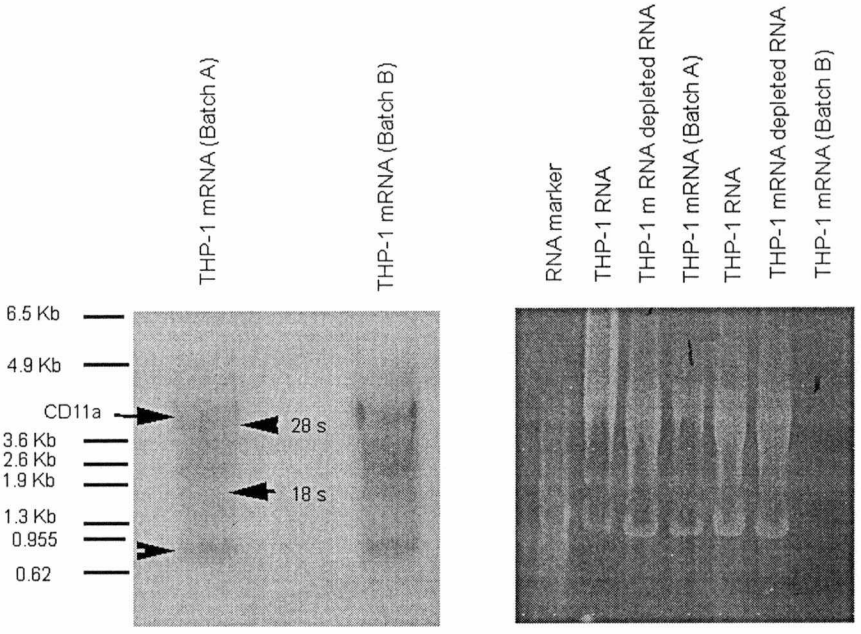


Figure 8: (a) Northern blot analysis of THP-1 mRNA. mRNA was isolated by using OdT cellulose. Twelve microgram of poly -A selected mRNA was electrophoresed on 1.1% agarose gel (denaturing). RNA marker ladder and rRNA were also electrophoresed on adjacent lanes to serve as molecular markers and as a control for integrity of RNA. Arrows show the alpha subunit of LFA-1, a band at approximately 1 Kb region, 28 s rRNA and 18 s rRNA. Batch A and B mRNA were isolated in 2 different batches. (b) Test for mRNA isolation. The RNA, mRNA depleted RNA (after several washes during mRNA isolation) and the mRNA were electrophoresed on 1.1% agarose gel (denaturing). This was done to compare changes that occur on various fractions on RNA as a result of mRNA isolation.

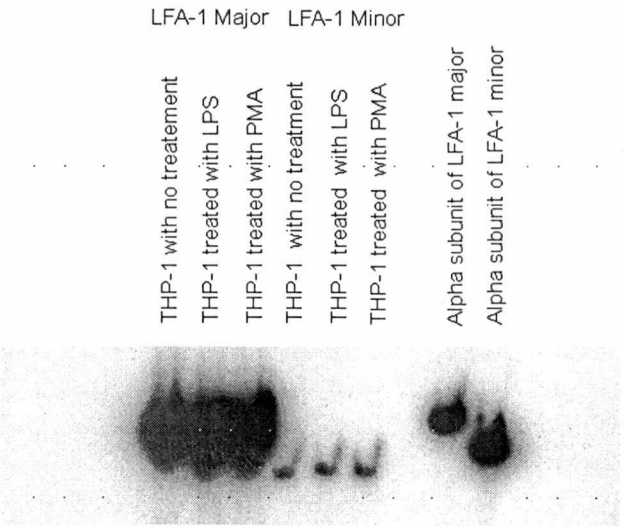


Figure 9: Studies on the expression of alpha subunit of LFA-1 major and LFA-1 minor. THP-1 cells were treated with various mitogens for 16-20 hours and RNA was isolated. RT-PCR and Southern blot analysis were done. The autoradiograms are shown above. Specific primers were used in PCR to enhance the amplification of alpha subunits of LFA-1 major and LFA-1 minor. Two positive controls (cloned fragments of alpha subunits of LFA-1 major and LFA-1 minor) were used to monitor the size of the PCR amplified fragments. Lane 7 contains positive control for the alpha subunit of LFA-1 major and lane 8 contains that of LFA-1 minor.

EXPRESSION OF ALPHA SUBUNIT OF LFA-1 MAJOR IN VARIOUS CELL LINES IN RESPONSE TO VARIOUS MITOGENS

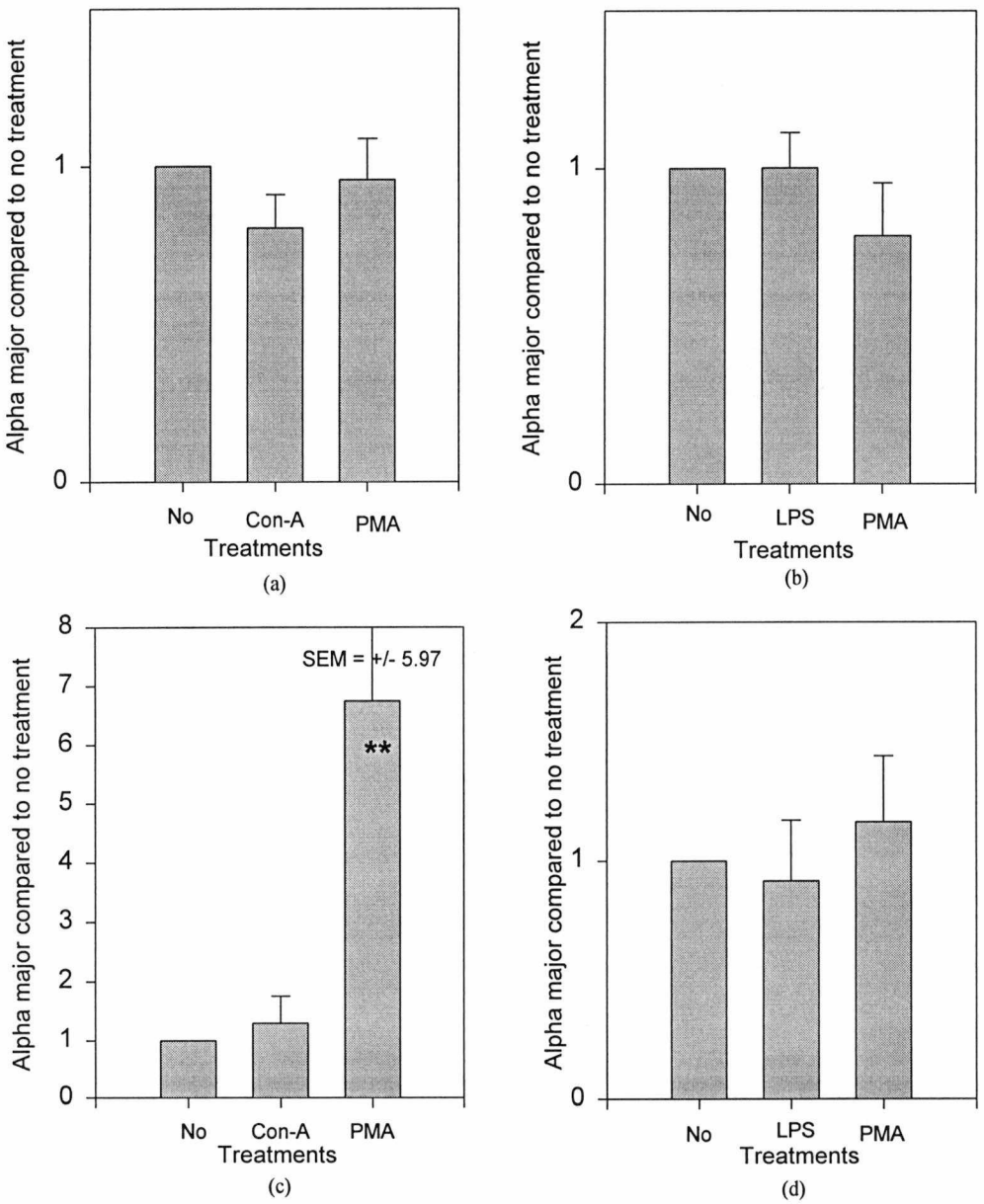


Figure 10: Expression of alpha subunit of LFA-1 major in various cell lines in response to various mitogens. Cells were treated with various mitogens for 16-20 hours and RNA was isolated. Analysis was done by RT-PCR and Southern blot. The results of densitometry on the autoradiograms were compared to that of no treatment. Error bars show standard error of the mean (SEM). The cell lines used were: (a) CEM, (b) DHL-4, (c) Molt-4, (d) THP-1. (**- high SEM).

EXPRESSION OF ALPHA SUBUNIT OF LFA-1 MINOR IN VARIOUS CELL LINES IN RESPONSE TO VARIOUS MITOGENS

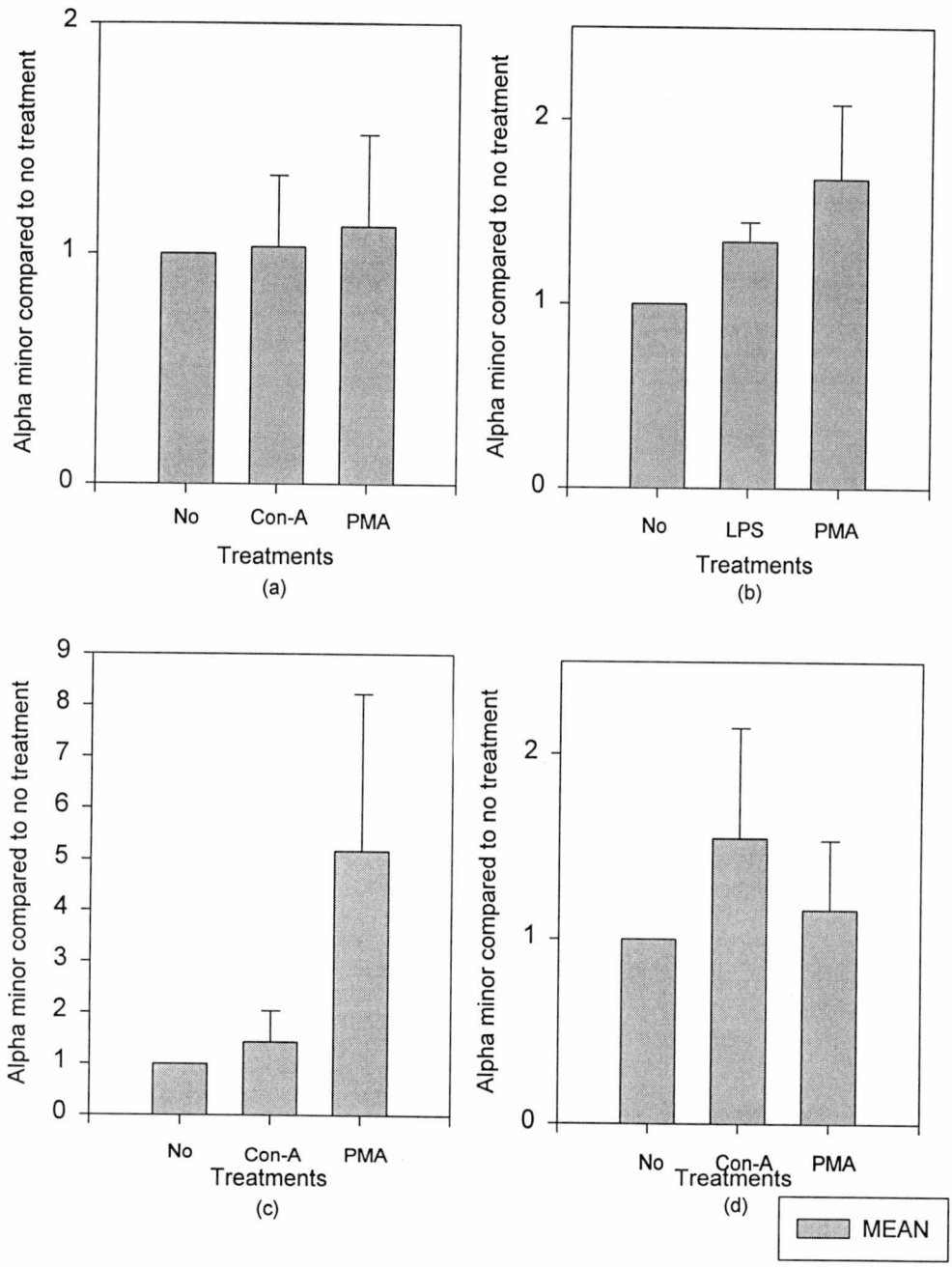


Figure 11: Expression of alpha subunit of LFA-1 minor in various cell lines in response to various mitogens. Cells were treated with various mitogens for 16-20 hours and RNA was isolated. Analysis was done by RT-PCR and Southern blot. The results of densitometry on the autoradiograms were compared to that of no treatment. Error bars show standard error of the mean (SEM). The cell lines used were : (a) CEM, (b) DHL-4, (c) Molt-4, (d) THP-1.

EXPRESSION OF ALPHA SUBUNITS OF LFA-1 MAJOR AND LFA-1 MINOR IN VARIOUS CELL LINES

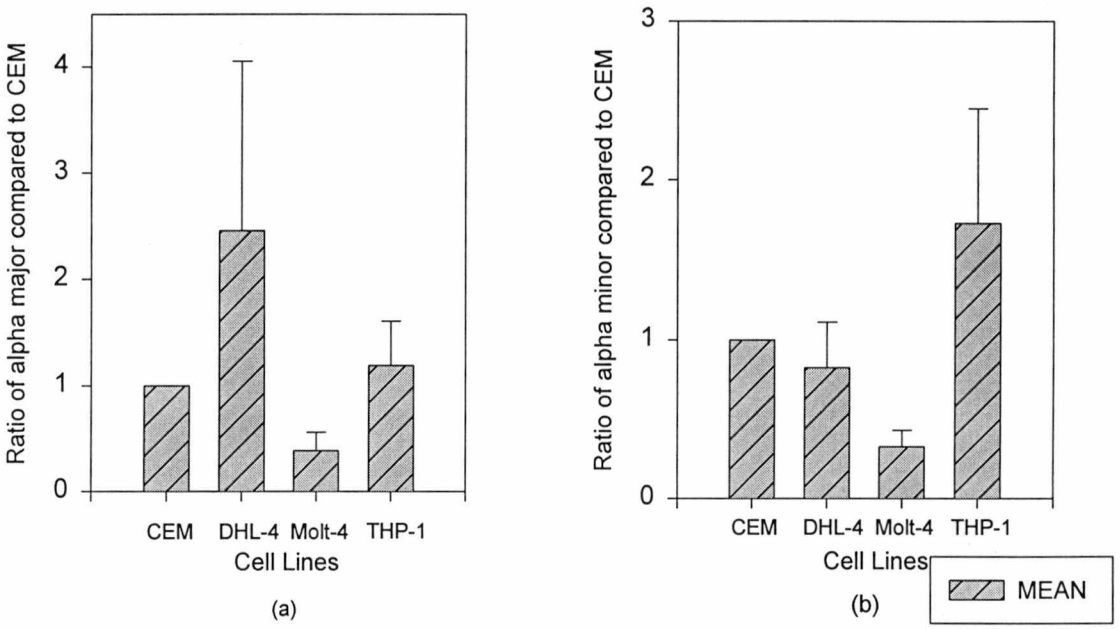


Figure 12: Expression of alpha subunits of LFA-1 major and LFA-1 minor in various cell lines. RNA was isolated from cells with no treatment and analysis was done by RT-PCR and Southern blot. The results of densitometry on the autoradiograms were compared to that of CEM cells. Error bars show standard error of the mean (SEM). (a) LFA-1 major, (b) LFA-1 minor.

EXPRESSION OF ALPHA SUBUNITS OF LFA-1 MINOR VS. LFA-1 MAJOR IN IN RESPONSE TO VARIOUS MITOGENS IN VARIOUS CELL LINES

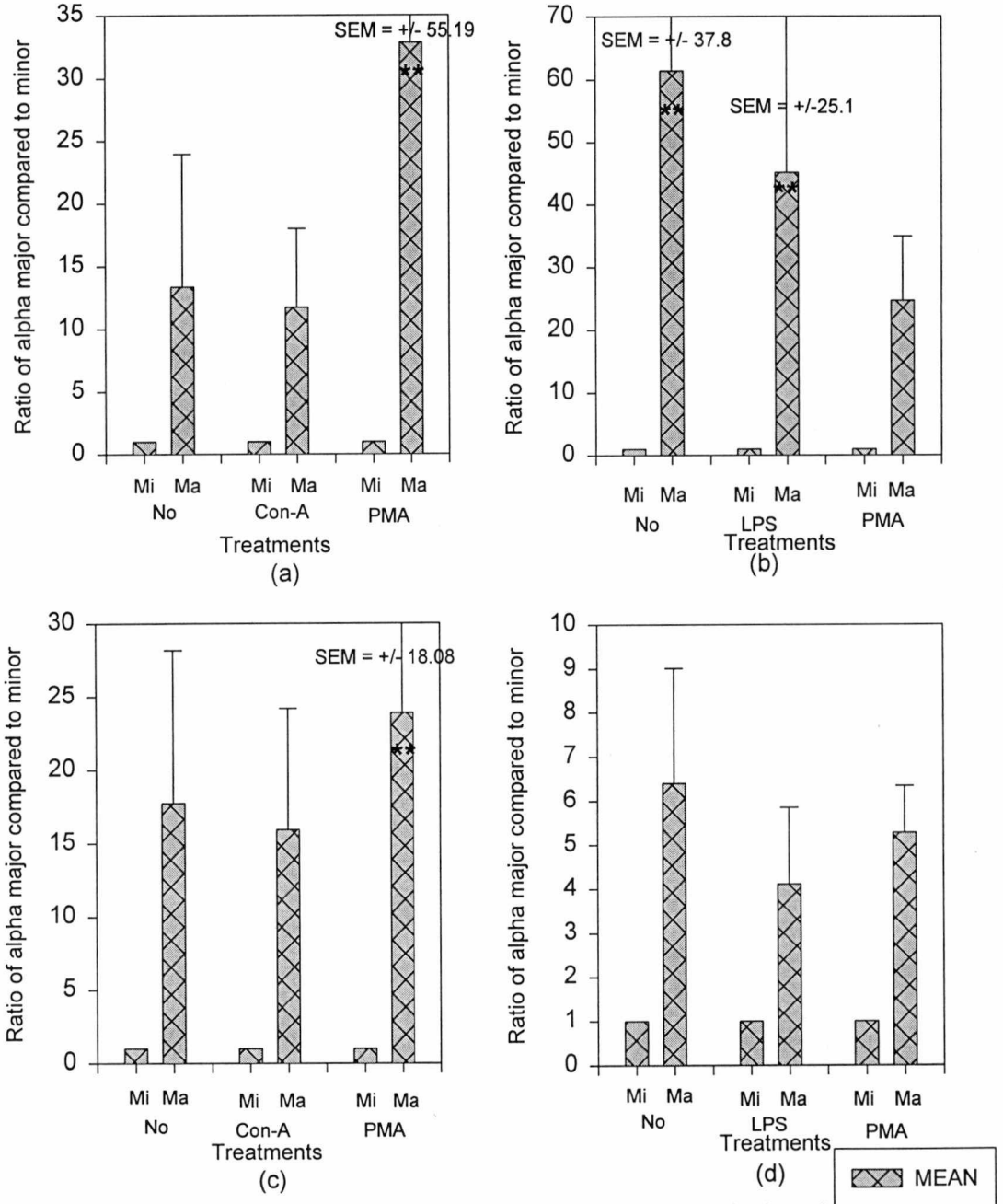


Figure 13: Expression of alpha subunits of LFA-1 minor Vs. LFA-1 major in various cell lines. Cells were treated with various mitogens for 16-20 hours and RNA was isolated. Analysis was done by RT-PCR and Southern blot. The results of densitometry were compared to that of no treatment. Error bars show standard error of the mean (SEM). The cell lines used were: (a) CEM, (b) DHL-4, (c) Molt-4, (d) THP-1. (**- high SEM, Mi = LFA-1 minor, Ma = LFA-1 major).

Table 1: The integrin family. Listed below are the integrin β -subunits, and their associated α -subunits, and known or potential ligands.

β -SUBUNIT	α -SUBUNIT	LIGANDS
β1	α1	collagens, laminin
	α2	collagens, laminin
	α3	laminin, fibronectin, epiligrin, collagen
	α4	VCAM-1, fibronectin
	α5	fibronectin,
	α6	laminin
	α7	laminin
	α8	?
	α9	?
	αV	vitronectin, fibronectin
β2	αL	ICAM-1, ICAM-2, ICAM-3
	αV	iC3b, ICAM-1, fibrinogen, factorX
	αX	fibrinogen, iC3b
β3	αIIb	fibrinogen, fibronectin, vWF, vitronectin, thrombospondin,
	αV	vitronectin, fibrinogen, fibronectin vWF, thrombospondin, osteopontin
β4	α6	laminin (?), basement, membrane protein (?)
β5	αV	vitronectin
β6	αV	fibronectin (?)
β7	α4	VCAM-1, fibronectin, MAdCAM-1
β8	αV	?

Table 2: Densitometer readings of CEM and DHL-4 cells.

CELL LINES	FRAGMENT	EXP. NO.	NO	CON-A	PMA
CEM	LFA-1 Major	I	589.64	319.72	607.52
		II	114.81	102.05	127.3
		III	65.1	37.57	30.06
		IV	295.9	283.76	297.51
		V	1511.78	1621.07	1812.03
	LFA-1 Major	I	159.65	37.03	104.94
		II	12.61	21.95	10.22
		III	2.09	3.52	4.84
		IV	20.48	21.63	35.28
		V	172.17	75.49	13.78
CELL LINES	**FRAGMENT**	**EXP. NO.**	**NO**	**LPS**	**PMA**
DHL-4	LFA-1 Major	I	178.75	135.14	82.6
		II	466.85	407.37	399.72
		III	294.51	353.14	360.84
		IV	158.89	187.89	97.54
	LFA-1 Minor	I	14.6	23.9	23.73
		II	2.7	3.46	7.66
		III	17.53	20.09	21.29
		IV	3.63	4.66	3.76

Table 3: Densitometer readings of Molt-4 and THP-1.

CELL LINES	FRAGMENT	EXP. NO.	NO	CON-A	PMA
MOLT-4	LFA-1 Major	I	589.21	287.01	793.92
		II	25.52	11.67	21.94
		III	9.94	15.86	6.94
		IV	161.47	166.62	36.33
		V	61.19	178.74	1874.2
	LFA-1 Minor	I	96.17	47.53	202.52
		II	4.88	2.02	26.19
		III	1.01	1.2	1.57
		IV	2.72	3.44	0.38
		V	7.58	28.59	128.37
CELL LINES	**FRAGMENT**	**EXP. NO.**	**NO**	**LPS**	**PMA**
THP-1	LFA-1 Major	I	337.33	630.84	635.98
		II	106.74	105.08	100.6
		III	176.73	89.64	139.79
		IV	108.03	62.56	187.25
		V	2111.15	1349.79	987.05
	LFA-1 Minor	I	49.25	191.76	131.03
		II	54.7	34.08	37.39
		III	38.39	43.23	34.54
		IV	44.84	51.47	32.54
		V	130.05	123.81	109.48

VITA

Lokesh Kumar Wuluvarana was born in Tiruchinapalli, India on May 15, 1969. He attended high schools at Central School Fort William, Calcutta, Central School ASC and Central School MEG, Bangalore, India. He joined the University of Agricultural Sciences, Bangalore, where he received the Bachelor of Science in Dairy Technology in the year 1992. He worked in a Dairy Industry and later enrolled for Master of Science in Dairy Microbiology. He published a paper in the course of this study (see below for details). In fall 1995, he joined the Master's Program in Biotechnology at the University of Tennessee, Knoxville and received a Master of Science in Life Sciences in the spring 1998. He was employed as a GTA (graduate teaching assistant) in the University of Tennessee from fall 1995 to spring 1998.

Published Paper

1. Jayaprakasha, H.M., Jayaraj, K.R., Lokesh Kumar, W.A. 1997. Studies on the influence of water activity (a_w) on the stability of foods – a critical appraisal. J. Food Sci. Technol. 34 (No. 4): 273-285.