REGULATION OF LYMPHOCYTE ADHESION:

MOLECULAR ANALYSIS

Presented by:

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MASTER OF SCIENCE

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

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ABBREVIATIONS

AA	---	amino acids
Ag	---	antigen
BCIP	---	5-bromo-4-chloro-3-indolyl phosphate
BIS	---	N,N'-methylene bisacrylamide
bp	---	base pair
BSA	---	bovine serum albumin
cDNA	---	complementary deoxyribonucleic acid
cpm	---	counts per minutes
Da	---	Dalton
ddH_2O	---	double distilled water
DNA	---	deoxyribonucleic acid
DNase	---	deoxyribonuclease
dNTP	---	2'-deoxynucleoside 5'-triphosphate
EDTA	---	ethylene diamine tetra-acetic acid
Fig	---	figure
hr	---	hour
Ig	---	immunoglobulin
IPTG	---	isopropyl-1-thio-β-D-galactoside
kDa	---	kilodalton

min	---	minute
MAb	---	monoclonal antibody
Mr	---	molecular mass
mRNA	---	messenger ribonucleic acid
PEG	---	polyethylene glycol
NBT	---	nitroblue tetrazolium chloride
PBS	---	phosphate buffered saline
pfu	---	plaque forming unit
poly(A)$^+$	---	polyadenylated mRNA
RNA	---	ribonucleic acid
RNase	---	ribonuclease
rpm	---	revolutions per minute
SDS	---	sodium dodecyl sulfate
SDS-PAGE	---	sodium dodecyl sulfate polyacrylamide gel electrophoresis
TEMED	---	N, N, N',N'-tetramethylethylene diamine
Tris	---	trihydroxymethylaminomethane
Tris-Cl	---	Tris hydrochloride
Tween 20	---	polyoxyethylene-sorbitan monolaurate
x-gal	---	5-bromo-4-chloro-3-indolyl-β-D-galactoside
^{125}I	---	iodine-125
ug	---	microgram

ml	---	milliliter
ul	---	microliter
M	---	molar
mM	---	millimolar

ABSTRACT

The induction of cellular adhesion involves multiple distinct steps. In an effort to identify some of the molecules which might be involved in the post receptor events leading to the generation of the adhesive phenotype, a panel of monoclonal antibodies to the surface of an adhesion competent leukaemic cell line were examined for their ability to modify the adhesive potential of these cells. Two such antibodies, B11E2, and A13D8 were observed to induce energy dependent intercellular adhesion. Two lambda gt11 libraries of the T cell leukaemia, Jurkat, cDNA have been screened by immunoselection using antibody to 30 kDa protein. Cloning and sequencing of the cDNA encoding the B11E2 antigen indicated almost complete ($>99\%$) sequence identity with the MIC2/E2 antigen which is expressed on the majority of human cell types and has been implicated in the adhesion of erythrocytes to human T cells. The B11E2 clone reacted with 12E7, an antibody which recognizes MIC2/E2, confirming the antigenic relationship between these two molecules.

The testing of functional activities of the B11E2 antigen indicated that induction of adhesion by B11E2 was not due to antibody and antigen crosslinking. Although the functional role of the B11E2 antigen is still unclear, the results of these studies demonstrate that induction of aggregation by B11E2 is independent of adhesion pathways mediated by LFA-1/ICAM-1, CD2/CD3 and β_1 integrin. Jurkat

cells lacking CD2 can be induced to aggregate yet the cells do not form rosettes under activating conditions. Soluble B11E2 antigen does not interfere with either normal T cell rosette formation or the aggregation of B11E2 stimulated cells, in addition, immobilized purified B11E2 does not act as adhesion substrate for B11E2 activated Jurkat cells. Intercellular adherence was not inhibited by the presence of B11E2 fusion proteins. Collectively these results suggest that B11E2 is not an adhesion molecule involved in the binding process but they do suggest a role in the regulation of adhesion. The widespread distribution of E2 suggests that this molecule may play a general role in the generation of adhesion competence of a variety of cell types.

INTRODUCTION

Roles of Adhesion in Lymphocyte Biology

Interactions of cells with cells or of cells with components of the cellular environment are an essential aspect of lymphocyte biology. An essential component of the body's defense system is the continuous recirculation of lymphocytes from the blood into lymphoid organs and back to the blood again. The recirculation of lymphocytes allows the full repertoire of antigenic specificities to be continuously represented throughout the body, and may also facilitate interactions between lymphocyte subsets and accessory cells required for antigen-specific effector functions. The first step required for lymphocyte entry into peripheral lymph nodes and mucosal lymphoid organs from the blood is recognition and adhesion to postcapillary venules lined by high endothelium. Lymphocytes as well as high endothelium employ specific molecules for this function(Jalkanen et al, 1986). Binding to lymph nodes is a cooperative process involving multiple receptors on the lymphocyte (Springer et al, 1990; Turunen et al, 1992) and counter receptors on the endothelium(Yednock et al, 1989). The interaction of lymphocyte receptor, LFA-1, and homing receptor, MEL-14 with counter-receptor , ICAM-1 and ELAM-1, on endothelial cells play a key role for leucocyte localization of circulating cells at an inflammatory site(Issekutz, 1992).

An adhesive interaction of lymphocytes with these so-called high endothelial venules(HEV) is mediated by selectins which are a family of cell surface molecules possessing three different structural motifs. MeL-14 is a selectin expressed on lymphocytes that has a critical role in interaction of lymphocytes with peripheral lymph node endothelial cells (Lasky et al, 1989; Walcheck et al, 1992). Other known members of this family are ELAM-1 and GMP-140 which are expressed on endothelial cells (Bevilacqua et al, 1989; Hattori et al, 1989). All three selectins appear to be involved in recruitment of neutrophils and other leucocytes to sites of inflammation. Evidence from several laboratories suggests that the selectins participate in the initial adhesion of neutrophils on the activated endothelium (Show et al, 1986). Several general cell adhesion molecules work in conjunction with the organ-specific receptors to mediate lymphocyte homing. Haman (1988) examined the potential role of LFA-1 in lymphocyte attachment to HEV. It was found that cell lines expressing the highest levels of the MEL-14 antigen bound better to PN HEV and was affected minimally by anti-LFA-1 and ICAM (Marlin et al, 1987). The LFA-1-ICAM pathway only plays an accessory role in lymphocyte-HEV interaction. Another molecule, VLA-4, and its ligand VCAM-1, also play a role in lymphocyte binding to HEV (Elices et al, 1990; Issekuts, 1991; Wysockj et al, 1992). Among these adhesion pathways of lymphocyte interaction with endothelial cells, the LFA-1/ICAM-1 and VLA-4/VCAM-1 are found to mediate T cell adhesion to activated endothelial cells (Dustin and Springer, 1988; Elices et al, 1990; Vachhula et al, 1992). MEL-14

contributes to the binding of resting $CD4^+$ T cells to endothelial cells(Graber et al, 1990). MEL-14 plays a very important role in the initial attachment of lymphocytes to inflamed endothelium and to the preferential migration of memory T cells into inflammatory sites (Shimizu et al, 1991).

The movement of lymphocytes into organized lymphoid tissue, the recruitment of leukocytes into inflammatory lesions, and the formation of platelet plugs at sites of vascular injury all begin with the attachment of circulating cells to modified regions of the vessel wall. Several families of adhesion molecules, including members of integrin, immunoglobulin and selectin families mediate lymphocyte migration. An important contribution of CD18 integrins was demonstrated by the finding that T cells from patients with CD18 deficiency migrated at about 50% of the level of normal T cells(Shimizu et al, 1991). LFA-1 appears to be an important integrin in this process, as monoclonal antibodies to CD11a, LFA-1 and CD54(ICAM) produced significant inhibition of migration of normal lymphocytes(Haman et al, 1988; Issekutz, 1992a; Marlin et al, 1987). The migration of neutrophils through confluent monolayers of endothelial cells into inflammatory sites has been characterized. The process of lymphocyte extravasation from postcapillary venules is mediated by the ELAM-1/LECCAM-1 , LFA-1/ICAM-1 and LPAM-1/2(alpha 4-integrin) adhesion pathways(Nakache 1989; Lewinsohn et al, 1987; Dustin et al, 1988; Elices et al, 1990; 1992; Buhrer et al, 1992). Several studies also demonstrated that lymphocytes interact with the surrounding extracellular matrix during migration in tissue; VLA-6-

mediated adhesion to laminin is likely to be critical to migration through the basement membrane(Yurchenco et al, 1990; Li et al,1992). Davis(1990) and Arencibia(1989) also identified that the interaction of integrins with collagen and FN provided functionally important for lymphocyte migration.

It is also well known that the interaction of lymphocytes are essential for cell mediated immune responses. T lymphocytes can be activated to produce lymphokines, to express new cell-surface molecules and to proliferate. This activation of T lymphocytes is the result of ligand-receptor interactions occurring at the plasma membrane. Immune responses or T lymphocyte-mediated lysis are driven by conjugation of lymphocyte with target cells. Antigen-dependent interactions of helper and cytolytic T lymphocytes show a dramatic spatial coordination of antigen recognition and adhesion. On CTLs, the membrane molecules, T cell receptor(TCR) and CD8 recognize foreign antigens associated with MHC class-I molecules, while helper T lymphocytes recognize foreign antigens associated with MHC class-II molecules in association with the TCR and CD4 molecules(Haskins et al, 1983; Bierer et al, 1989; Shaw et al, 1986). The T cell receptor complex binding to the antigen MHC complex does not appear to be a major adhesion pathway. Four adhesion molecules, LFA-1/ICAM-1 and CD2/LFA-3, seem to contribute a major component of the adhesive potential of helper and killer T lymphocytes with target cells(Kupfer et al, 1989; Springer et al, 1987; Kishimoto et al, 1987).

Intercellular Adhesion Molecules

As detailed above, cytoadhesion of cell to cell or to components of the cellular environment plays an important role in lymphocyte biology. Many cell surface adhesion molecules have been identified which are involved in diverse adhesion systems. These molecules are listed in Table 1(from Dustin and Springer, 1991).

Table 1 Guide to lymphocyte adhesion molecules

Adhesion receptors/counter-receptors					
Name[a]	Synonyms	Size[b] (kd)	Name	Synonyms	Size (kd)
LFA-1	Integrin α_L/β_2 CD11a/CD18	α, 180 β, 95	ICAM-1 ICAM-2	CD54	74–114 70
CD2	E rosette receptor, T11, leu 5, LFA-2	50	LFA-3	CD58	55–70
CD4	T4, leu 3	55	MHC class II		α, 34 β, 29
CD8	T8, leu 2	α-α & α-β 30–38	MHC class I		α, 44 β, 12
CD44	ECMR-III, Pgp-1, Hermes	90 & 200	(?) (collagen)		
VLA-4	Integrin α_4/β_1 CD49d/CD29 LPAM-1	α, 150 β, 110	VCAM-1 Fibronectin CS-1 region	INCAM-110	110
LPAM-2	Integrin α_4/β_P CD49d/CD-	α, 150 β, 110	(?)		
(?)			Mucosal addressin (MECA-367)		58–66
Mel-14	LAM-1, leu 8 TQ1	90	Phosphorylated oligosaccharides		(?)

As shown in table 1, multiple receptor systems mediate a variety of lymphocyte adhesion capabilities. In this introduction, only some adhesion molecules, β_1 , β_2 integrins, CD2 and CD3 are described.

The largest number of integrins are members of the β_1 or VLA subfamily. Seven receptors, each with different ligand specificities, have been identified in this family(Albelda et al,1990). The classical fibronectin receptor ($\alpha_5\beta_1$), the VCAM and fibronectin receptor($\alpha_4\beta_1$), and the laminin receptor($\alpha_6\beta_1$) appear to have unique ligands. Other members of this subfamily bind to more than one ligand and can exhibit different ligand specificities depending on the cell in which they are expressed. The stability of adhesive structures may require cooperative interactions between multiple integrins binding to alternative sites on the same or different matrix molecules, or each integrin may transmit different information from the extracellular environment which determines the morphology and physiology of the cell. Unlike other members of the β_1 integrin family which are only receptors for extracellular matrix, $\alpha_4\beta_1$, and $\alpha_5\beta_1$ have been functionally implicated in cell to cell and cell to matrix adhesion(Szekanecz et al, 1992). On both mouse and human lymphocytes, $\alpha_4\beta_1$ acts as a homing receptor, facilitating attachment to Peyer's Patch HEV(Holzmann et al, 1989; Wysocki et al, 1992). The $\alpha_4\beta_1$ mediates adhesion of human lymphocytes to activated endothelial cells by binding to an inducible endothelial cell surface protein called VCAM-1(Elices et al, 1990; Vonderheide et al, 1992). The $\alpha_4\beta_1$ also has a role in heterotypic adhesion between cytolytic T cells and B cell targets(Takada et al,

1989).

Another important adhesion subfamily of integrins is β_2, also known as LEUCAMS or CD18 antigens, consists of three leukocyte adhesion receptors, LFA-1, MAC-1, and GP150,95 which share a common β subunit with different α subunits. LFA-1 is expressed on most types of white blood cells. It plays an important role in leukocyte-leukocyte interactions and leukocyte-endothelial cell adhesion(Martz, 1987; Lauscinskas et al, 1991; Denger et al, 1992). LFA-1 is involved in T cell, killer cell, and antibody-dependent cytotoxicity, as well as in helper T cell function(Springer et al, 1987). The ligand for LFA-1 is ICAM-1, a member of the immunoglobulin superfamily(Wawryk et al, 1989). The LFA-1/ICAM adhesion shows both short and long time-scale regulation through LFA-1 and ICAM-1, respectively.

Studies have shown that LFA-1 can play a role on both inside-out and outside-in signal transduction. An antibodies to LFA-1 block many T lymphocyte responses by interfering with reception of signals through the TCR and other surface molecules(van Noesel et al, 1988; van Kooyk et al, 1989). The other members of β_2 integrin family, MAC-1 and gp150,95, are found on neutrophils, monocytes, and some lymphocytes. They are particularly important in the adhesion of myeloid cells to other cells and to ligands that become insolubilized during activation of the complement and clotting cascades(Kishimoto et al, 1989a). The importance of the β_2 integrins is illustrated in congenital leukocyte adhesion deficiency(LAD) in which they are deficient in β_2 integrins because of mutations in the common β_2 subunit(

Kishimoto et al, 1989; Aderson et al, 1987).

Another two adhesion molecules which are involved in interaction of lymphocytes to lymphocytes, lymphocytes to endothelial and lymphocytes to erythrocytes are CD2(Kamoun et al, 1981; Seed et al, 1987) and LFA-3(Huing, 1985; Tiefenthaler et al, 1987). The primary structures of CD2 and LFA-3 resemble those of the immunoglobulin superfamily. CD2 is expressed only on T lymphocytes and their progenitors. In contrast, LFA-3 is expressed on virtually all cells. Adhesion mediated by CD2 interaction with LFA-3 is independent of divalent cations. Once contact between cells is established, the CD2/LFA-3 mechanism appears to be significantly more efficient in mediating adhesion at 4°C than 37°C. It suggests that cell motility or other active processes may work against stable adhesion mediated by CD2/LFA-3(Shaw et al, 1986). As a lymphocyte receptor, CD2 also plays a role in signal transduction. It cooperates with a signal mAb to the TCR in the activation of lymphocytes(Yang et al, 1986). Interaction of CD2 with LFA-3 may deliver a co-signal with the TCR that increases adhesion of CTL with target cells and enhances CTL-mediated lysis. In sheep, a ligand for CD2 has been identified on sheep red blood cell surface as being a 42 kDa protein, termed T11Ts(Hunig et al, 1987). Besides the T11Ts , two other molecules from the sheep erythrocyte surface involved in rosette formation have been identified, termed S14 and S110-220 according to their molecular weight(Bernard et al, 1987). In human, CD2 also interacts with more than one ligand. A human erythrocyte surface protein, termed H19, a 19 kDa protein, was

identified as being involved in rosette formation (Groux et al, 1989). The H19 molecule is not limited to the erythrocyte surface, but is also present on many nucleated cells. Like LFA-3, the H19 molecule is required for T lymphocyte activation.

Control of Cellular Adhesion

Studies with different adhesion molecules as described above indicated that cellular adhesion is mediated by many different mechanisms. Studies with the integrins and other adhesion molecules indicate that the adhesion process is a multistep one. It can be regulated at different levels, viz; 1) modulation of the expression of receptor and ligands; 2) activation processes may modify adhesion molecules or enzymatically clip molecules from the cell surface; 3) specific activation of adhesion receptors can affect their ligand binding affinity; 4) multiple receptors interacting with the cytoskeleton can also affect cell adhesion.

Several observations show that the number of adhesion receptors expressed by a cell can be modulated by different factors, such as cytokines and products from infectious agents. Among the adhesion molecules expressed on endothelial cells and lymphocytes, only very few are constitutively expressed on cells, like ICAM-2, one of the counter receptors of LFA-1(Dustin et al, 1988; Staunton et al 1989). Most adhesion molecules expressed on cells are inducible. Several counter-receptors, such

as ICAM-1, and LFA-3, under normal physiological conditions, are not or only minimally expressed by endothelial cells and other kind cells. High level expression is induced by stimulation with TNF-α, IL-1β and IL-4(Dustin et al, 1986; Renkonen, 1989; Osborn et al, 1989; Shimizu et al, 1990; Piela-Smith et al, 1992). Several lymphocyte receptors, such as, LFA-1, CD2 and VLA-4 also can be induced by IL-2(Dustin et al, 1986; Hemler et al, 1987; Hughes et al, 1990) . FMLP, IL-8, or ATP can activate CR-3 mediated adhesion to endothelium or homotypic aggregation of granulocytes (Buyon et al, 1988; Vedder et al 1988). Also, VLA-4, VLA-5 and VLA-6 expressed on CD4 cells can be activated through PMA(Wilkins et al, 1991; Shimizu et al, 1990). Some lymphocyte receptors can be down regulated, like the homing receptor, LECAM-1. Its expression can be rapidly inhibited by phorbol ester(PMA) and IL-1β(Smith et al,1991; Kishmoto et al, 1989b).

The expression level of adhesion receptors does not necessarily correlate with adhesiveness. The second mechanism to control cell adhesion is modulation of the affinity of the receptor for its ligand. Resting leucocytes or platelets do not adhere spontaneously. A variety of stimuli can induce integrin-mediated cell-cell interactions. Exposure of lymphocytes and platelets to phorbol esters strongly induce cell aggregation(Dustin et al, 1988; Wright et al, 1986). Similarly, FMLP can stimulate CR3-mediated adhesion of granulocytes to endothelial cells(Buyon et al, 1988) and thrombin can cause IIB/IIIA-mediated aggregation of platelets(Shatti et al 1987). These adhesion processes are mediated through transactivation of adhesion molecules.

It has been found that some membrane molecules can play dual roles as adhesion molecules and signalling molecules, such as CD2, CD3 and LFA-1. Van Kooyk (1989) found that T lymphocyte surface molecules CD2 and CD3 can trigger LFA-1-mediated cell adhesion. They demonstrated by using cells from an LFA-1-deficient patient that induction of adhesion by anti-CD2 and anti-CD3 antibodies is a consequence of activation of the LFA-1 molecule, rather than activation of its ligand or other adhesion molecules,such as CD2 and CD3. Stimulation by anti-CD2 and CD3 antibodies probably results in a conformational modification of the LFA-1 molecule and leading to high-affinity ligand binding(Van Kooyk et al, 1991). Like anti-CD2 and CD3, antibodies directed against CD14, CD15, CD40, CD43 and CD44 also can induce LFA-1-mediated adhesion (Lauener et al, 1990; Forsyth et al, 1989; Barrett et al, 1991; Axelsson et al, 1988; Koopman et al, 1990). The phenomena of cellular adhesion regulated by intracellular messengers is not well understood. Some reports indicate that anti-LFA-1 antibody together with anti-CD3 antibodies enhance lymphocyte adhesion (Alcover et al, 1988). Similarly, binding of lymphocytes to immobilized affinity purified ICAM-1 and anti-CD3 antibodies induce cell aggregation (Dustin et al, 1989 and 1988), supporting the notion that ICAM-1 can provide a costimulatory signal through LFA-1. Also anti-CD2 and anti-CD3 antibodies induce T- lymphocyte proliferation through costimulatory activity of VLA-4 and VLA-5 binding to fibronectin (Davis, et al 1990; Yamada, et al 1991). Other extracellular matrix components, like collagen and laminin have also been shown to provide

costimulatory signals together with PMA(Wilkins et al,1991; Mercurio et al, 1988).

Studies have also shown that for cell-cell aggregation, the cell surface molecules can be activated to directly bind to their ligands. An anti-LFA-1 antibody, NKI-L16, which induces lymphocyte homotypic aggregation has been described(Van Kooyk et al, 1991). NKI-L16 induces aggregation by directly binding to LFA-1 and modulating the conformation of the LFA-1 molecule. Van Kooyk (1991) suggested that the adhesion molecule, LFA-1, can be expressed on the cell membrane in three distinct forms. The first is an inactive form of LFA-1 in which the NKI-L16 epitope is absent or only partially exposed. The second form is an intermediate form that expresses the Ca^{++}-dependent NKI-L16 , this form can be rapidly converted by NKI-L16 antibody into the third form, activated LFA-1, which is created by a conformational change of LFA-1. They also indicated that cell aggregation induced by NKI-16 antibody is an extracellular event leading to an increased binding to ICAM-1 which is not due to crosslinking or to demonstrable induction of signalling pathways that stimulate other cellular adhesion molecules. Similarly, an anti-VLA-4 antibody, L-125, which induces aggregation among human T and B cells has been identified (Takada et al, 1989). Aggregation induced by L125 antibody is independent of the LFA-1, CD2, CD4, CD8 adhesion pathway, but metabolic energy as well as an intact cytoskeleton are required. So, Bednarczyk suggested that the VLA-4 molecule is involved directly in binding to a ligand during mAb L-125-mediated

aggregation. Just like LFA-1 induced by NKI-L16, VLA-4 would reside on the lymphocyte membrane in an inactive or low affinity form with binding of mAb L-125 increasing its affinity for the ligand. Other antibodies that induce adhesion molecules to bind ligand have also been described. mAb specific for the CD9 has been shown to induce platelet aggregation(Higashihare et al, 1985). Acting on the same kind cells, an anti-IIb/IIIa antibody, CD33, induces a conformational change in IIb/IIIa and results in increasing binding to its ligand(Gulino et al, 1990).

Besides the transactivation and conformational change of adhesion molecules, clustering and reorganization of adhesion molecules and interaction of adhesion receptors with the cytoskeleton also can control the cellular adhesion processes. Clustering of integrins at the cell surface by their ligand on an adjacent cell may generate signals that maintain or extend the active state of the receptor(Detmers et al, 1987). Studies have shown that multiple receptor-ligand interactions could overcome the relatively low affinity of the receptor for its ligand and may result in stable adhesion(Hermanowski et al,1988). Several studies have revealed that integrins are attached to cytoskeleton components, including talin, vinculin, and actin filaments(Burn et al, 1988; Haverstick et al, 1992; Shaw et al, 1990). Experiments have also shown that PMA which activates protein kinase C and induces cellular adhesion, has a dramatic effect on cytoskeleton organization(Haverstick et al,1992).

Studies with different adhesion molecules indicate that the process is a multistep one in which induction of receptor activation and multiple subsequent steps

are involved in generating an adhesion competent cell. However the nature of the biochemical and molecular events are unknown at this time.

Purpose of Project

In an effort to identify some of the molecules which might be involved in the post receptor events leading to the generation of the adhesive phenotype, a panel of monoclonal antibodies which induced cell adhesion were prepared against the Jurkat leukemia cell line and a number of antibodies were identified and used to determine the target antigens by radioimmunoprecipitation(RIP) SDS PAGE analysis of ^{125}I labelled surface antigens. These antibodies defined four groups of molecular species: 1), 30 kDa antigen, 2), 90-110 kDa molecules, 3), 130-150 kDa family of heterodimers which belong β_1 integrin and 4), 180-220 kDa antigens which have been identified as members of the CD45 family. The 30 kDa and 90-110 kDa antigens were unknown species, possibly representing new molecules involved in cell adhesion. This project was designed charactering to the 30 kDa antigen and determine its potential roles in the cellular aggregation of lymphocytes.

MATERIALS AND METHODS

MATERIALS

1. Chemicals

Chemicals were purchased from Sigma Chemical Company (ST. Louis, MO USA) and Fisher Scientific Company (Fair Lawn, NJ. USA). Reagents for cell fusion and hybridoma production and fetal calf sera were purchased from Gibco (Gaitherburg, MD. USA).

2. Radioisotopes

Deoxycytidine 5'-[α-^{32}P]triphosphate(dCTP-α-^{32}P), 10 mCi/ml, >3000 Ci/mmol, Adenosine 5'-[γ-^{32}P]triphosphate(ATP-γ-^{32}P), >7000 Ci/mmol, Deoxyadenosine 5'-[α-^{35}S] thiotriphosphate(dATP-α-^{35}S), >1000Ci/mmol and ^{125}I-labeled $F(ab)_2$ sheep anti-mouse Ig were purchased from Amersham (Arlinton Heights, IL. USA).

3. Membranes

Nitrocellulose membranes(0.45 μm) used for western blotting were obtained from Bio-Rad Lab(Richmond, CA. USA). Hybond-nylon membranes(0.45 μm) used for northern blotting and plaque blotting were purchased from Amersham (Arlington

Heights, IL.USA).

4. Enzymes And Kits

All restriction enzymes were purchased from Gibco (Gaitherburg, MD. USA) . The ligation kit, random primer labelling kit, were purchased from Stratagene (LA. Jolla, CA. USA). The immunoscreening Kit was obtained from Clontech (Palo Alto, CA. USA). The DNA sequencing kits were purchased from United State Biochemical Corporation (Cleveland, Ohio.USA). The fusion protein purification kit was obtained from New England Biolabs.

5. cDNA Libraries

A cDNA expression library of the human leukaemic T lymphocyte cell line, Jurkat, prepared in the vector λ ZAPII^R was purchased from Stratagene (Jolla, CA. USA). This library was constructed by priming with oligo(dT). The number of independent clones was more than 1 x 10^6, with an average insert size was 0.8 kb.

A cDNA expression library of Jurkat cell line, constructed in λ gt11, was purchased from Clontech (Palo Alto, CA. USA). This library was made by the method of random-priming and contained 1.8 x 10^6 independent clones. The insert size range is 0.4-3.5 kb, average size is 1.0 kb.

6. Antibodies

Hybridomas to CD2(OKT11), CD3(OKT3), CD4(OKT4) and CD8(OKT8) were purchased from the American Type Culture Collection(ATCC)(Rockville, MD. USA). Monoclonal antibody, 12E7 specific for MIC2, was provided by Dr. R. Levy(Stanford University, School of Medicine).

7. Cell lines

HSB2, CEM, Molt 3, Molt 4, RPMI 8226, Ramos, U266, Colo 205 and K562 were purchased from ATCC (Rockville, MD. USA). Jurkat was subcloned from a stock provided by Drs. C. Bleakley and V. Paetkaeu, University of Alberta. All cell lines were maintained in RPMI-1640 10% fetal calf serum at 37^{0}C in a 5% CO_2 environment.

METHODS

1. Cell culture

Jurkat cells were grown in RPMI 1640 supplemented with 10% fetal calf serum at 37^{0}C in a 5% CO_2 and subcultured by dilution in culture medium every 3-4 days.

2. Monoclonal antibody production and characterization

Monoclonal antibodies were produced in the laboratory by Shen Caixia and John Wilkins as described(Shen et al, 1991). The initial screening was performed by selecting those cultures which produced antibodies that bound to B cell line, JR-2B10. The binding was detected using a sandwich technique in which the cells were incubated with test supernatant for 30 minutes, washed three times in PBS-1% BSA , and reacted with a ^{125}I-labelled F(ab)$_2$ goat anti-mouse Ig for 30 minutes. The cells were washed three times and the level of bound radioactivity was determined. Positive clones were expanded, and tested for induction of homotypic aggregation, as well as immunoprecipitation of ^{125}I-labelled Jurkat surface proteins.

3. Aggregation Assay

Washed Jurkat cells were suspended(2 x 10^6/ml) in RPMI-1640 containing 10% fetal calf serum and 100 μl incubated with an equal volume of hybridoma supernatant in flat bottom microtiter trays for three hours at 37^0C. The numbers of free cells(non free is clusters of $\geq$ 3 cells) in the control and treated groups were counted in 3 fields using an ocular fitted with a 10 x 10 square graticule. This represented on average a total of 200-250 cells per control sample. The percentage of aggregation was calculated using the following formula:

$$1 - \frac{\text{[No. of free cells in treated group]}}{\text{[No. of free cells in control group]}} \times 100$$

In those cases where the effects of antibodies or inhibitors on aggregation were under examination, the cells were preincubated for 30 minutes before addition of the aggregation stimulus.

4. Flow Cytometry

Cells were washed in PBS containing 1% BSA, 0.01% sodium azide(PBS-A) and resuspended in the same medium at 10^7 cells/ml. The cells were incubated on ice for 30 min with a saturating concentration of mAb and washed three times with

PBS-A then reacted with an FITC-conjugated $F(ab')_2$ rabbit anti-mouse immunoglobulin for 30 min on ice. The cells were washed three times with PBS-A and fixed for 10 min with PBS containing 1% paraformaldehyde. Samples were analyzed using a Coulter Epice IV cytometer. Background levels of binding were determined using cells which had been treated with a control mouse IgG.

5. FACS

10^6 cells were pelleted, resuspended in 100 μl of PBS/0.1% azide/1%FBS. The cells were placed into V-bottom 96 well plate and spun down. Antibody was added and shaken on ice one hour. The cells were washed 3 times in PBS/0.1% azide/1% FBS then reacted with FITC-conjugated $F(ab)_2$ goat antimouse immunoglobulin. Cells were analyzed by a Coulter Epics IV FACS system.

6. Immunoprecipitation

The cells surface were iodinated using lactoperoxidase, solubilized in 0.5% NP-40/1 mM phenylmethysulfonylfluoride/PBS. The cell lysates were precleared by incubating with an equal volume of 10% formalin-fixed S.aureus for 30 min and the monoclonal antibodies were added and incubated overnight at 4^0C. The proteins were immunoprecipitated with S. aureus preloaded with rabbit anti-mouse immunoglobulin.

The immunoprecipitated proteins were separated through 7% to 10% polyacrylamide gels. Autoradiography was performed to visualize the proteins.

7. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis(SDS-PAGE)

The discontinuous buffer system of Laemmli(1970) was used with all reagents being of electrophoresis purity grade. The gels were run in 0.025 M Tris/0.129 M glycine/0.1% SDS at 100 volts until the tracking dye reached the bottom of the gel. Estimation of molecular weights was performed using the prestained molecular weight standards lysozyme(14,000), soya bean trypsin inhibitor(21,500), carbonic anhydrase(31,000), ovalbumin(46,000), bovine serum albumin (66,000), phosphorylase b(92,500) and myosin(200,000)(Amersham). The gels were then either stained with Commasie blue or blotted to transfer the protein to a nitrocellulose membrane.

8. Western Blotting

The procedure involved the transfer of proteins separated by SDS-PAGE(described as above) onto a nitrocellulose membrane(NCM). An LKB electroblot apparatus was used for the protein transfer. The transfer was performed using 1 mAMP/1cm^2 for one hour. The NCM with bound protein was immersed in a blocking buffer(20% calf serum in PBS pH7.5) for 90 minutes at room temperature

with shaking. This was done to prevent non-specific binding of immunoglobulin to areas on the NCM where protein had not been attached. The hybridoma supernatant was diluted in 20% serum-PBS-0.05% Tween 20 solution. The amount of antibody added to the NCM depended upon the titer of the hybridoma supernatant. The NCM with antibody was incubated for one to two hours at room temperature with shaking. It was then washed three times over 40 minutes in wash buffer(0.1% Tween 20-PBS pH7.5) and then the second antibody was added. The second antibody was rabbit anti-mouse immunoglobulin conjugated alkaline phosphatase at a dilution of 1:2500 in a 20% serum-PBS-0.05% Tween buffer. This incubation step was carried out at room temperature for one hour followed by three washes in wash buffer. The blots were developed with 10ml buffer C (0.1M NaCl, 0.1M Tris-HCl, 50 mM $MgCl_2$, pH9.5) mixed 33 ul(50 mg/ml) 5-bromo-4-chloro-3-indolyl phosphate (BCIP) in combination with 66 ul(50 mg/ml) nitro blue tetrazolium (NBT). The colour was developed for 20 to 40 minutes and the NCM was washed with dH_2O and blotted dry.

9. Screening and Isolation of B11E2, A13D8 Reactive Materials for Jurkat cDNA Libraries

Growth of plating cells. E. coli strain Y1090 r- was streaked on LB plates (5g NaCl, 5g yeast extract, 10g tryptone and 17g agar per liter, pH7.5). A single colony was selected after overnight incubation at 37°C and incubated in 10 ml LB

mediumt 37°C with aeration.

Infecting of cells with phage. An overnight culture of host cells was centrifuged and then suspended in a 10 mM $MgSO_4$ solution to an OD=1. If plating was done on 90-mm plates, 0.2 ml of the adjusted host cells was mixed with 0.1 ml of the appropriate dilution containing 5 x 10^4 pfu of the lambda gt11 cDNA library for each plate. If plating was done on 150-mm plate, 0.6 ml of host cells was used and mixed with 2.5 to 3 x 10^4 phages for each plate. The mixed cells with phages were incubated for 20 minutes at 37°C.

Plating cells. A volume of 2.5 ml (for a 90-mm plate) or 7 ml(for 150-mm plate) LB soft agar(0.7% agar, 0.2% maltose, 5mM $MgCl_2$ in LB, pH 7.5) was added to the culture and poured onto an LB plate(1.5% agar, 0.2% maltose, 5mM $MgCl_2$ in LB, pH 7.5). Slightly dry plates(i.e., 2 days old) were used so that the soft agar would stick to the plate. The plates were incubated at 42°C for 4 hours.

Overlaying nitrocellulose membrane. The plates were removed to a 37°C incubator and each plate overlaid with a dry nitrocellulose membrane disk which had been saturated previously in 10 mM IPTG solution. The overlaid plates were incubated for 6-12 hours at 37°C.

Preparing antigen-bound membrane for antibody screen. The membranes were placed in TBST (10mM Tris-HCl at pH7.9,150 mM NaCl,0.05% Tween 20) and rinsed briefly to remove any remnants of agar. In some cases, a second membrane was overlaid after the first had been removed and then was incubated for

an additional 6-12 hours at 37°C.

Blocking nonspecific protein binding sites. The membranes were put on the surface of TBST in individual petri dishes until they were evenly wet and gently agitated for 20 min with one buffer change. To saturate non-specific protein binding sites, the membranes were incubated in TBST + 20% calf serum for one hour with shaking. 7.5 ml blocking buffer was used for 82-mm membrane and 15 ml for 137-mm membrane.

Binding of primary antibody. The membranes were incubated in TBST + primary antibody (hybridoma supernatant diluted to 5 to 10 times) for 40 minutes with gentle shaking. The diluted primary antibody was reused several times.

After primary antibody binding, the membranes were washed in 20 ml TBST three times for 5-10 minutes each.

Binding anti-IgG alkaline phosphatase conjugate. The membranes were transfered to the buffer A(0.2 M NaCl, 0.1 M Tris-HCl pH 7.9, 0.05% Triton X-100) containing the alkaline phosphatase conjugated rabbit anti-mouse immunoglobulin(1:2500 dilution) and incubated for 30 minutes at room temperature with shaking.

The membranes were washed in buffer A, as above.

Colour development. The membranes were blotted damp dry on filter paper and transfered to the colour development substrate solution. For 5 ml solution, 33 ul of 50 mg/ml NBT and 16ul of 50mg/ml BCIP were added to 5ml buffer C (0.1 M

NaCl, 0.1M Tris-HCl pH 9.5, 50mM $MgCl_2$). The membranes were incubated in development solution for 30- 60 min. The reaction was stopped by rinsing the membranes with several changes of deionized water.

10. Fusion Protein Preparation From λgt11 Recombinants

Fusion proteins were prepared(Snyder et al, 1987) from 3 positive recombinants and 1 negative lambda gt11 cDNA clone by infecting 3 x 10^8 Y1089 E. coli cells with 1.5 x 10^9 phages to 3 x 10^8 E.coli Y1089 cells in a total of 5ml. The cultures were incubated at 37^0C for 1 hour, then 0.3mM IPTG was added and incubation continued until the bacteria lysed. The lysates were collected by centrifuging at 20,000 rpm for 20 minutes. The supernatants were frozen at -70^0C and lyophilized overnight. The powder containing the fusion proteins was resuspended in ddH_2O.

11. Determination of Insert Size and Subcloning

Proof that the λgt11 positive cDNA recombinants contained inserts was accomplished by in vitro cloning using the PCR technique (Sambrook et al, 1989). Two primers, λ gt11 forward, 5'-d(GGTGGCGACGACTCCTGGAGCCCG)-3' and λ gt11 reverse, 5'-d(TTGACACCAGACCAACTGGTAARG)-3' were purchased from

University of Calgary. Prior to amplification, a plaque was placed in 200 μl of water and agitated for one hour to uncoat phage DNA. Amplification conditions were performed as follows; 50 μl of uncoated phage DNA, 10 μl of 10x reaction buffer, 100 ng of each primers, 1 mM of dNTPs, 2.5 units Taq DNA polymerase, ddH_2O was added to 100 μl. Reactions were cycled as follows; denaturation- 95 ^{0}C x 1 min: annealing- 55-60 ^{0}C x 1 min: extension- 72 ^{0}C x 2-3 min; for a total of 35 cycles. An aliquot of 10 ul was removed from each PCR reaction and separated by electrophoresis in agarose gel to check the quality of the products. The fragments from PCR reaction were kinased and ligated with sequencing vector, pBS-M13+ (Stratagene), which was previously restricted and dephosphatased with Sma I and Calf alkaline phosphatase respectively. The ligation reactions were incubated at 15^{0}C overnight, then the reaction was precipitated and transformed into E. coli strain, XL1-Blue, which had been prepared for competent by the method described by Sambrook et al (1989).

12. Nucleotide Sequencing

The Sanger(1977) dideoxy-mediated chain-termination method was used for sequencing plasmids that contained the cloned cDNA. The procedure was followed as described by manufacture(UBS Corp. Cleveland, Ohio. USA).

13. Protein Fusion And Purification

(a). Construction of fusion vector

The cDNA fragments of B11E2CLONE1 which were obtained directly from λ gt11 were digested with EcoRI, then ligated with the fusion protein vector, pMAL-cRI(BioLabs, New England), in which the EcoRI site is in the same reading frame as λ gt11. The procedures for ligation, transformation and positive clone selection are the same as previously described. Positive transformants were screened by restriction digestion followed by immunoblot.

(b). Fusion protein preparation

Ten ml of an overnight culture of cells which contained fusion plasmids were incubated in one liter rich broth + glucose and ampicillin(10 g tryptone, 5 g yeast extract, 5 g NaCl, 2g glucose, and 50 mg of ampicillin). Once the cells had grown to an OD_{600} of = 0.4, IPTG was added to a final concentration of 0.1 mM. The cells were incubated at 37°C for 2 hours, harvested by centrifugation at 5000g for 10 min, and resuspended in 50 ml lysis buffer. The sample was frozen overnight at -20°C. The cells were sonicated(Modle VC375 Sonicator, Sonics & Meterias INC. Dnabury,

CT USA) 6-10 1 min intervals setting. NaCl was added to 0.5 M, dissolved, and centrifuged at 10,000g for 30 min. The supernatant was stored in -70^{0}C until required for purification.

(d). Fusion protein purification

1.5g of amylose resin(New England Biolabs) was swollen for 30 min in 50 ml column buffer(10 mM sodium phosphate, 0.5 M NaCl, 1 mM sodium azide, 10 mM ß-mercaptoethanol, 1 mM EGTA, pH 7.2), degassed with an aspirator, and the amylose resin was poured into a 50 ml syringe. The column was washed with 3 column volumes of column buffer containing 0.25% Tween 20. The crude sample was diluted to 1:5 with column buffer containing 0.25% Tween 20 and the diluted sample was loaded into the column at a rate of about 1 ml/min. The loaded column was washed with 3 column volumes of column buffer containing 0.25% Tween 20, then washed with 5 column volumes of column buffer. The fusion proteins were eluted with low salt buffer(30 mM NaCl, 10 mM maltose) and collected in 20-30 1.5 ml fractions.Protein concentration was determined by absorbency at 260 nm. The protein-containing fractions were pooled and concentrated by freeze drying and resuspended to yield a final concentration of 0.5mg/ml. The samples were stored in -70°C.

14. Recloning Of Expression Vector

A set of Epstein-Barr virus episomal expression vectors were provided by Dr.M Tykocinski(Case Western Reserve University). pREP4(Guinng, 1987) was selected for task of subcloning. Two pBS-M13+ clones which contained the B11E2 inserts in opposite orientations were selected. Both clones were digested with BamHI and HindIII and subcloned into pREP4 which had been digested with same enzymes. Thus pREP4 constructs contained the insert in either sense or antisense orientation. The ligation, transformation and screening were performed as described by Sambrook et al(1989).

15. Northern Blotting

(a). Isolation of total RNA from Jurkat cells

1.5 x 10^8 cells were homogenized in 5 ml GITC buffer(4.0 M guanidinium thiocyanate, 0.1 M Tris-HCl (pH 7.5), 1% ß-mercaptoethanol) for 10 min. The cell lysates were passed through a No.23 guage needle several times(to shear the DNA) and centrifuged at 10,000 rpm for15 min. 2.5 ml of supernatant were transferred to an ultracentrifuge tube containing 9 ml of 5.7 M CsCl. The samples were centrifuged at 38K rpm (Ti50) for 22 hours at room temperature. The fluid above the cushion was carefully removed and the tubes were inverted and drained to dryness. The pellet was

resuspended in 1 ml ddH_2O, placed on ice and vortexed. The sample was incubated at 65^{0}C for 20 minutes, vortexing occasionally, then centrifuged with microcentrifuge at 13,000g for 5 min. To the supernatant was added 1/10 volume 3 M sodium acetate(pH 5.2), 2.5 volumes of ice-cold ethanol, mixed and stored at -70 ^{0}C for 30 min. Total RNA was collected by centrifugation at 13,000g for 15 min at 4^{0}C. The pellet was washed with 70% ethanol and vacuum dried.

(b). mRNA purification

Two oligo(dT)-cellulose columns were prepared by loading two 5-ml hypodermic syringes with presoaked oligo(dT)-cellulose, each with a bed volume of 2 ml. The columns were washed with 10 bed volumes of sterile distilled water, 5 bed volumes of 0.1 M NaOH, and 10 bed volumes of binding buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.5 M NaCl, 0.5% SDS). Total RNA was resuspended in 1 ml ddH_2O, incubated at 65^{0}C for 10 minutes, and placed on ice. An equal volume of 2x loading buffer (20 mM Tris-HCl, pH 7.5, 2 mM EDTA, 1 M NaCl, 1% SDS) was added to the RNA solution. This mixture was applied to one of the oligo(dT)-cellulose columns at room temperature. The effluent was collected and reapplied to the column which was then incubated for 20 min and washed with 5 bed volumes of wash buffer(10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.1 M NaCl). The sample was eluted with sterile TE buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA) and collected in 0.5 ml fractions. The RNA- containing fractions were determined by absorbency

at 260 nm. The pooled sample was heated at 65^{0}C for 10 minutes and brought to room temperature before applying to the second oligo(dT)-cellulose column. Sample application, elution, collection were as previously described.

(d). Hybridization

2 μg of mRNAs were electrophoresed through a 1.2% agarose formaldehyde gel as described by Amersham(1985). mRNAs were then transferred to nylon Hybond N^+ (Amersham) in 20× SSPE (3.6 M NaCl, 0.2 M NaH_2PO_4, pH 7.7, 0.02 M EDTA) overnight at room temperature. The membrane was then fixed by the method described in " protocols for nucleic acid blotting and hybridization" (Amersham, 1988).

The fixed membrane was prehybridized in a solution composed of 50% formamide, 10% dextran sulphate, 1% SDS, 20 ug denatured non-homologous salmon sperm DNA/ml at 42^{0}C for 5 hours. Labelled probes were prepared by random primer labelling as described by manufacture (Gibco). The synthesized probes were denatured by boiling for 10 min and chilled on ice prior to addition to the membrane. Hybridization was carried out at 42^{0}C least 12 hours. The membrane was then washed serially in 5x SSPE, 2x SSPE,(1x SSPE + 0.1% SDS) for 15 min each at 42^{0}C and finally washed in (0.1x SSPE + 0.1% SDS) for 15 min at room temperature. The membrane was blotted dry, covered with saran wrap and exposed to X-ray film at -70^{0}C overnight.

RESULTS

Immunoblot Analysis Monoclonal Antibodies

A large panel of monoclonal antibodies which induce cell adhesion were prepared against the T cell leukaemia line, Jurkat. These antibodies were used to determine the target antigen by radioimmunoprecipitation/SDS PAGE analysis of ^{125}I labelled surface antigens. These antibodies defined four groups of antigens depending on the molecular weight: 1), 180-220 kDa molecules, 2), 130-150 kDa family of heterodimers, 3), 90-110 kDa antigens, and 4), a 30 kDa membrane glycoprotein. Although these monoclonal antibodies can induce cellular aggregation, the functions they play in such a process are very difficult to understand without knowledge of their molecular structure. Using mAb to screen a cDNA expression is one approach for gene isolation. Because not all mAbs can produce good signals for cDNA library screening, it is necessary to test the panel of mAb by immunoblot analysis. Generally, if a mAb produces good signal on immunoblot, it may also yield a good signal in cDNA library immunoscreening. A total of 96 monoclonal antibodies were tested by immunoblot analysis of Jurkat cell lysates. The results are shown in Table 2 . From a total of 96 mAbs, 12 mAbs reacted on immunoblot analysis. One reacted with a 30 kDa antigen, Seven reacted with a 110 kDa antigen and four reacted with 130-150 kDa molecules(Fig. 1)

Table 2. Immunoblot analysis of mAb Affecting Jurkat Adhesion

mAbs	Blotting	mAbs	Blotting	mAbs	Blotting	mAbs	Blotting
A16F3	-	A9F10	-	A11D3	-	B11E2	+
19G8	-	A8F3	-	A8C10	-	A10C10	-
22E8	+	A8D6	-	A10B4	-	A16D7	-
A7E4	-	A5G6	-	A9G9	-	A8C2	-
A7G3	-	A4B4	-	A16G6	+	A13E9	-
A1G6	-	A19E3	-	A13E10	+	A1G5	-
13B9	-	A12C3	-	A14E9	-	A6G11	+
A15E8	-	A16D7	-	B9C3	-	A19F2	+
A7G4	-	BB16G5	-	B2E9	-	A7G3	+
A4B7	-	B7C11	-	A2F7	-	A11B4	+
A16D17	-	B5D9	-	A9E2	-	A13D8	+
4F6	-	B1B5	-	A5E6	-	B5D9	+
10F2	-	A19E6	-	A10E2	-	A8D6	+
6F4	-	A18E10	-	A6G6	-	A12F3	-
10F5	-	A17C9	-	A20D5	-	A15E9	-
3D8	-	B8E4	-	A16E9	-	A16D11	-
21C8	-	B6D2	-	A13C5	-	A14D10	-
6B3	-	B5C4	-	B6E9	-	A6B8	-
4C10	-	B4D2	-	B19E6	-	10E2	-
19F5	-	B17C9	-	A8E8	-	19G8	-
B3B11	+	B4D5	-	A6G8	-	6F5	-
A14G6	-	A15F10	-	A9F10	-	19F5	-
A14D10	-	A15E6	-	A10B4	-	5F6	-
A12G11	-	A14G10	-	A6D7	-	21C8	-

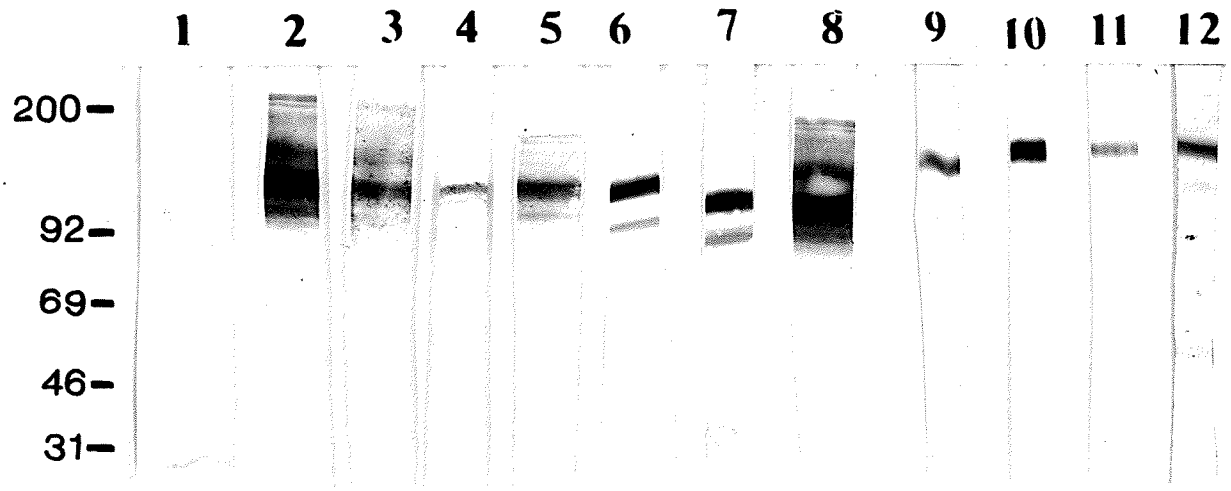
Figure 1. Western blot analysis of monoclonal antibodies

Jurkat cell lysates were subjected to 10% SDS-polyacrymide gel separation and the proteins were transferred to nitrocellulose membrane for western blotting. Each strip was probed with a different monoclonal antibody.

Lane 1; Immunostained with B11E2(30 kDa).

Lane 2-8; Immunostained with A13D8, A19F2, A7G3, A11B4, B5D9,A8D6, A6G11(110 kDa).

Lane 9-12;Immunostained with 22E8, B3B11, A16G6, A13E10 (135-150 kDa).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Biological properties of Abs B11E2 and A13D8

1, Induction of Homotypic Aggregation

Antibodies, B11E2 A13D8 were identified because of the intense cellular aggregation which they induced in Jurkat cells (Fig. 2). The aggregation was not a general response to the binding of antibodies to the cell surface as antibodies to CD3 did not stimulate aggregation. The B11E2 and A13D8 effects were rapid with maximal aggregation occurring within 30 minutes of exposure to antibodies (Fig. 3 and Fig. 4). The aggregation persisted for at least 48 hours with no apparent release of cells from the clusters during this period. The aggregation was dependent upon the continuous presence of the antibody as removal of the B11E2 stimulus resulted in rapid dissociation of the aggregates (data not shown).

Active cellular metabolism was necessary for aggregation induced by B11E2 as incubation at 4°C or culture in the presence of the metabolic inhibitors 2-deoxyglucose and sodium azide completely prevented aggregation (Table 3). The process was also inhibited by incubation with cytochalasin b. In contrast colchicine did not affect B11E2 induced aggregation. Table 3 summary the properties of B11E2 induced aggregation.

2, Distribution of B11E2 and A13D8 antigens

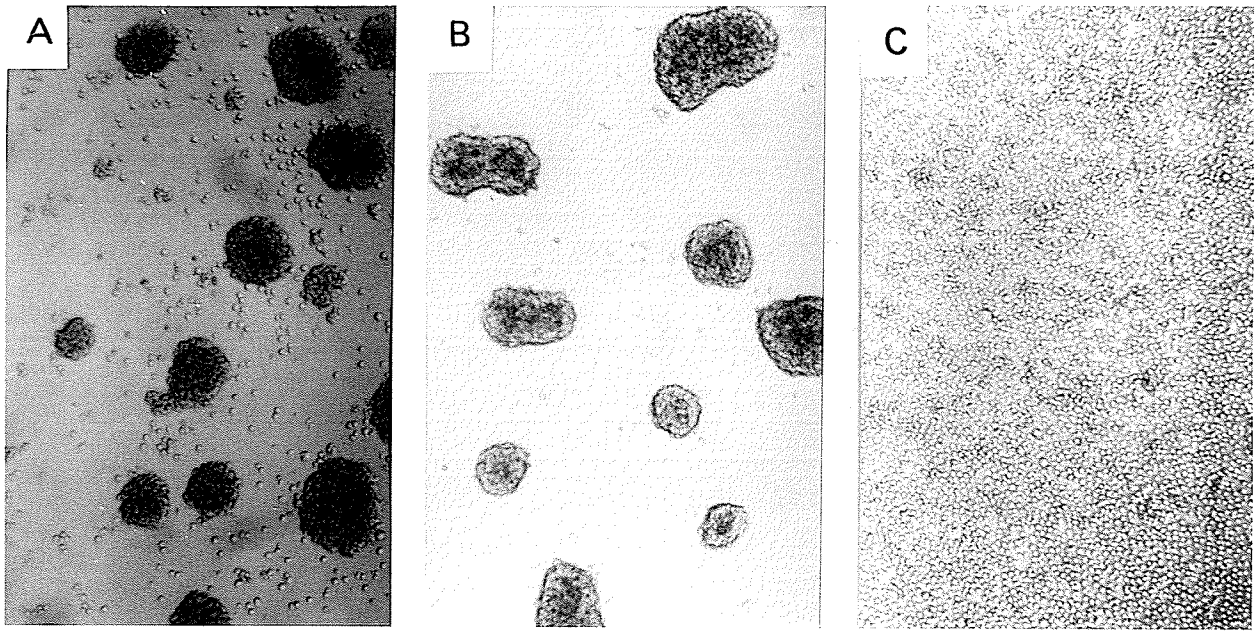
Figure 2. Induction of Jurkat aggregation by antibodies B11E2 and A13D8

Jurkat cells were cultured with and without monoclonal antibody for 30 min. Homotypic cellular aggregations were induced by the monoclonal antibodies (panels A and B).

(A) Treated with B11E2.

(B) Treated with A13D8.

(C) No antibody


A
B
C

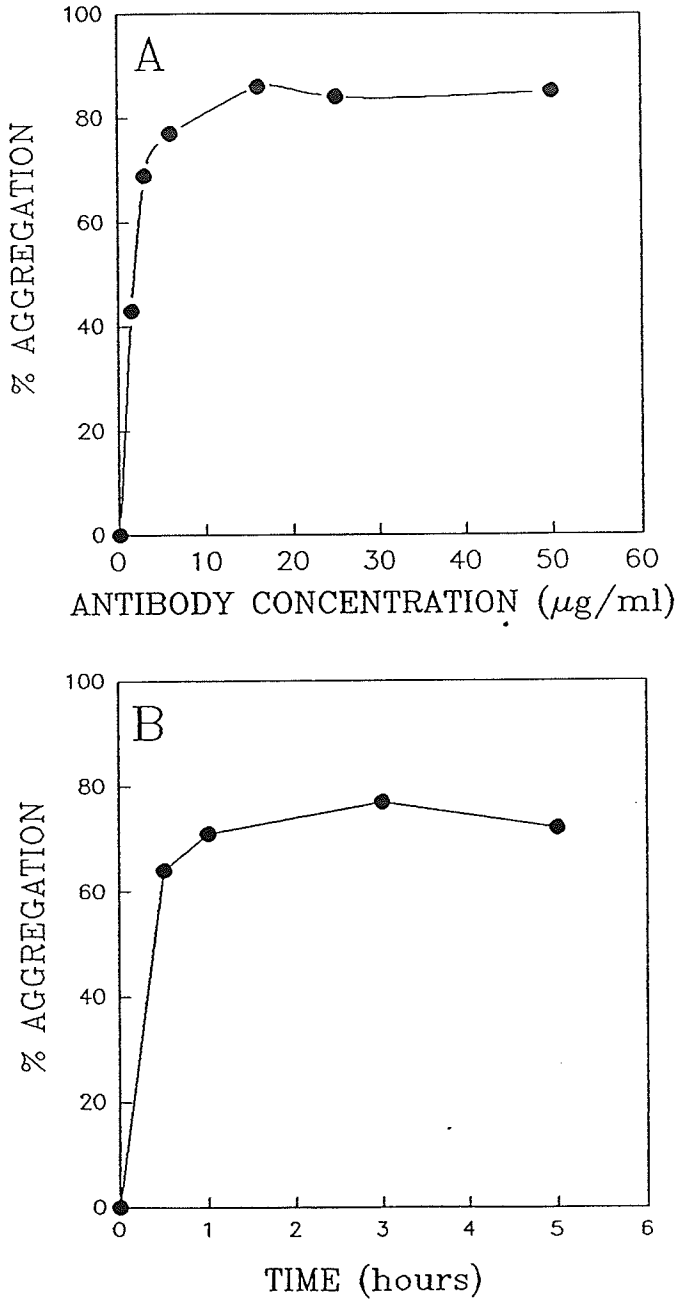


Figure 3. The kinetics and dose responses of aggregation induced by B11E2 (A), Dose-response curve for B11E2 induced aggregation. (B), Kinetics of homotypic aggregation induced by B11E2.

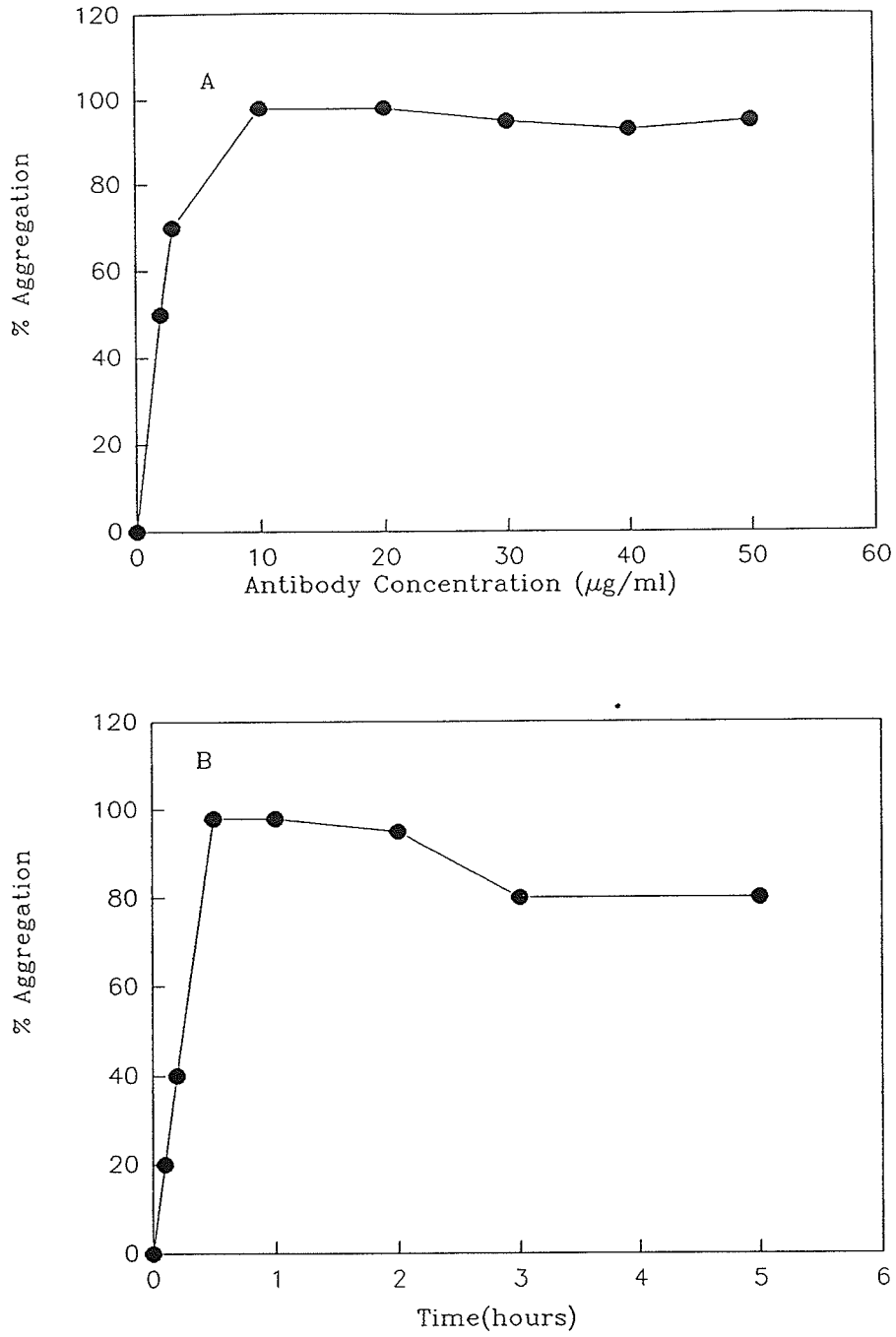


Figure 4. Dose response and kinetics of homotypic aggregation induced by A13D8 (A), Dose response curve for A13D8 induced aggregation. (B), Kinetics of homotypic aggregation induced by A13D8.

Using indirect immunofluoresence analysis, the antigen reacting with B11E2 was present on a variety of cell types including all T cell lines examined, fresh PBL, IL-2 dependent T cell lines, and some B cell lines (Table 4). A limited number of non-lymphoid cell lines were also examined and the antigen reacting with B11E2 was detected to varying degrees on these cells suggesting that the molecule was not restricted to lymphoid cells.

A13D8 antigen is present on Jurkat cells but is not present on other T cell lines, fresh PBL, IL-2-dependent T cell lines and some B cell lines examined.

Table 3. Properties of B11E2 Induced Aggregation

Treatment	Effect on B11E2 Induced Aggregation
4°C	INHIBITION
2-deoxyglucose + sodium azide	INHIBITION
Colchicine	NONE
Cytochalasin b	INHIBITION
EDTA	PARTIAL INHIBITION(< 60%)
EGTA	PARTIAL INHIBITION(< 60%)

Table 4. Cellular Distribution of B11E2

	PBMC	+++
	CD3 T Cells	++++
	Jurkat	++++
T Cells	Molt 3	++++
	Molt 4	++++
	HSB2	++++
	CEM	++++
	JR2B10	++++
	JR2D3	+++
	JY	++++
B Cells	RPMI8866	++
	RPMI8266	-
	U266	-
	Ramos	-
Non Lymphoid Cells	Colo 205	++++
	K562	+++

Molecular Characterization of B11E2 and A13D8

Two approaches were used in this study to determine the biochemical nature of the antigen recognized by mAb B11E2 on Jurkat cells. First, cultured Jurkat cells were surface labelled with ^{125}I, extracted with Nonidet P-40 lysis buffer, and used for immunoprecipitation experiments described in " Materials and Methods". However, no specific immunoprecipitates were obtained. Second, detergent extracts of whole Jurkat cells were separated by SDS-PAGE, transferred to nitrocellulose membranes, and tested by immunoblot with the indirect immunophosphatase method. As shown in Fig 5A, B11E2 detected a band of a 30-32 kDa molecule. A 70 kDa species was often seen when the cell lysates were not prepared freshly(Fig. 5B). The presence of reducing agents did not affect the relative proportions of the 30 and 70 kDa species, suggesting that the higher molecular weight species were not disulphide linked complexes of the 30 kDa forms. The band of B11E2 antigen was not identified by immunoprecipitation.

The B11E2 antigen is sensitive to trypsin and disappeared from the cell surface as determined by FACS(data not shown). This result suggested that the B11E2 antigen was a cell surface protein.

Figure 5 . Immunoblot analysis of B11E2 antigen

Jurkat cells lysates was electrophoresed through 10% polyacrylamide/SDS gels. Proteins were electroblotted onto nitrocellulose membranes and immunostained with B11E2 antibody. Molecular standards are given in kDa.

(A) Freshly prepared cell lysates

(B) Stocked cell lysates

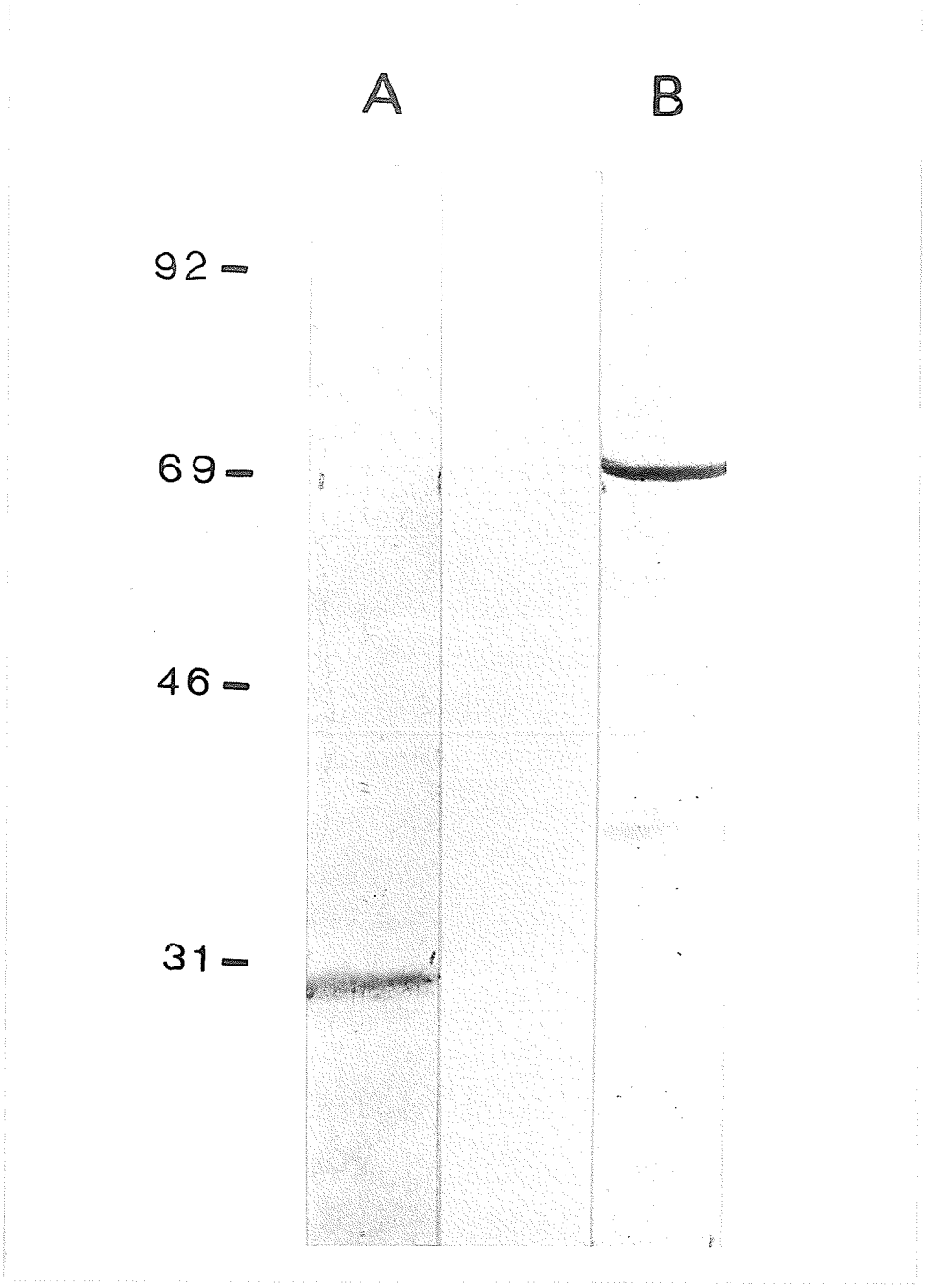

A
B
92
69
46
31

Figure 6. Immunoprecipitation of A13D8 antigen

Jurkat cells were surface-labeled with ^{125}I,immunoprecipitated and electrophoresed through 10% acrylamide/SDS gel under reducing conditions. Molecular standards are given in kDa.

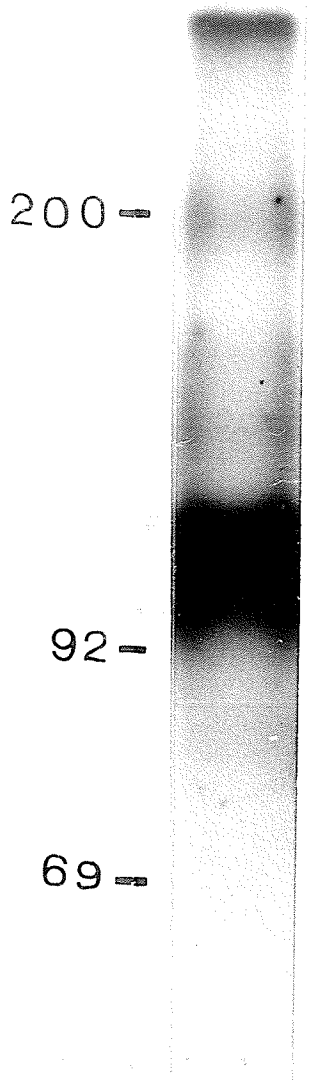
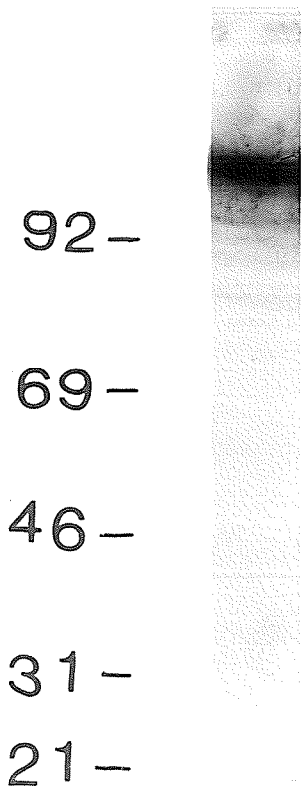

200
92
69

Figure 7. Immunoblot analysis of A13D8 antigen

Jurkat cell lysates were subject to 10% SDS-PAGE and the protein bands were transferred to a nitrocellulose membrane. The membranes were stained with antibody A13D8. Molecular standards are given in kDa.


92–
69–
46–
31–
21–

Identification of B11E2 Positive Clones

1. Isolation of the B11E2 Antigen cDNA Clones

A λ cDNA expression library of Jurkat cells was immunoscreened with monoclonal antibody B11E2. A total of 6 x 10^5 primary phage were screened and three positive clones were identified and plaque purified (Fig. 8).

2. Determination of Insert Size by PCR

Recovery of the cDNA inserts from three positive clones was carried out by PCR(polymerase Chain Reaction). Ten microliter from PCR reaction was removed and electrophoresed in a 1.2% agarose gel and stained with ethidium bromide. The insert of B11E2CLONE1 was 1.15 kilobases and the inserts of B11E2CLONE2 and B11E2CLONE3 were 0.9 kilobases(Fig. 9).

3. Fusion Protein Production

Fusion protein production had two objectives: First, additional support for the presence a biologically active protein encoded within the positive clones. Second, to determine if a fusion protein would inhibit cellular aggregation.

Figure 8. Immunoscreening of cDNA Library.

The original cDNA library was plated and immunoscreened with B11E2 monoclonal antibody by the procedure described in " Materials and Methods".

A: Positive B11E2CLONE1

B: Plaque purified B11E2CLONE1

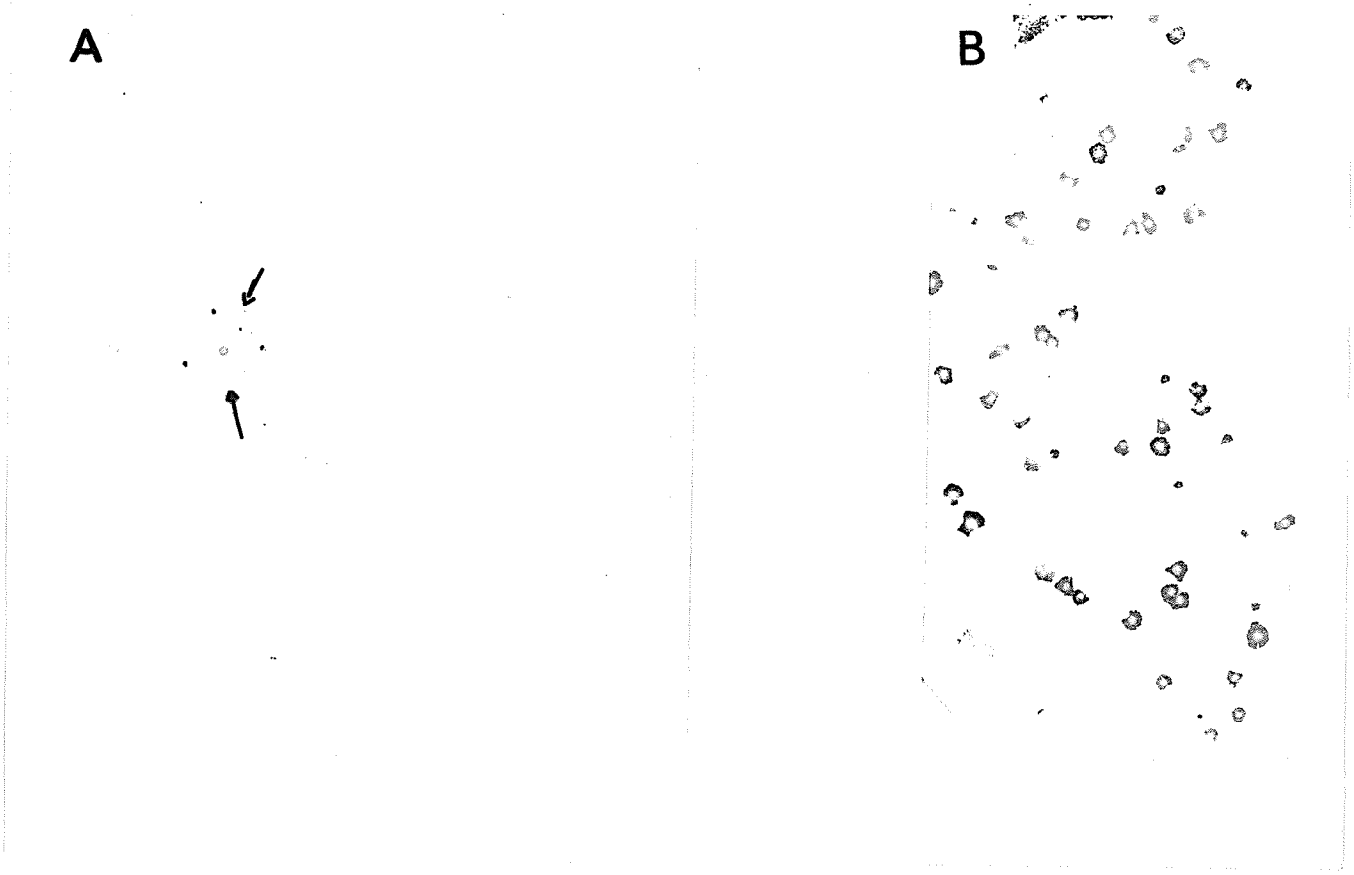

A
B

Figure 9. Determination of the size of the cDNA inserts in the three positive clones

The cDNA inserts were generated by PCR (see Materials and Methods). The products were analyzed by electrophoresis in a 1.2% agarose gel.

Lane 1, 0X174 RF DNA/Hae III; the sizes are (kb): 1.353, 1.078, 0.872, 0.603, 0.310, 0.271, 0.234.

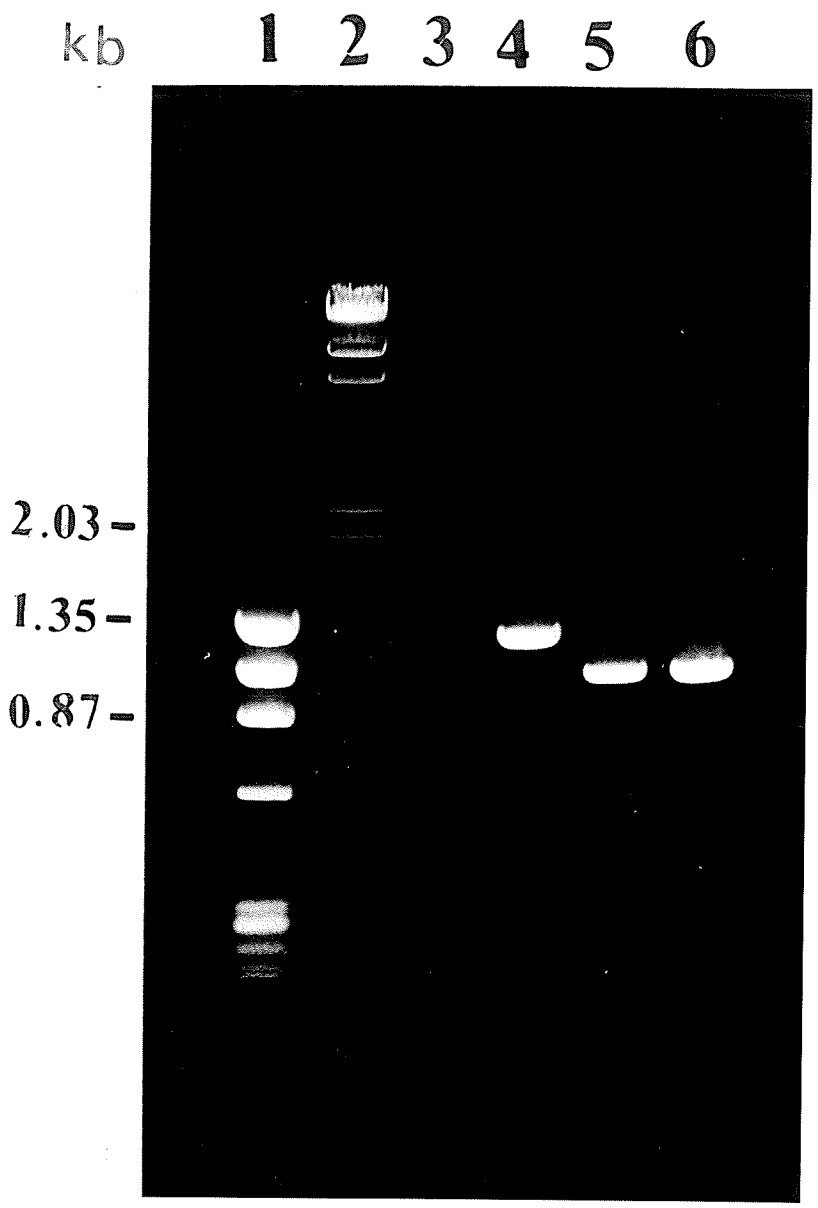
Lane 2, λDNA/Hind III; the sizes are(kb): 23.13, 9.416, 6.557, 4.361, 2.322, 2.027.

Lane 3, PCR of λgt11 which has no inserts.

Lane 4, PCR products of B11E2CLONE1.

Lane 5, PCR products of B11E2CLONE2.

Lane 6, PCR products of B11E2CLONE3.


kb
1 2 3 4 5 6
2.03
1.35
0.87

Fusion protein-containing samples (2 positives and 1 control) were electrophoresed on SDS-PAGE (7% gel) in duplicate. One set of the samples was stained with Comassie Blue R-250 (data not shown). The other was electroblotted onto nitrocellulose membrane and immunostained with mAb B11E2(Fig. 10). The amount of fusion proteins produced by B11E2CLONE1 and B11E2CLONE2 were almost the same(Fig. 10, lanes 3 and 2). No fusion products were visualized in the negative control(Fig. 10, lane 1). Preincubation of the antibody B11E2 with the fusion protein completely inhibited cellular aggregation (Fig.11 lane B). While the negative control samples did not inhibit induced aggregation(Fig. 11 lane C).

4. Subcloning and Nucleotide Sequencing

The inserts of clones, B11E2CLONE1 and B11E2CLONE2,were subcloned into plasmid pBS-M13+. The complete coding sequence of the B11E2 gene was obtained by sequencing both strands of the B11E2CLONE1(1.15 kb) clone. Partial sequence of B11E2CLONE2(0.9 kb) was also performed. The 1150 bp sequence of the B11E2 insert contains a short 5' untranslated region (92 bp) and a long 3' untranslated region(500 bp). The open reading frame begins with the first ATG at position 93 and encodes a predicted polypeptide of 185 amino acids (Fig. 12). Comparison of the sequences with those in the Genebank data base indicated that the clone was related to the molecule E2/MIC-2 (Banting et al, 1989; Aubrit et al, 1989)

Figure 10. Detection of fusion protein expressed by cDNA clones

Cultures of E.coli Y1089 were infected independently with B11E2CLONE1 and B11E2CLONE2 and lysates were electrophoresed through an 8% polyacrylamide gel/SDS gel, electroblotted and immunostained with B11E2 antibody. Molecular standards are given in kDa.

lane 1: negative conttrol(λ gt11);

lane 2: B11E2CLONE2 infection;

lane 3: B11E2CLONE1 infection;

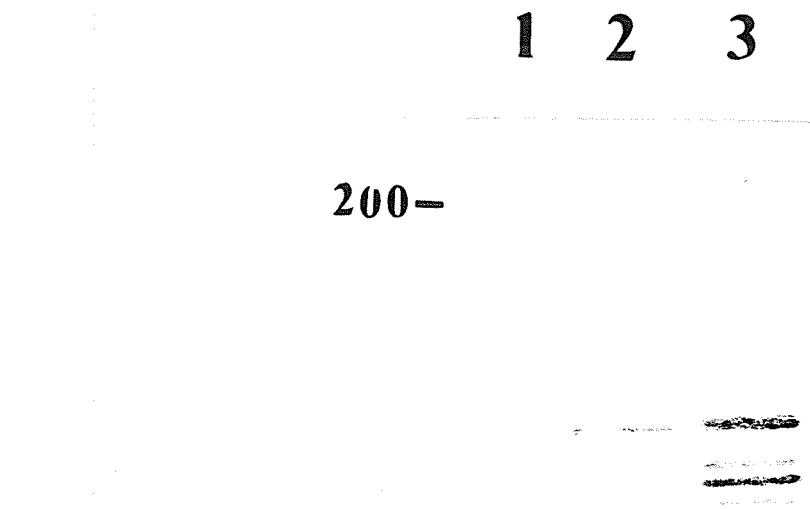
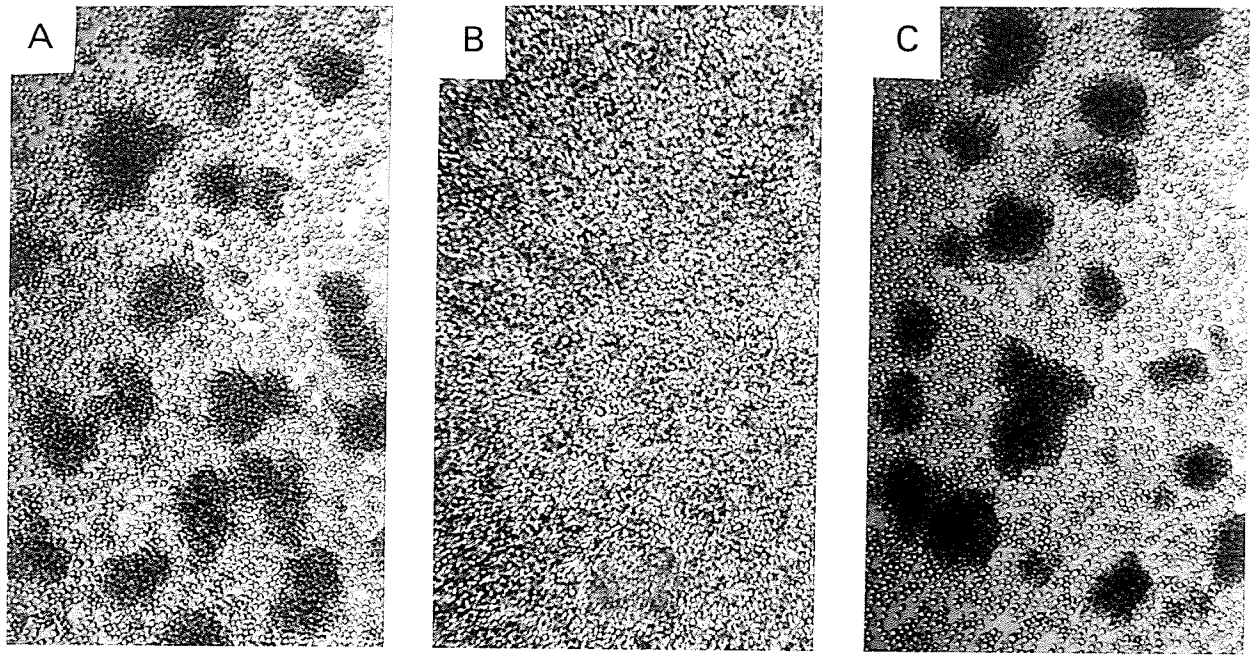

1 2 3
200–
92–
69–
46–
31–

Figure. 11 B11E2 fusion protein inhibition of aggregation induced by mAb B11E2

(A), Jurkat cell aggregation induced by mAb B11E2.

(B), B11E2 fusion protein and B11E2 antibody were added to cultured Jurkat cells.

(C), Absence of aggregation by λ gt11 induced β-galactosidase.


A
B
C

sharing >99% nucleotide sequence identity and a >98% predicted amino acid identity with E2 (Gelin et al,1989) and MIC2(Darling et al, 1986) . The full length B11E2 protein has a predicted molecular weight of 18.4 kDa which is considerably less than the value obtained by SDS-PAGE analysis. However, MIC2/E2 have been reported to be heavily O-glycosylated such that the final product has an apparent molecular weight of 32 Kda (Gelin et al, 1989). There are ten potential O-glycosylated linked sites(9 Ser and 1 Thr).

5. The Relationship between B11E2 and 12E7

In order to determine the relationship between E2b and E2/MIC2, the reactivity of an mAb to MIC2\E2, 12E7, with the E2b fusion protein was examined. mAb 12E7 recognize the E2b fusion protein indicating that two antigens were antigenically related (Fig. 13). Immunoblot analysis of Jurkat cell lysates was carried out using both 12E7 and B11E2 antibodies. B11E2 detected a single 30 kDa band, but 12E7 revealed three bands at 28 kDa, 30 kDa and 32 kDa (Fig. 14 lane A and B respectively). Banting et al (1985) showed that 12E7 can recognize an antigen which has two forms, a 32 kDa cell surface antigen and a 29 kDa cytoplasmic molecule. The 30 kDa molecule of B11E2 was also present on the cell membrane and may be the same as the 32 kDa of 12E7. The exact relationship of both 30 kDa band and 32 kDa band has not been clarified. We have demonstrated that mAb B11E2 antibody can induce homotypic cellular aggregation of Jurkat cells. However the mAb

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AATTCCCCCCACCCGGCCCGTGGGGGAGTATCTGTCCTGCCGCCTTCGCCCACGCCCTGCACT    63
CCGGGACCGTCCCTGCGCGCTCTGGGCGACC ATG GCC CGC GGG GCT GCG CTG GCG    118
                                Met Ala Arg Gly Ala Ala Leu Ala      8

CTG CTG CTC TTC GGC CTG CTG GGT GTT CTG GTC GCC GCC CCG GAT GGT    166
Leu Leu Leu Phe Gly Leu Leu Gly Val Leu Val Ala Ala Pro Asp Gly     24

GGT TTC GAT TTA TCC GAT GCC CTT CCT GAC AAT GAA AAC AAG AAA CCC    214
Gly Phe Asp Leu Ser Asp Ala Leu Pro Asp Asn Glu Asn Lys Lys Pro     40

ACT GCA ATC CCC AAG AAA CCC AGT GCT GGG GAT GAC TTT GAC TTA GGA    262
Thr Ala Ile Pro Lys Lys Pro SER Ala Gly Asp Asp Phe Asp Leu Gly     56

GAT GCT GTT GTT GAT GGA GAA AAT GAC GAC CCA CGA CCA CCG AAC CCA    310
Asp Ala Val Val Asp Gly Glu Asn Asp Asp Pro Arg Pro Pro Asn Pro     72

CCC AAA CCG ATG CCA AAT CCA AAC CCC AAC CAC CCT AGT TCC TCC GGT    358
Pro Lys Pro Met Pro Asn Pro Asn Pro Asn His Pro Ser Ser Ser Gly     88

AGC TTT TCA GAT GCT GAC CTT GCG GAT GGC GTT TCA GGT GGA GAA GGA    406
Ser Phe Ser Asp Ala Asp Leu Ala Asp Gly Val Ser Gly Gly Glu Gly    104

AAA GGA GGC AGT GAT GGT GGA GGC AGC CAC AGG AAA GAA GGG GAA GAG    454
Lys Gly Gly Ser Asp Gly Gly Gly Ser His Arg Lys Glu Gly Glu Glu    120

GCC GAC GCC CCA GGC GTG ATC CCC GGG ATT GTG GGG GCT GTC GTG GTC    502
Ala Asp Ala Pro Gly Val Ile Pro Gly Ile Val Gly Ala Val Val Val    136

GCC GTG GCT GGA GCC ATC TCT AGC TTC ATT GCT TAC CAG AAA AAG AAG    550
Ala Val Ala Gly Ala Ile Ser Ser Phe Ile Ala Tyr Gln Lys Lys Lys    152

CTA TGC TTC AAA GAA AAT GCA GAA CAA GGG GAG GTG GAC GTG GAG AGC    598
Leu Cys Phe Lys Glu Asn Ala Glu Gln Gly Glu Val Asp Val Glu Ser    168
                                                    ***
CAC CGG AAT GCC ATC GCA GAG CCA GCT GTT CAG CGT ACT CTT TTA GAG    646
His Arg Asn Ala Ile Ala Glu Pro Ala Val Gln Arg Thr Leu Leu Glu    184
                ***
AAA TAG AAGATTGTCGGCAGAAACAGCCCAGGCGTTGGCAGCAGGGTTAGAACAGCTGCCT    707
Lys  *                                                              185

GAGGCTCCTCCCTGAAGGACACCTGCCTGAGAGCAGAGATGGAGGCCTTCTGTTCACGGCGGA    770
TTCTTTGTTTTAATCTTGCGATGTGCTTTGCTTGTTGCTGGGCGGATGATGTTTACTAACGAT    833
GAATTTTACATCCAAAGGGGGATAGGCACTTGGACCCCCATTCTCCAAGGCCCGGGGGGGCGG    896
TTTCCCATGGGATGTGAAAGGCTGGCCATTATTAAGTCCCTGTAACTCAAATGTCAACCCCAC    959
CGAGGCACCCCCCCGTCCCCCAGAATCTTGGCTGTTTACAAATCACGTGTCCATCGAGCACGT    1022
CTGAAACCCCTGGTAGCCCCACTTCTTTTTAATTAAAATAAGGTAAGCCCTTCAATTTGTTTC    1085
TTCAATATTTCTTTCATTTGTAGGGATATTTGTTTTTCATATCAGACTAATAAAAAGAAATTA    1148
GAAA                                                                1152
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Fig. 12. Nucleotide sequence of the B11E2 cDNA and deduced amino acid structure of the protein. The signal and transmembrane sequences are underlined. * represents a stop cod. *** represents amino acids which differ from the previously isolated E2 protein.

12E7 did not induce homotypic aggregation in a two hour assay and generated weak aggregates after 24 hours of culture.

6. Subcloning of B11E2 cDNA into Protein Expression Vectors

The B11E2CLONE1 cDNA was ligated into the EcoRI site of the fusion protein vector, pMAL-cRI. Ten transformants which contained the cDNA inserts were analyzed by immunoblot with B11E2 mAb. Four out of ten clones produced a B11E2-fusion protein. Prior to large scale fusion protein purification through an amylose resin column, a 60-65 kDa band was visualized by immunoblot analysis. After purification through amylose resin column, the fusion protein was not visualized by immunoblot(data not shown). Expression of the soluble domain of the B11E2 molecule by direct PCR subcloning was unsuccessful(data not shown).

7. Functional Activities of B11E2 Antigen.

The E2 molecule was originally identified with monoclonal antibodies which prevented the rosette formation of thymocytes with sheep erythrocytes. It was therefore suggested that the E2 molecule might function as an alternate receptor for erythrocytes or be involved in some aspect of intercellular adhesion (Aubrit et al, 1989). The Jurkat cells used in the present study did not spontaneously

Figure 13. Antibodies B11E2 and 12E7 were used to probe λgt11 B11E2 clones

The phages from cDNA positive clone B11E2CLONE1 were grown in host cells. The proteins were transferred to membrane for immunostaining. The method was described in "Materials and Methods".

(A). The clones were stained by mAb B11E2

(B). The clones were stained by mAb antibody

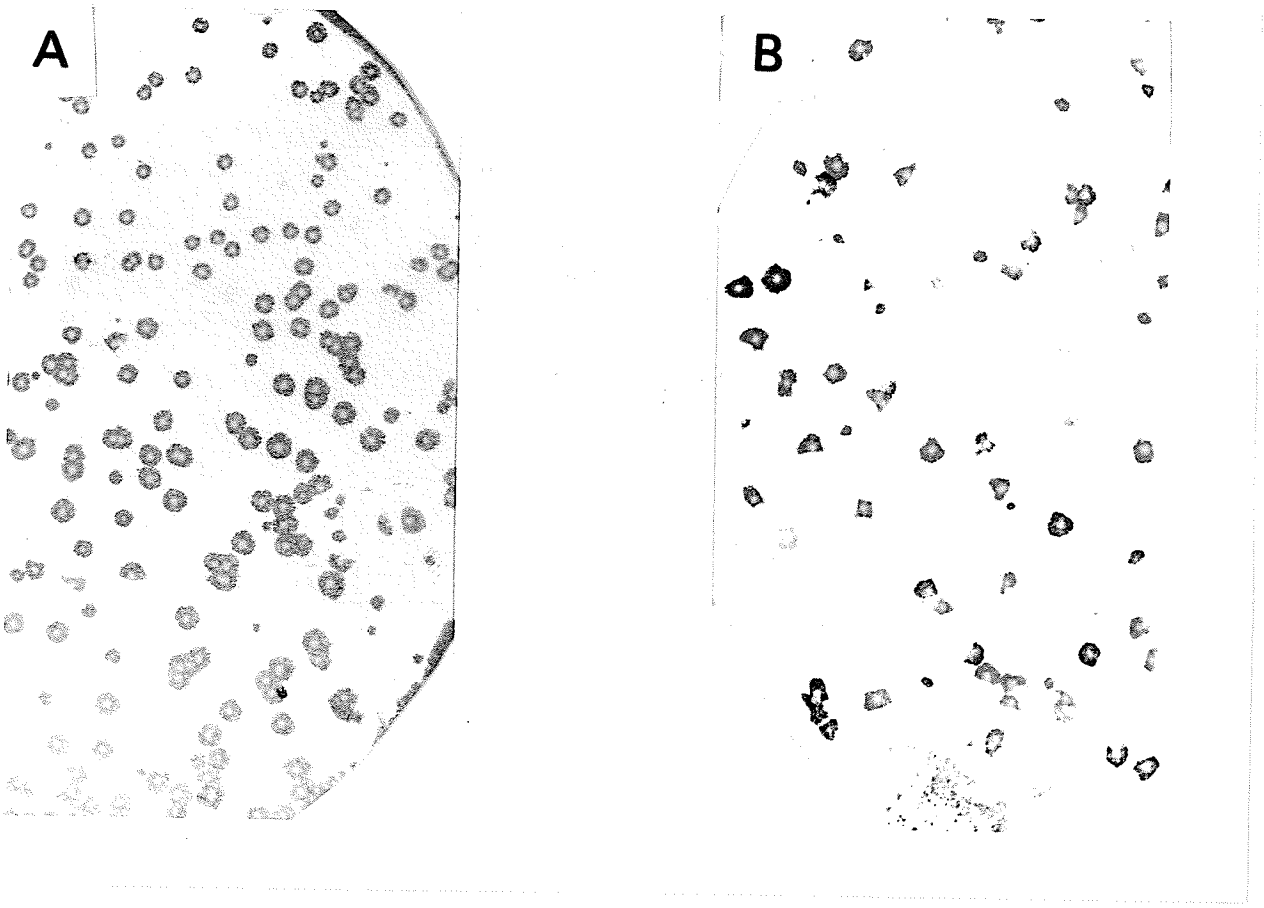
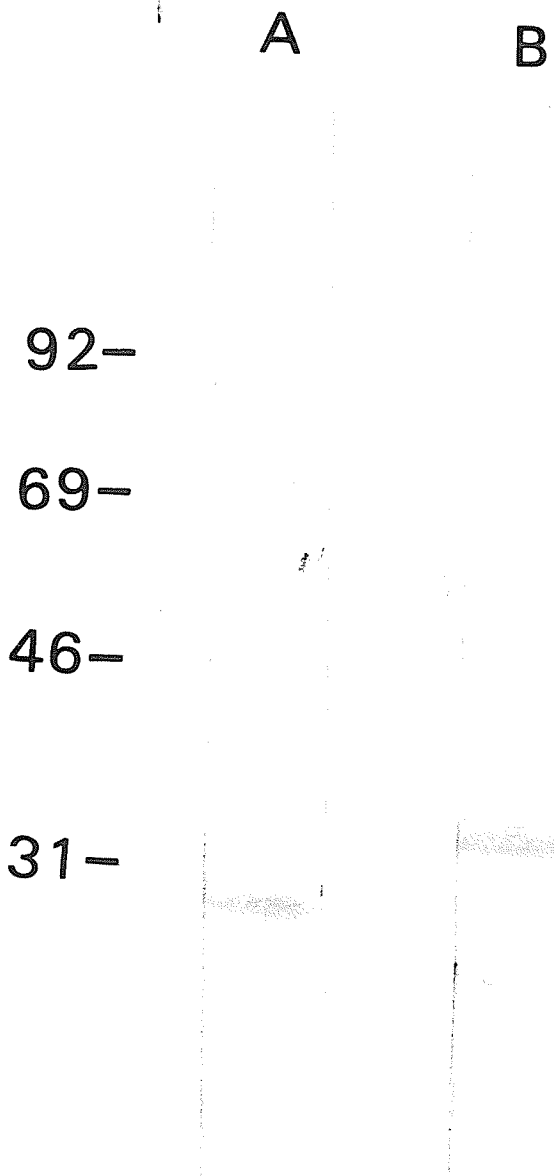

A
B

Figure 14. Western blot analysis of B11E2 and 12E7

Jurkat cell lysates were electrophoresed in 10% SDS-PAGE. Proteins were electroblotted onto nitrocellulose membrane and immunostained with B11E2 and 12E7 antibodies. Molecular standards are given in kDa.

Strip (A); Immunostained with mAb B11E2

Strip (B); Immunostained with mAb 12E7


A
B
92–
69–
46–
31–

form rosettes with AET treated SRBC and they expressed very low levels of CD2. Treatment of Jurkat cells with B11E2 resulted in strong aggregation yet the cells failed to form rosettes with SRBC. These results suggested that the cells did not employ E2 as an erythrocyte receptor. Preabsorption of the B11E2 antibody with the E2b containing fusion product completely blocked the induction of aggregation, while a control fusion protein had no effect. In contrast, addition of the fusion protein to a dissociated single cell suspension of B11E2 stimulated cells did not affect the reaggregation of these cells implying that the soluble product did not influence the adhesive process (data not shown).

Normal PBL and IL-2 dependent T cells also express significant levels of B11E2 and they spontaneously form rosette with SRBC. However these cells did not aggregate following treatment with mAb B11E2 and antibody treatment did not influence the level or intensity of rosette formation.

Equal numbers of T cell clones or PBL were mixed with Jurkat cells in the presence or absence of mAb B11E2 and incubated as described. Due to the differences in size and morphology of the Jurkat and the two other cell types it was possible to distinguish the cell types in coculture. The B11E2 treatment resulted in aggregation of the Jurkat cells but the other cell types were not incorporated into the aggregates (data not shown).

8. B11E2 Antigen mRNA Detection

Polyadenylylated Jurkat mRNA probed with the B11E2 insert revealed the presence of a single mRNA species at 1.2 kb (Fig. 15 lane 1 & 2).

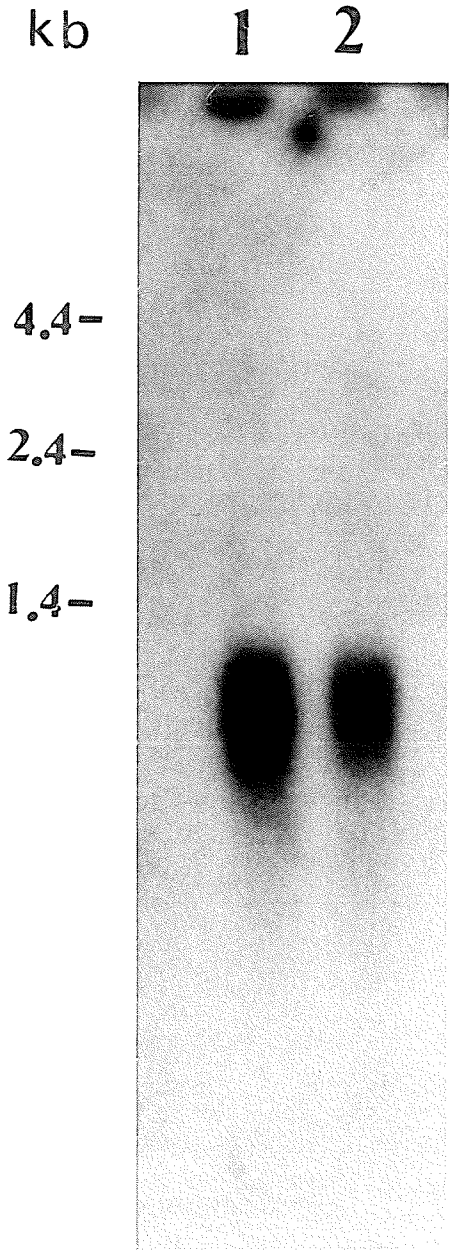
Figure 15. Jurkat cell mRNA probed with the B11E2 cDNA insert

Total RNAs and poly(A)$^+$ were prepared and purified from Jurkat cells.The poly(A)$^+$RNA was electrophoresed in an agarose denaturing gel and blotted onto Hybond N$^+$ membrane. The membranes were probed with labelled PCR products of the B11E2CLONE1 and B11E2CLONE2 inserts.

Molecular size(kb) was determined by comparing the developed film with the agarose gel picture which contained markers obtained from BRL stained with ethidium bromide.

lane 1: Probed by B11E2CLONE1;

lane 2: Probed by B11E2CLONE2;


kb
1
2
4.4–
2.4–
1.4–

DISCUSSION

Cellular adhesion is important for many lymphocyte activities. There is considerable interest in the identification of molecules involved in mediating or controlling the process. The present approach employed a panel of monoclonal antibodies which modified lymphocyte adhesive potentials. These antibodies were used to screen a cDNA expression library in an effort to identify the relevant molecules. A total 96 monoclonal antibodies which induced lymphocyte aggregation were analyzed and 12 of these antibodies reacted on immunoblot analysis.

Previous studies with different antigens indicated that approximately 15% of mAb to a given protein are blot positive, presumably detecting linear epitopes(Geysen et al, 1987). It may be that some of these monoclonal antibodies detect native but not the denatured antigen used in the immunoblot analysis.

B11E2 Antigen Sequence and Related Protein

The present studies were undertaken to characterize mAb, B11E2 which induces homotypic cellular aggregation of lymphoid cell lines. The isolation and sequencing of cDNA clones expressing antigen B11E2 reactive with monoclonal antibody indicated that the target antigen shared almost complete sequence identity with the sequences previously identified as MIC2 or E2 (Banting et al, 1989; Aubrit

et al, 1989). This relationship is further supported by the observations that mAb 12E7, the prototypic anti-MIC2/E2 reagent, reacts with the fusion protein generated from the E2b clone. The molecule detected by B11E2 is closely related to E2/MIC2 in that there are four base pair substitutions in a total sequence of 555 bases (Gelin et al, 1989). Two of the substitutions are conservative while another is semi-conservative (methionine to valine, position 166). The final substitution at position 173 is nonconservative (asparginine to isoleucine). Both of the changes are in the cytoplasmic domain of the molecule. Based upon the high degree of sequence identity between B11E2 reactive material and MIC2/E2, we propose that the former be tentatively designated E2b; both may be the MIC2 gene product. It has been demonstrated that the MIC2/E2 is an antigen which is encoded by a gene on the short arms of the X and Y chromosomes(Goodfellow et al, 1983). The 12E7 antigen determinant is not found on the cell surface of mouse, the human-mouse polymorphism has been exploited to study the genetic control of expression of 12E7 antigen(Goodfellow et al, 1980).

Although cDNA sequences of B11E2 and E2 are almost identical and anti-MIC2 antibody can react with the B11E2 clone and give identical cell staining, the immunoblot analysis of Jurkat cell lysates revealed a 30 kDa antigen was defined by antibody B11E2 and a 30 kDa and a 28 kDa species were defined by antibody 12E7. Immunofluorescence assays and biochemical studies by Aubrit et al(1989) have confirmed that antibodies to E2 can define two molecules of 28 kDa and 32 kDa. The

28 kDa antigen is a cytoplasmic component, that is absent from the cell surface. Antibody 12E7 can recognize both the 32 kDa cell membrane antigen and 28 kDa cytoplasmic antigen, while antibody B11E2 only recognizes a 30 Kda membrane protein but not the 28 Kda cytoplasmic protein. The functional significance of these differences are unknown at this time. The differences between B11E2 and 12E7 effects could be due to antibody specificity (different epitopes on E2/E2b) or different carbohydrates may mask epitopes. To define the antibody specificity, peptide mapping and competition binding analysis will be useful to address this speculation. Also a O-glycanase could be used to cut out the carbohydrates and the digested molecules and the undigested molecules will be analyzed with B11E2 and 12E7 antibodies. Also the distribution of the isoforms on different cell types may be useful in assigning functional roles.

B11E2 recognized a 70 kDa protein when stored cell lysates were used for immunoblot analysis. The nature of the 70 kDa species is unknown. Variably seen, it may represent aggregation of E2b monomers or an association with other molecules to cause a mobility shift. Variability suggests that this may be an artifact of preparation.

Functions of E2/B11E2 Antigen

The first red blood cell receptor was identified as a T cell surface molecule designated as CD2(Kamoun et al, 1981; Bernard et al, 1984; Bernard et al, 1982; Verbi et al, 1982) which mediates T cell interaction with erythrocytes by binding its ligand CD58/LFA-3(Shaw et al, 1986; Vollger et al, 1987). The other T cell surface molecules are involved in the lymphocyte-erythrocyte adhesion including CD44(Scheeren et al, 1991) and CD59(Hahn et al, 1992; Whitlow et al, 1990). E2, as a T cell surface antigen involving in rosette formation was defined by Bernard et al (1988). They confirmed that the antibodies for E2 are able to block rosetting after preincubation with T cells in a dose dependent manner. The dose dependency was identical when they used either untreated SRBC or SRBC pretreated with AET. They also determined that the E2 mAb inhibition of rosette formation was temperature dependent. At 37^0C, mAb could completely inhibit the rosette formation, whereas at 4^0C, the inhibition was only partial, regardless of whether or not SRBC had been pretreated with AET. The pattern of rosette inhibition was compared using mAb against E2 and CD2. Most CD2 antibodies are very efficient in blocking rosette formation, regardless of the temperature of the assay and whether or not SRBC have been pretreated with AET. The blocking effect of the mAb for E2 on T cell rosetting is not restricted to SRBC, they are also able to block T cell rosetting with red blood cells from wide variety of species. Preincubation of mAb to E2 with erythrocytes before rosette formation does not induce any inhibition(Bernard et al, 1986). Although, all of the T cell populations reacted with E2 mAb, there are striking

differences in the surface density among these populations. All medullary type thymocytes carry low amounts of E2, whereas all thymocytes carrying high amounts of E2 are of the cortical type. Peripheral blood T cell display an intermediate surface density. Following stimulation with various factors, including lectins, allogenic cells, CD3 and CD2 mAbs, the surface density of E2 did not vary. This phenotype is markedly different from the CD2 molecule(Banting et al, 1988).

Although E2 antigen is involved in rosette formation, the mechanism and function of this E2 antigen in such interactions is unknown. Aubrit et al (1989) suggested that two schematic possibilities exist; either anti-E2 mAb could block rosettes indirectly, since E2 and CD2 may be linked on the T cell surface, or E2 antigen binds, independently from the CD2-LFA-3 linkage, to a particular ligand on the erythrocyte surface. Three different molecules, N4, N23 and N 212, involved in rosette formation on erythrocyte were identified(Bernard et al 1987).

B11E2 was identified because of induced intense cellular aggregation. This homotypic aggregation does not appear to be a consequence of antibody mediated crosslinking of the cells for a number of reasons. The process is temperature and energy dependent, not all cell types are induced to aggregate, despite equivalent levels of E2 expression and not all antibodies to E2 induce the aggregation.

In contrast the E2 molecule was identified by monoclonal antibodies to a non CD2 molecule which inhibited the rosette formation of thymocytes with erythrocytes(Seed et al, 1987). The B11E2 antigen does not induce aggregation

between erythrocyte and Jurkat cells. These observations imply that E2b may not function as an alternate receptor for erythrocytes but rather that the molecule is involved in the acquisition of adhesive phenotype.

Although the functional role of E2 is unclear at this time, the results of the present study do not support a role for E2b as an erythrocyte receptor or an adhesion molecule. Jurkat cells lacking CD2 can be induced to aggregate yet the cells do not form rosettes under these activating conditions. Soluble E2b does not interfere with either normal T cell rosette formation or the aggregation of B11E2 stimulated cells. Despite the fact that the soluble B11E2 does interfere with the induction of aggregation by the antibody presumably as a competitive inhibitor of antibody binding. Immobilized purified E2b does not act as an adhesion substrate for B11E2 activated Jurkat cells or T lymphocytes (data not shown). Collectively these results suggest that interaction with E2 may be activating an adhesion system which does not involve E2 as one of the cognates in the process.

Previous studies using antibodies to the $\alpha 4$ or $\alpha 5$ chains of the ß1 integrins to induce homotypic aggregation indicated that the expression of these molecules was not sufficient for the generation of the cytoadherence (Davis et al, 1990). Similarly the expression of E2 on a cell does not in itself confer responsiveness to B11E2 mediated stimulation as normal T cells or IL-2 dependent T cells which express significant levels of E2 are not induced to aggregate by this antibody. Furthermore mixtures of Jurkat cells and IL-2 dependent T cells do not coaggregate in the

presence of B11E2 indicating that it is not the absence of the expression of a counter receptor on normal T cells which prevents their induction by B11E2.

There is increasing evidence to indicate that there is interplay between distinct adhesion systems. Antibodies to CD2 or CD3 cause the transient activation of ß1 and ß2 integrin adhesive potentials of T cells (Davis et al, 1990; Yamada et al, 1991; van Kooky et al, 1989). Similarly antibodies to the ß1 integrins can induce homotypic cellular aggregation (Shen et al, 1991; Bednarczyk et al 1990) apparently mediated by non homologous adhesion systems. However the fact that E2 does not appear to function as an adhesion molecule raises the possibility that it may play a role in other events related to the generation of the adhesion competent state.

Observations in a number of integrin mediated adhesive processes indicate that there may be multiple steps in the acquisition of the cytoadhesive phenotype. At least three states have been described for CD11a, some conformations correlating with adhesive potential(Van Kooky et al, 1991). There are also changes in the distribution of the CD11a molecules in the plane of the membrane following stimulation with fMLP in the absence of ligand (Hassall et al, 1991). Similarly the stimulation of neutrophils with PMA and FMLP results in the reorganization of CR3 in the absence of ligand interaction (Detmers et al, 1987). Furthermore the process appears to be independent of the changes in the interaction between these molecules and the cytoskeleton . It is only subsequent to ligand interaction that increased associations with the cytoskeleton are observed (Beckerle et al, 1989).

Although the potential role and mechanism of B11E2 antigen mediated cellular aggregation is unclear, the data seems to suggest that the molecule may be involved in regulation of adhesion rather than in mediating adhesion. The possible mode of induced aggregation may be similar that of anti-β_1 integrin activation of adherence in that it depends on the properties of the target cells(Shen et al, 1991).

The widespread distribution of E2 suggests that this molecule may play a more general role in the generation of adhesion competence of a variety of cell types. As such it may represent an ancillary molecule in the adhesion process. Recently there has been considerable interest in MIC2 as it is heavily expressed on a subset of small round cell carcinomas known as primitive neural ectodermal cancers(pNET). It could be speculated that the high levels of the E2 molecules present on pNET may relate to their invasive phenotypes (Kovar et al, 1990).

To define the biological function role of B11E2 antigen several strategies would be helpful. Purification of the B11E2 protein; a purified B11E2 antigen may inhibit aggregation or induce aggregation. Further investigation may define the biological role of B11E2 antigen, such as, a cell receptor or a factor of signal transduction.

To produce a B11E2 antigen negative cell line, transfection of antisense of B11E2 cDNA is one choice. A negative cell line and normal cell line will be treated with antibodies which are recognize some adhesion proteins, such as, CD2, CD3, and integrin antigens. Also the negative cell line and normal cell line could be

microinjected with mAbs to known adhesion antigens antibodies or purified B11E2 protein and used to compare the effects on adhesion phenotype.

Transfection of the B11E2 cDNA into some known cell line. The B11E2 antigen overexpressed cell line, normal cell line and negative cell line would be treated with B11E2 antibody and other antibodies which induce intercellular adhesion, such as, CD45, CD2, CD3 and LFA-1 then analyzed the effects on aggregation.

Another strategy to define the function of B11E2 antigen is that B11E2 antigen may be involved in signal transduction. This study could be related to G protein/tyrosine kinase/phosphorylation. Some anti-G protein and anti-tyrosine antibodies could be used to treat B11E2 antigen negative and normal cell lines to look for effects on cellular aggregation. If these antibodies block B11E2 induced cellular aggregation, this suggests that B11E2 induced cellular aggregation may be related to signal transduction. Further investigations would define the relationship between B11E2 antigen and signal transduction. These studies could be useful to address the biological role of B11E2 antigen in cellular aggregation.

The results of the present study suggest that the identification of monoclonal antibodies which modify cellular adhesive properties may provide an approach for defining molecular species related to the generation and regulation of adhesion.

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