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Interleukin-2 Activated Lymphocytes Use CD11B/CD18 for Adhesion to Candida Albicans

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INTERLEUKIN-2 ACTIVATED LYMPHOCYTES USE CD11B/CD18 FOR ADHESION TO *CANDIDA ALBICANS.*

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To Rachel Elizabeth Forsyth

In my hunt for the secret of life , I started my research in histology. Unsatisfied by the information that cellular morphology could give me about life, I turned to physiology. Finding physiology too complex I took up pharmacology. Still finding the situation too complicated I turned to bacteriology. But bacteria were even too complex, so, I descended to the molecular level, studying chemistry and physical chemistry. After twenty years' work, I was led to conclude that to understand life we have to descend to the electronic level, and to the world of wave mechanics. But electrons are just electrons, and have no life at all. Evidently on the way I lost life; it had run out between my fingers.

Albert Szent-Györgyi, *Personal Reminiscences*

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LIST OF ABBREVIATIONS

ATCC	American Type Culture Collection
BSA	bovine serum albumin
C3	complement component 3
C3bi	cleavage fragment of C3
$[Ca^{2+}]_i$	intracellular calcium concentration
CD	cluster of differentiation antigen
cDNA	complementary deoxyribonucleic acid
Ci	curies
CMI	cell mediated immunity
cpm	counts per minute
^{51}Cr	chromium-51
CR3	complement receptor type 3
CR4	complement receptor type 4
EC3bi	erythrocytes coated with C3bi
ECM	extracellular matrix
DMEM	Dulbecco's Modified Eagle Medium
DNA	deoxyribonucleic acid
dpm	disintegrations per minute
DTH	delayed type hypersensitivity
FBIP	fibrinogen binding inhibitory peptide

FBS	fetal bovine serum
FEP	fibronectin-like engineered protein
FHA	filamentous hemagglutinin
FITC	fluorescein conjugated
fMLP	N-formyl-methionyl-leucyl phenylalanine
G-CSF	granulocyte colony stimulating factor
GM-CSF	granulocyte-monocyte colony stimulating factor
GPI	glycosyl-phosphatidylinositol
h	hour
HBSS	Hank's balanced salt solution
HIAL	human IAL
I domain	inserted domain
IAL	Interleukin-2 activated lymphocytes
IC_{50}	concentration causing 50% inhibition of adhesion
ICAM	intercellular adhesion molecule
IEL	intraepithelial lymphocytes
IFN-γ	interferon-γ
IL	interleukin
i. p.	intraperitoneal
i. v.	intravenous
Kd	dissociation constant
kD	kilodaltons
LAD	leukocyte adhesion deficiency
LAK	lymphokine activated killer

LFA-1	lymphocyte function associated antigen-1
LGL	large granular lymphocyte
LIBS	ligand-induced binding site
LPS	lipopolysaccharide
mAb	monoclonal antibody
mAbs	monoclonal antibodies
Mac-1	macrophage-1 antigen (same as CD11b/CD18)
MASH	multiple automated sample harvester
2-ME	2-mercaptoethanol
MHC	major histocompatability complex
μg	microgram
μl	microliter
μM	micromolar
mIAL	murine IAL
MIDAS	metal ion-dependent adhesion site
min	minute
ml	milliliter
mM	millimolar
mRNA	messenger ribonucleic acid
NADG	N-acetyl-D-glucosamine
NIF	neutrophil inhibitory factor (hookworm)
NIH	National Institutes of Health
NK	natural killer
nM	nanomolar

PAGE	polyacrylamide gel electrophoresis
PBL	peripheral blood lymphocytes
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PE	phycoerythrin conjugated
PHA	phytohemagglutinin
PMA	phorbal 12-myristate 13-acetate
PMN	polymorphonuclear leukocyte
PMSF	phenylmethyl-sulfonylfluoride
PT-2000	PepTite-2000
RGD	arginine-glycine-aspartate
RNA	ribonucleic acid
S. D.	standard deviation
SDA	Sabouraud's dextrose agar
TBS	Tris-buffered saline
TGF-β	transforming growth factor-β
TNF-α	tumor necrosis factor-α
U	units of activity
uPA	urokinase-type plasminogen activator
uPAR	uPA receptor

CHAPTER 1.

INTRODUCTION AND OVERVIEW OF RELATED LITERATURE

The incidence of life-threatening fungal infections is increasing at an alarming rate with increased immunosuppressive therapies and the AIDS epidemic (Fox, 1993). Fungal infections now are responsible for 40% of deaths due to hospital-acquired infections (Sternberg, 1994). Cell-mediated immunity (CMI) by lymphocytes is an important form of host defense against fungi, and is probably the principal defense at mucosal and epidermal surfaces (Ashman, 1990; Fidel and Sobel, 1994; Calderone *et al.*, 1994; Levitz *et al.*, 1995). During the CMI response to fungi, lymphocytes can release cytokines that not only enhance CMI but also modulate the antifungal activity of polymorphonuclear leukocytes (PMN) and macrophages (Spaccapelo *et al.*, 1995). In addition, natural killer cells (NK) and IL-2 activated lymphocytes have been shown to interact directly with and inhibit the growth of certain fungi (Beno and Mathews, 1992; Beno *et al.*, 1995; Murphy *et al.*, 1993; Levitz and Dupont, 1994). The role of each of these forms of lymphocyte-mediated, antifungal host defense is dependent upon the immune status of the host and upon the individual fungal pathogen (Murphy, 1990; Murphy, 1991; Calderone *et al.*, 1994).

The dimorphic fungus *Candida albicans* is responsible for about 80% of all deaths due to hospital-acquired fungal infections (Sternberg, 1994). The adaptability of the organism is emphasized by the fact that oral *C. albicans* infection, commonly called 'thrush,' was also the first yeast infection described: by Hippocrates in the fifth century B.C. (Hazen, 1995).

Heat inactivated *C. albicans* injected intraperitonealy in mice (Scaringi *et al.*, 1991), or cultured with peripheral blood lymphocytes without exogenous IL-2 (Ausiello *et al.*, 1993), evoke a population of IL-2 activated lymphocytes (IAL) very similar to those used in this dissertation stimulated *in vitro* with IL-2 alone (Ausiello *et al.*, 1993). Previous work from this laboratory has shown that IAL inhibit the hyphal growth of *C. albicans* (Beno and Mathews, 1992; Beno *et al.*, 1995). These IL-2 activated lymphocytes have a large granular lymphocyte (LGL) appearance and have been shown to require direct contact with *C. albicans* hyphae to effect growth inhibition (Beno and Mathews, 1992). The interaction between lymphocytes and *C. albicans* has been demonstrated in a number of ways including; competitive inhibition of mammalian cell binding to the fungus (Beno and Mathews, 1992; Zunino and Hudig, 1988), direct measurement of adhesion of lymphocytes to fungal hyphae (Levitz *et al.*, 1995), and by yeast cell stimulation of cytokine synthesis in LGL (Blanchard *et al.*, 1991; Ausiello *et al.*, 1993). However, the lymphocyte surface structures that mediate adhesion to *C. albicans* or any fungus during the CMI response are unknown. The goal of this dissertation research has been to identify the principal molecular structures on murine IL-2 activated lymphocytes (mIAL) mediating adhesion to *C. albicans* hyphae during growth inhibition of the fungus. To achieve this goal, three Specific Aims were formulated:

Aim 1.) Develop an *in vitro* assay system to quantify lymphocyte binding to *C. albicans* hyphae.

Aim 2.) Identify the principal adherence molecules on murine IL-2 activated lymphocytes that mediate binding to *C. albicans*.

Aim 3.) Confirm and/or prove the identified lymphocyte adherence molecules mediate binding

of murine IL-2 activated lymphocytes to *C. albicans* hyphae.

The CD11b/CD18 heterodimer is one of three members of the leukocyte (restricted to white blood cells) integrins or β_2 family of integrins. Each has the β_2 integrin chain (CD18, 95 Kd) in common which is noncovalently associated with a unique CD11 α chain: LFA-1 (CD11a/CD18), Mac-1 (CD11b/CD18,CR3) and p150,95 (CD11c/CD18, CR4) (Arnaout, 1990). The CD11a,b,c α chains all contain a 200 amino-acid insert critical for binding protein ligands referred to as the I (inserted) or A domain. A fourth member of the β_2 family, designated $\alpha_d\beta_2$, has not yet been described in mice, but has very recently been cloned in dogs and humans (Danilenko *et al.*, 1995; Van Der Vieren *et al.*, 1995). Mac-1 is found on most PMN, monocytes, macrophages, NK cells, LGL (including IL-2 activated lymphocytes), basophils, eosinophils, and subsets of mast cells, B cells, and T cells (Arnaout, 1990; Anderson, 1994; Van der Vieren *et al.*, 1995).

The structure of the I (or A) domain of Mac-1 was recently determined and provides an illuminating first view of CD11b/CD18-ligand interactions (Lee *et al.*, 1995). That data demonstrates the I domain contains a magnesium ion dependent 'MIDAS' (metal ion-dependent adhesion site). In light of this structure, those authors propose a new paradigm for integrin-ligand interaction which identifies a critical aspartate (D) residue and β-looped structure (Main *et al.*, 1992) to be essential in ligands of integrins containing MIDAS motifs. Such ligands are termed 'RGD-mimetic', that is, they can mimic the conventional RGD (Arg-Gly-Asp) integrin binding motif in their ability to interact with the I domain during integrin mediated adhesion (Du *et al.*, 1993). This critical acidic aspartate (and also, rarely, glutamate), and not an exact "RGD" sequence *per se*, are now seen as essential for ligand binding by integrins which contain the MIDAS structure (Bergelson and Hemler, 1995). This MIDAS motif also forms the RGD binding region of the β_3 integrin chain (Loftus *et al.*,

1994), which clarifies how anti-β_3 integrin monoclonal antibodies (Mabs) like 7E3 which also binds to recombinant Mac-1 I domain (Zhou *et al.*, 1994), could inhibit Mac-1. It also could clarify why Mac-1 protein ligand recognition sequences are often RGD-mimetic and contain a key aspartate, for example: fibrinogen (RLD; Zhou *et al.*, 1994), C3 complement fragment C3bi (DE...EE; Taniguchi-Sidle and Isenman, 1994), and Factor X (GYD..QED; Rozdzinski *et al.*, 1995) as well as *Bordatella pertussis* FHA (RGD; and QED; Relman *et al.*, 1990; Rozdzinski *et al.*, 1995) and *Leishmania* gp63 (RYD; Soteriadou *et al.*, 1992). This Mac-1 structural data clarifies how Mac-1 adhesion to both FHA and gp63 as well as C3bi (Talamas-Rohanas *et al.*, 1990) has been blocked with GRGDSP sequence peptides and virtually all Mac-1 ligands are inhibitable with RGD-mimetic peptides derived from that ligand.

The β_2 integrins require activation to become adhesive to protein ligands (Li *et al.*, 1995; Stewart *et al.*, 1995). Activated Mac-1 undergoes quantitative and qualitative changes including release from intracellular stores, aggregation on the cell surface (Detmers *et al.*, 1987), association with the cytoskeleton (Rabb *et al.*, 1993) and changes in conformation resulting in expression of activation specific neoepitopes (Altieri and Edgington, 1988; Diamond and Springer, 1993; Elemer and Edgington, 1994). CD11b/CD18 possesses two distinct extracellular adhesion domains (Wright *et al.*, 1989; Arnaout, 1990). One is a protein ligand domain (partly composed of the I domain) which binds C3bi, Factor X, fibrinogen, FHA, and other protein ligands and is inhibitable with RGD-mimetic peptides. The other is a lectin-like ligand domain proximal to the cell membrane which binds polysaccharide ligands like LPS, *Saccharomyces cerevisiae*, yeast zymosan, yeast mannoproteins, and β-glucan and is inhibitable with N-acetyl-D-glucosamine (NADG) (Ross *et al.*, 1985; Anderson, 1994; Thornton *et al.*, 1996).

In this dissertation, a human LGL cell line (YT) that binds to and directly inhibits the

growth of *C. albicans* was employed as a model system to evaluate the interaction between lymphocytes and *C. albicans*. This cell line has been valuable in other studies of MHC-unrestricted cytotoxicity (Azuma *et al.*, 1992). Utilizing these effector lymphocytes in this study, RGD-peptide and carbohydrate inhibition data confirm the more definitive Mab blocking data which demonstrates CD11b/CD18 is the principal structure on effector lymphocytes mediating adhesion to *C. albicans* hyphae. These studies were followed by similar studies using murine and human IL-2 activated lymphocytes (HIAL) which also identified Mac-1 (CD11b/CD18) as the structure on MIAL and HIAL which mediates adhesion to *C. albicans* hyphae. The ability of Mac-1 to mediate adhesion to *C. albicans* hyphae was confirmed using murine NIH-3T3 fibroblast transfectants expressing human CD11b/CD18 which mediated specific adhesion to *C. albicans* hyphae that was blocked by Mabs to CD11b/CD18 and RGD-mimetic peptides. Finally, Mabs to murine CD11b/CD18 were demonstrated to completely eliminate mIAL mediated growth inhibition of *C. albicans* hyphae in a concentration dependent manner, while mAbs to murine CD11a/CD18 and CD11c/CD18 had no effect. When viewed as a whole, the data from these studies clearly demonstrate that the integrin CD11b/CD18 is the structure on mIAL which mediates adhesion to *C. albicans* hyphae.

The ligand(s) on the surface of *C. albicans* that bind mammalian cell surface structures have not been fully characterized. The current view of the cell wall of *C. albicans* identifies six "layers". From outermost moving inward these layers are: 1.) fibrillar (composed of fimbriae and filamentous carbohydrates), 2.) glucomannoproteins (proteins covalently linked to mannan, a polymer of mannose and β-glucan, a glucose polymer), 3.) β-glucan (both β-(1,3) and some β-(1,6) linkages), 4.) β-glucan and chitin (a polymer characteristic of fungi of N-acetyl-D-glucosamine (NADG), 5.) mannoprotein, and 6.) plasma

membrane (Hostetter, 1994a). While this simple model conveys the basic elements, a more recent model depicts the outermost five elements embedded in a "β-(1,3)-glucan/chitin mesh" (Georgopapadakou and Tkacz, 1995). *C. albicans* adherence to host tissues, mediated by *C. albicans* molecules designated as "adhesins", correlates with the rank order of virulence (Odds, 1994; Santoni *et al.*, 1994; Calderone and Braun, 1991; Hostetter, 1995b) and is considered to be a major virulence factor. In general, *C. albicans* hyphae express more adhesins than the yeast form of the fungus (Hostetter, 1994a) and these greater adhesive properties, combined with a filamentous hyphal morphology suited to invasion between mammalian cells, are probably the major reasons for hyphal formation closely correlating with invasive *C. albicans* infections and thus being considered a major virulence factor (Calderone and Braun, 1991; Odds, 1994). All of the *C. albicans* adhesins characterized so far (except one designated as Factor 6) are mannoproteins (Hostetter,1994; Calderone *et al.*, 1994). *C. albicans* adherence to mammalian tissues is complex and involves multiple mechanisms that are grouped into three major categories designated as: lectin-like, those that are still incompletely defined, and the largest group: protein-protein interactions (Calderone and Braun, 1991; Hostetter, 1994). Until recently, much of the focus has been on identifying *C. albicans* molecules which enable the fungus to invade mammalian epithelial and endothelial (when blood-borne) surfaces (Calderone and Braun, 1991; Hostetter, 1994a), rather than receptors on mammalian leukocytes (Murphy, 1990; Murphy, 1991).

Studies of a mannoprotein lectin-like receptor on *C. albicans* mediating adhesion to epithelial cells indicate a fucose inhibitable lectin (Cutler, 1991). In this dissertation, 150 mM concentrations of either L-fucose or D-fucose had no effect on lymphocyte adhesion to *C. albicans* hyphae. Another study indicated mannan oligosaccharide structures (designated Factor 6) on *C. albicans* serotype A mediate some adhesion to epithelial cells (Pendrak and

Klotz, 1995). Undefined mechanisms of *C. albicans* adherence to epithelial and endothelial cells include isolation of a *C. albicans* DNA sequence encoding a putative *C. albicans* adhesin (Barki *et al.*, 1994). When the protein encoded by this DNA was expressed in a nonadherent strain of the yeast *S. cerevisiae* it conferred the ability to adhere to epithelial cells. The specific protein conferring adherence has not been characterized. Other studies have isolated a *C. albicans* 66 kD fimbrial mannoprotein with 50% of it's proteins composed of hydrophobic amino acids implicated in adhesion to mammalian epithelial/endothelial cells via asialogangliosides (Calderone *et al.*, 1994). Hydrophobicity of *C. albicans* has also been implicated as a general mechanism for adhesion to endothelial and epithelial cells *in vivo* (Hazen, 1989). A *C. albicans* aspartyl proteinase has been implicated in adhesion to human epithelial corneocytes, and protease inhibitors block *C. albicans* yeast adhesion by up to 53% (Frey *et al.*, 1990). Interestingly, it is known that murine and human endothelial and epithelial cells secrete C3 complement component. A more recent study showed that the major targets inactivated by *C. albicans* proteinase were the Fc receptor of immunoglobulin and C3, both critical elements of receptor mediated phagocytosis by macrophages (Kamanishi *et al.*, 1995). The NADG polymer chitin is also considered as a *C. albicans* adhesin (Calderone *et al.*, 1994).

Among protein-protein interactions, several investigations have shown that *C. albicans* has receptors that mimic integrins in ability to bind C3 complement components, ECM proteins, and even cross-react with some mAbs to mammalian integrins. *C. albicans* also expresses surface structures which serve as integrin ligands. Consistent with these findings, *C. albicans* adhesion to some mammalian cells can be inhibited with RGD peptides, including the GRGDSP and PepTite-2000 peptides used in this dissertation research (Bendel and Hostetter, 1993; Santoni *et al.*, 1994, Hostetter, 1994a). However, upon close examination,

there are several points where the *C. albicans* integrin-like molecules and the actual mammalian cell integrins differ. Integrin-like molecules on *C. albicans* have been described that cross-react with some mAbs to the mammalian integrin $\alpha_5\beta_1$ "fibronectin receptor" (Hemler, 1990; Santoni *et al.* 1994), $\alpha_M\beta_2$ (Arnaout, 1990) and $\alpha_X\beta_2$ (Arnaout, 1990; Hostetter, 1994b). It is not known whether these Mabs react with the same structure or different structures on *C. albicans* because competition with the reactive mAbs has not been performed. Anti-fibronectin antibodies block adhesion described by one group (Santoni *et al.*, 1994) but have no effect on *C. albicans* adhesion measured by others (Hostetter, 1994b). Different mAbs to mammalian integrin $\alpha_5\beta_1$ label *C. albicans* yeasts in a range from only 34% of cells to 77% (Santoni *et al.*, 1994). The fibronectin receptor on *C. albicans* originally described (Skerl *et al.*, 1984) and later studied further (Klotz and Smith, 1991; Klotz *et al.*, 1992) has been isolated and found to be a 60-70 kD protein monomer which occurs on the surface of *C. albicans* as a dimer or trimer (Klotz *et al.*, 1994). Several candidal mannoproteins have been partially characterized in the 60-70 kD range that bind ECM proteins including fibronectin (Skerl *et al.*, 1984; Klotz *et al.*, 1994), laminin (Bouchara *et al.*, 1990), and fibrinogen (Casanova *et al.*, 1992). It is not known whether a single fungal structure or multiple fungal structures mediate this binding (Calderone and Braun, 1991; Calderone *et al.*, 1994). Evidence that laminin and fibrinogen compete for binding (Bouchara *et al.*, 1990) suggests these two ECM proteins may bind to the same structure. *C. albicans* yeast cells preincubated with fibronectin were shown to be inhibited in their adhesion to buccal and vaginal epithelial cells (Skerl *et al.*, 1984). Weak blocking of *C. albicans* binding to human keratinocytes by PT-2000 and fibronectin has been described by one group (Ollert *et al.*, 1993). In summary, the genetic cloning and definitive identification of these *C. albicans* receptor(s) mediating adhesion to ECM components, as well as the CR3/CR2-like

molecule(s) still remains to be demonstrated, although data from this dissertation and other studies support the concept that lymphocytes use separate molecules to adhere to *C. albicans* than do mammalian epithelial and endothelial cells previously examined.

Murine and human PMN's and macrophages probably constitute the major host defenses against disseminated *C. albicans* (Calderone *et al.*, 1994). In contrast to *C. albicans* adherence mechanisms to epithelial and endothelial cells, recent evidence indicates murine and human macrophages also use CD11b/CD18 as the principal receptor for adhesion to *C. albicans*. The identity of the principal receptor for *C. albicans* was investigated using a *C. albicans* cell wall extract which blocked adhesion to macrophages (Szabo *et al.* 1995). These authors have observed that the phagocytosis of *C. albicans* is mediated by a combination of mannose specific lectin-like receptor, Fc receptor, and complement receptor-type 3 (CR3)-dependent processes. Their conclusion was, however, that the most efficient and major uptake of this organism is dependent on CR3-mediated phagocytosis and concluded that the principal receptor for *C. albicans* on murine macrophages is CR3 (CD11b/CD18) (Szabo *et al.*, 1995). A mannose specific lectin-like receptor on splenic and lymph node macrophages has been described by others as mediating adhesion to *C. albicans* (Cutler, 1991). Using an *ex vivo* binding assay, adhesion of *C. albicans* yeast to lymph node macrophages was not blocked with laminin or fibronectin (final concentrations 100 nM)(Han *et al.*, 1993). However, *C. albicans* adherence in the *ex vivo* assay was blocked by *C. albicans* purified carbohydrates which were eluted from a Con A column with α-methyl-D-mannopyranoside (Han *et al.*, 1993). The Han *et al.* study was consistent with earlier studies which the authors believed showed that integrins were not involved in *C. albicans* adherence to splenic zone macrophages (Kanbe *et al.*, 1993). Murine and human PMN also appear to utilize Mac-1 as the principal receptor for adhesion to *C. albicans* similar to macrophages (Szabo *et al.*, 1995).

Bober *et al.* also recently demonstrated that CR3, CR1 (CD35; an immunoglobulin superfamily member which binds C3b, and Fc receptors were involved in PMN-mediated killing of *C. albicans* blastospores (Bober *et al.*, 1995). These data are consistent with the extensive data demonstrating *S. cerevisiae* binding by Mac-1 on macrophages and PMN (Ross *et al.*, 1985; Sehgal *et al.*, 1993; Thornton *et al.*, 1996). A recent allergy study supporting the similarity between *S. cerevisiae* and *C. albicans* cell wall carbohydrates demonstrated that specific murine serum IgE directed against *S. cerevisiae* glucomannans was 100% eliminated by preadsorption with *C. albicans* glucomannans (Nermes *et al.*, 1995). It is known that a common cross-reactive carbohydrate epitope exists between *Pneumocystis carinii* and 15 other opportunistic fungi such as *S. cerevisiae* and *C. albicans* (Lundgren *et al.*, 1992). In contrast to the ECM and RGD peptide data discussed above for epithelial and endothelial cells, these data support the concept that lymphocytes, macrophages, and PMN all utilize CD11b/CD18 for adhesion to *C. albicans*.

Finally, and relevant to the discussion which follows, Senet *et al.* have demonstrated that murine and human platelets adhere to *C. albicans* yeast and hyphae via the integrin $\alpha_{IIb}\beta_3$ (Calderone *et al.*, 1994). Interestingly, $\alpha_{IIb}\beta_3$ exhibits "ligand induced activation of binding" in which this β_3 integrin is activated by and then binds to specific ligands (Du *et al.*, 1993), as has been recently shown for Mac-1 adhesion to ICAM-2 (Li *et al.*, 1995), and is proposed for Mac-1 adhesion to *C. albicans* in this study. Consistent with a picture of ligand induced activation of adhesion, pre-activated platelets were found to adhere far less than non-activated platelets (Mahaza *et al.*, 1991; Robert *et al.*, 1992) and not by $\alpha_{IIb}\beta_3$ but by ECM proteins expressed on the activated platelet surface. A 45 kD mannoprotein on the surface of *C. albicans* has recently been identified as the ligand for resting platelets (Calderone *et al.*, 1994). Whether this same 45 kD mannoprotein is a ligand for Mac-1 as well remains to be

investigated. However, it represents an excellent candidate ligand considering the fact that two separate groups have identified specific mAbs that bind only to CD11b/CD18 and $\alpha_{IIb}\beta_3$. One mAb (7E3) blocks function of both integrins but reacts with them only in their activated state (Altieri and Edgington, 1988). A second newly described mAb (25E11) directed against $\alpha_{IIb}\beta_3$ also blocks adhesion function of both integrins and does not require integrin activation for reactivity (De Nichilo *et al.*, 1996). Such cross-reactivity between functional blocking mAbs for two distinct integrins in different β-chain subfamilies is unique for these two integrins (De Nichilo *et al.*, 1996). Another mAb (mAb 24) is known to react with multiple β_2 integrins and recognizes an activation specific epitope common to CD11a, CD11b, and CD11c, but does not block adhesion (Dransfield and Hogg, 1989). However, it does appear to block deadhesion. Like the β_2 integrins, the β_3 integrins require activation to bind specific ligands (Du *et al.*, 1993). The integrin $\alpha_{IIb}\beta_3$ is considered an RGD-inhibitable integrin when activated (Hynes, 1992; Du *et al.*, 1993; Loftus *et al.*, 1993). A 45% overall sequence homology exists between CD11b and α_{IIb} (Corbi *et al.*, 1988). In addition, greater than 80% sequence homology exists in the sequences involved in CD11b/CD18 and $\alpha_{IIb}\beta_3$ adhesion to RGD-mimetic peptides through a MIDAS domain in the CD11b I domain and a MIDAS domain found on the β_3 chain (Corbi *et al.*, 1988; Loftus *et al.* 1994; Bajt *et al.*, 1995). These mAb and sequence data provide solid physical evidence for a functionally important epitope shared by these two integrins (De Nichilo *et al.*, 1996), and provide evidence that activated Mac-1 could interact with an RGD-mimetic ligand on the surface of *C. albicans* in a manner similar to activated $\alpha_{IIb}\beta_3$ on platelets.

It should not be too surprising that the CR3 (Mac-1) complement receptor mediates adhesion of lymphocytes to *C. albicans*, or that *C. albicans* expresses complement binding proteins. Several complement receptors have been demonstrated to mediate adhesion to

microorganisms including CR1, CR2, CR3, CR4 (p150,95), MCP, and DAF (Cooper, 1994). Conversely, most microorganisms including viruses, bacteria, fungi, and protozoan parasites also express a continually growing list of molecules to co-opt, subvert, and evade the mammalian complement defense system (Joiner, 1988; Cooper, 1994). Also, other integrins in addition to CD11b/CD18 have been demonstrated to mediate adhesion to a wide variety of nonopsonized and opsonized microbial pathogens. At least 21 integrins have been identified to date and 12 of these have now been proven to bind microorganisms and viruses (Isberg and Nhieu, 1994). Organisms that have been shown to bind to the CD11b/CD18 integrin directly without opsonization include the fungi *Blastomyces dermadititis* (Newman *et al.*, 1995), *Histoplasma capsulatum* (Bullock and Wright, 1987), *Saccharomyces cerevisiae* (Ross *et al.*, 1985), and probably *Cryptococcus neoformans* (Levitz *et al.*, 1994). Other pathogens binding directly to Mac-1 include HIV (Thieblemont *et al.*, 1993), *Bordatella pertussis* (Relman *et al.*, 1990), *Leishmania* spp. (Mosser and Edelson, 1985), *Escherichia coli* (Gbarah *et al.*, 1991), *Mycobacterium* spp. (Rao *et al.*, 1993), and the hookworm *Ancyclostoma caninum* (Muchowski *et al.*, 1994). Microorganisms binding directly to other integrin receptors include *Yersinia* spp. (Isberg and Leong, 1990), *Borrelia burgdorferi* (Coburn *et al.*, 1993), Echovirus 1 (Bergelson *et al.*, 1992), Adenovirus 2 (Wickham *et al.*, 1993), and foot-and-mouth disease virus (Mason *et al.*, 1994). In addition a number of microorganisms have been shown to bind to the CD11b/CD18 integrin after opsonization including *Cryptococcus neoformans* (Levitz and Tabuni, 1991), *Leishmania* sp. (Mosser *et al.*, 1992), *Rhodococcus equi* (Hondalus *et al.*, 1993), West Nile virus (Cardosa *et al.*, 1983) and HIV (Dierich *et al.*, 1993; Thieblemont *et al.*, 1993). In the cases of *Bordatella* and *Leishmania* adhesion to CD11b/CD18 and adhesion of *Yersinia* (β_1 integrins), *Borrelia* ($\alpha_{IIb}\beta_3$ integrin), and foot-and-mouth disease virus (β_3 integrins) to integrins, inhibition of binding with RGD-mimetic

peptides has been demonstrated. An RGD-containing sequence mediates attachment of *Bordatella* FHA glycoprotein to CD11b/CD18 on macrophages (Relman *et al.*, 1990), while it is also clear that *Yersinia* species interact with β_1 integrins via the β_1 GRGDSPK binding site via the protein invasin using an RGD-mimetic sequence with a key aspartate that contains no "RGD" sequence *per se* (Nhieu and Isberg, 1991). Of relevance to the present study, the WI-1 molecule with homology to invasin has been cloned from another dimorphic fungus *Blastomyces dermatiditis* (Klein *et al.*, 1993), and shown to mediate adhesion to CD11b/CD18 on macrophages (Newman *et al.*, 1994). The expression of WI-1 correlates with adhesion and virulence of *B. dermatiditis* and is a major target of cell mediated immunity to *B. dermatiditis* (Klein *et al.*, 1992; Klein *et al.*, 1994). Relevant to the model for Mac-1 activation by carbohydrates on *C. albicans* proposed later, the expression of surface *B. dermatiditis* α-1,3-glucan also correlates with virulence (Hogan and Klein, 1994). In summary, integrins in general and specifically CD11b/CD18 mediate adhesion of a growing list of microorganisms to mammalian cells.

The β_2 leukocyte integrins are only expressed on cells of the immune system (Kishimoto *et al.*, 1989; Springer, 1990; Arnaout, 1990; Anderson, 1994). Mac-1 (Macrophage-1 Antigen) was originally defined as a myeloid cell specific marker (Springer *et al.*, 1979) but is now known to be expressed on macrophages, dendritic cells, PMN's, eosinophils, basophils, mast cells, large granular lymphocytes (including NK cells), B cells (especially $CD5^+$), and T cells (especially $CD8^+$) (Hoshino *et al.*, 1993; Anderson, 1994). The expression of CD11b/CD18 on cells of the murine immune system (Timonen *et al.*, 1990; McFarland *et al.*, 1992; Razvi *et al.*, 1995) and the human immune system (Hoshino *et al.*, 1993, Jääskeläinen *et al.*, 1992; Robertson *et al.*, 1990) can be considered the same. CD11b/CD18 is expressed by virtually all monocytes/macrophages and PMN's, and 45% of

peripheral blood lymphocytes (PBL) overall including: 85-95% of NK cells, 15-30% of peripheral T cells (about 90% $CD8^+$/10% $CD4^+$), and 10-30% of peripheral B cells (Patel *et al.*, 1983; McFarland *et al.*, 1992; Diamond and Springer, 1993; Hoshino *et al.*, 1993). CD11b/CD18 expression is dramatically upregulated in both murine and human IL-2 activated lymphocytes to expression of 60% or greater on large granular lymphocytes (Timonen *et al.*, 1991; McFarland *et al.*, 1992; Dianzani *et al.*, 1989). Interestingly, Mac-1 has several potential glycosylation sites (18 human/17 murine) and may be differentially glycosylated in different cell types (Kishimoto *et al.*, 1989). The structure of murine and human CD11b/CD18 are also very similar. There is a 74% sequence identity (over 85% if conservative substitutions are allowed) between murine and human CD11b and each is 1137 amino acids in length with a 26-amino acid transmembrane domain and a 19-amino acid cytoplasmic domain (Corbi *et al.*, 1988; Pytela, 1988; Arnaout *et al.*, 1988). Both murine and human Mac-1 contain seven "homologous repeat" regions in which 13 conserved cysteines are found with homology to the 'EF-hand' calcium binding proteins calmodulin and troponin. Both human and murine Mac-1 contain a 200 amino acid inserted domain ("I domain") in the N-terminal portion (amino acids 128-314) containing the MIDAS adhesion motif (Lee *et al.* 1995; Bergelson and Hemler, 1995) and hydrophobic residues with homology to collagen binding proteins (Corbi *et al.*, 1988; Arnaout, 1990). In the cytoplasmic, transmembrane, homologous repeats, and I domain regions implicated in modulating direct ligand interactions, human and murine CD11b have greater than 90% sequence identity (Fleming *et al.*, 1993). In addition, both the human and mouse cDNA contain a conserved 5'-untranslated region containing consensus sequences for interferon response elements (Pytela, 1988).

The β_2 integrin subunits of murine CD18 (770 amino acids) and human CD18 (769

amino acids) proteins are also very similar with 82% sequence identity overall and 100% conservation of 56 characteristic cysteine residues (Wilson *et al.*, 1989). Little comparison has been made of mAbs which react with murine versus human CD11b/CD18 except the initial mAb (M1/70) used to identify Mac-1 in mice also reacts with and blocks function of human Mac-1 (Springer *et al.*, 1979). Similarly, this laboratory has previously demonstrated both mIAL and hIAL are equally capable of adhesion to and growth inhibition of *C. albicans* hyphae (Beno and Mathews, 1992). Recently, gene targeting has yielded a CD18 'knockout' mouse which is intended as a valuable model of human Leukocyte Adhesion Deficiency (LAD) (Wilson *et al.*, 1993). Overall then, the critical structural and functional characteristics of murine and human Mac-1, as well as that of other mammals (Fleming *et al.*, 1993), have been tightly conserved during evolution and the murine model is a relevant representation of human Mac-1 function and vice versa.

CHAPTER 2.

MATERIALS AND METHODS

Mice

C57Bl/6 and BALB/c female mice, ages 6 to 7 weeks, were obtained from Jackson Laboratory, Bar Harbor, ME. Mice were 6 to 12 weeks of age when used in experiments. BALB/c mice were used solely for the preparation of monoclonal antibody containing ascites fluid. All mice used were housed in an AAALAC approved facility at Loyola University Medical Center.

Fungal Culture

Candida albicans (#58716, ATCC, Rockville, MD) was obtained from Dr. T. Hashimoto, Loyola University of Chicago, Maywood, IL, and used throughout this investigation. Cultures were stored at 25°C on Sabouraud's dextrose agar (SDA) (Becton Dickinson, Lincoln Park, NJ). Cells used for experimentation were cultured overnight at 37°C on SDA, collected as isolated colonies, and washed once in complete Hank's Balanced Salt Solution (HBSS) containing sodium bicarbonate, pH 7.4 (GIBCO, Grand Island, NY). Yeast cultures were enumerated microscopically and those with greater than 15% budding were discarded. Clinical isolates of *C. albicans* were obtained from the Clinical Microbiology Laboratories of the Loyola University Medical Center, Maywood, IL.

Mammalian Cells

Mouse polymorphonuclear leukocytes (PMN) were elicited by intraperitoneal (i.p.) injection of 1.0 ml Thioglycollate Broth (Difco Labs, Detroit, MI). Three hours later the peritoneal cavity was washed with 10 ml of HBSS, the elicited cells enumerated and placed in culture medium at a concentration of 2.5 x 10^6 cells/ml in multiwell plates for 18 h at 37°C and 5% CO_2. Non-adherent cells were recovered and were found to be greater than 90% PMN as judged by Wright-Giemsa staining. Human erythrocytes were obtained from peripheral blood using a one-step Ficoll-Hypaque density gradient (Sigma Chemical Co., St. Louis, MO), washed twice in HBSS, and were greater than 99.99% erythrocytes as judged by Wright-Giemsa staining. The murine tumor cell lines EL-4 (thymoma; ATCC, Rockville, MD) and NYC (B cell; a gift from Hans-Martin Jäck, Loyola University, Chicago, IL), and P3-X63-Ag8.653 (myeloma; a gift from Charles F. Lange, Loyola University of Chicago, Maywood, IL) are maintained routinely in this laboratory as detailed for YT lymphocytes, except without the addition of conditioned medium. For ^{51}Cr labeling of mammalian cells, 100 μCi of ^{51}Cr (NEN Dupont, Wilmington, DE) were added to 10^7 mammalian cells in a final volume of 0.2 ml of HBSS. The cells were incubated at 37°C with 5% CO_2 for 1 h with agitation every 10 min, washed 3 times in HBSS, and enumerated with a hemocytometer.

LGL-like YT Lymphocyte Cell Line

Human leukemia, large granular lymphocyte (LGL)-like "YT" lymphocytes were originally obtained from E. Kovacks, Loyola University of Chicago, Maywood, IL. A subline of these original lymphocytes was selected in our laboratory as described previously (Forsyth and Mathews, 1993). These lymphocytes were cultured at 5 x 10^4 cells/ml in Falcon 24-well plates (Becton Dickinson, Lincoln Park, NJ) in RPMI 1640 media supplemented with

10% fetal bovine serum (FBS) (Sigma Chemical Co., St. Louis, MO), 50 μM mercaptoethanol (2-ME), 100 U/ml penicillin, 100 μg/ml streptomycin, 100 μM nonessential amino acids, and 2 mM L-glutamine (all from GIBCO, Grand Island, NY). Lymphocytes were passaged every 2 days with the addition of 2.5% 2-day spent culture conditioned medium. YT lymphocytes may also be referred to as lymphocytes or LGL throughout.

IL-2 Activation of Murine and Human Lymphocytes

For murine IL-2 activated lymphocytes (mIAL) spleens from untreated mice were aseptically removed. Single cell suspensions were prepared by dissociating the spleen through a 60-gauge wire mesh with the hub of a syringe. Spleen cells were washed once in HBSS prior to placement in culture medium containing 50 μM 2-ME at a concentration of 2.5 x 10^6 cells/ml with 1500 U/ml IL-2 (Hoffman-LaRoche, Nutley, NJ) in Falcon, 24-well plates (Becton Dickinson, Lincoln Park, NJ). Non-adherent cells were harvested following incubation for 4-6 days at 37°C, overlaid onto Lymphocyte Separation Medium (Litton Bionetics, Kensington, MD) and centrifuged at 1000 x *g* for 20 min. The cells at the interface were washed twice with HBSS prior to assessment of growth inhibitory activity. These splenocytes were >99% lymphocytes as judged by Wright-Giemsa staining.

For human IL-2 activated lymphocytes (hIAL), human peripheral blood mononuclear cells were obtained by venipuncture from normal healthy volunteers and isolated with Lymphocyte Separation Medium as described above. The cells were placed in culture with IL-2 and processed identically as described above for mouse splenocytes.

NIH 3T3 Fibroblast Transfected Clones

The experiments described herein with NIH-3T3 cells (National Institutes of Health,

Bethesda, MD) utilize two transfected clones of this murine fibroblast cell line which were the gift of Robert F. Todd, III (The University of Michigan School of Medicine, Ann Arbor, MI). The description and characterization of these cells has been detailed previously (Krauss *et al.*, 1994). Briefly, NIH-3T3 cells were transfected by calcium phosphate precipitation with a mixture of 1.5 μg of pSV2*neo*, 5 μg of pBACD11b containing human CD11b cDNA (a gift of D. Hickstein, University of Washington, Seattle, WA), 5 μg of pCMVBACD18 containing human CD18 CDNA, and 5 μg of human CD16 cDNA (a gift of Brian Seed, Massachusetts General Hospital, Boston MA) as described previously (Krauss *et al.*, 1994). Twenty-five G418-resistant colonies were expanded and screened for their expression of CD11b, CD18 and CD16 by indirect immunofluorescence.

Of the 3T3 fibroblast clones characterized by those authors, two clones were sent to this laboratory as frozen aliquots and immediately thawed and grown to numbers sufficient for use in the *C. albicans* hyphae adhesion assay protocol and maintained in continuous culture as adherent cells. Clone 3-1 (3T3-1), while subjected to the complete transfection protocol expressed no surface CD11b/CD18 or CD16, but must as a minimum contain the transfected PSV2*neo* plasmid for G418 selection. Clone 3-19 (3T3-19) expressed abundant surface CD11b/CD18 and no CD16. These phenotypes were confirmed upon receipt and at regular intervals with immunofluorescent microscopy using the anti-human CD11b mAb OKM1 and the anti-human CD18 mAb TS1/18 as described below.

3T3 cells were maintained at 37°C as described for lymphocytes and were grown in Falcon 75 mm^2 tissue culture flasks (Becton Dickinson, Lincoln Park, NJ) in Dulbecco's Modified Eagles's Medium (DMEM) with 4500 mg glucose/L with sodium pyruvate and L-glutamine (GIBCO, Grand Island, NY) supplemented with 10% FBS (Sigma Chemical Co., St. Louis, MO), 50 μM 2-ME, 100 U/ml penicillin, 100 μg/ml streptomycin, 25 ng/ml

amphotericin B, 100 μM nonessential amino acids, and 2 mM L-glutamine (all from GIBCO, Grand Island, NY). Cells received fresh media every 3 d and were passaged every 7-14 d when nearly confluent. Cells for passage or use in experiments were removed from the tissue culture flasks with a solution of HBSS without Ca^{2+} or Mg^{2+} ($HBSS^{-/-}$; GIBCO, Grand Island, NY) pH 7.4 containing Tris-base to adjust pH and 10 mM EDTA (Sigma Chemical Co., St. Louis, MO). Non-adherent 3T3 fibroblasts were than washed once in $HBSS^{-/-}$ pH 7.4 without EDTA and resuspended in DMEM media for passage or $HBSS^{-/-}$ to minimize clumping during labeling with ^{51}Cr for use in the adhesion assay. Cells were ^{51}Cr labeled as described below for lymphocytes and then washed and resuspended in HBSS (for at least one hour) also as described below for lymphocytes with resultant ^{51}Cr uptake and radioactive labeling approximately equal to that seen with YT lymphocytes (i.e. 5 x 10^4 cpm $\pm$ 2000 / 5 x 10^4 cells).

Phenotypic Analysis Using Monoclonal Antibodies

The monoclonal antibodies (mAbs) used in this study were verified to bind to lymphocytes and 3T3-19 fibroblasts before and after the mAbs were used in experimentation by indirect immunofluorescence with mAbs by utilizing them with an appropriate anti-mouse, anti-rat, or anti-hamster secondary antibody. Each secondary antibody was titrated for each cell type and number to achieve minimal background baseline immunofluorescence. In these microscopic determinations, randomly chosen groups of 100 cells were scored on an arbitrary scale of 0-4^+ in which 0 represented negative fluorescence equivalent to background and 1^+-4^+ were increasing magnitudes of brightness.

C. albicans Growth Inhibition

The anti-fungal activity of lymphocytes for *C. albicans* was determined as described previously (Beno and Mathews, 1993). For mAb blocking studies, cells were preincubated with mAbs as described for adhesion inhibition below. Briefly, fungal cells used for experimentation were collected from isolated, overnight SDA colonies, and washed once in HBSS. Yeast form cells were resuspended to 2 x 10^5/ml in RPMI 1640 (without serum unless specified). 10^4 cells were then added to individual wells of 96 well, flat bottom plates (#25861; Corning, Corning, NY). *C. albicans* hyphal forms were obtained by incubation at 37°C in 5% CO_2 for 2 h. Effector cells were then added at ratios from 100:1 to 2.5:1. After 3 h incubation at 37°C in 5% CO_2, effector cells were lysed and removed by washing with water using a PHD cell harvester (Cambridge Technology, Cambridge, MA). RPMI 1640 (50 μL) containing 1 μCi of ^{3}H-uridine (ICN Radiochemicals, Irvine, CA) was added to individual wells. Following 1 h incubation at 37°C, 5% CO_2, 25 U lyticase (Sigma Chemical Co., St. Louis, MO) in 50 μL HBSS was added to individual wells for 0.5 h at 25°C. Hyphae were harvested and associated radioactivity determined. Growth inhibition was determined and expressed as percentage inhibition of *C. albicans* growth as judged by associated ^{3}H-uridine as follows:

$$\% \text{ Inhibition} = 1 - \frac{(\text{experiment dpm} - \text{background dpm})}{(\text{maximum dpm} - \text{background dpm})} \times 100.$$

Data are calculated from mean % inhibition for triplicate values of two or more experiments. Maximum dpm was obtained from wells in which effector lymphocytes were not added.

^{51}Cr-Labeling of Mammalian Cells

100 μCi of ^{51}Cr (NEN, Dupont Inc., Wilmington, DE) were added to 1 x 10^7

mammalian cells in a final volume of 0.2 ml of HBSS. The cells were incubated at 37°C with 5% CO_2 for 1 h with agitation every 10 min, washed 3 times in HBSS and enumerated with a hemocytometer.

Adhesion of Lymphocytes and 3T3 Fibroblasts to C. albicans Hyphae

This assay is an adaptation of previously described procedures which utilize ^{51}Cr labeled cells to quantify cellular binding to substrate (Dustin and Springer, 1989; Van Seventer *et al.*, 1991). Briefly, *C. albicans* hyphae were prepared by growth in RPMI 1640 without serum for 3 h at 37°C in flat-bottomed 96-well plastic plates (Corning, Corning, NY). Preliminary studies also utilized test tubes (12 x 75) of borosilicate glass and polystyrene (Corning, Corning, NY). After 3 h 90%-100% confluence of hyphae was obtained when 10^5 yeast were delivered initially to each well of these assay plates. ^{51}Cr (NEN, Dupont Inc., Wilmington, DE) labeled lymphocytes were added to individual wells of the assay plates and incubated in a 5% CO_2 incubator at 37°C for 1 h. The assay was terminated by washing and removal of unbound lymphocytes from each well either with a pasteur pipet ("hand washing") with subsequent 3 x wash with 200 μl of HBSS, or by use of a multiple automated sample harvester (MASH) (PHD Cell Harvester, Cambridge Scientific, Cambridge, MA). The assay wells were washed three times with HBSS or 0.9% saline and 200 μl of 0.5% NP-40 (Sigma Chemical Co., St. Louis, MO) was added to each well for 20 min. The 0.5% NP-40 containing supernates were removed with a pasteur pipet and associated radioactivity determined.

Results are expressed as percentage cells bound as judged by the associated [^{51}Cr] as follows:

$$\% \text{ Bound} = \frac{(\text{experimental cpm}) - (\text{background cpm})}{(\text{maximum cpm}) - (\text{background cpm})} \times 100.$$

Maximum cpm release was obtained by adding 0.5% NP40 directly to mammalian cells. Experimental means were calculated from triplicate values from two or more experiments. Maximum cpm release was obtained by adding 0.5% NP-40 directly to radioactively labeled lymphocytes. Typical maximum cpm for 5 x 10^4 YT lymphocytes or 5 x 10^4 and NIH 3T3 fibroblasts was 5 x 10^4 cpm $\pm$ 3%, while typical maximum cpm for 10^5 murine and human IAL was 4 x 10^4 cpm $\pm$ 3%. Typical background values were usually less than 1% of maximum cpm for all the experiments and were typically determined to be 500 cpm $\pm$ 100 for the YT and 3T3 fibroblast experiments and 400 cpm $\pm$ 100 for the IAL experiments by adding equivalent ^{51}Cr to wells containing only *C. albicans* hyphae, washing as described above, and adding 0.5% NP-40 and determining the associated radioactivity. Data are calculated from mean % adhesion for triplicate values of two or more experiments.

Competition for Binding of Lymphocytes and 3T3-19 Fibroblasts to C. albicans Hyphae

This procedure was performed as described previously for the adhesion of lymphocytes to *C. albicans*, except that 5 x 10^4 radiolabeled YT lymphocytes or 3T3 cells, or 10^5 IAL were preincubated for 1 h with the indicated unlabeled YT cells, proteins, peptides, carbohydrates or antibodies at 37°C in 200 μl HBSS. This preincubation step was carried out in a 96-well polystyrene plate (Corning, Corning, NY) that had been pretreated with sterile 1% bovine serum albumin (BSA) (Sigma Chemical Co., St. Louis, MO) in HBSS at 25°C overnight and washed once with HBSS prior to addition of lymphocytes or 3T3 cells. The entire 200 μl preincubation mixture was transferred to wells containing *C. albicans* unless otherwise noted. In experiments in which free ligand was removed by washing, 5 x 10^5 YT lymphocytes in 1 ml HBSS were preincubated in 12 x 75 mm polystyrene culture tubes (Baxter Healthcare Corp., McGaw Park, IL) that had been treated at 4°C overnight with

sterile 1% BSA in HBSS and washed once with HBSS. Incubation with proteins, peptides or antibodies was for 1 h in HBSS at 37°C at the indicated concentrations. YT lymphocytes were then washed twice in HBSS, resuspended in HBSS and aliquots of 5 x 10^4 YT lymphocytes were added to individual wells containing *C. albicans* and the assay completed as described above. In complementary experiments, hyphae were preincubated with proteins, peptides or antibodies for 1 h at 37°C in HBSS and then washed twice with HBSS and the capacity of untreated YT lymphocytes to bind to these treated hyphae was determined as described above.

For all inhibition experiments, associated radioactivity was determined and expressed as percentage inhibition of cells bound to hyphae as judged by associated [^{51}Cr] as follows:

$$\% \text{ Inhibition} = 1 - \frac{(\text{experiment CPM} - \text{background CPM})}{(\text{maximum CPM} - \text{background CPM})} \times 100.$$

Maximum cpm release was obtained by adding 0.5% NP40 directly to radioactively labeled YT cells. Data are calculated from mean % inhibition for triplicate values of two or more experiments.

Scanning Electron Microscopy

Scanning electron microscopy was accomplished with a JEOL 64OA scanning electron microscope. Specimens of YT lymphocytes and *C. albicans* hyphae were allowed to adhere for one hour, then dehydrated with graded concentrations of acetone and critical point dried before microscopy.

Western Blot Analysis of YT Lymphocyte CD11b

Immunopurification was carried out as described previously (Altieri and Edgington, 1988). YT lymphocytes (2 x 10^7/ml) were lysed in buffer containing: 1% NP-40, 1% Triton

X-100, 0.05 M Tris-HCl, 0.15 M NaCl, 0.5% BSA, 2 mM PMSF, and 0.5 U/ml aprotinin (all from Sigma Chemical Co., St. Louis, MO) at pH 8.3 for 30 min at 4°C. Cell nuclei and other cellular debris were removed by centrifugation at 15,000 x *g* for 30 min and dialyzed overnight to remove Triton X-100. Antigen-antibody complexes were formed by incubating 1 ml of lysate with 50 μl of undiluted anti-CD11b (LM2/1) mouse ascites at 4°C for 4 h. The LM2/1 mAb is known to immunoprecipitate the intact CD11b/CD18 heterodimer (Altieri and Edgington, 1988). This solution was then passed over a recombinant protein A/G column (Pierce, Rockford, IL) at pH 8.0., and bound material was eluted with 0.1 M sodium acetate pH 2.0 (adjusted quickly to pH 7.4 with 2 M Tris-base), monitored for protein content by uv absorption at 206 nm, and collected as 2 ml fractions. Fractions containing peak protein concentration at 206 nm were adjusted to 1 mg/ml in a solution containing 2% SDS with TBS pH 6.8, containing 50 mM 2-dithiothreitol (Sigma Chemical Co., St. Louis, MO) as a reducing agent. Samples were boiled for 5 min at 100°C, and then clarified by centrifugation at 14,000 x *g* for 5 min. This solution was loaded, 20 μg per lane, and electrophoretically separated on 6% polyacrylamide gels with SDS at 200 V as described (Laemmli, 1970). Sample buffer solutions containing molecular weight standards (Gibco, Grand Island, NY) were similarly analyzed in adjoining lanes. Proteins were transferred to PolyScreen PVDF membranes (DuPont NEN Research Products, Wilmington, DE) at 4°C and 80 mA overnight. Blot transfers were blocked for 2 h at room temperature in 50 mM Tris-HCl (pH 7.5), 200 mM NaCl, 0.05% Tween 20 (TBST) plus 5% BSA (Sigma Chemical Co., St. Louis, MO), followed by overnight incubation with anti-CD11b (OKM1) (0.5 mg/ml PBS) diluted 1:150 in TBST plus 5% BSA at 4°C. After three 10 min washes in TBST, membranes were incubated for 1 h at room temperature with horseradish peroxidase labeled sheep-anti-mouse IgG antibody, diluted 1:4000 (Sigma Chemical Co., St Louis, MO). Following three washes in

TBST, membranes were incubated for 1 min with the Chemiluminescence Reagent (NEN, Dupont Inc., Wilmington, DE). Emitted light was captured on Dupont Reflection autoradiography film for a 30 sec exposure. Only the portions of the membrane developed above the 66 kD molecular weight are shown.

Proteins, Peptides, and Carbohydrates

Proteins and peptides used in this investigation were: human albumin, human complement component C3, echistatin, human Factor X, human fibrinogen, human fibronectin (from plasma), Fibronectin-like Engineered Protein (FEP), Fibrinogen Binding Inhibitory Protein (FBIP: HHLGGAKQAGDV, residues 400-411 from human fibrinogen-γ fragment), heparin sulfate, chicken egg lysozyme, MHC antigen H-2K^b fragment 163-174: TCVEWLRRYLKN, and the peptides GRGDSPK, GRGDTP, GRYDS, RGD, and RGDS (Sigma Chemical Co., St. Louis, MO); human laminin from placenta (laminin$_P$), human vitronectin, GRGDSP peptide, PepTite-2000 (PT-2000) and Cyclic GPenGRGDSPCA (GRGDSP$_C$) peptide (Telios Pharmaceuticals, San Diego, CA); mouse laminin from EHS cells (Gibco, Grand Island, NY); GRGDSP peptide (Peninsula Laboratories, Belmont, CA). The carbohydrates used were: N-acetyl-D-glucosamine (NADG), D-galactose, β-glucan (from bakers yeast and barley, prepared as in: Ross *et al.*, 1985; and D-glucose, D-mannose, methyl α-D-mannopyranoside, sucrose, and zymosan (Sigma Chemical Co., St. Louis, MO); and LPS type B from *S. enteritidis* (Difco Laboratories, Detroit, MI).

Monoclonal Antibodies

The mAbs used in these experiments have been summarized for clarity (Table 1.) Anti-human murine mAbs were purchased or purified from hybridoma culture supernates, or

Table 1.--Monoclonal Antibodies Used

Antigen	MAb	Origin	Isotype	Purified
murine CD11b	M1/70	rat	IgG_{2b}	yes
murine CD11b	5C6	hamster	IgG	yes
murine CD18	M18/2.A	rat	IgG_{2a}	yes
murine CD18	2E6	hamster	IgG	yes
murine CD11a	M17/4.4	rat	IgG_{2b}	yes
murine CD11c	N418	hamster	IgG	yes
murine CD29	anti-β_1	rat	IgG_{2b}	yes
human CD11b	OKM1	mouse	IgG_{2b}	yes
human CD11b	MY904	mouse	IgG_1	yes
human CD11b	MN-41	mouse	IgG_1	yes
human CD11b	LM2/1	mouse	IgG_1	yes
human CD18	IB4	mouse	IgG_{2a}	yes
human CD18	TS1/18	mouse	IgG_1	yes
human CD11b	TMG6-5	mouse	IgG_1	ascites
human CD11b	LPM19c	mouse	IgG_{2a}	ascites
human CD11a	TS1/22	mouse	IgG_1	yes
human CD51	anti-α_V	mouse	IgG_1	yes
human CD11c	SHCL-3	mouse	IgG_1	yes
human CD29	anti-β_1	mouse	IgG_1	yes
human CD58	anti-LFA3	mouse	IgG_1	yes
human CD30	HEF1	mouse	IgG_1	yes
human CD61	anti-β_3	mouse	IgG_1	yes

as noted below for LPM19c and TMG6-5 were used as ascites. All other mAbs utilized for blocking studies were used as purified mAb. Hybridoma cells were grown under conditions

as specified by ATCC (ATCC, Rockville, MD), from which all were obtained. The anti-murine mAb hybridomas and animal of origin used were as follows: M1/70.15 (rat anti-mouse CD11b, IgG_{2b}), 5C6 Clone 1 (hamster anti-mouse CD11b, IgG), M18/2.A (rat anti-mouse CD18, IgG_{2a}, kappa), 2E6 (hamster anti-mouse CD18, IgG), M17/4.4.11.9 (rat anti-mouse CD11a, IgG_{2b}, kappa), N418 (hamster anti-mouse CD11c, IgG).

Purchased anti-murine mAbs were: rat anti-mouse CD29 (β_1 integrin),IgG_{2b}, and FITC labeled secondary goat anti-rat IgG (Becton Dickinson, Lincoln Park, NJ). The FITC secondary used for hamster IgG antibody was FITC goat anti-mouse (Accurate Chemical and Scientific Corp., Westbury, NY).

The mouse anti-human mAbs used were as follows: OKM1 (anti-CD11b, IgG_{2b}), MY904 (anti-CD11b, IgG_1), LM2/1 (anti-CD11b, IgG_1) and TS1/18 (anti-CD18, IgG_1) (all from ATCC, Rockville, MD). These antibodies were purified from mouse ascites using a column of recombinant protein A/G as described by the manufacturer (Pierce, Rockford, IL). TMG6-5 (anti-CD11b, IgG_1) was used as ascites and was a generous gift of Dr. Istvan Ando (Hungarian Academy of Sciences, Szeged, Hungary). LPM19c (anti-CD11b, IgG_{2a}) was used as ascites and was a generous gift of Dr. K. Pulford (Radcliffe Hospital, Oxford, UK). MN-41 (anti-CD11b, IgG_1) was used as purified antibody generously provided by Dr. Gordon Ross (Univ. of Louisville, Louisville, KY). IB4 (anti-CD18, IgG_{2a}) was used as purified antibody and was the generous gift of Dr. Samuel Wright (Rockefeller Univ., New York, NY) and Dr. Elaine Tuomanen (Rockefeller Univ., New York, NY). HEF1 (anti-CD30, IgG_1) was used as purified antibody and was a generous gift of Dr. Hans-Martin Jäck (Loyola Univ. Chicago, Maywood, IL). TS1/22 (anti-CD11a, IgG_1) and TS2/9 (anti-CD58, IgG_1) were used as purified antibodies and a generous gift of Dr. Tom Ellis (Loyola Univ. Chicago, Maywood, IL). Purified monoclonal antibodies which were purchased included: anti-human

integrin α_V (CD51), IgG_1, clone VNR147 and anti-human β_1 integrin (CD29), IgG_1, clone P4C10 (both from Telios Pharmaceuticals, San Diego, CA); and anti-human p150,95 (CD11c), IgG_1, clone SHCL-3, and anti-human β_3 (CD61), IgG_1, clone #550036, (Becton Dickinson, Lincoln Park, NJ). All anti-human antibodies were of mouse origin and the secondary antibody used for immunofluorescence analysis was FITC goat anti-mouse (Accurate Chemical and Scientific Corp., Westbury, NY). Protein concentrations were determined by the BCA assay (Pierce, Rockford, IL).

Statistical Analysis

Linear regression plots were constructed from data for the labeled individual matrix proteins and Student's t-tests were performed for the data in Figure 13 using the computer graphing software Sigmaplot (Sigmaplot 4.1 © 1991, Jandel Corp.). The concentration of inhibitor which resulted in 50% inhibition of cell adhesion to hyphae was estimated by dropping a perpendicular to the x-axis from the point of intersection of the linear regression plot for that inhibitor and 50% inhibition using Sigmaplot 4.1 and was designated as the IC_{50}.

CHAPTER 3.

RESULTS

Aim 1. Develop an *in vitro* Assay System to Quantify Lymphocyte Binding to *C. albicans* Hyphae.

Radiometric Binding Assay for Quantifying Mammalian Cell Adhesion to C. albicans Hyphae

In order to identify the principal molecular structures which mediate adhesion of mIAL to *C. albicans* hyphae, it was first necessary to develop a method to consistently and reproducibly quantify this adhesion. The purpose of this series of experiments was to develop an *in vitro* assay with which to quantifiably measure mammalian cell adhesion to *C. albicans* hyphae and to investigate the usefulness of this assay for evaluating the identity of the principal adhesion molecules mediating this interaction.

The adhesion of several mammalian cell populations to the hyphal form of *C. albicans* was examined using the radiometric binding assay which was developed (Figure 1). Optimal lymphocyte adhesion to hyphae was demonstrated with the human LGL-like cell line YT and with murine IL-2 activated lymphocytes (mIAL) and human IL-2 activated lymphocytes (hIAL). For YT lymphocytes 15% of maximum total cell numbers bound was achieved with 10^4 cells added per well and 50% of maximum cells bound was obtained with 6 x 10^4 cells added per well. For 10^5 YT per well, the mean percent bound of added cells relative to the maximum counts bound was 80% $\pm$ 10%. Maximum values of total cells bound to hyphae

were attained with between 8 x 10^5 and 10^6 cells added per assay well. For mIAL and hIAL the mean percent bound of added cells was 30% $\pm$ 10% when 5 x 10^4 cells were added while the number of cells required to achieve 50% of maximum total cells bound to hyphae was higher at 10^5 lymphocytes added with maximum numbers of total cells bound using between 10^6 and 3 x 10^6 lymphocytes per well. Therefore, for the later inhibition experiments, the cell numbers which yielded 50% binding were used for comparison: 5 x 10^4 for YT lymphocytes and 10^5 cells per well for mIAL and hIAL. No apparent adhesion to hyphae was observed with human erythrocytes, murine thymocytes, non-activated murine splenocytes, the murine T cell thymoma EL-4 or the murine B cell leukemia NYC. Visual inspection of the assay wells revealed hyphae bound lymphocytes prior to the addition of NP-40. Associated radioactivity correlated visually with the number of lymphocytes bound to the hyphae. No lymphocytes appeared to adhere to the plastic of the assay well surface.

The conditions employed for these assessments were determined to be optimal. To do this, the YT lymphocyte cell line was used as a model in experiments to determine the optimal conditions for evaluating lymphocyte adhesion to *C. albicans* hyphae. YT lymphocytes have been demonstrated to inhibit growth of *C. albicans* hyphae in a manner comparable to mIAL and hIAL and therefore represent a relevant model to study IAL adhesion to *C. albicans* hyphae. In the development of this assay procedure comparisons were made of methods for immobilization of *C. albicans, C. albicans* initial cell concentration, degree of *C. albicans* confluence after culture, time and temperature of lymphocyte interaction with *C. albicans*, and the culture medium in which the binding assay was performed. Also, tubes (12 x 75) of borosilicate glass and polystyrene plastic were evaluated and did not provide the surface necessary for reproducible immobilization of *C. albicans*. The flat surface of the 96 well polystyrene,

Figure 1.: Adhesion of various cell types to *C. albicans*. 10^5 ^{51}Cr labeled cells of each type were added to individual wells of 96 well plates containing 10^5 *C. albicans* and allowed to bind 1 h at 37°C before washing. Adhesion of different cell types to *C. albicans* was assessed by the retention of ^{51}Cr labeled cells. Data are presented as mean % bound ± the standard deviation (SD) of 2 or more experiments. selected YT, ◆-◆; original YT, ■-■; mIAL, □-□; hIAL, ●-●; murine PMN, ◇-◇; human PMN^r, ▽-▽; human erythrocytes, ○-○; EL-4, ▼-▼; murine splenocytes, △-△; human PBMC, ▲-▲.

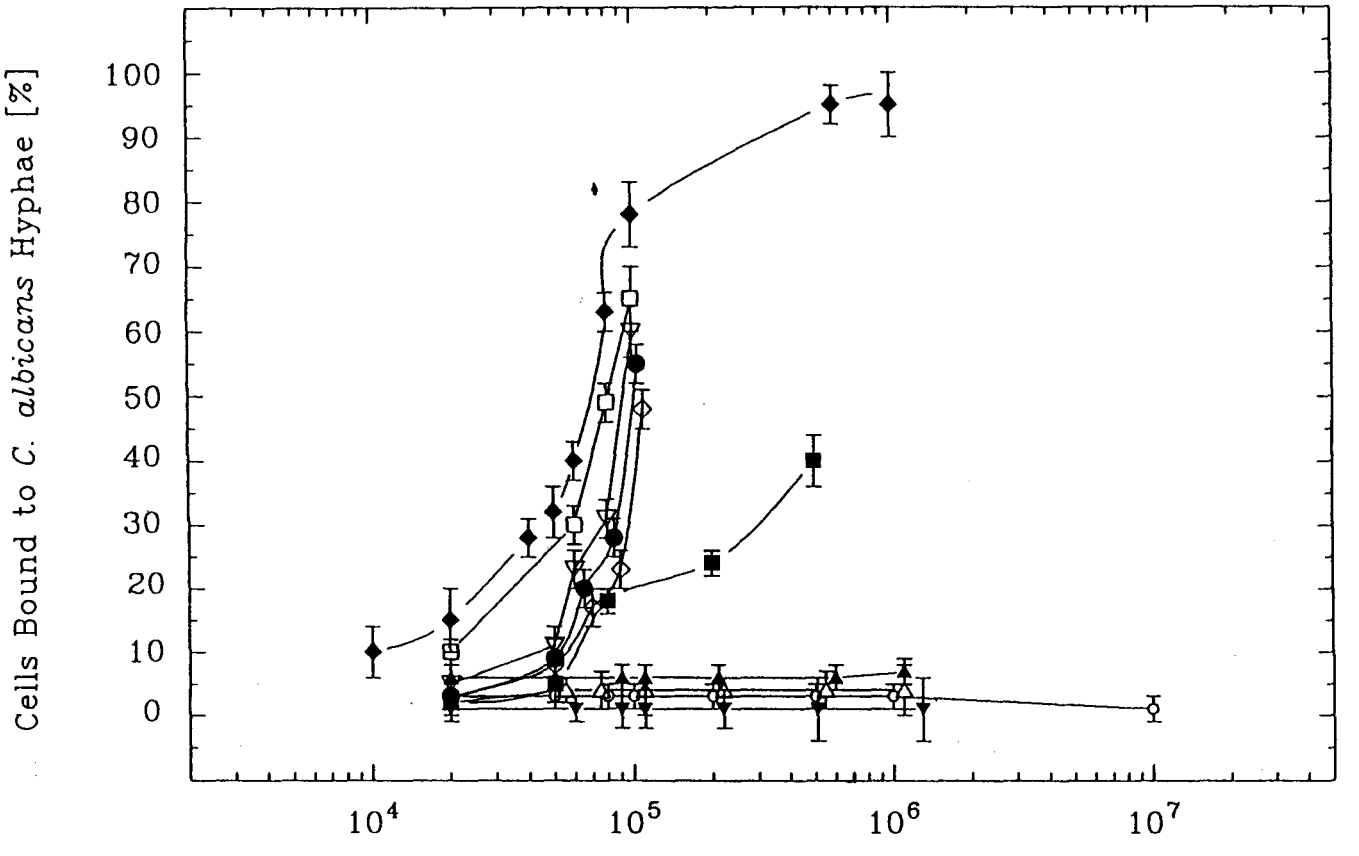
Cells Bound to C. albicans Hyphae [%]
100
90
80
70
60
50
40
30
20
10
0
10^4
10^5
10^6
10^7
Number of Cells Added

Figure 1.

tissue culture treated cluster plates provided highly reproducible results and was simpler and easier to manipulate. Comparisons of varying cell numbers of *C. albicans* (10^4 - 5 x 10^5) immobilized to the plastic well surfaces showed that optimal growth of hyphae and maximum lymphocyte adhesion to the fungal hyphae was achieved by incubation of 10^5 yeasts/assay well and incubating at 37°C and 5% $C0_2$ for 3 h. This initial yeast cell number produced approximately 100% hyphal confluency during the assay with hyphal interdigitation across the entire surface of each well. Maximal lymphocyte adhesion occurred at 60 min of incubation with hyphae while shorter periods of time resulted in less lymphocyte adhesion to hyphae. Lymphocyte adhesion to hyphae was virtually abolished at 4°C, however no difference in lymphocyte binding was observed at 25°C compared to 37°C. Experiments that compared YT lymphocyte binding to *C. albicans* hyphae in either HBSS, RPMI 1640, or RPMI 1640 with either 0.1% or 1.0% FBS were performed. There was no difference in adhesion using HBSS versus RPMI 1640, however those wells containing FBS averaged (from 10-50%) fewer bound YT cells.

To further establish the specific nature of the YT lymphocyte interaction with *C. albicans* hyphae experiments similar to the "cold target inhibition" assay for cytotoxic lymphocytes were conducted. Non-radioactively labeled YT and murine thymocytes were used to compete for the adhesion of radioactively labeled YT to the fungal surface (Figure 2). No competitive binding of radioactively labeled YT was seen with non-radioactive thymocytes. Cell number dependent inhibition was observed with non-radioactive YT, which effectively competed for radioactive YT binding to hyphae, demonstrating a specific interaction of YT lymphocytes with *C. albicans* hyphae. Inspection of assay wells revealed no homotypic aggregation of YT cells.

Another important aspect of the assay developed was the use of a multiple

Figure 2. Competition by non-radiolabeled cell types with ^{51}Cr-labeled YT lymphocytes for adhesion to *C. albicans*. Adhesion of YT lymphocytes to *C. albicans* was assessed by the retention of ^{51}Cr-labeled lymphocytes in the presence of non-labeled YT lymphocytes or thymocytes as in Fig. 1. Data are presented as mean % inhibition ± SD of two or more experiments. YT, o-o; mouse thymocytes, •-•.

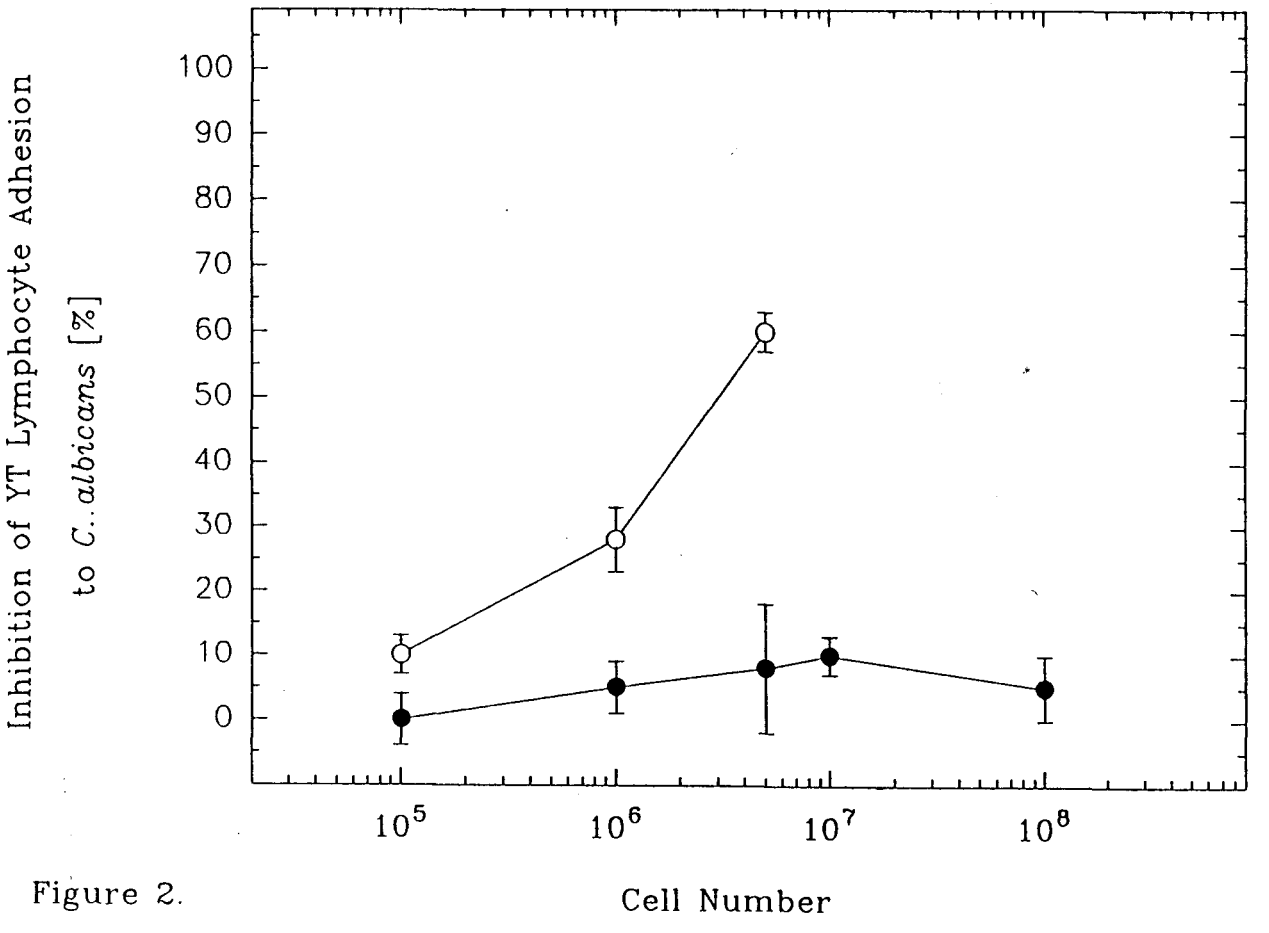
Inhibition of YT Lymphocyte Adhesion
to C. albicans [%]
100
90
80
70
60
50
40
30
20
10
0
10^5
10^6
10^7
10^8
Cell Number

Figure 2.

Table 2.--Comparison of Hand Washing and Multiple Automated Sample Harvester Recovery of Radioactivity Associated with Lymphocytes Bound to *C. albicans*

Lymphocyte Cell Number[a]	MASH[b] (mean cpm ± SD)	Hand Wash (mean cpm ± SD)
10^5	88,008 ± 3,974	80,536 ± 7,092
8 x 10^4	75,142 ± 1,446	66,771 ± 4,203
6 x 10^4	49,103 ± 2,272	48,030 ± 3,820
4 x 10^4	37,222 ± 2,822	39,164 ± 3,737

[a] YT cells were radiolabeled with [^{51}Cr].
[b] MASH (= multiple automated sample harvester.

Figure 3. Binding of ^{51}Cr-labeled YT lymphocytes to different clinical isolates of *C. albicans*. Binding of YT lymphocytes to *C. albicans* isolates from 6 different patients was assessed by the retention of ^{51}Cr-labeled cells. Data are presented as mean % bound ± SD of 2 or more experiments. ATCC 58716 = (●···●), all others lines are the 6 separate clinical isolates of *C. albicans*.

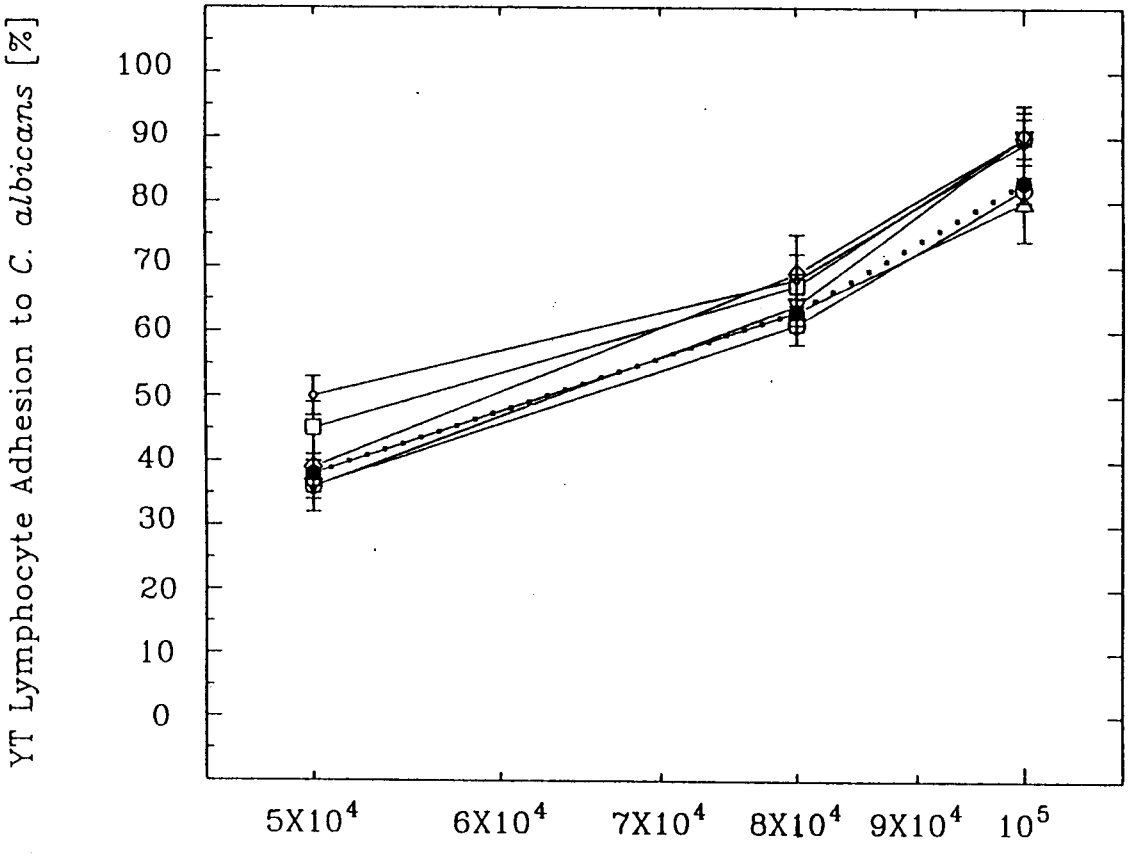
YT Lymphocyte Adhesion to C. albicans [%]
100
90
80
70
60
50
40
30
20
10
0
5X10^4
6X10^4
7X10^4
8X10^4
9X10^4
10^5
YT Lymphocyte Cell Number

Figure 3.

automated sample harvester (MASH) to wash and remove unbound mammalian cells. Data from a representative experiment in which "hand washing" with a pasteur pipet was compared to the use of the MASH using separate aliquots of the same batch of labeled YT lymphocytes (Table 2). Not only is the MASH more simplistic and easy to use but also the use of the MASH results in less variability as judged by comparison of standard deviation (SD) for the MASH versus that for the hand washing. For comparative purposes, percentage of adhesion and percentage of inhibition of adhesion are used throughout. The data in Table 2 are included to illustrate typical cpm $\pm$ SD for an individual experiment.

To make certain that the utility of the binding assay described herein is not restricted to a particular strain of *C. albicans*, the adhesion of YT lymphocytes to six clinical isolates of *C. albicans* was evaluated (Figure 3). Clearly, the six clinical isolates of the microorganism were bound by YT lymphocytes in a similar if not identical manner as was strain ATCC 58716 (Figure 3). These data demonstrate that the utility of the assay is therefore not limited to the strain of *C. albicans* (ATCC 58716) used in these experiments.

Aim 2.) Identify the Adherence Molecules on Murine IL-2 Activated Lymphocytes that Mediate Binding to *C. albicans* Hyphae

Extracellular Matrix and Blood Proteins Block YT Lymphocyte Adhesion to C. albicans

In order to identify the probable family of cell adhesion molecules involved in adhesion of mIAL to *C. albicans* hyphae, YT lymphocytes were employed as a model. Because of their interaction with a range of cell adhesion molecules, extracellular matrix (ECM) proteins were tested for their ability to inhibit adhesion of YT lymphocytes to hyphae (Figure 4). The purpose of this was to determine whether ECM proteins could

Figure 4. Extracellular matrix proteins inhibit YT lymphocyte adhesion to *C. albicans*. Adhesion of YT lymphocytes to *C. albicans* in the presence of the indicated proteins was assessed by the retention of ^{51}Cr labeled lymphocytes as in Fig. 1 with 5 x 10^4 lymphocytes per well. Lymphocytes were preincubated for 1 h at 37°C with the indicated proteins or with no protein before adding the entire mixture to *C. albicans* hyphae: vitronectin, •-•; EHS-laminin, ■-■; fibronectin, ▼-▼; fibrinogen, ▲-▲; C3 complement component, ◇-◇; placental laminin, ○-○. Solid lines (/) represent linear regression plots constructed from data for the labeled individual matrix proteins using Sigmaplot (Sigmaplot 4.1 © 1991, Jandel Corp.). Dotted lines (...) represent verticals dropped to the x-axis from a point where the regression plots intersect the 50% inhibition value to determine the respective matrix protein concentration. Dashed line (--), represents linear regression plot if vitronectin, EHS-laminin, fibrinogen, and C3 data points are considered to all be data for a single hypothetical matrix component. Data are presented as mean % inhibition ± SD of 2 or more experiments.

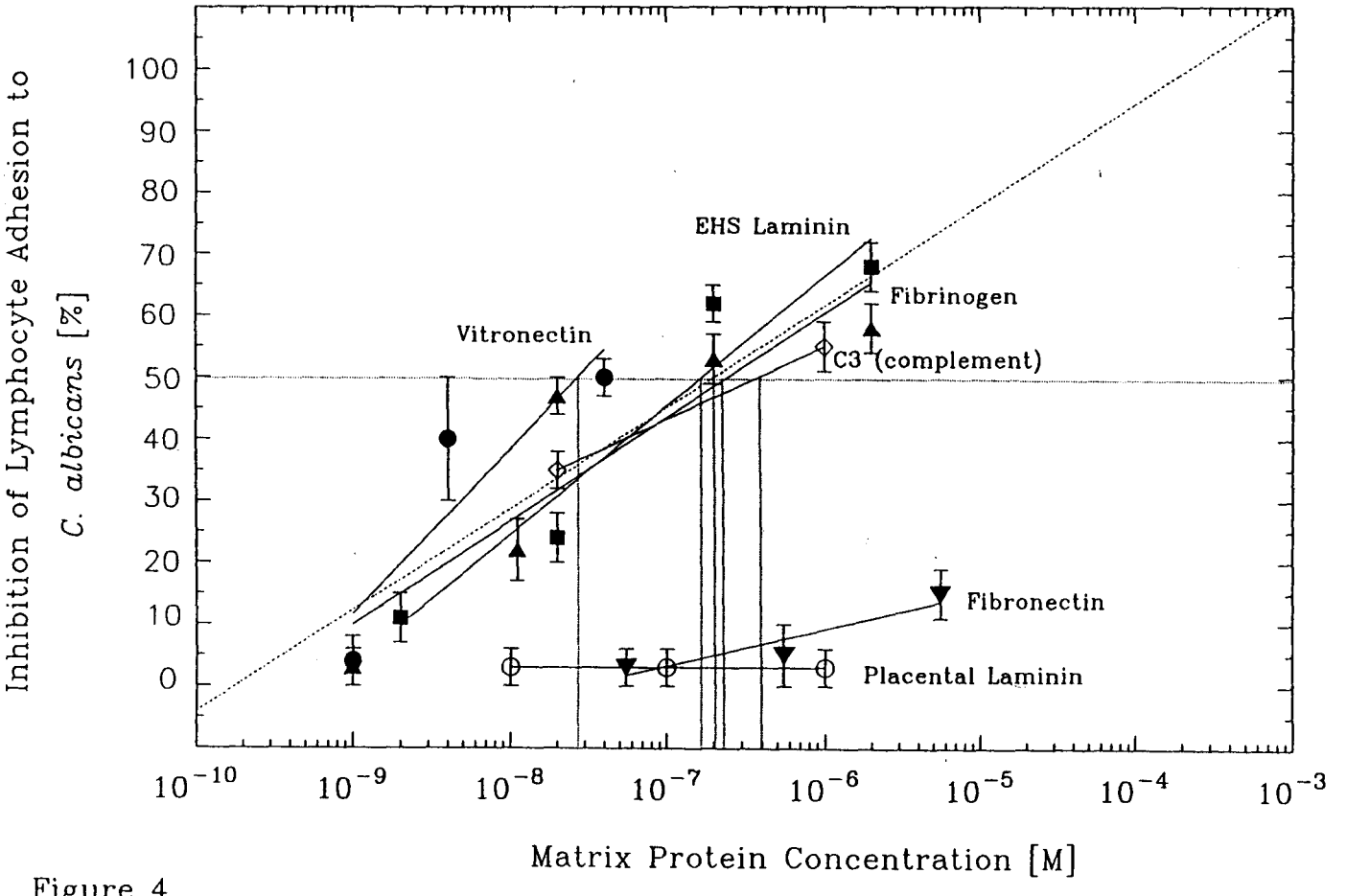
Inhibition of Lymphocyte Adhesion to C. albicans [%]
100
90
80
70
60
50
40
30
20
10
0
EHS Laminin
Fibrinogen
Vitronectin
C3 (complement)
Fibronectin
Placental Laminin
10^{-10}
10^{-9}
10^{-8}
10^{-7}
10^{-6}
10^{-5}
10^{-4}
10^{-3}
Matrix Protein Concentration [M]

Figure 4.

inhibit YT adhesion to hyphae and thereby identify candidate lymphocyte adhesion molecule(s) or a family of adhesion molecules mediating this binding.

In order to more accurately compare the inhibitory effect of individual ECM and blood protein and RGD-mimetic peptides, a linear regression plot was constructed from inhibition data for each protein or peptide using a computer graphing system (Sigmaplot 4.1 © 1991 Jandel Incorporated). These linear regression plots were then used to graphically estimate the concentration of each protein or peptide at which 50% inhibition of YT lymphocyte and later mIAL adhesion to hyphae could be achieved (dotted vertical lines in Figures 4, 5, and 6). This value for YT and mIAL was termed the 'estimated 50% inhibition concentration' and abbreviated as IC_{50}. Some proteins or peptides for which an IC_{50} was obtained were subsequently tested at this concentration on hIAL and/or 3T3-19 transfectants and produced either 50% or no (parentheses) inhibition and are included in Table 3. Known IC_{50} or Kd values for proteins and peptides in Table 3 are noted for comparison in the fifth column.

The most potent ECM protein inhibitor of this YT adhesion on a molar basis was vitronectin which inhibited YT lymphocyte adhesion to *C. albicans* with an IC_{50} of 30 nM. The most potent blood protein inhibitor of YT lymphocyte adhesion to hyphae on a molar basis was Factor X. Human clotting Factor X (Anderson, 1994) and C3 (Arnaout, 1990) are documented ligands for CD11b/CD18 as is heparin (Diamond *et al.*, 1995). Factor X was tested at a single concentration of 50 nM, this concentration was found to equal the mIAL IC_{50} for adhesion to hyphae. This concentration of Factor X inhibited YT adhesion by 85%, resulting in an estimated IC_{50} for YT cells of "less than" (<) 50 nM (Table 3). Concentrations of 200 nM fibrinogen and 200 nM mouse EHS laminin each produced 50% inhibition of YT adhesion to hyphae. A hypothetical linear regression plot was

Table 3.--Comparison of ECM Proteins and RGD-Mimetic Peptide Inhibition of Lymphocyte and 3T3-19 (CD11b/CD18) Transfectant Adhesion to *C. albicans*

Protein/peptide (concent.)	MIAL IC_{50}[a]	YT IC_{50}	HIAL IC_{50}	3T3-19 IC_{50}	Mac-1 Kd/IC_{50}
Factor X	50 nM	<50 nM[b]	50 nM	50 nM	44.0 nM [Kd]
C3	1 μM	400 nM	ND[c]	ND	3.5 μM [IC_{50}]
Fibrinogen	400 nM	200 nM	ND	ND	2.0 μM [Kd]
Echistatin	2 μM	2 μM	2 μM	ND	-[d]
EHS-Laminin	300 nM	200 nM	ND	ND	-
Heparin	100 μM	100 μM	ND	ND	9.0 μM [IC_{50}]
FBIP	300 μM	300 μM	300 μM	(300 μM)[e]	600 μM [IC_{50}]
RGD	ND	6 mM	ND	ND	-
GRGDSPK	300 μM	ND	300 μM	(300 μM)	-
GRGDSP	ND	500 μM	ND	ND	-
GRADSP	ND	1 mM	ND	ND	-
FEP	700 nM	500 nM	ND	ND	-
PT-2000	ND	500 μM	ND	ND	-
$GRGDSPCA_c$	(40 mM)	(40 mM)	(40 mM)	ND	-
MHC Peptide	(400 μM)	ND	ND	(400 μM)	-
GRYDS	(700 μM)	(700 μM)	(700 μM)	ND	-
$Laminin_p$	(1 μM)	(1 μM)	(1 μM)	ND	-

[a] IC_{50} is the estimated concentration which inhibits mammalian cell adhesion to *C. albicans* hyphae by 50%.

[b] Denotes single concentration tested with 85% inhibition so IC_{50} is estimated as less.

[c] Not Done (no concentration tested).

[d] (-) denotes no value specific for CD11b/CD18 (Mac-1) has been established.

[e] Numbers in parentheses denote highest concentration tested although no inhibition was seen

Figure 5. Specific RGD-mimetic peptides inhibit YT lymphocyte adhesion to *C. albicans*. Adhesion of lymphocytes to *C. albicans* was assessed by the retention of ^{51}Cr labeled lymphocytes in the presence of various RGD-mimetic peptides and polymers as described in Fig. 4 RGD sequences of each are shown in parentheses: FEP (Fibronectin-like Engineered Protein),(◆), is a polymer of 13 repetitions of the sequence VTGRGDSPAS and 9 repetitions of GAGAS sequence spacers; PepTite-2000, (■); GRGDSP, (▲); RGD, (●); GRYDS (▽); $GRGDSP_c$ is a cyclical molecule comprised of the sequence C-PenGRGDSPCA where Pen=penicillimine. Data are presented as in Fig. 4 and represent mean % inhibition ± SD of 2 or more experiments.

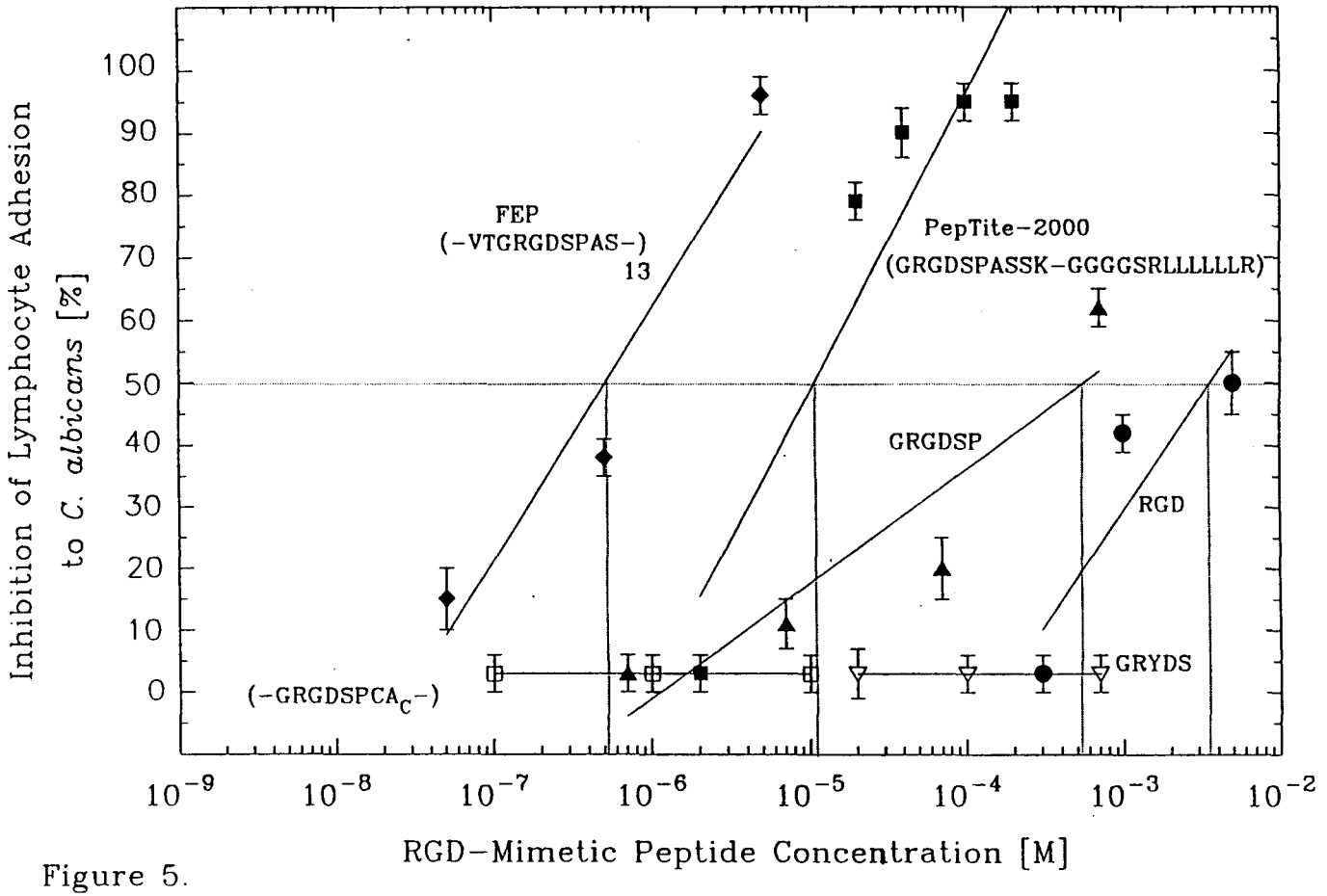
Inhibition of Lymphocyte Adhesion to C. albicans [%]
100
90
80
70
60
50
40
30
20
10
0
FEP
(-VTGRGDSPAS-)
13
PepTite-2000
(GRGDSPASSK-GGGGSRLLLLLLR)
GRGDSP
RGD
GRYDS
(-GRGDSPCA$_C$-)
10^{-9}
10^{-8}
10^{-7}
10^{-6}
10^{-5}
10^{-4}
10^{-3}
10^{-2}
RGD-Mimetic Peptide Concentration [M]

Figure 5.

also constructed from combined data for vitronectin, fibrinogen, EHS-laminin and C3 because of the clustering of the plots for these four proteins. The IC_{50} of this hypothetical ECM component was also 200 nM. Complement component C3 inhibited by only 34% at the 55 nM concentration, but did inhibit adhesion by 55% at a concentration of 2 μM resulting in an IC_{50} of 400 nM. Heparin sulfate tested at a single concentration of 100 μM (the mIAL IC_{50}), inhibited adhesion by 48%. Fibronectin from human plasma was a relatively poor inhibitor of lymphocyte adhesion to hyphae with maximal inhibition of 20% at a final concentration of 6 μM. Human laminin from placenta ($Laminin_P$, Table 3) which lacks the RGD-containing "A" chain present in EHS-laminin had no inhibitory effect at concentrations ranging from 10 nM - 1 μM (Figure 4). Two proteins not known to be recognized by integrins: chicken egg lysozyme and human albumin each had no effect on lymphocyte adhesion to hyphae at concentrations as high as 1.0 mM thereby demonstrating inhibition was not due to non-specific protein blocking. YT lymphocytes were examined microscopically prior to and after treatment with proteins and no homotypic aggregation was observed.

The relative inhibition of YT lymphocyte adhesion to *C. albicans* was not a direct function of the molecular size of the proteins (in order of potency: vitronectin 75 kD, Factor X 63 kD, fibrinogen 350 kD, EHS-laminin 850 kD, C3 190 kD, heparin 7.5 kD, and fibronectin 540 kD,). However, ECM and blood components that competed for YT lymphocyte adhesion to *C. albicans* hyphae all contained at least one RGD (arginine-glycine-aspartic acid) or RGD-like (containing a key aspartate residue) sequence (Yamada and Kleinman, 1992), while human laminin from placenta lacks the RGD-containing A-chain present in EHS-laminin (Tryggvason, 1993). Therefore, while these data do not identify a single adhesion molecule candidate, the potential pattern of inhibition by proteins containing RGD-like or "RGD-mimetic" (Du *et al.*, 1991) sequences suggested a member of the integrin

family of cell adhesion molecules as a candidate molecule mediating lymphocyte adhesion to *C. albicans* hyphae and for this reason the inhibitory capacity of RGD-mimetic peptides was investigated next.

RGD-mimetic Peptides Inhibit YT Lymphocyte Adhesion to C. albicans

The purpose of these experiments was to determine if lymphocyte adhesion to hyphae could be blocked using peptides containing RGD-mimetic sequences known to specifically inhibit integrins (Pierschbacher and Ruoslahti, 1984). The contribution to inhibition of this integrin signature adhesion motif was examined using several RGD-mimetic peptides. Fibrinogen Binding Inhibitory Peptide (FBIP) from human fibrinogen -γ fragment 400-411 is also a documented ligand for Mac-1 (Wright *et al.*, 1989). Like other RGD-mimetic peptides it contains a critical aspartate residue within the RGD-mimetic sequence:HHLGGAKQAGDV. Echistatin, which contains two RGD sequences, is a circular peptide (5.4 kD) member of the disintegrin family of highly specific integrin inhibitors isolated from venoms (Garsky *et al.*, 1989). Echistatin is not an established Mac-1 antagonist *per se*, but disintegrins are known to exert their integrin specific inhibition of adhesion through RGD-mimetic sequences which they contain. Four of the peptides tested contained the identical sequence: GRGDSP, yet these peptides differ in their specificity of integrin receptors (Piersbacher and Ruoslahti, 1987) and in their ability to inhibit lymphocyte adhesion to *C. albicans* (Figure 5 and Table 3).

The least potent RGD peptide inhibitor tested was the RGD tripeptide which inhibited YT lymphocyte adhesion to *C. albicans* weakly with an IC_{50} of 6 mM (Figure 5). The peptide GRADSP inhibited by 40% at a concentration of 200 μM with an IC_{50} of 1 mM (Table 3). The hexapeptide GRGDSP (the fibronectin RGD sequence) was a better inhibitor with an IC_{50} of 400 μM consistent with the 500 μM reported to inhibit integrin $\alpha_{IIb}\beta_3$ by 50%

(D'Sousa *et al.*, 1991). FBIP was tested only at the concentration of 340 μM which inhibited YT lymphocyte adhesion by 45%, slightly better than the 600 μM reported to inhibit Mac-1 adhesion to C3bi coated erythrocytes (EC3bi) by 50% (Wright *et al.*, 1989) (Table 3). PepTite-2000 (PT-2000) is a peptide of 2 kD that consists of the sequence: GRGDSPASSK-GGGGSRLLLLLLR with a single GRGDSP motif. PT-2000 was a potent inhibitor of lymphocyte binding to *C. albicans* hyphae with an IC_{50} of 10 μM and 98% inhibition of adhesion at a final concentration of 100 μM. Echistatin was a more potent inhibitor although tested only at the concentration of 2 μM, which inhibited YT adhesion to hyphae by 47% (Table 3). Fibronectin-like Engineered Protein (FEP), a 72 kD protein which contains 13 identical repeats of the VTGRGDSPAS human fibronectin sequence 10-mer (-VTGRGDSPAS-$_{13}$, Figures 5 and 9) separated by 9 GAGAS structural linker regions, was the most potent GRGDSP-containing inhibitor tested with an IC_{50} of 500 nM and 100% inhibition of adhesion to *C. albicans* at a final concentration of 5.5 μM. The circular GRGDSP containing peptide: GPenGRGDSPCA ($GRGDSP_C$, Table 3 and Figure 5), which specifically inhibits the vitronectin receptor but not the fibronectin receptor (Piersbacher and Ruoslahti, 1987), had no capacity to inhibit the binding of YT lymphocytes to hyphae at concentrations ranging from 100 nM - 40 mM. No homotypic aggregation was observed after cells were treated with the above peptides or engineered proteins.

Identification of the cell population inhibited by ECM/RGD-peptides

It is known that *C. albicans* hyphae express receptors for some ECM proteins and RGD containing peptides (Calderone and Braun, 1991; Hostetter, 1994b). Therefore, experiments were carried out to determine whether the ECM proteins and RGD-peptides exerted an inhibitory effect by binding to the surface of the lymphocytes

Figure 6. Comparative inhibition of lymphocyte adhesion to *C. albicans* by preincubation of either YT lymphocytes or *C. albicans* with ECM proteins or RGD-peptides. Adhesion of ^{51}Cr labeled YT lymphocytes to *C. albicans* was assessed after pretreatment of either lymphocytes (shaded bars) or *C. albicans* (solid bars) with the indicated agent followed by washing. Concentrations were: 10 μM laminin and fibrinogen, and 1 mM for RGD, GRGDSP, and PT-2000. Assessment and data presentation was as described in Fig. 4 except pretreatment was followed by washing with HBSS before addition of lymphocytes. Data represent mean % inhibition $\pm$ SD of 2 or more experiments.

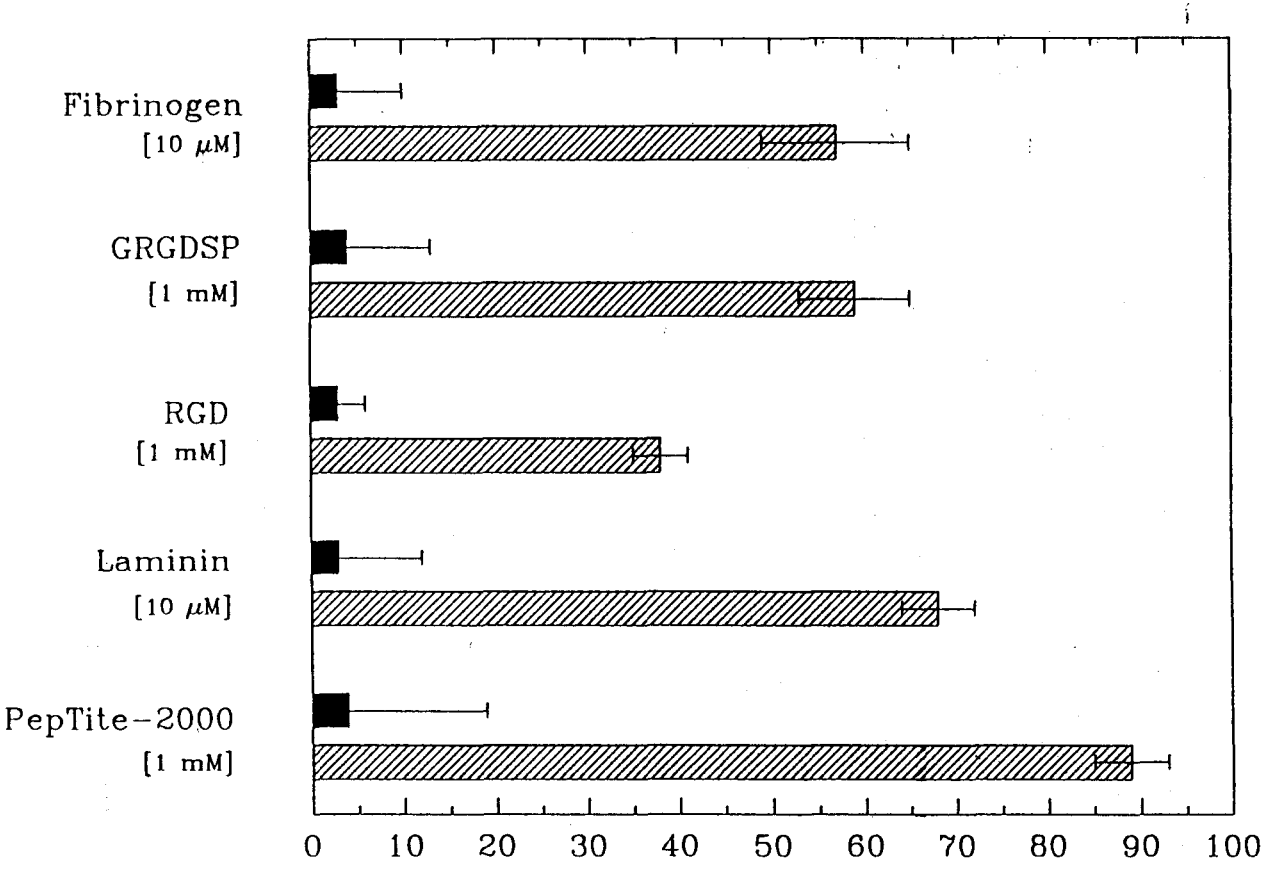
Fibrinogen
[10 μM]
GRGDSP
[1 mM]
RGD
[1 mM]
Laminin
[10 μM]
PepTite-2000
[1 mM]
0
10
20
30
40
50
60
70
80
90
100
Inhibition of Lymphocyte Adhesion to C. albicans [%]

Figure 6.

Table 4.--Summary of Microscopic Phenotypic Analysis

MAb	Antigen	YT	mIAL	hIAL	3T3-19	3T3-1
M1/70	m-CD11b	$4^+/76$[a]	$4^+/40$	$4^+/40$	$4^+/50$	0+/100
OKM1	h-CD11b	$3^+/76$	$3^+/40$	$3^+/40$	$3^+/50$	0+/100
5C6	m-CD11b	$0^+/100$	$3^+/40$	$0^+/100$	$0^+/100$	0+/100
MY904	h-CD11b	$3^+/76$	ND[b]	$3^+/40$	ND	ND
MN-41	h-CD11b	$2^+/76$	ND	ND	ND	ND
LM2/1	h-CD11b	$3^+/76$	ND	$3^+/40$	$3^+/50$	0+/100
TMG65 ascites	h-CD11b	$3^+/76$	ND	ND	ND	ND
LPM19 ascites	h-CD11b	$3^+/76$	ND	ND	ND	ND
M18/2.	m-CD18	$0^+/100$	$3^+/90$	ND	ND	ND
2E6	m-CD18	$0^+/100$	$3^+/90$	ND	ND	ND
TS1/18	h-CD18	$3^+/76$	$0^+/100$	$3^+/90$	$3^+/50$	0+/100
IB4	h-CD18	$3^+/90$	ND	ND	ND	ND
M17/4.	m-CD11a	$0^+/100$	$3^+/80$	ND	ND	ND
TS1/22	h-CD11a	$0^+/100$	$0^+/100$	$3^+/80$	ND	ND
N418	m-CD11c	$0^+/100$	$2^+/60$	$0^+/100$	ND	0+/100
SHCL3	h-CD11c	$3^+/76$	ND	ND	ND	ND
β_1	m-CD29	ND	$3^+/90$	ND	$2^+/90$	2+/90
β_1	h-CD29	$0^+/100$	ND	$2^+/80$	ND	ND
LFA-3	h-CD58	$3^+/90$	ND	$3^+/90$	ND	ND
β_3	h-CD61	$0^+/100$	ND	ND	ND	ND
HEF1	h-CD30	$2^+/90$	ND	ND	ND	ND
α_V	h-CD51	$0^+/100$	ND	ND	ND	ND

[a] Given as brightness score($^+$)/ percent of cells expressing antigen.
[b] ND = not done

and/or *C. albicans* (Figure 6). Lymphocytes or hyphae were pretreated with the indicated proteins or RGD-peptides and washed before adhesion was evaluated. The concentrations of inhibitors employed represented doses which had resulted in 50% or greater inhibition when no washing step was employed. The concentrations utilized were: 10 μM laminin and fibrinogen, and 1 mM for RGD, GRGDSP, and PT-2000. Pretreatment of *C. albicans* hyphae with ECM or RGD-peptides resulted in no inhibition of lymphocyte adhesion to hyphae. However, pretreatment of lymphocytes with the same ECM or RGD-peptides resulted in substantial inhibition of adhesion. The RGD tripeptide was the least potent inhibitor of LGL binding to *C. albicans* while PT-2000 was the most effective inhibitor of the proteins and peptides tested. Because the washing step may introduce undefined variability, a more quantitative comparison of relative inhibition by each element was not performed. The important conclusion from these experiments is that clearly the ECM proteins and RGD-mimetic peptides are exerting their inhibitory effects on the YT lymphocyte cell population directly and not by interaction with *C. albicans* hyphae.

Phenotypic Analysis Using Monoclonal Antibodies

All the mAbs utilized were found to label the YT lymphocytes and respective mouse and human IAL equally well ranging from 2^+-4^+ brightness (mean 3^+) at the concentrations of mAb employed in these experiments. These data have been summarized (Table 4). One exception was found: the lymphocytes labeled with the M1/70 mAb tended to be the brightest at 4^+ and sometimes brighter than the same lymphocytes labeled using the other mAbs at 3^+ and consistently brighter than lymphocytes labeled with OKM1 at 3^+ despite variations in primary and secondary antibodies which were attempted. This consistent difference between M1/70 and OKM1 labeling occurred with YT lymphocytes and murine and human IAL as

well and was the only consistent labeling difference which was observed. However, it should be noted that these were the only two mAbs which positively labeled all three groups of cells. The difference in labeling exhibited by these two mAbs was in the magnitude of brightness of those cells which were positive for Mac-1, not in the percentage of positive cells in each case. These differences were not observed for the 3T3-19 Mac-1 positive fibroblasts which were consistently very bright (4^+) for all mAbs tested. Lymphocytes which were positive for respective anti-murine or anti-human Mac-1 mAbs were found to exhibit a similar uniformly distributed punctate pattern of immunofluorescent staining at 4°C with extensive capping of staining if the same cells were warmed to 25°C. The Mac-1 positive 3T3-19 fibroblasts exhibited a less punctate pattern of staining at 4°C with less pronounced capping at 25°C. These differences in fluorescence may reflect partial masking of the epitope for OKM1 resulting from association between CD11b/CD18 with other receptors such as CD16 involving the CD11b/CD18 lectin-like domain. Similar diminished fluorescence of CD11b/CD18 on neutrophils and monocytes using OKM1 has been demonstrated to be due to Mac-1 association with CD16 (Sehgal *et al.*, 1993) and carbohydrates (Thornton *et al.*, 1996).

For all cases except the Mac-1^+ transfected 3T3 fibroblasts (3T3-19) the percentage of cells adhering to hyphae correlated well with the percentage which were positive for Mac-1 ± 10% (Table 4). The mean percentage of YT lymphocytes which adhered to *C. albicans* hyphae was 70% ± 10% (5 x 10^4/well) which correlated closely with the 76% ± 5% which were Mac-1 positive. The mean percentages of murine and human IAL adhering (10^5/well) was found to be virtually the same at 30% ± 10%, while the mean percentage of IAL positive for CD11b/CD18 was consistently slightly higher than the percent adherent for any given sample with a mean percentage positive of 40% ± 5% for both mIAL and hIAL. The 3T3 fibroblast control cells (3T3-1) exhibited very poor adhesion to hyphae of 3% ± 3% and

were clearly all negative (0^+) for Mac-1 surface expression. The transfected 3T3-19 fibroblasts adhesion to hyphae was 20% ± 10%, 50% ± 15% were Mac-1 positive.

Anti-CD11b/CD18 Monoclonal Antibodies Inhibit YT Lymphocyte Adhesion to C. albicans

These ECM and RGD-peptide inhibition data presented in Figures 4, 5, 6, and in Table 3, suggest that an RGD-inhibitable integrin may mediate lymphocyte adhesion to hyphae. Essentially 50% of the known integrins have been demonstrated so far to be inhibitable with GRGDSP sequence RGD-mimetic peptides (Pierschbacher and Ruoslahti, 1984; D'Souza *et al.*, 1991). An evaluation of possible candidate integrins on YT lymphocytes was performed using immunofluorescence microscopy. YT lymphocytes evaluated by immunofluorescent microscopy did not express the α_V(CD51), β_1(CD29), or the β_3(CD51) integrin subunits which are one or both subunits of the most widely recognized RGD-inhibitable integrins (Hynes, 1992), suggesting that another integrin on the surface of YT lymphocytes mediates adhesion to *C. albicans*. However, immunofluorescence did show the β_2 integrins CD11b/CD18 and CD11c/CD18 but not CD11a/CD18 to be on the surface of these YT lymphocytes. CD11c/CD18 adhesion has never been shown to be inhibitable with RGD peptides (Arnaout, 1990; Anderson, 1994). In contrast, CD11b/CD18 (Mac-1) has been inhibited with RGD-containing peptides (Russell *et al.*, 1989; Wright *et al.*, 1989; Relman *et al.*, 1990; Anderson, 1994) and was the best candidate, although the direct inhibition of CD11b/CD18 by RGD-mimetic peptides remains controversial. Therefore, several mAbs to CD11b and also to CD18 were tested for their ability to block YT lymphocyte adhesion to *C. albicans* hyphae (Figure 7).

Anti-CD11b antibodies inhibited adhesion of YT lymphocytes to *C. albicans* hyphae. The mAbs tested were used in purified form unless sent from another investigator as ascites.

Figure 7. Competitive inhibition of YT lymphocyte adhesion to *C. albicans* with monoclonal antibodies. Adhesion of 5 x 10^4 ^{51}Cr labeled YT lymphocytes to *C. albicans* hyphae was assessed in the presence of the indicated mAbs. Lymphocytes were preincubated in HBSS containing purified murine mAbs or ascites (*) containing mAbs. All cells were incubated with the same quantity (45 μg) of protein unless shown otherwise. The mAbs shown are to the designated CD antigens: CD11b: *TMG6-5, OKM1, MY904, *LPM19c, LM2/1, and MN-41; CD18: TS1/18 and IB4; CD-58 (LFA-3): TS2/9; CD30: HEF1. Conditions and adhesion assessment were as described in Fig. 4. Data are presented as mean % inhibition $\pm$ SD of 2 or more experiments.

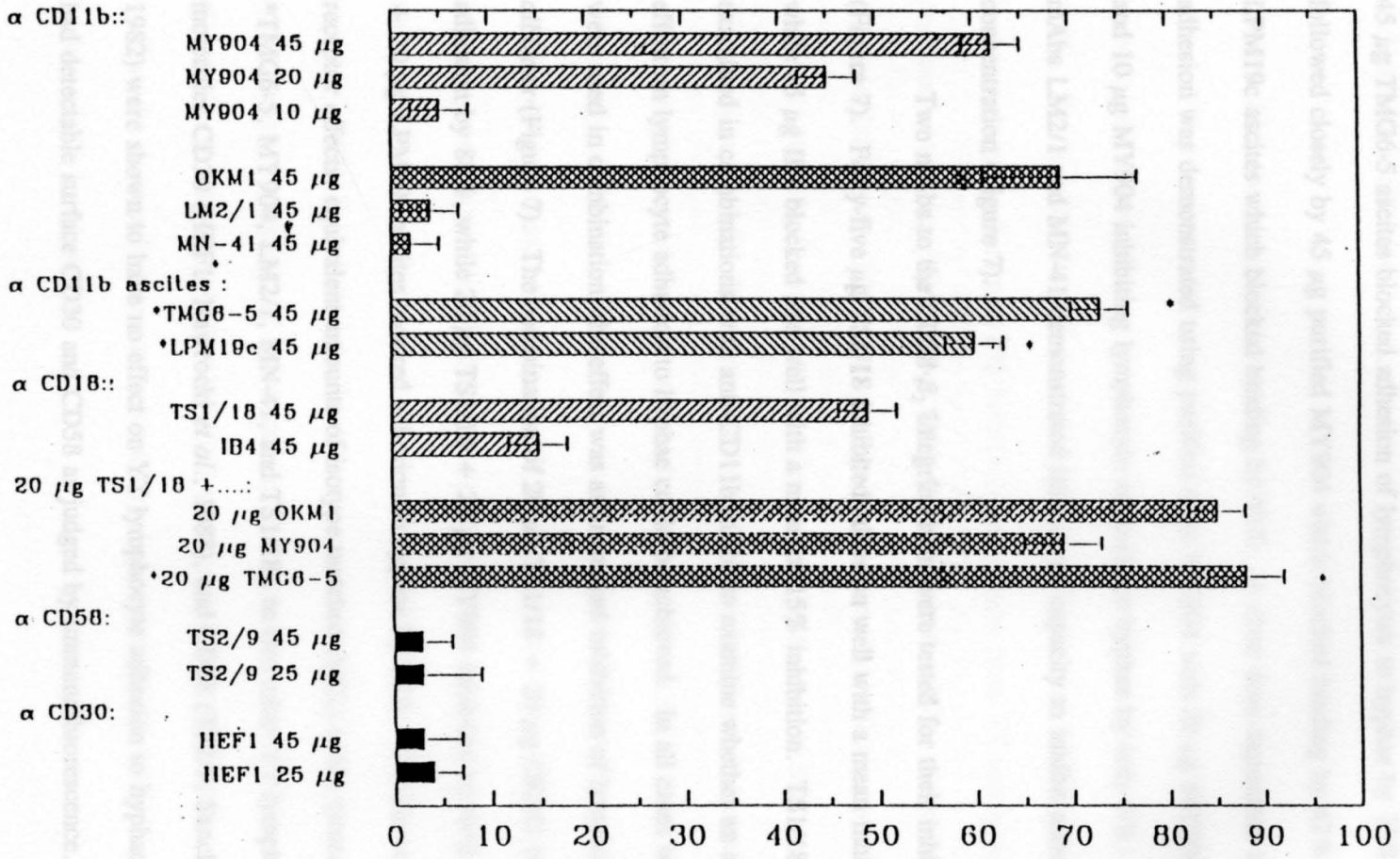
α CD11b::
MY904 45 µg
MY904 20 µg
MY904 10 µg
OKM1 45 µg
LM2/1 45 µg
MN-41 45 µg
α CD11b asciles :
•TMG8-5 45 µg
•LPM19c 45 µg
α CD18::
TS1/18 45 µg
IB4 45 µg
20 µg TS1/18 +....:
20 µg OKM1
20 µg MY904
•20 µg TMG8-5
α CD58:
TS2/9 45 µg
TS2/9 25 µg
α CD30:
HEF1 45 µg
HEF1 25 µg
0
10
20
30
40
50
60
70
80
90
100
Inhibition of Lymphocyte Adhesion to C. albicans [%]

Figure 7.

Those used as ascites were received as amounts too small to be purified, and their use as ascites is denoted with a (*) in Figure 7. At the highest concentration tested of 45 μg protein, the most potent purified anti-CD11b mAb was OKM1 which blocked adhesion by 69% while 45 μg TMG6-5 ascites blocked adhesion of lymphocytes to hyphae by 73%. These were followed closely by 45 μg purified MY904 which blocked binding by 62% and 45 μg LPM19c ascites which blocked binding by 59%. A clear dose dependent inhibition of adhesion was demonstrated using purified mAb MY904 with 20 μg MY904 blocking by 45% and 10 μg MY904 inhibiting lymphocyte adhesion to hyphae by only 5%. The anti-CD11b mAbs LM2/1 and MN-41 demonstrated little or no capacity to inhibit adhesion at the 45 μg concentration (Figure 7).

Two mAbs to the CD18 β_2 integrin chain were tested for their inhibitory capacity (Figure 7). Forty-five μg TS1/18 inhibited adhesion well with a mean inhibition of 49% while 45 μg IB4 blocked less well with a mean of 15% inhibition. TS1/18 (20 μg) was also examined in combinations with anti-CD11b mAbs to examine whether an additive inhibitory effect on lymphocyte adhesion to hyphae could be achieved. In all cases when the antibodies were used in combination, the effect was an increased inhibition of lymphocyte adhesion to *C. albicans* (Figure 7). The combination of 20 μg TS1/18 + 20 μg OKM1 inhibited lymphocyte adhesion by 88%, while 20 μg TS1/18 + 20 μg MY904 inhibited by 69%, and 20 μg TS1/18 + 20 μg LPM19c ascites blocked adhesion to hyphae by 88%. To rule out steric and Fc receptor effects, equivalent amounts of isotype matched (IgG_1) mAbs (matching IgG_1 mAbs: *TMG6-5, MY904, LM2/1, MN-41, and TS1/18), to two other YT lymphocyte surface molecules CD30 (HEF1; Nawrocki *et al.*, 1988), and CD58 (TS2/9; Sanchez-Madrid *et al.*, 1982) were shown to have no effect on YT lymphocyte adhesion to hyphae. YT lymphocytes had detectable surface CD30 and CD58 as judged by immunofluorescence. Specific labeling

by all mAbs of the YT lymphocyte surface was verified before andafter adhesion experiments with FITC goat anti-mouse IgG and immunofluorescence analysis by microscopy. Preincubation of hyphae with equivalent amounts of mAbs followed by washing had no effect on lymphocyte adhesion.

Western Blot Analysis of YT Lymphocyte CD11b

The presence of CD11b/CD18 on the surface of the YT lymphocytes was verified by immunopurification followed by western blot analysis (Figure 8)(Forsyth and Mathews, 1996). The anti-CD11b mAb LM2/1 used to specifically bind to CD11b/CD18 is known to immunoprecipitate the intact CD11b/CD18 heterodimer from YT cell lysates as shown previously (Altieri and Edgington, 1988; Diamond *et al.*, 1993). Under reducing conditions, the anti-CD11b mAb OKM1 (which only binds to the CD11b lectin domain) was then used to visualize a broad band at 165 kD on a blot transferred from a 6% SDS gel which identifies CD11b (Lane 1., left). This broad band for CD11b on SDS gels is characteristic for CD11b, and is thought to result from extensive glycosylation of native CD11b (Altieri and Edgington, 1988; Arnaout, 1990). An identically loaded lane from the same gel was cut from the blot transfer and processed using only the FITC labeled rat anti-mouse secondary IgG as a control indicating no non-specific labeling by the secondary IgG under these conditions (Lane 2., right). Only the membrane developed above the 66 kD molecular weight cut-off to verify the presence of CD11b is included. Faint bands appearing at lower molecular weights characteristic for dissociated IgG immunoglobulin chains were therefore not included. Clearly, these data identify CD11b/CD18 as present in YT whole cell lysates, and serve to confirm the immunofluorescence microscopy data showing CD11b/CD18 present on the surface of YT lymphocytes.

Figure 8. Western blot analysis of YT lymphocyte CD11b. Lane 1 contains a sample from the Mac-1 fraction, and Lane 2 contains an identical sample treated only with the secondary antibody as a control. The broad band at 165 kD is characteristic of CD11b, which is heavily glycosylated. YT lymphocyte whole cell lysates were incubated with anti-CD11b (LM2/1) ascites for 4 h at 4°C, then passed over a recombinant protein A/G column at pH 8.0 and eluted with 0.1 M Na acetate pH 2.0. Column fractions were run on 6% SDS gels and transferred to PVDF membranes and incubated overnight with anti-CD11b (OKM1) mAb and then processed with peroxidase conjugated goat anti-mouse secondary antibody followed by chemiluminescence reagent and autoradiography. Data are presented from a representative experiment from multiple experiments.

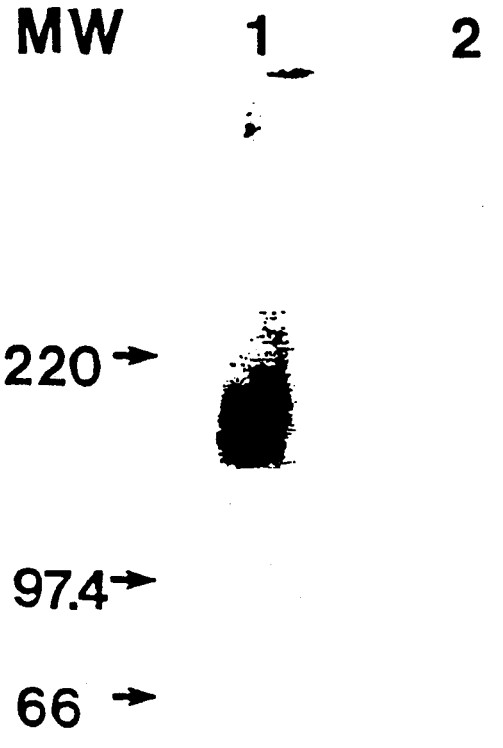

Figure 8.

Extracellular Matrix and RGD-mimetic Peptides Inhibit mIAL Adhesion to C. albicans Hyphae

The data from the studies with the YT lymphocyte cell line demonstrate that the integrin receptor Mac-1 is the principal adhesion molecule mediating adhesion of these human LGL-like lymphocytes to hyphae of *C. albicans*. Experiments were next carried out utilizing the unique adhesion assay developed previously to determine whether this receptor also mediates adhesion of murine IL-2 activated lymphocytes (mIAL) to *C. albicans* hyphae.Experimental methodology was the same as for YT lymphocytes with the exception that the optimal cell number per well was raised from 5 x 10^4 YT per well to 10^5 mIAL and hIAL per well.

To examine adhesion of mIAL to *C. albicans* hyphae, extracellular matrix proteins and RGD-mimetic peptides were tested for their ability to inhibit binding of mIAL to *C. albicans* hyphae (Figure 9). The two ECM proteins examined were murine EHS laminin and human fibrinogen. A clear dose dependent inhibition of mIAL adhesion to hyphae was obtained with both of these ECM proteins. The EHS laminin was a slightly more potent inhibitor of mIAL adhesion with an IC_{50} of 200 nM while a concentration of 500 nM fibrinogen was required for 50% inhibition of murine IAL adhesion. In addition, the complex glycoprotein heparin sulfate, which was recently shown to be a ligand for Mac-1 (Diamond *et al.*, 1995), inhibited adhesion of mIAL to *C. albicans* hyphae with an IC_{50} of 100 μM. The Mac-1 ligand Factor X (Anderson, 1994), of the blood coagulation cascade, was the most potent inhibitor on a molar basis and showed a clear dose dependent inhibition of mIAL adhesion to *C. albicans* hyphae with 50% inhibition at 55 nM and 25% inhibition at a concentration of 27 nM.

Several RGD-mimetic peptides were also examined for their ability to inhibit mIAL adhesion to *C. albicans* hyphae (Figure 9). The GRGDSPK peptide demonstrated a dose

Figure 9. Extracellular matrix proteins and certain RGD-mimetic peptides inhibit adhesion of murine IL-2 activated lymphocytes to *C. albicans*. Adhesion of murine IL-2 activated lymphocytes to *C. albicans* was assessed by the retention of ^{51}Cr labeled lymphocytes in the presence of the indicated proteins and RGD-peptides: GRGDSPK (●); GRGDSPK plus 30 μg αCD11b monoclonal antibody M1/70 (▲); FEP (◆); fibrinogen (■); EHS-laminin (◇); Factor X (▼); -GRGDSPCA$_C$- (▽); H-2k^b MHC peptide: TCVEWLRRYLKN. Conditions and adhesion assessment were as described in Fig. 4 with 10^5 cells added per well. Data are presented as mean % inhibition ± SD of 2 or more experiments.

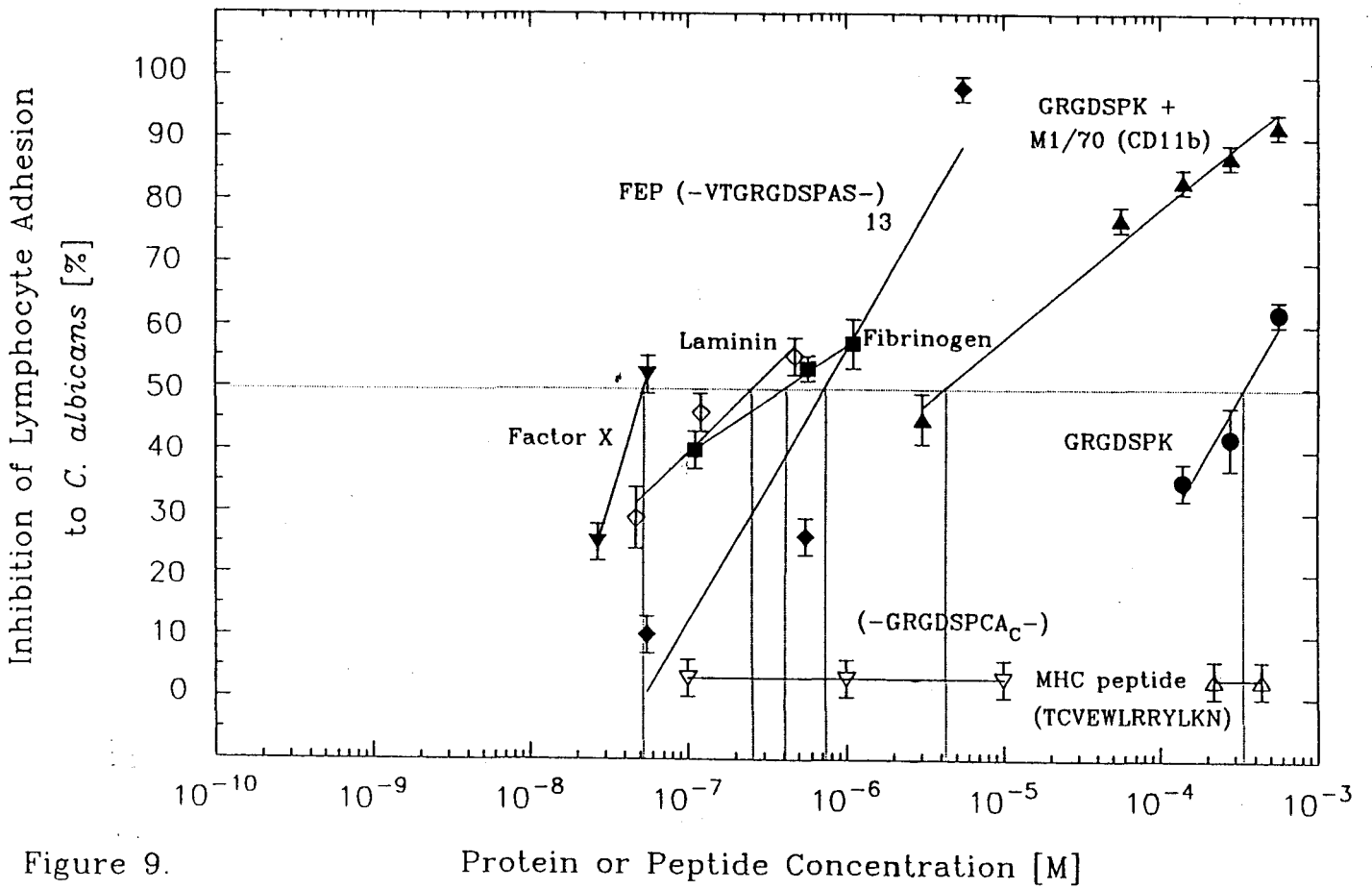
Inhibition of Lymphocyte Adhesion to C. albicans [%]
100
90
80
70
60
50
40
30
20
10
0
GRGDSPK +
M1/70 (CD11b)
FEP (-VTGRGDSPAS-)
13
Laminin
Fibrinogen
Factor X
GRGDSPK
(-GRGDSPCA$_C$-)
MHC peptide
(TCVEWLRRYLKN)
10^{-10}
10^{-9}
10^{-8}
10^{-7}
10^{-6}
10^{-5}
10^{-4}
10^{-3}
Protein or Peptide Concentration [M]

Figure 9.

dependent inhibitory effect on mIAL adhesion to *C. albicans* hyphae with an IC_{50} of 300 μM almost identical to the 500 μM concentration required for 50% inhibition of YT lymphocyte adhesion to hyphae by the GRGDSP peptide (Figure 5). The FEP multimer peptide was also examined for dose response and demonstrated clear dose dependent inhibition of mIAL adhesion to hyphae with a concentration of 5.5 μM completely eliminating adhesion of mIAL to *C. albicans* hyphae and an IC_{50} of 800 nM. Again, these concentrations were examined in light of data showing very similar FEP inhibition of YT lymphocytes with a 500 nM IC_{50} (Figure 5). Two additional RGD-mimetic peptides which were examined significantly inhibited mIAL adhesion by 50%. These were the human fibrinogen 12-mer fragment termed Fibrinogen Binding Inhibitory Peptide (FBIP; sequence: HHLGGAKQAGDV) with an IC_{50} of 300 μM equal to the value for YT lymphocytes and the snake venom disintegrin echistatin with an IC_{50} of 2 μM also equal to that for YT lymphocytes (Table 3).

While PepTite 2000 (sequence:GRGDSPASSK-GGGGSRLLLLLLR) was no longer available commercially, its potent RGD-mimetic inhibition has been attributed in part to the hydrophobic: GGGGRLLLLLLR containing portion of the peptide as well as the GRGDSPKASSK sequence which it also contains (Craig *et al.*, 1995). Therefore, experiments were carried out in this study to examine whether an additive inhibitory effect could be obtained with the GRGDSPK peptide and the M1/70 mAb to an epitope on murine CD11b outside the RGD-binding I domain which may be associated with Mac-1 adhesion to hydrophobic ligands. This combination of mAb M1/70 (30 μg) and the same concentrations of GRGDSPK peptide used alone did result in a dramatic dose dependent increase in inhibition by the GRGDSPK peptide with a concentration achieving 50% inhibition of adhesion of 4 μM virtually identical to that of 1 μM obtained with PepTite 2000 and YT lymphocytes (Figure 5).

Several other RGD-mimetic peptides were poor inhibitors of mIAL adhesion to *C. albicans* with 900 μM RGDS and 700 μM GRGDTP each inhibiting by only 30%. Also, concentrations as high as 700 μM of the GRYDS peptide, which partly mimics the SRYDS Mac-1 adhesion motif in *Leishmania* gp63 protein, had no effect on mIAL adhesion to *C. albicans* hyphae. An "irrelevant" bioactive peptide fragment containing 12 amino acids (MW=1580; sequence: TCVEWLRRYLKN) which inhibits allorecognition and adhesion of C57BL/6 TCR to the murine MHC H-2K^b receptor (Schneck *et al.*, 1989) was found at the concentrations of 400 μM and 200 μM to have no effect on mIAL adhesion to *C. albicans*. The H-2K^b MHC receptor is expressed by the C57BL/6 murine cells utilized in these experiments and the peptide used has been documented to block allorecognition at the concentrations utilized as well, and it's lack of inhibition demonstrates that the inhibition by ECM and RGD-mimetic peptides is specific. These ECM and RGD-mimetic peptide data provided strong evidence that mIAL utilize the same receptor employed by YT lymphocytes for adhesion to *C. albicans* hyphae.

Monoclonal Antibodies to Murine CD11b/CD18 Block Adhesion of mIAL to C. albicans

Unlike the human CD11b/CD18 heterodimer an extensive epitope mapping of murine Mac-1 has not been carried out. OKM1 and M1/70, which also bind to human CD11b, have been mapped using human CD11b/CD18. M1/70 has been mapped outside the RGD-binding I domain to an epitope distinct from the OKM1 lectin binding domain epitope which also maps outside the I domain of CD11b. The other anti-murine CD11b/CD18 mAbs used in this study did not bind to the human lymphocytes or 3T3-19 fibroblasts and are characterized simply as either anti-CD11b or anti-CD18 (Figure 10).

The mAb which inhibited mIAL adhesion to *C. albicans* hyphae most completely

Figure 10. Monoclonal antibodies to CD11b/CD18 inhibit adhesion of murine IL-2 activated lymphocytes to *C. albicans*. Adhesion of murine IL-2 activated lymphocytes to *C. albicans* was assessed by the retention of ^{51}Cr labeled lymphocytes in the presence of the indicated monoclonal antibodies to the noted CD antigens: CD11b: OKM1 (mouse anti-human, IgG_{2b}), M1/70 (rat anti-mouse, IgG_{2b}), 5C6 (hamster anti-mouse, IgG); CD 18: M18/2.A (rat anti-mouse, IgG_{2a}, kappa), 2E6 (hamster anti-mouse, IgG); CD11a: M17/4.4 (rat anti-mouse, IgG_{2b}, kappa); CD11c: N418 (hamster anti-mouse, IgG). Conditions and adhesion assessment were as described in Fig. 4 with 10^5 cells added per well. Data are presented as mean % inhibition $\pm$ SD of 2 or more experiments.

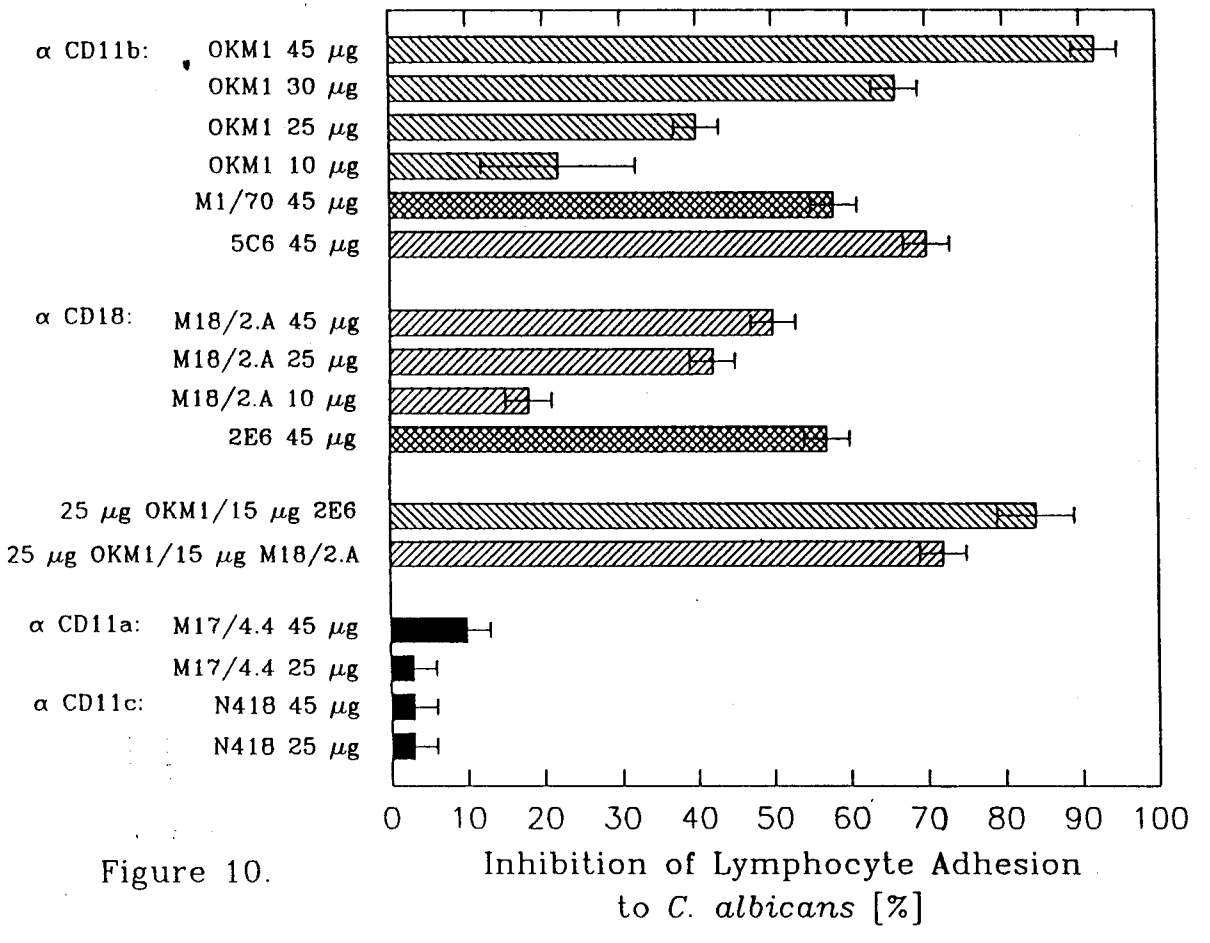
α CD11b:
OKM1 45 µg
OKM1 30 µg
OKM1 25 µg
OKM1 10 µg
M1/70 45 µg
5C6 45 µg
α CD18:
M18/2.A 45 µg
M18/2.A 25 µg
M18/2.A 10 µg
2E6 45 µg
25 µg OKM1/15 µg 2E6
25 µg OKM1/15 µg M18/2.A
α CD11a:
M17/4.4 45 µg
M17/4.4 25 µg
α CD11c:
N418 45 µg
N418 25 µg
0 10 20 30 40 50 60 70 80 90 100
Inhibition of Lymphocyte Adhesion
to C. albicans [%]

Figure 10.

when used alone was OKM1 (mouse anti-human, which also binds to murine, CD11b, IgG_{2b}). OKM1 virtually eliminated mIAL adhesion to hyphae with inhibition by greater than 90% at 45 μg (1.2 μM). OKM1 demonstrated a clear dose dependent inhibition of mIAL adhesion to hyphae with 50% inhibition of adhesion at a concentration of 30 μg (800 nM) and 30% inhibition at 15 μg (400 nM). The next most potent anti-murine CD11b mAb was the 5C6 mAb (hamster anti-mouse CD11b, IgG) which inhibited adhesion of mIAL by 70% at the 45 μg concentration and by 60% when only 25 μg (670 nM) were present. Finally, the mAb M1/70 (rat anti-mouse CD11b, IgG_{2b}), which also binds to human CD11b, also demonstrated clear dose dependent inhibition of adhesion of mIAL to *C. albicans* hyphae and inhibited by 60% at the 45 μg concentration, by 50% at 30 μg and by 25% when 15 μg were present. Two anti-murine CD18 mAbs were tested and each showed a dose dependent inhibition of mIAL adhesion to *C. albicans* hyphae. The anti-CD18 mAb 2E6 (hamster anti-mouse CD18, IgG) blocked adhesion by 60% at 45 μg while inhibiting adhesion of mIAL by 40% at 25 μg and 20% at 10 μg. The anti-CD18 mAb M18/2.A (rat anti-mouse CD18, IgG_{2a}, kappa) inhibited mIAL adhesion to hyphae by 50% at 45 μg and blocked adhesion by 40% at 25 μg and 20% at 10 μg. Combinations of mAb were tested to determine whether additive effects in inhibition of adhesion could be demonstrated. Interestingly, 25 μg OKM1 + 15 μg M1/70 yielded only 53% inhibition and 25 μg OKM1 + 15 μg 5C6 also only inhibited mIAL adhesion to hyphae by 57%. However, combinations of anti-CD11b and anti-CD18 mAbs proved very effective at inhibiting mIAL adhesion with 25 μg OKM1 + 15 μg 2E6 inhibiting adhesion by 80% and 25 μg OKM1 + 15 μg M18/2.A inhibiting mIAL adhesion to hyphae by 70%. The anti-murine "irrelevant" mAbs used for the mIAL mAb adhesion inhibition experiments were M17/4.4 (rat anti-mouse CD11a, IgG_{2b}, kappa) and N418 (hamster anti-mouse CD11c, IgG). At concentrations of 45 μg, 35 μg, 25 μg, and 15 μg neither of these

Table 5.--Carbohydrates as Competitive Blockers of the Adhesion of YT Lymphocytes to *C. albicans*

Carbohydrate	Concentration (mg/ml or [M])	% Inhibition of Binding ± SD
D-Mannose	150 mM	55 ± 5
D-Galactose	150 mM	20 ± 12
α-Methyl D-Mannopyranoside	150 mM	59 ± 2
LPS	4.0 mg/ml	3 ± 7
β-Glucan (yeast)	4.0 mg/ml (40 μM)	62 ± 4
PepTite-2000 (peptide)[a]	1.0 mM	98 ± 4
Sucrose	150 mM	0.0 ± 5
Zymosan	4 mg/ml	85 ± 3
N-Acetyl-D-Glucosamine	150 mM	66 ± 8

Note(s): The effects of several carbohydrates on adherence were assessed by the competitive binding assay detailed in Figure 2. Data shown are mean % inhibition ± SD of triplicate values of 2 or more experiments and represent the maximum concentration tested for multiple carbohydrate concentrations.
[a] PepTite-2000 is a peptide (not a carbohydrate) shown for comparison.

two antibodies demonstrated any inhibitory activity towards mIAL adhesion to hyphae other than the 10% inhibition observed with 45 μg M17/4.4 (Figure 10). Lack of inhibition by these antibodies demonstrates an absence of non-specific and Fc mediated inhibition. These mAb inhibition data demonstrate that, like YT lymphocytes, mIAL use CD11b/CD18 for adhesion to *C. albicans* hyphae.

Carbohydrates Inhibit YT Adhesion to Hyphae

Carbohydrate-lectin interactions have been shown to be important in adherence of CD11b/CD18 to *S. cerevisiae* and other microbial ligands (Arnaout, 1990; Ross *et al.*, 1985; Thornton *et al.*, 1996). Lymphocyte adhesion to hyphae was blocked with the saccharides: 0.15 M N-acetyl-D-glucosamine (NADG), α-methyl-D-mannopyranoside, D-mannose and β-glucan in the concentrations indicated (Table 5). The form of LPS used has been shown not to block CD11b/CD18 which was confirmed in our experiments. Galactose had slight and sucrose had no inhibitory effect on lymphocyte adhesion to hyphae as shown previously for CD11b (Ross *et al.*, 1985). The most inhibitory carbohydrate was zymosan (2 mg/ml) which inhibited binding by 85% and virtually eliminated YT lymphocyte adhesion to *C. albicans* hyphae. The next most inhibitory carbohydrate was NADG with 66 ± 8 % inhibition. This profile of carbohydrate inhibition at these concentrations is characteristic of adhesion mediated by the CD11b/CD18 lectin-like domain (Ross *et al.*, 1985; Sehgal *et al.*, 1993; Thornton *et al.*, 1996).

Carbohydrates Inhibit mIAL and hIAL Adhesion to C. albicans Hyphae

As with the YT lymphocyte cell line, selected carbohydrates that have been documented to block adhesion of neutrophil CD11b/CD18 to the yeast *S. cerevisiae* were examined for their ability to inhibit mIAL adhesion to *C. albicans* hyphae at concentrations of 150 mM (Ross *et al*, 1985; Thornton *et al.*, 1996) (Table 6). A clear dose dependent inhibition was obtained using N-acetyl-D-glucosamine (NADG) which was also the most potent inhibitor of mIAL adhesion with 85% inhibition at the 150 mM concentration. In addition, NADG inhibited mIAL adhesion to hyphae by 50% at 75 mM and by 25% at 7.5 mM and not at all at 2.5 mM. Although 150 mM D-mannose inhibited mIAL adhesion by

Table 6.--Carbohydrates as Competitive Blockers of the Adherence of Murine and Human IL-2 Activated Lymphocytes to *C. albicans*[*]

Carbohydrate	Concentration: mg/ml (Molar)	% Inhibition of Binding ± SD
Mouse IAL:		
N-Acetyl-D-Glucosamine	7.5 mM	25 ± 3
N-Acetyl-D-Glucosamine	75 mM	50 ± 2
N-Acetyl-D-Glucosamine	150 mM	85 ± 3
D-Mannose	150 mM	40 ± 5
N-Acetyl-D-Glucosamine + D-Mannose	75 mM 75 mM	74 ± 2
α-Methyl mannoside	150 mM	45 ± 2
α-Methyl mannoside	75 mM	19 ± 4
D-Glucose	150 mM	5 ± 3
Sucrose	150 mM	0 ± 5
Human IAL:		
D- Glucose	150 mM	3 ± 5
β-Glucan (yeast)	4 mg/ml (40 μM)	70 ± 5
N-Acetyl-D-Glucosamine	150 mM	66 ± 2

Note(s): The effects of several carbohydrates on adherence were assessed utilizing the competitive binding assay detailed in Figure 2. Data shown are mean % inhibition ± SD and represent the maximum concentration tested for multiple carbohydrate concentrations.

only 40%, the combination of 75 mM NADG + 75 mM D-mannose inhibited mIAL adhesion to hyphae by 75%. This combination of inhibitors was tested because it was previously noted to have a "synergistic" inhibitory effect on Mac-1 mediated adhesion to yeast (Ross *et al*, 1985). The carbohydrate α-methyl mannoside also demonstrated a dose dependent inhibitory effect with 150 mM inhibiting by 45% and 75 mM inhibiting mIAL adhesion to hyphae by 19%. Yeast β-glucan when tested at 4 mg/ml strongly inhibited mIAL adhesion by 75%. The "irrelevant" carbohydrates (Ross *et al*., 1985) sucrose and D-glucose showed no inhibition of mIAL adhesion to hyphae at either 150 mM or 75 mM concentrations.

Carbohydrates which characteristically inhibit neutrophil Mac-1 mediated adhesion to yeast (Ross *et al.*, 1985) also inhibited hIAL adhesion to hyphae at concentrations which inhibited mIAL adhesion to *C. albicans* (Table 6). The carbohydrate NADG at 150 mM inhibited hIAL adhesion to *C. albicans* hyphae by 66% and β-glucan from *S. cerevisiae* yeast at 4 mg/ml inhibited adhesion of hIAL to hyphae by 70%. D-glucose again had no effect on adhesion. Taken as a whole, the mIAL and hIAL carbohydrate inhibition data is consistent with the YT lymphocyte data and with inhibition of Mac-1 mediated adhesion by these carbohydrates in other published studies (Ross *et al.*, 1985; Sehgal *et al.*, 1993; Thornton *et al.*, 1996). This characteristic inhibition by selected carbohydrates serves to further confirm that CD11b/CD18 is the principal adhesion molecule on YT, mIAL, and hIAL mediating adhesion to *C. albicans* hyphae.

Human IAL Adhesion to C. albicans Hyphae is Inhibited by ECM/RGD-mimetic Peptides and Monoclonal Antibodies to CD11b/CD18

Human IAL (hIAL) were not the direct subject of this dissertation, but data from experiments with blood from human donors could have more direct clinical relevance to

Figure 11. Monoclonal antibodies to CD11b/CD18 and RGD-mimetic proteins inhibit adhesion of human IL-2 activated lymphocytes to *C. albicans*. Adhesion of human IL-2 activated lymphocytes to *C. albicans* was assessed by the retention of ^{51}Cr labeled lymphocytes in the presence of the indicated monoclonal antibodies to the noted CD antigens and RGD-mimetic proteins: CD11b: OKM1 (mouse anti-human, IgG_{2b}), M1/70 (rat anti-mouse, IgG_{2b}); CD18: TS1/18 (mouse anti-human, IgG_1); CD58: TS2/9 (mouse anti-human LFA-3, IgG_1); CD11a: TS1/22 (mouse anti-human LFA-1, IgG_1); Proteins: FBIP (fibrinogen binding inhibitory peptide: HHLGGAKQAGDV); Echistatin, a disintegrin RGD-specific integrin inhibitor; heparin, an RGD-containing extracellular matrix protein; $GRGDSP_C$ is a cyclical molecule comprised of the sequence GPenGRGDSPCA where Pen=penicillimine. Conditions and adhesion assessment were as described in Fig. 4 with 10^5 cells added per well. Data are presented as mean % inhibition $\pm$ SD of 2 or more experiments.

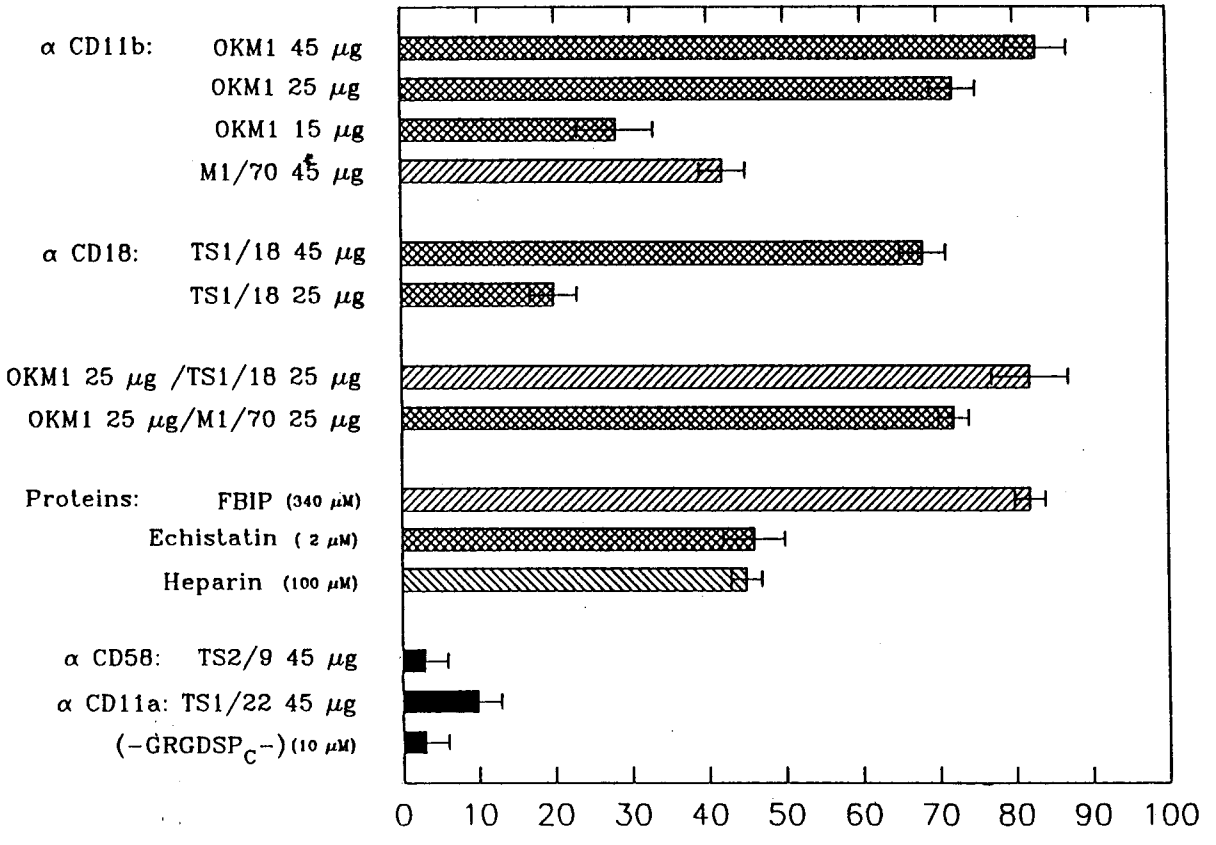
α CD11b:
OKM1 45 μg
OKM1 25 μg
OKM1 15 μg
M1/70 45 μg
α CD18:
TS1/18 45 μg
TS1/18 25 μg
OKM1 25 μg /TS1/18 25 μg
OKM1 25 μg/M1/70 25 μg
Proteins:
FBIP (340 μM)
Echistatin (2 μM)
Heparin (100 μM)
α CD58:
TS2/9 45 μg
α CD11a:
TS1/22 45 μg
(-GRGDSP$_C$-) (10 μM)
0 10 20 30 40 50 60 70 80 90 100
Inhibition of Lymphocyte Adhesion
to *C. albicans* [%]

Figure 11.

treatment of human fungal infections and would substantiate data from the mIAL studies. Therefore, experiments were conducted using a subset of the reagents and anti-human CD11b/CD18 mAbs detailed previously to examine whether human IAL from peripheral blood of twelve healthy donors also utilize Mac-1 for adhesion to *C. albicans* hyphae (Figure 11). ECM protein and RGD-mimetic peptide inhibition analysis was not as extensive as with mIAL, however the fibrinogen fragment FBIP inhibited hIAL adhesion to hyphae with the same IC_{50} of 340 μM. The disintegrin echistatin also blocked adhesion of hIAL to hyphae by 50% at a concentration of 2 μM, an effect identical to it's inhibition of mIAL adhesion to hyphae. The Mac-1 ligand and complex glycoprotein heparin sulfate was found to inhibit hIAL by 45% at the concentration of 100 μM which also inhibited mIAL adhesion to hyphae by 50% (Table 3). The $GRGDSP_C$ cyclic peptide had no effect on hIAL adhesion to hyphae, the same as seen for YT lymphocytes and mIAL.

Experiments using a restricted number of the anti-human CD11b/CD18 mAbs previously described for the YT lymphocyte experiments were examined for their ability to inhibit hIAL adhesion to *C. albicans* hyphae (Figure 11). The OKM1 anti-CD11b mAb again demonstrated a dose dependent inhibition of adhesion with 45 μg virtually eliminating hIAL adhesion to hyphae with inhibition of 83% and concentration dependent inhibition of 72% at 25 μg, 28% at 15 μg, and 9% at 10 μg. The M1/70 mAb was tested only at the 45 μg concentration which inhibited hIAL adhesion to hyphae by 42%. The combination of 25 μg OKM1 + 25 μg M1/70 inhibited adhesion of hIAL by 75%. The anti-human CD18 mAb TS1/18 exhibited a dose dependent inhibition of hIAL adhesion with 45 μg inhibiting hIAL adhesion by 68% while 25 μg inhibited adhesion of these lymphocytes to hyphae by 22%. The combination of 25 μg OKM1 + 25 μg TS1/18 inhibited hIAL adhesion to *C. albicans* hyphae by 82%. "Irrelevant" mAbs utilized in the hIAL experiments were the anti-human

CD58 (LFA-3) mAb TS2/9 (Sanchez-Madrid *et al.*, 1982) as well as the anti-human CD11a mAb TS1/22 (Diamond *et al.*, 1993) which are both blocking antibodies for interactions by these receptors. The TS1/22 mAb inhibited hIAL adhesion by 10% at 45 μg and had no effect on hIAL adhesion at 25 μg. The TS2/9 mAb had no effect on hIAL adhesion to *C. albicans* hyphae at either 45 μg or 25 μg. Overall, these hIAL data are in agreement with the YT lymphocyte and mIAL inhibition data and identify CD11b/CD18 as mediating lymphocyte adhesion to *C. albicans* hyphae.

Aim 3.) Confirm and/or Prove the Identified Lymphocyte Adherence Molecules Mediate Binding of IAL to *C. albicans* Hyphae.

Murine 3T3 Fibroblasts Expressing Transfected Human CD11b/CD18 Specifically Bind to C. albicans Hyphae

Experiments detailed above with YT lymphocytes and murine and human IAL indicate that Mac-1 (CD11b/CD18) is the principal adhesion molecule mediating binding of these activated lymphocytes to hyphae of *C. albicans* during growth inhibition of the fungus by these lymphocytes. To confirm the ability of Mac-1 to mediate this adhesion, mouse NIH 3T3 fibroblasts expressing transfected human CD11b/CD18 (designated 3T3-19) were examined using the adhesion assay and by competitive inhibition with a restricted number of the ECM proteins, RGD-peptides, and anti-CD11b/CD18 mAbs previously found to inhibit YT lymphocyte and hIAL adhesion to *C. albicans* hyphae (Figure 12). To demonstrate that adhesion of 3T3-19 transfectants to hyphae is Mac-1 specific, NIH 3T3 fibroblasts subjected to the transfection protocol but not expressing Mac-1 (designated 3T3-1) were also examined using the adhesion assay. These 3T3-1 fibroblasts demonstrated no specific adhesion

Figure 12. Monoclonal antibodies to CD11b/CD18 inhibit adhesion of 3T3-19 (Mac-1^+) transfectants to *C. albicans*. Adhesion to *C. albicans* of NIH-3T3 fibroblasts expressing transfected human CD11b/CD18 (3T3-19) and not expressing CD11b/CD18 (3T3-1) was assessed by the retention of ^{51}Cr labeled transfectants in the presence of the indicated monoclonal antibodies to the noted CD antigens: CD11b: OKM1 (mouse anti-human, IgG_{2b}); CD18: TS1/18 (mouse anti-human, IgG_1); murine (3T3 cell) CD29: clone #551125 (rat anti-mouse β_1 integrin, IgG_1). Conditions and adhesion assessment were as described in Fig. 4 with 5 x 10^4 cells added per well. Data are presented as mean % inhibition $\pm$ SD of 2 or more experiments.

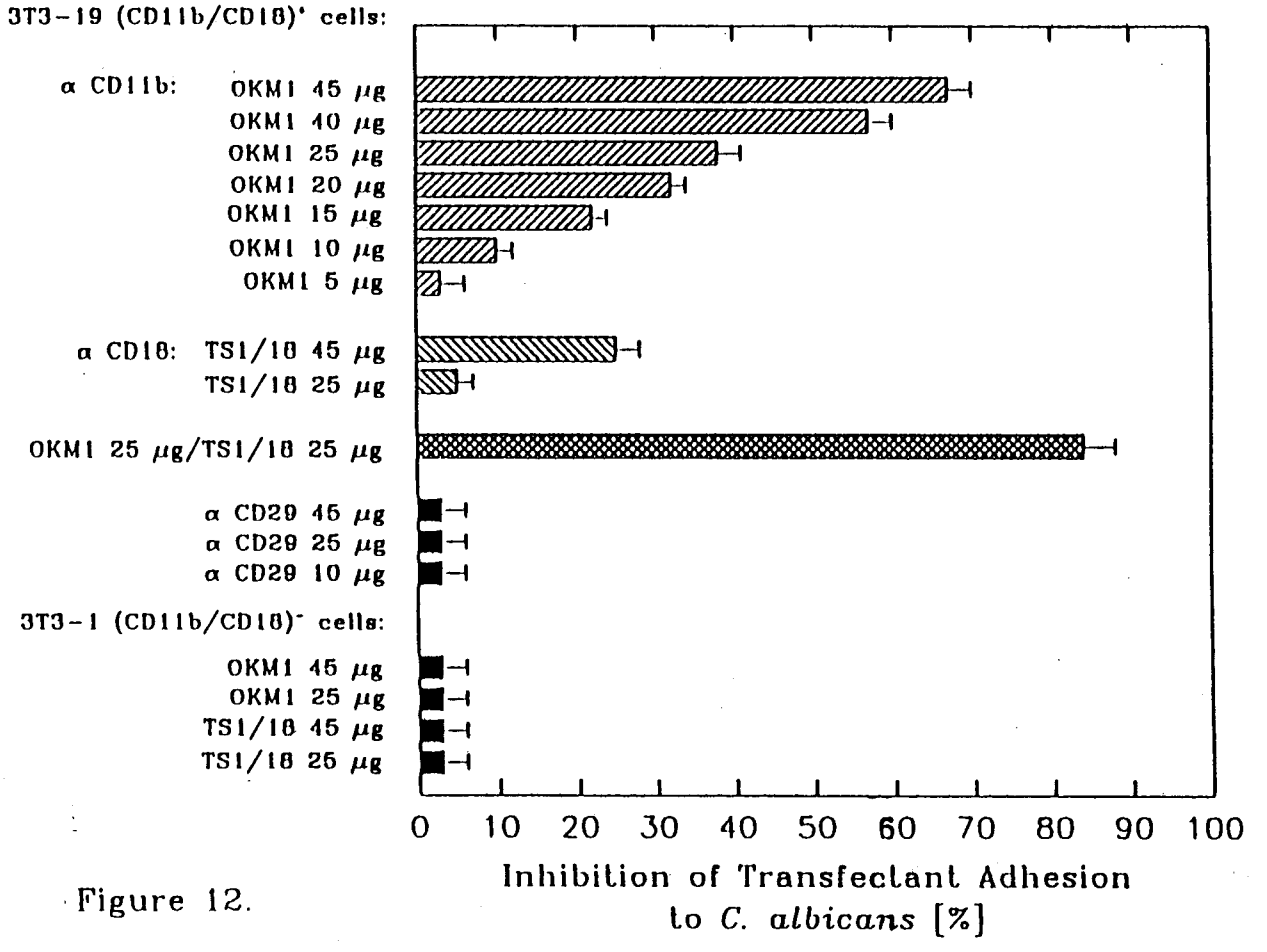
3T3-19 (CD11b/CD18)+ cells:
α CD11b:
OKM1 45 μg
OKM1 40 μg
OKM1 25 μg
OKM1 20 μg
OKM1 15 μg
OKM1 10 μg
OKM1 5 μg
α CD18:
TS1/18 45 μg
TS1/18 25 μg
OKM1 25 μg/TS1/18 25 μg
α CD29 45 μg
α CD29 25 μg
α CD29 10 μg
3T3-1 (CD11b/CD18)- cells:
OKM1 45 μg
OKM1 25 μg
TS1/18 45 μg
TS1/18 25 μg
0 10 20 30 40 50 60 70 80 90 100
Inhibition of Transfectant Adhesion
to C. albicans [%]

Figure 12.

to *C. albicans* hyphae above background levels of 3% ± 3%, which was not different with addition of the anti-CD11b mAb OKM1 or the anti-CD18 mAb TS1/18 (Figure 12, solid bars). These data demonstrate that only transfectants expressing CD11b/CD18 bind to *C. albicans* hyphae.

The CD11b/CD18 specific adhesion of 3T3-19 transfectants was further demonstrated by a concentration dependent inhibition of 3T3-19 adhesion to *C. albicans* hyphae by anti-CD11b/CD18 mAbs. The anti-CD11b mAb OKM1 was tested over a broad range of concentrations and was found to inhibit adhesion of 3T3-19 fibroblasts to *C. albicans* in a clearly concentration dependent manner (Figure 12). The OKM1 mAb inhibited by 67% at 45 μg, 57% at 40 μg, 38% at 25 μg, 32% at 20 μg, 22% at 15 μg, 10% at 10 μg, and had no effect on 3T3-19 fibroblast adhesion to hyphae at a concentration of 5 μg. The anti-CD18 mAb TS1/18 also demonstrated concentration dependent inhibition of 3T3-19 adhesion to *C. albicans* with 45 μg TS1/18 inhibiting adhesion by 28% while 25 μg TS1/18 inhibited 3T3-19 adhesion to hyphae by only 9%. That 3T3-19 adhesion to *C. albicans* hyphae is CD11b/CD18 specific was also demonstrated by the dramatic 85% inhibition of adhesion by the combination of 25 μg OKM1 with 25 μg TS1/18. A further verification of specificity is demonstrated by the anti-murine CD29 mAb, which reacts with the murine β_1 integrin chain on the surface of these 3T3-19 fibroblasts, and which had no effect on 3T3-19 adhesion to *C. albicans* (Figure 12, solid bars). These data confirm that CD11b/CD18 can mediate specific adhesion to *C. albicans* hyphae.

Monoclonal Antibodies to CD11b/CD18 Synergize with RGD-Mimetic Peptides to Inhibit 3T3 Transfectant Adhesion to C. albicans.

Because of the controversy surrounding inhibition of Mac-1 by RGD-mimetic

peptides, experiments were undertaken using 3T3-19 transfectants to determine whether the inhibition of lymphocyte adhesion to *C. albicans* hyphae by RGD-mimetics was in fact specific for Mac-1 mediated adhesion (Figure 13). The Mac-1 ligand clotting Factor X, containing the RGD-mimetic sequence: GYD..QED which blocks Mac-1 adhesion (Rozdzinski *et al.*, 1995), inhibited 3T3-19 adhesion to hyphae by 48% at 55 nM comparable to Factor X inhibition of lymphocyte adhesion to hyphae (Table 3). However, the FBIP peptide at a concentration of 340 μM and the GRGDSPK peptide at 560 μM had no effect on 3T3-19 adhesion to *C. albicans* hyphae. Because these two peptides had clearly inhibited adhesion of IAL to hyphae by 50% at these concentrations, these peptides were tested with low concentrations of mAb to CD11b in an attempt to "activate" the transfected CD11b/CD18 molecules expressed by the 3T3-19 fibroblasts to a state where RGD-mimetic peptides other than Factor X might inhibit adhesion. The anti-CD11b mAb M1/70 inhibited adhesion of 3T3-19 fibroblasts to hyphae by 25% at 30 μg but when combined at this concentration with the FBIP peptide demonstrated a synergistic effect by inhibiting 3T3-19 adhesion by 70% with 340 μM FBIP and inhibited by 53% with 170 μM FBIP. The anti-CD11b mAb OKM1 inhibited 3T3-19 adhesion to hyphae by 22% at 15 μg when used alone but when combined with 140 μM GRGDSPK peptide inhibited 3T3-19 fibroblast adhesion to hyphae by 40%. A similar synergistic effect was observed between the OKM1 mAb and the anti-human CD18 mAb TS1/18 (Figure 13). At a concentration of 45 μg TS1/18 inhibited 3T3-19 adhesion by 25% and had no effect on 3T3-19 adhesion to hyphae at 25 μg. However, the combination of 15 μg OKM1 + 25 μg TS1/18 achieved a synergistic type inhibition of 84% of 3T3-19 fibroblast adhesion to *C. albicans* hyphae. An additive effect was seen for 15 μg OKM1 + 15 μg M1/70 which together blocked 3T3-19 transfectant adhesion by 58%. These data confirm that the inhibition of lymphocyte adhesion by RGD-mimetic peptides is

Figure 13. RGD-mimetic peptides in combination with monoclonal antibodies to CD11b/CD18 but not alone inhibit adhesion of 3T3-19 (Mac-1^+) transfectants to *C. albicans*. Adhesion to *C. albicans* of NIH-3T3 fibroblast transfectants expressing human CD11b/CD18 (3T3-19) was assessed by the retention of ^{51}Cr labeled transfectants in the presence of the indicated monoclonal antibodies to the noted CD antigens and RGD-mimetic peptides: CD11b: OKM1 (mouse anti-human, IgG_{2b}), M1/70 (rat anti-mouse, IgG_{2b}); CD18: TS1/18 (mouse anti-human, IgG_1); Peptides: GRGDSPK; FBIP (fibrinogen binding inhibitory peptide: HHLGGAKQAGDV). Conditions and adhesion assessment were as described in Fig. 4 with 5×10^4 cells added per well. Data are presented as mean % inhibition $\pm$ SD of 2 or more experiments.

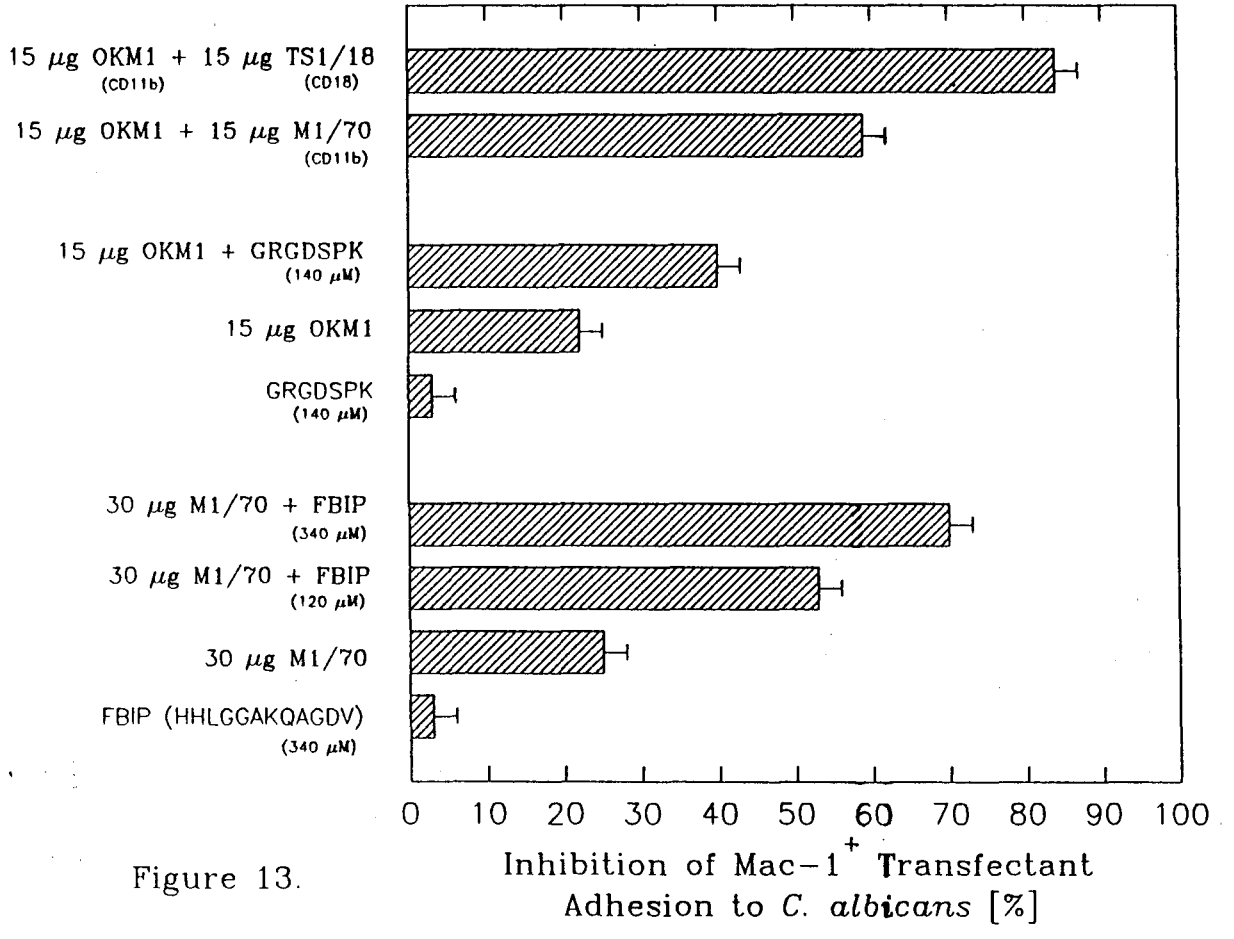
15 μg OKM1 + 15 μg TS1/18
(CD11b) (CD18)
15 μg OKM1 + 15 μg M1/70
(CD11b)
15 μg OKM1 + GRGDSPK
(140 μM)
15 μg OKM1
GRGDSPK
(140 μM)
30 μg M1/70 + FBIP
(340 μM)
30 μg M1/70 + FBIP
(120 μM)
30 μg M1/70
FBIP (HHLGGAKQAGDV)
(340 μM)
0 10 20 30 40 50 60 70 80 90 100
Inhibition of Mac-1+ Transfectant
Adhesion to C. albicans [%]

Figure 13.

specifically an inhibition of Mac-1 mediated adhesion to *C. albicans* hyphae.

Monoclonal Antibodies to CD11b/CD18 Block mIAL Growth Inhibition of C. albicans

Finally, experiments were carried out to investigate whether inhibition of CD11b/CD18 adhesion of mIAL to *C. albicans* hyphae was functionally relevant to the growth inhibition of the fungus by these IL-2 activated lymphocytes (Figure 14). The growth inhibition assay utilizes uptake of ^{3}H-uridine to compare growth of treated and untreated hyphae (Beno and Mathews, 1993). The mIAL utilized for these experiments were either preincubated with no mAb or the indicated mAb for 1 h, then added to hyphae for 3 h, and finally removed and ^{3}H-uridine added for 2 h. Baseline values were obtained using wells with no mIAL and wells with mIAL but no mAb (Figure 14, bars 1 and 2). Some *C. albicans* hyphae also were treated with the noted anti-CD11b mAbs, M1/70 or OKM1, without mIAL present (Figure 14, bars 3 and 6, respectively). This had no significant effect on fungal growth (Student's t-test, $p > .05$), ruling out a direct effect of the mAbs on hyphal growth. Similarly, mIAL treated with the "irrelevant" mAbs to murine CD11a (M17/4.4; Figure 14, bars 9 and 10) and CD11c (N418; Figure 14, bars 11 and 12) were able to inhibit hyphal growth with no significant difference ($P > .05$) from untreated mIAL (Figure 14, bar 2). For both anti-CD11b mAbs, a dose dependent blocking of mIAL growth inhibition of *C. albicans* was obtained. OKM1 and M1/70, each tested at 45 μg separately, completely eliminated mIAL growth inhibition of *C. albicans* hyphae (Figure 14, M1/70 bar 4, OKM1 bar 7). There was no significant difference ($p > .05$) between these values and the baseline values without added mIAL either with or without mAbs (Figure 14, bars 1, 3, and 6). Thereby demonstrating a significant ($*^{a}$) ($p < .05$; bar 4 versus bar 2) blocking effect of the anti-CD11b mAbs on mIAL growth inhibition of *C. albicans*.

Figure 14. Monoclonal antibodies to CD11b/CD18 block growth inhibition of *C. albicans* hyphae by murine IL-2 activated lymphocytes. *C. albicans* growth inhibition was assessed by the incorporation of ^{3}H-uridine after treatment with murine IL-2 activated lymphocytes. Where indicated, monoclonal antibodies were added either to hyphae alone or were preincubated with MIAL (murine IL-2 activated lymphocytes) as detailed in Fig. 4. Monoclonal antibodies used to the noted CD antigens were: CD11b: OKM1 (mouse anti-human, IgG_{2b}), M1/70 (rat anti-mouse, IgG_{2b}); CD11a: M17/4.4 (rat anti-mouse, IgG_{2b}, kappa); CD11c: N418 (hamster anti-mouse, IgG). An asterisk (*) denotes statistical significance ($p < .05$) between the designated data as determined using the Student's independent t-test (SigmaPlot 4.1 © 1991 Jandel Incorporated). ($*^{a}$ denotes statistically significant blocking of mIAL mediated growth inhibition by 45 μg mAb M1/70: bar 4, or OKM1: bar 7); ($*^{b}$ denotes statistically significant growth inhibition by mIAL treated with 25 μg M1/70, bar 5 versus bars 2 or 3, or 25 μg OKM1, bar 8 versus bars 2 or 6). Note that the baseline mean of 58% growth inhibition (bar 2) was compared to the four indicated means (bars 9, 10, 11, and 12) to test significance and they were not found different ($p > .05$). Data are presented as the mean % inhibition $\pm$ SD of 2 or more experiments.

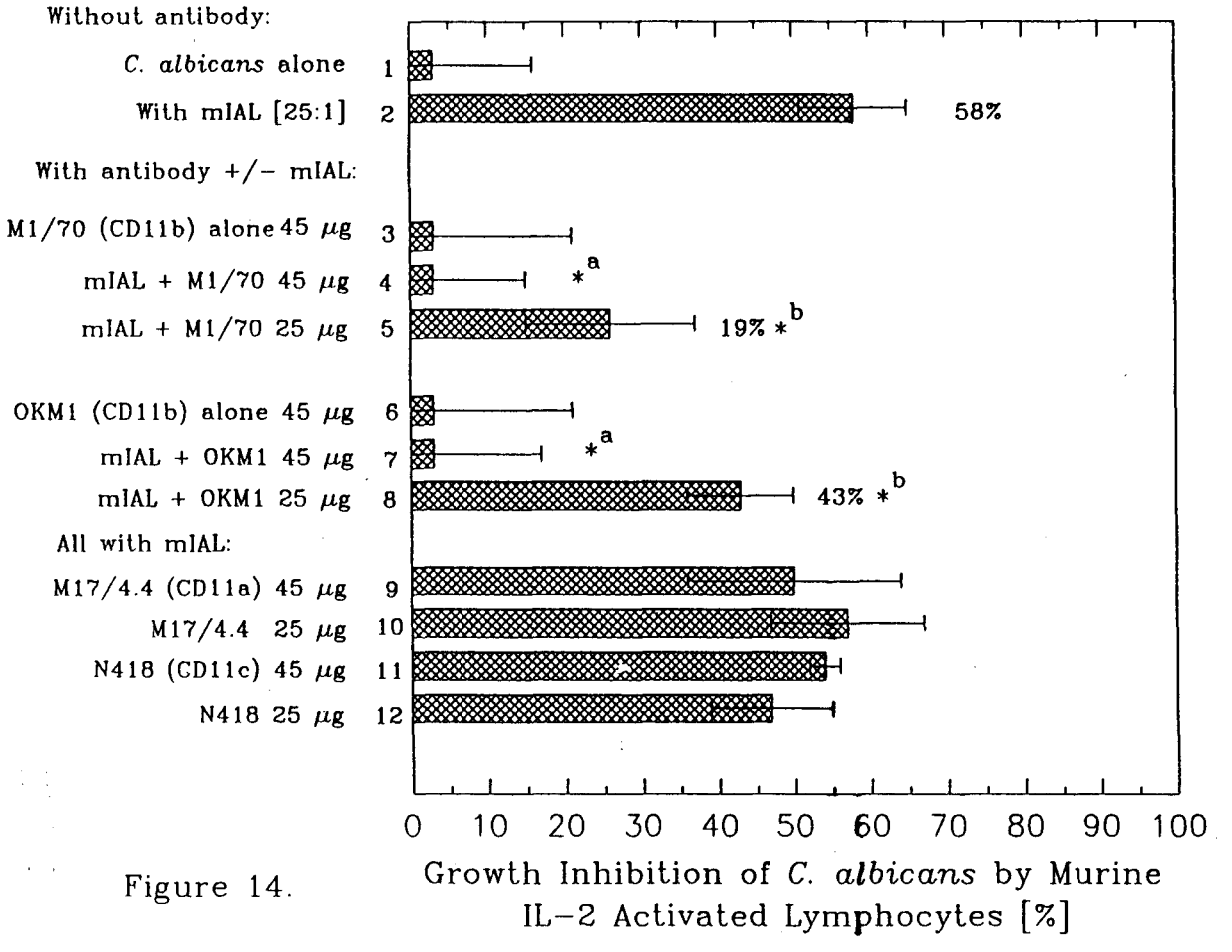
Without antibody:
C. albicans alone 1
With mIAL [25:1] 2
58%
With antibody +/- mIAL:
M1/70 (CD11b) alone 45 µg 3
mIAL + M1/70 45 µg 4
*a
mIAL + M1/70 25 µg 5
19% *b
OKM1 (CD11b) alone 45 µg 6
mIAL + OKM1 45 µg 7
*a
mIAL + OKM1 25 µg 8
43% *b
All with mIAL:
M17/4.4 (CD11a) 45 µg 9
M17/4.4 25 µg 10
N418 (CD11c) 45 µg 11
N418 25 µg 12
0 10 20 30 40 50 60 70 80 90 100
Growth Inhibition of C. albicans by Murine IL-2 Activated Lymphocytes [%]

Figure 14.

However, a significant (*[b]) ($p < .05$) growth inhibition by mIAL was evident at the 25 μg concentration of both OKM1 (43%; bar 8 versus bar 6) and M1/70 (19%; bar 5 versus bar 3), although the percentage of growth inhibition as noted for each is also significantly less ($p < .05$) than the 58% seen with mIAL alone (Figure 14, bar 2) thus demonstrating a dose dependent blocking by mAbs of mIAL growth inhibition of hyphae. These data, taken together with the specific adhesion to *C. albicans* demonstrated with Mac-1^+ 3T3-19 fibroblasts, confirm that the integrin CD11b/CD18 is the principal adhesion molecule utilized by mIAL during growth inhibition of *C. albicans* hyphae.

CHAPTER 4.

DISCUSSION

In this dissertation, the human YT lymphocyte cell line was utilized for the initial characterization of cytotoxic lymphocyte adhesion to *C. albicans* (Figure 15). The epitopes for the anti-human CD11b mAbs used in this study have been 'mapped' by assessing the binding of each mAb to fragments of CD11b expressed by transfected cells. Each mAb maps to either the I-domain or the lectin-like domain of CD11b or the CD18 β_2 chain of Mac-1 (Diamond *et al.*, 1993; Zhou *et al.*, 1994; Violette *et al.*, 1995). Epitope specific inhibition is not purely steric, since nonblocking versus blocking mAbs to the same integrin initiate different intracellular phosphorylation signalling patterns (Miyamoto *et* al., 1995), and possibly bind to distinct subdomains within the I domain of CD11b (Violette *et al.*, 1995; Champe *et al.*, 1995; Zhang and Plow, 1996). The mAbs TMG6-5, MY904, and LPM19c block lymphocyte adhesion to *C. albicans* by as much as 73%, 62%, and 59% respectively. They each recognize epitopes in the I domain of Mac-1 (Diamond *et al.*, 1993). However, two other mAbs to I domain epitopes, LM2/1 and MN-41, have little effect on YT lymphocyte adhesion to hyphae. The reason for these differences is not known (Diamond *et al.*, 1993; Violette *et al.*, 1995). However, it may be that these mAbs bind to the different functional subdomains within the I domain which were recently demonstrated for human and murine β_2 integrin CD11a/CD18 (Champe *et al.*, 1995). Most recently, evidence for this subdomain structure for the CD11b I domain has been demonstrated using peptide ligands of

Figure 15. Scanning electron micrograph of YT lymphocytes. Two YT lymphocytes are shown adhering to a *C. albicans* hyphal segment after 30 min of interaction (x 12,500).

Figure 15.

Mac-1 and a library of fifteen '293' type cell transfectants expressing single point mutations in the CD11b/CD18 I domain and by showing binding sites for these ligands are overlapping, but not identical (Zhang and Plow, 1996). Based on work by these and other authors and data from this study a tentative model of the CD11b/CD18 heterodimer was formulated (Figure 16). In support of this model, LPM19c and MY904 both require the entire I domain intact to bind, while LM2/1 will bind to an octamer peptide from the C-terminal end of the I domain (Violette *et al.*, 1995). The epitopes for TMG6-5 and MN-41 have not yet been assigned to a region(s) of the I domain. In contrast to these mAbs, the mAb OKM1 recognizes an epitope on Mac-1 associated with the lectin-like domain (Diamond *et al.*, 1993; Thornton *et al.*, 1996). Both β-glucan and NADG (as the chitin polymer) are ligands for this domain and predominant components of the surface of *C. albicans* hyphae and other fungi (Georgopapadakou and Tkacz, 1995). In addition, the carbohydrate specificity of the Mac-1 lectin domain has been broadened to include fungal mannoprotein (Thornton *et al.*, 1996). By adhering to the lectin-like domain, polymeric β-glucan, mannoprotein, (and/or NADG) on the *C. albicans* surface may cross-link Mac-1 into clusters of receptors resulting in their activation. Activated Mac-1 has been demonstrated to cluster (Detmers *et al.*, 1987). In addition, cross-linking of Mac-1 with anti-CD11b mAbs results in transient intracellular calcium ($[Ca^{2+}]_i$) increase and an activation of Mac-1 to bind fibrinogen (Altieri *et al.*, 1992). Interestingly, cross-linking CD11a or CD11c with mAbs did not have this effect, while cross-liking CD18 using mAbs also resulted in transient $[Ca^{2+}]_i$ increase (Altieri *et al.*, 1992). Clustering has thus been established as a fundamental mechanism of Mac-1 activation (Altieri *et al.*, 1992), and for β_2 integrin activation by extracellular calcium mediated clustering for CD11a/CD18 (Van Kooyk *et al.*, 1994). Aggregation also results in activation of β_1 integrins using cross-linking by mAb coated beads and multimeric RGD-mimetic peptides.

Figure 16. Schematic diagram of the CD11b and CD18 subunits of Mac-1, showing the possible locations of subdomains. The I domain contains three subdomains with the OKM1 lectin domain nearby.

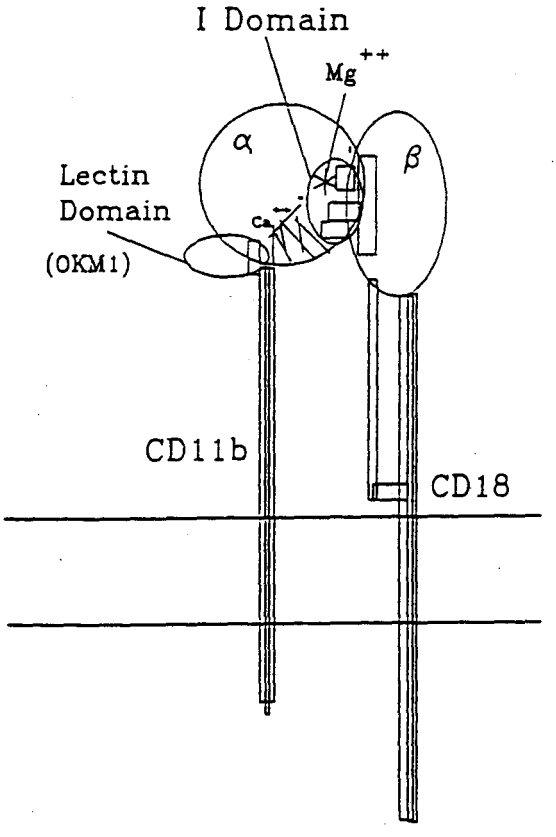

Figure 16.

(Miyamoto *et al.*, 1995a; Miyamoto *et al.*, 1995b). Following aggregation, Mac-1 activation would then be followed by adhesion to an RGD-mimetic ligand on the surface of *C. albicans* to trigger cytotoxic LGL degranulation (Figure 17). In this way, receptor clustering and ligand occupancy synergize during integrin adhesion as demonstrated for β_1 integrins (Miyamoto *et al.*, 1995a; Miyamoto *et al.*, 1995b). Such synergy has been shown for Mac-1 by soluble β-glucan activating Mac-1 mediated tumoricidal function on NK and cytotoxic lymphocytes (CTL) (Di Renzo *et al.*, 1991; Muto *et al.*, 1993). In addition, it was recently demonstrated that the Mac-1 lectin domain represents the cellular receptor for an entire class of polysaccharide immunomodulators such as lentinan which potentiate cytotoxic anti-tumor activity through an unknown mechanism and are currently in clinical trials for anti-cancer therapy (Thornton *et al.* 1996). This explains previous data showing that an anti-Mac-1 mAb coupled to forty-six repeated units of muramyldipeptide/gluconolactone activated macrophage non-MHC restricted tumor cytotoxicity similar to zymosan (Midoux *et al.*, 1992). Thus Mac-1 probably represents a broadly specific non-MHC restricted receptor for recognition of microbial polysaccharides bound by C3bi or displaying RGD-mimetic virulence factors like *B. pertussis* FHA (and possibly tumors with aberrant surface glycoproteins). This model is supported by a recent description of the Mac-1 activating mAb VIM12 (Stöckl *et al.*, 1995). VIM12 mAb binding to Mac-1 can be inhibited with NADG, and the epitope has been mapped to the lectin-like domain of CD11b (Diamond *et al.*, 1993; Stöckl *et al.* 1995). Only intact mAb VIM12 or cross-linked Fab' fragments, not monomeric Fab', result in activation of Mac-1 adhesion, which was also shown to require intact microfilaments (Stöckl *et al.*, 1995). This model of Mac-1 activation extends one proposed for Mac-1/CD16 (FcRIII) mediated phagocytosis of IgG-coated particles (Petty and Todd, 1993; Sehgal *et al.*, 1993).

Figure 17. Schematic model for lymphocyte (IAL or YT) Mac-1. Activation of Mac-1 is proposed to occur via cross-linking of single Mac-1 molecules (at left) by carbohydrates on the surface of a *C. albicans* hypha (above) to form activated clusters (a cluster of three at right). The single Mac-1 receptor is unable to bind to the RGD-mimetic ligand (large arrowhead) until undergoing a conformational change due to cross-linking and association with cytoskeletal proteins (below).

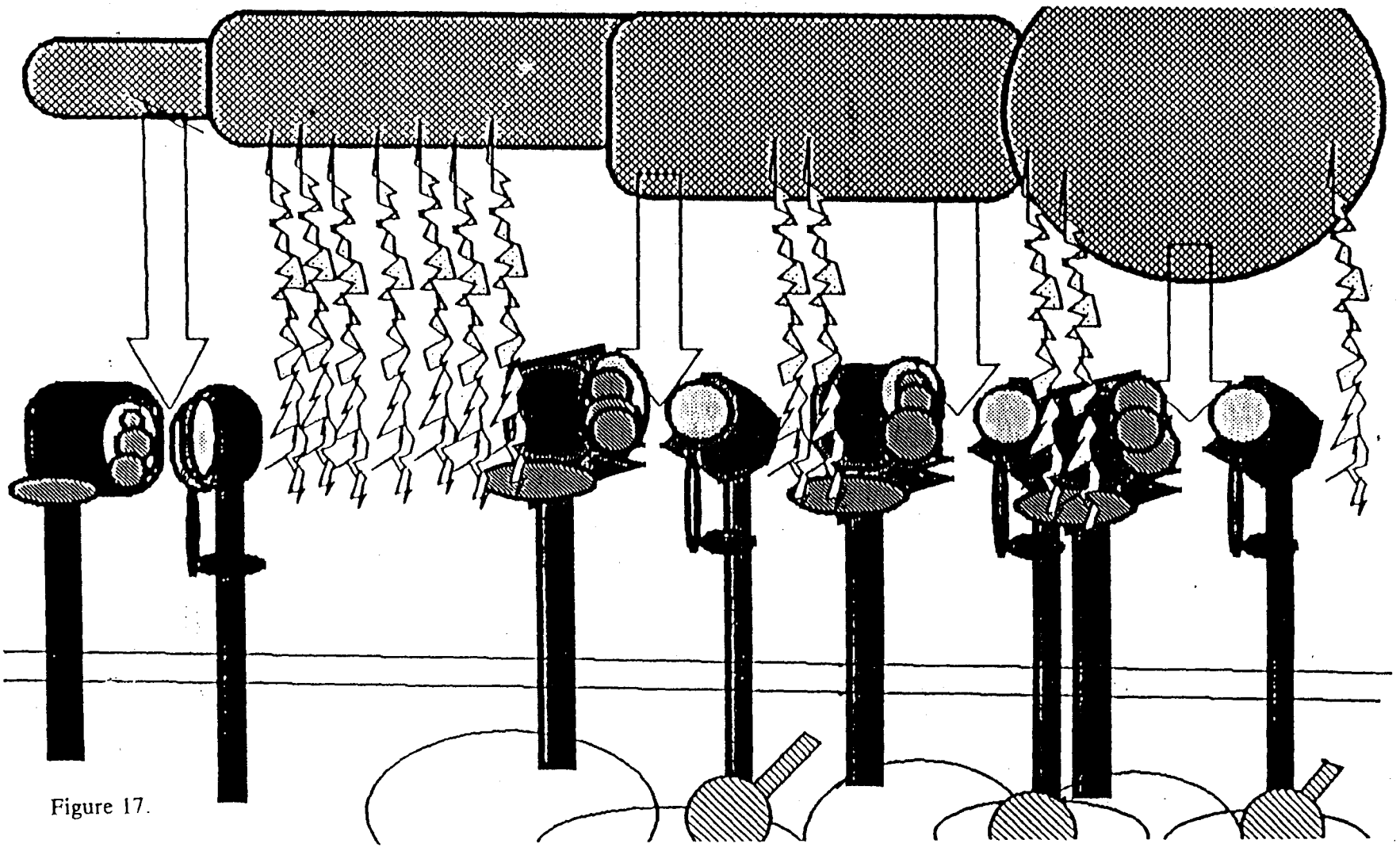

Figure 17.

The CR3-like molecule on *C. albicans* has been defined as a single receptor for C3bi with saturable binding to C3bi and an affinity for C3bi said to be similar to the CR3 integrin (CD11b/CD18, Mac-1) (Hostetter, 1994a). However, the concentration of C3bi required for 50% inhibition to HELA epithelial cells was 300 mM (Bendel and Hostetter, 1993), far above the CR3 Kd of 3.5 μM for C3bi (Arnout, 1990), or the 400 nM IC_{50} for YT lymphocytes and 1 μM IC_{50} for mIAL seen in this dissertation using C3 complement (Table 3). The *C. albicans* CR3-like molecule may also be inhibitable with GRGDSP-sequence peptides (Hostetter, 1994b), however the washing experiments in this dissertation demonstrate the RGD-inhibitable integrin to be on the surface of lymphocytes (Figure 6). Besides directly mediating adhesion, expression of this receptor may be a device utilized by *C. albicans* to evade detection by the host immune system. *C. albicans* expression of this C3 complement binding protein inversely correlates with phagocytosis of *C. albicans* yeasts (Gilmore *et al.*, 1988) and correlates directly with virulence (Hostetter, 1994a; Bendel *et al.*, 1995). The CR3-like molecule has also been implicated in iron acquisition by *C. albicans* (Moors *et al.*, 1992). Of the mAbs to mammalian $\alpha_M\beta_2$ (CD11b/CD18, Mac-1) tested for reactivity with *C. albicans,* several bind (OKM1, M1/70, Mo1, mAb 17, mAb 44, Mn-41, and OKM10) while several others which are potent blockers of mammalian Mac-1 do not bind (Leu15, 60.1, 95G8)(Mayer *et al.*, 1990). Of the mAbs to Mac-1 which bind to *C. albicans*, only mAb 17 and mAb 44 block adhesion of *C. albicans* to mammalian cells, even though the other mAbs including OKM1 were tested at concentrations as high as 2 mg/ml (Bendel and Hostetter, 1993; Hostetter, 1994a). This is far above the 180 μg/ml of OKM1 which blocked murine and human IAL adhesion to *C. albicans* by 70-85% in the present study. It is also consistent with experiments performed in this dissertation in which pre-incubation of *C. albicans* hyphae with 200 μg/ml OKM1, M1/70 or TS1/18 followed by washing had no effect on lymphocyte

adhesion to hyphae. Conversely, a polyclonal rabbit IgG antiserum which reacts specifically with the *C. albicans* CR3-like molecule and blocks C3bi and OKM1 adhesion does not react with CR3 expressed on U937 cells (Calderone and Braun, 1991). What is believed to be the same integrin-like molecule on *C. albicans* also reacts with mAb to $\alpha_X\beta_2$ (CR4, p150,95) with no demonstrated effect on adhesion of the fungus to mammalian cells (Hostetter, 1994b). Notably, no reactivity of any mAb to the Mac-1 β_2 integrin subunit (including testing of mAb TS1/18 used in this dissertation) has ever been detected on *C. albicans* yeast or hyphal forms (Hostetter, 1994b). In contrast, the TS1/18 mAb to human β_2 blocked adhesion of lymphocytes to *C. albicans* by 49% in the present study.

Two other differences between the mammalian integrins and the *C. albicans* integrin-like protein(s) are that carbohydrates, including NADG, at concentrations greater than 65 mM have no effect on *C. albicans* adhesion to endothelial or epithelial cells, while NADG blocked in this study by 50-75% at those concentrations (Tables 3 and 4) (Hostetter, 1994b). Also, the candidal proteins do not require divalent cations, while the need for divalent cations in most adhesion mediated by mammalian integrins is clear (Springer, 1991, Arnaout, 1990; Hostetter, 1994b). Finally, the C3bi binding protein of *C. albicans* has been identified using affinity chromatography as a 42 kD protein apparently also occurring on *C. albicans* in other increasingly glycosylated forms of 55 kD and 66 kD (Eigentler *et al.*, 1989; Alaei *et al.*, 1993). The isolated proteins bound C3bi, cross-reacted with the mAb OKM1, and rabbit antiserum to the purified 42 kD protein blocked adhesion of C3bi coated erythrocytes to *C. albicans* pseudohyphae (Alaei *et al.*, 1993). This data is consistent with a 60-70 kD CR3/CR2-like molecule described by other groups to also bind to mAbs to mammalian CR2, a member of the immunoglobulin superfamily which also binds C3 fragments (Eigentler *et al.*, 1989; Wadsworth *et al.*, 1993; Calderone *et al.*, 1994). Nevertheless, a small group of

researchers continues to search for the gene for the "integrin-analog" C3 receptor on *C. albicans* (Bendel *et al.*, 1995). Clearly, the characteristics of the candidal "CR3/CR2-like protein" (Calderone *et al.*, 1994) differ from the native mammalian CR3 (CD11b/CD18) shown to mediate adhesion of YT lymphocytes and murine and human IAL to *C. albicans* hyphae in this dissertation.

A mannose specific lectin-like receptor on splenic and lymph node macrophages has been described by others as mediating adhesion to *C. albicans* (Cutler, 1991). Using an *ex vivo* binding assay, adhesion of *C. albicans* yeast to lymph node macrophages was not blocked with laminin or fibronectin (final concentrations 100 nM)(Han *et al.*, 1993). This concentration of laminin was sufficient to block YT lymphocyte and mIAL adhesion to *C. albicans* by 40% in this dissertation, although fibronectin at this concentration also had no effect on lymphocyte adhesion. However, *C. albicans* adherence in the *ex vivo* assay was blocked by *C. albicans* purified carbohydrates which were eluted from a Con A column with α-methyl-D-mannopyranoside (Han *et al.*, 1993), a carbohydrate which inhibited adhesion of lymphocytes to *C. albicans* in this dissertation and is a well documented inhibitor of adhesion mediated by the lectin-like domain of Mac-1 (Ross *et al.*, 1985; Thornton *et al.*, 1996). Another study using the U937 macrophage cell line also claimed that a β-glucan receptor other than CR3 mediated adhesion to *C. albicans* (Janusz *et al.*, 1988). However, in light of the carbohydrate inhibition data in this study, as well as recent mapping of the U937 β-glucan receptor to the Mac-1 lectin-like domain (Thornton *et al.*, 1996), it seems possible that those authors were describing Mac-1 mediated adhesion of *C. albicans* to macrophages. These data are consistent with the inhibition of Mac-1 mediated adhesion by lymphocyte effectors in this dissertation by the same profile of carbohydrates known to block CD11b/CD18 mediated adhesion to *S. cerevisiae*, including β-glucan from *S. cerevisiae* itself (Tables 3 and

4)(Forsyth and Mathews, 1996; Thornton *et al.*, 1996). However, it is known that macrophages from patients with leukocyte adhesion deficiency (LAD) can still mediate a reduced level of phagocytosis of *C. albicans* by a lectin receptor (Szabo *et al.*, 1995). Which of these two lectin-like receptors has been described in the series of studies by Han *et al.* remains to be determined (Han *et al.*, 1993). With regard to inhibition of *C. albicans* adhesion to epithelial and endothelial cells, the majority of investigations have shown that fibronectin fragments of the GRGDSP sequence and PT-2000 used in this dissertation do not block adhesion of *C. albicans* yeast cells to mammalian cells including HELA S3 epithelial cells (Hostetter, 1994a) or rabbit aortic endothelium (Klotz *et al.*, 1992). This was true even when used at final concentrations of 1 mg/ml (Bendal and Hostetter, 1993). In this dissertation, PT-2000 at a final concentration of 40 μg/ml blocked binding of YT lymphocytes to *C. albicans* by 98% (Forsyth and Mathews, 1996).

The β_2 leukocyte integrins must become activated in order to become adhesive (Anderson, 1994; Li *et al.*, 1995; Stewart *et al.*, 1995). Two general pathways of cell signaling have been characterized as mediating the activation of β_2 integrins (as well as β_1 and β_3 integrins) and have received the terms "outside-in" signalling and "inside-out" signalling. The classic example of β_2 integrin inside-out signaling, is that cross-linking of the T cell receptor (TCR) results in an activation of CD11a/CD18 (LFA-1) for adhesion to ICAM-1 (Dustin and Springer, 1989). The molecular events underlying these two pathways are not well understood for any integrin, but progress is slowly being made, especially in the area of β_1 integrin signaling. Two synergistically related processes that are believed to regulate integrin outside-in signalling are ligand occupancy and receptor cross-linking (Miyamoto *et al.* 1995a). A molecular hierarchy has been constructed for β_1 integrin signaling for redistribution of cytoplasmic proteins by integrins. The hierarchy describes increasing

association of intracellular proteins with the cytoplasmic domains of integrins beginning with weak ligand occupancy, then aggregation/cross-linking followed by ligand occupancy plus cross-linking (Miyamoto *et al.*, 1995b). The initial step (ligand occupancy) results in no significant intracellular signals. Cross-linking alone with non-inhibitory mAbs results in significant intracellular signaling with induction of some 20 intracellular signaling molecules including kinase phosphorylated proteins and initial cytoskeletal interactions. However, if integrins are cross-linked and ligand occupancy occurs (or cross-linked with inhibitory mAbs), then a synergistic intracellular response is elicited with an increase in affinity of the receptor for ligand accompanied by induction of some 50 intracellular proteins including increased kinase phosphorylated proteins and actin polymerization with full integrin association to the cytoskeleton (Miyamoto *et al.*, 1995a; Miyamoto *et al.*, 1995b).

What is known about β_2 integrins and Mac-1 signaling is in agreement with this model. Mac-1 has been shown to mediate degranulation in PMN (Walzog *et al.*, 1994), Macrophages (Klegeris and McGeer, 1994; Hellberg *et al.*, 1995), NK and T cells (Muto *et al.*, 1993), and eosinophils (Kaneko *et al.*, 1995). Cross-linking of CD18 on lymphocytes results in transient intracellular calcium ($[Ca^{2+}]_i$) currents but no increase in Mac-1 affinity for fibrinogen, while cross-linking of CD11b also results in increased $[Ca^{2+}]_i$ accompanied by activation of Mac-1 adhesion to fibrinogen (Altieri *et al.*, 1992). Mac-1 affinity for ligand has been demonstrated to be modulated by the cytoplasmic domains of the CD11b and CD18 subunits (Rabb *et al.*, 1993). Truncation of either the CDllb or CD18 cytoplasmic tails results in constitutive activation of Mac-1 adhesion (Rabb *et al.*, 1993). This truncation may allow easier aggregation, but also may remove constitutive inhibition due to phosphorylation and/or dephosphorylation of cytoplasmic residues (Valmu and Gahmberg, 1995). No intracellular signal is elicited by Mac-1 contact with monomeric ligand alone (Li *et al.*, 1995).

However, cross-linking Mac-1 has been shown to result in several signals. These signals include increased $[Ca^{2+}]_i$ (Altieri *et al.*, 1992; Hellberg *et al.*, 1995; Walzog *et al.*, 1994). Interestingly, *in vitro* studies done with adhesion of PMN to *C. albicans* hyphae demonstrate transient changes in $[Ca^{2+}]_i$ in PMN as they move the length of the hyphae. Furthermore, that study measured a difference in time before contact to when the transient changes in $[Ca^{2+}]_i$ occurred with unopsonized (4 min) versus serum opsonized (1 min) hyphae (Levitz *et al.*, 1987). Cross-linking Mac-1 with opsonized bacteria and other ligands in PMN and lymphocytes has also been shown to result in phosphorylation of paxillin (a key cytoskeletal linker protein), upregulation of Mac-1 from intracellular stores, shedding of L-selectin, granule exocytosis, and actin polymerization (Muto *et al.*, 1993; Walzog *et al.*, 1994; Fuortes *et al.*, 1994). The initial calcium signal was not inhibitable with tyrosine kinase inhibitors, although PMA (which is known to cause clustering of Mac-1) did inhibit it (Walzog *et al.*, 1994). More extensive events resulting from anti-CD18 cross-linking, such as granule exocytosis, were inhibitable with tyrosine kinase inhibitors. The PMN respiratory burst was not affected by soluble anti-CD18 mAb, but was blocked by solid phase mAb, indicating an effect of cross-linking (Walzog *et al.*, 1994). Similarly, the ICAM-2 peptide fragment that activates Mac-1 mediated NK cell killing evokes a 35 KD and 150 KD phosphorylated protein but mAbs to Mac-1 evoke only the 150 kD protein (Somersalo *et al.*, 1995). Also, cytokines that "prime" Mac-1 (TNF-α, fMLP, GM-CSF, but not G-CSF or IFN-γ) result in phosphorylation of paxillin in the absence of ligand and result in a synergistic respiratory burst response in PMN when ligand does engage Mac-1 (Lilles *et al.*, 1995). These data are in agreement with the hierarchical model for integrin activation (Miyamoto *et al.*, 1995). The fact that opsonized bacteria and opsonized zymosan evoke these responses, including degranulation, is virtually the same as *C. albicans* evoking them, as indicated by the study of

$[Ca^{2+}]_i$ currents in *C. albicans* adherent PMN (Levitz *et al.*, 1987). Therefore, the model proposed herein of Mac-1 activation via cross-linking through the lectin domain has a basis in theory and Mac-1 experimental data.

Finally it should be noted that ligation of the CD11b versus CD18 subunits of Mac-1 has been shown to have different effects. This is in agreement with data showing different β_2 integrin "activating" mAbs can result in affinity for different ligands (Ortlepp *et al.*, 1995). Regulation of Mac-1 adhesion by the differential phosphorylation status of the alpha and beta chains has been reported (Buyon *et al.*, 1990; Valmu and Gahmberg, 1995). Similarly for Mac-1, cross-linking with anti-CD11b mAbs (using secondary antibodies) results in different cellular signals than when anti-CD18 mAbs are used (Crockett-Torabi *et al.*, 1995). In one study with PMN, mAbs specific for CD11b or CD18 blocked Mac-1 mediated adhesion to zymogen, while only mAbs to CD18 blocked phagocytosis of these particles (Peterson *et al.*, 1994). Of great interest for $CD8^+$ cytotoxic cells is the subject of deadhesion from a target that allows recycling of cytotoxic lymphocytes. Mac-1 has been demonstrated to be instrumental in the deadhesion of PMN (Bohnsack *et al.*, 1991). A very recent paper shows a synergistic relationship between CD3 receptor aggregation and integrin ligand occupancy in deadhesion triggered by LFA-1 from tumor targets (Rovere *et al.*, 1996). It also agrees with data showing ligand binding to Mac-1 is necessary but not sufficient for function, because full function requires deadhesion (Dransfield *et al.*, 1992). Thus the synergistic "trigger" for cytotoxic lymphocyte degranulation as well as deadhesion mediated by Mac-1 may come via cross-linking mediated by the CD11b lectin domain followed by adhesion to ligand mediated by both subunits with signaling via the CD18 subunit also required for degranulation as well as deadhesion. Consistent with this view are activating mAbs for Mac-1 which are specific for the CD11b lectin domain (Stöckl *et al.*, 1995) and another specific for CD18 (Petruzzelli

et al., 1995). In the model proposed in this dissertation, most natural ligands of Mac-1 are proposed here to fulfill the requirements for cross-linking and ligand occupancy by different portions (often discontinuous) of the same molecule. Such multiple, noncontiguous sites within a Mac-1 ligand which block Mac-1 adhesion have been described for fibrinogen, Factor X, *S. cerevisiae*, FHA, and other microbial ligands noted below as well as *C. albicans* hyphae in the present study.

This two step adhesion cascade of polysaccharide/hydrophobic adhesion resulting in activation followed by RGD-mimetic recognition has been demonstrated for other microbial ligands of Mac-1. Binding of the *Bordatella pertussis* RGD-containing FHA glycoprotein to macrophages is inhibitable by GRGDSP peptides and also by 50% with NADG (Relman *et al.*, 1990). *Leishmania mexicana* also expresses two distinct structures that bind to Mac-1: a surface glycolipid (LPG) that can be blocked with OKM1 and not RGD and a second protein (gp63, containing RYD) which binds to Mac-1 in an RGD-inhibitable manner (Russell *et al.*, 1989). Mac-1 was also recently shown to mediate macrophage binding to another dimorphic fungus *Blastomyces dermatitidis* and, like adhesion to *C. albicans*, mAb MY904 is inhibitory while MN-41 is not (Newman *et al.*, 1995). RGD peptide inhibition studies have not been reported but inhibition by RGD-mimetic peptides would be expected. This is supported by these authors identification of the principal Mac-1 ligand on *B. dermatitidis* as WI-1, with homology to invasin of *Yersinia* species. The invasin protein is an RGD-mimetic integrin ligand which contains a critical aspartate but lacks an RGD sequence (Leong *et al.*, 1995). Interestingly, not only does WI-1 expression correlate with adhesion and virulence of *B. dermatiditis* (Klein *et al.*, 1994), but so does expression of the Mac-1 lectin domain ligand β-glucan by *B. dermatiditis* (Hogan and Klein, 1994). Such a two-step adhesion cascade has been demonstrated for Mac-1 activation by P and E selectins on endothelium which probably

interact with the carbohydrate sialyl-Lewis^x on Mac-1 to cross-link Mac-1 during leukocyte rolling on endothelium at sites of inflammation (Kotovuori *et al.*, 1993).

The epitope mapping for murine specific anti-CD11b/CD18 mAbs has not yet been carried out. However, the anti-murine CD11b mAb M1/70 has been mapped to bind outside the I domain of human CD11b to an area proximal to the I domain possibly responsible for Mac-1 hydrophobic adhesion and also blocked by mAb MY904 (Dana *et al.*, 1986; Zhou *et al.*, 1994). Also, the anti-human CD11b mAb OKM1 is known to bind the lectin domain of human Mac-1 (Thornton *et al.*, 1996). Both mAbs blocked YT lymphocytes and murine and human IAL in a comparable manner, with maximum inhibition by M1/70 in the 35-45% range and maximum inhibition by OKM1 of 84% for hIAL and 92% for mIAL (Figures 7, 10 and 11). This is strong evidence that the cross-reactive epitopes recognized by these antibodies represent similar functional portions of the respective Mac-1 molecules. Further support for similar functions is the potent additive effect of OKM1 and anti-CD18 mAbs for both mIAL and hIAL resulting in greater than 80% inhibition (Figures 10 and 11). The anti-murine CD18 mAbs inhibited mIAL adhesion by 52% (M18/2.A) and 59% (2E6) and in a dose dependent manner similar to YT lymphocyte inhibition of 49% by TS1/18, and slightly greater but comparable inhibition of 69% was seen for hIAL by TS1/18. These data correlate well with the close conservation of sequences in the functional areas of Mac-1 that has been observed between mouse and human Mac-1 (Pytela, 1988). The specificity of inhibition by these mAbs is further demonstrated by the negligible effects on YT and IAL adhesion exerted by equal amounts of isotype matched mAbs to other adhesion molecules verified to be present on these lymphocytes (solid bars in Figures 7, 10, and 11). This is especially significant when considering treatment of mIAL (M17/4.4) and hIAL (TS1/22) with mAbs to LFA-1 (CD11a/CD18) which have been shown to block adhesion of cytotoxic lymphocytes to targets

mediated by LFA-1 in the respective species (Anderson, 1994). No surface reactivity to anti-LFA-1 mAbs was found for the YT lymphocytes. These data taken together with the dose dependent inhibition of lymphocyte adhesion by CD11b/CD18 mAbs, and with maximum inhibition of over 80% for both mIAL and hIAL adhesion to *C. albicans* by mAbs to CD11b/CD18, strongly indicate that these lymphocytes from both species utilize CD11b/CD18 as the principal structure for adhesion to *C. albicans* hyphae.

The data show that for the YT lymphocytes and murine and human IAL, combinations of anti-CD11b and anti-CD18 mAbs had an additive inhibitory effect on lymphocyte adhesion to *C. albicans* hyphae (Figures 7, 10, and 11). Maximum inhibition of adhesion observed for these combinations of purified mAb were 85% for YT lymphocytes, 84% for mIAL, and 82% for hIAL. These data combined with the inhibition demonstrated by mAbs to CD18 alone suggest both subunits of the Mac-1 heterodimer participate in adhesion of lymphocytes to *C. albicans* hyphae. This concept is consistent with mutations in the CD18 chain eliminating Mac-1 adhesion to C3bi (Bajt *et al.*, 1995) even though C3bi binds to the purified CD11b subunit (Van Strijp *et al.*, 1993). As noted above, mAbs to murine and human CD11a/CD18 showed no inhibitory effect on IAL adhesion to *C. albicans*. The only other integrin identified on the YT lymphocyte subline is p150,95 (CD11c/CD18) which also utilizes the CD18 β_2 integrin subunit. However, p150,95 has not been shown to be inhibitable with RGD-mimetic peptides or NADG (Anderson, 1994; Thornton *et al.*, 1996). In addition, mAb N418 to murine CD11c had no effect on mIAL adhesion to hyphae (Figure 10). The $\alpha_d\beta_2$ integrin reported in canines and humans recently has not yet been described in murine cells (Van der Vieren *et al.*, 1995). Specific mAbs to test for the $\alpha_d\beta_2$ integrin on IAL will be utilized when available. However, evidence for $\alpha_d\beta_2$ not mediating human lymphocyte adhesion to *C. albicans* is that of the anti-Mac-1 mAbs utilized in this dissertation, MY904

has been tested for cross-reactivity with human $\alpha_d\beta_2$ and no cross-reactivity to $\alpha_d\beta_2$ was found (Danilenko *et al.*, 1995), while MY904 demonstrated a dose dependent inhibition of YT lymphocyte adhesion to *C. albicans* (Figure 7). Therefore, the additive effects of mAbs reactive with CD11b and CD18 are probably due to the contribution by CD18 to Mac-1 adhesion. Collectively, these data demonstrate the principal structure that mediates lymphocyte adhesion to *C. albicans* is CD11b/CD18.

The ECM and RGD-peptide inhibition data support the mAb blocking data. Fragments from ECM peptides containing aspartate residues have been shown to activate lymphocytes in a manner similar to β-glucan (Lopez-Moratalla *et al.*, 1995). ECM proteins were evaluated for their capacity to inhibit lymphocyte adhesion to *C. albicans*. On a molar basis, the ECM proteins fibrinogen, EHS-laminin, and vitronectin were similarly effective. Human placental laminin was ineffective at blocking adhesion, possibly because it lacks the RGD-containing laminin A chain (Tryggvason, 1993). Several ECM and blood proteins which are documented ligands for Mac-1 demonstrated excellent inhibition of YT lymphocyte, mIAL and hIAL adhesion to *C. albicans* hyphae (Table 3). The complex glycoprotein heparin has recently been defined as a Mac-1 ligand and inhibited adhesion well, but with a YT and mIAL IC_{50} of 100 μM compared to an IC_{50} for blocking Mac-1 adhesion to heparin coated plastic of 9.0 μM (Diamond *et al.*, 1995). The higher concentration required might be due to the higher density of carbohydrate ligands on *C. albicans*. Fibrinogen has been documented by many laboratories to be a ligand for Mac-1, but only is bound by activated Mac-1 (Wright *et al.*, 1988; Altieri *et al.*, 1990; Altieri *et al.*, 1993; Languino *et al.*, 1993). The IC_{50} values for YT and mIAL agree closely with the published value of 2.0 μM (Altieri and Edgington, 1988). For mIAL, the documented Mac-1 ligand Factor X (Anderson, 1994) showed a clear dose dependent inhibition of adhesion to hyphae with an IC_{50} of 50 nM

virtually identical to the documented kD for Mac-1 of 44 nM (Altieri and Edgington, 1988). The interaction of Mac-1 with Factor X is so specific that inhibition of transendothelial migration of leukocytes in a rabbit model of meningitis using a Factor X RGD-mimetic peptide: ETKEVDG (that also mimics a motif in *Bordatella pertussis* FHA) was recently accepted as evidence that Mac-1 recognizes RGD-mimetic sequences *in vivo* (Rozdzinski *et al.*, 1995). These data confirm *in vivo* that RGD-mimetic peptides based on a microbial Mac-1 ligand can directly inhibit adhesion mediated by activated Mac-1. Inhibition of at least 50% by this concentration of 50 nM Factor X of YT, mIAL, and hIAL as well as the 3T3-19 transfectants (Table 3) is strong evidence in itself that Mac-1 mediates lymphocyte adhesion to *C. albicans* (Rozdzinski *et al.*, 1995). The Mac-1 ligand complement C3 also demonstrated a dose dependent inhibition of YT lymphocyte adhesion to *C. albicans* with an IC_{50} for YT lymphocytes (400 nM) and mIAL (1 μM) remarkably close to the Mac-1 kD for C3bi of 3.5 μM (Arnaout, 1990). This is also strong evidence that Mac-1 mediates adhesion of mIAL to *C. albicans*. These data are consistent with β_2 integrins being crucial for migration of IL-2 activated T and NK lymphocytes through complex ECM models containing all of the ECM components employed in the present study (Jääskeläinen *et al.*, 1992). Also, while controversial, several studies specifically demonstrate Mac-1 mediates PMN adherence to solid phase ECM components consistent with our data including vitronectin, EHS-laminin, fibrinogen, fibronectin, collagens type II and VI, thrombospondin, and undulin (Nathan *et al.*, 1989; Bohnsack *et al.*, 1992; Walzog *et al.*, 1995). The "irrelevant" proteins BSA and chicken egg lysozyme tested in nanomolar-millimolar concentrations demonstrated no inhibition of lymphocyte adhesion to *C. albicans*.

Several RGD-mimetic peptides also markedly inhibited lymphocyte adhesion to *C. albicans*. The GRGDSP and GRGDSPK peptide sequences from fibronectin (Kleinman and

Yamada, 1993) demonstrated dose dependent inhibition of YT lymphocyte and mIAL adhesion to hyphae (Figure 9). The IC_{50} values for these peptides of 500 μM and 300 μM respectively are in the range of 400 μM IC_{50} for inhibition of C3bi coated erythrocytes (EC3bi) with an RGD-mimetic peptide containing the C3bi RGD sequence: TRYRGDQDATMS (Wright *et al.*, 1989). In the same study, an "L10" peptide portion of the FBIP (HHLGGAKQAGDV) composed of the LGGAKQAGDV segment demonstrated an IC_{50} to block adhesion to EC3bi of 600 μM, being virtually identical to the 300 μM IC_{50} value obtained for FBIP inhibition of mIAL as well as YT lymphocytes and hIAL to *C. albicans* (Table 3). Interestingly, the L10 peptide enhanced Mac-1 adhesion to erythrocytes coated with the LPS lipid core (rough LPS), indicating that cross-linking through the I domain can activate Mac-1 adhesion to purely hydrophobic surfaces, and may indicate an obligatory role for Mac-1 adhesion to an RGD-like ligand(s) on *C. albicans* (Wright *et al.*, 1989). Two engineered GRGDSP-containing peptides, PT-2000 and FEP, eliminated lymphocyte adhesion to *C. albicans* hyphae. Tertiary structure is clearly important as emphasized by the $\alpha_V\beta_3$ specific circular GRGDSP peptide with an identical RGD sequence that had no effect on adhesion (Pierschbacher and Ruoslahti, 1987). The potent inhibitory activity of FEP may also be due to the multiple GRGDSP repeats contained within each molecule which can interact with clustered integrins (Miyamoto *et al.*, 1995a; Miyamoto *et al.*, 1995b). The potent inhibition by PT-2000 has been attributed to the two domain structure of the GRGDSPASSK fibronectin sequence linked to the hydrophobic GGGGSRLLLLLLR sequence (Craig *et al.*, 1995). The sequence: ASSK-GGGGS serves a necessary role as a "spacer sequence" between the fibronectin RGD sequence and six hydrophobic lysines (Craig *et al.*, 1995). More than six lysines results in solubility problems (indicating some polymerization occurs) and less than six lysines decreases bioactivity (Craig *et al.*, 1995). Remarkably, the RGD-inhibitable

"integrin $\alpha_V\beta_3$ clusters in the membrane at the sites of cell attachment" to PT-2000 coated surfaces with formation of integrin associated F-actin bundles (Craig *et al.*, 1995). The two domain structure of PT-2000 may thus potently inhibit clustering or prematurely stimulate clustering of Mac-1 on the lymphocyte surface in the presence of an RGD-mimetic inhibitor of adhesion to *C. albicans* as demonstrated for GRGDSPK and thus block interaction of Mac-1 with *C. albicans* hyphae. Finally, the integrin inhibitor echistatin inhibited adhesion of YT, mIAL, and hIAL potently, with an IC_{50} of 2.0 μM comparable to C3 inhibition of Mac-1 (Table 3) (Gan *et al.*, 1988; Garsky *et al.*, 1989). This is the first demonstrated inhibition of Mac-1 by any disintegrin, echistatin contains two RGD-mimetic sequences within a circular peptide: CKRARGD...DMDDYC (Blobel and White, 1992). The "irrelevant" bioactive fragment from the murine class I MHC molecule H-$2K^b$ had no effect on mIAL (or YT lymphocyte and hIAL) adhesion to hyphae at concentrations (0.2-0.4 mM) which block allorecognition by 50% or greater (Kd = 0.1 mM) (Schneck *et al.*, 1989). In addition, the GRYDS peptide demonstrated no inhibition of lymphocyte adhesion to *C. albicans*. This peptide is not known to block Mac-1, but was tested because it is similar to the SRYDQL sequence in *Leishmania* gp63 protein which is a ligand for Mac-1 (Soteriadou *et al.*, 1992). In summary, the RGD-mimetic peptide, ECM, and blood protein inhibition data strongly support a principal role for Mac-1 in mediating YT lymphocyte, mIAL and hIAL adhesion to *C. albicans* hyphae. Taken together with the large body of data demonstrating specific blocking of lymphocyte adhesion to *C. albicans* by mAb to CD11b/CD18, these data demonstrate CD11b/CD18 is the principal structure on mIAL, hIAL and YT lymphocytes which mediates adhesion to *C. albicans* hyphae.

To further confirm the principal role of CD11b/CD18 in mediating adhesion of mIAL to *C. albicans* hyphae experiments were carried out using NIH-3T3 murine fibroblasts

expressing transfected human CD11b/CD18 (3T3-19) (Figures 12 and 13). The purpose was to verify that CD11b/CD18 was capable of mediating specific adhesion *in vitro* to *C. albicans* hyphae. Parallel experiments were performed using 3T3 cells subjected to the transfection protocol which did not express CD11b/CD18 (3T3-1). The 3T3-1 cells have been previously characterized as not binding to *S. cerevisiae* yeasts, while the 3T3-19 Mac-1^+ cells spontaneously phagocytose *S. cerevisiae* (Krauss *et al.*, 1994). Both cell lines were found not to express Fc receptors for immunoglobulin (Krauss *et al.*, 1994). To confirm that Mac-1 was mediating this phagocytosis FITC labeled β-glucan was shown to not bind to 3T3-1 cells, but bound to 3T3-19 cells extensively in a punctate pattern. Those authors concluded that one possibility is that the yeast provides both the ability to bind and the necessary activation signal (Krauss *et al.*, 1994). In this dissertation, these two 3T3 cell lines were compared for their ability to bind *C. albicans* hyphae after preincubation with mAbs to CD11b/CD18 or isotype matched irrelevant mAbs (Figure 12). The 3T3-1 fibroblasts demonstrated only background adhesion of 3% to *C. albicans* hyphae which was unaffected by mAb OKM1 (anti-human CD11b) or mAb TS1/18 (anti-human CD18). Adhesion of 3T3-1 and 3T3-19 cells was also unaffected by a mAb to murine CD29 (β_1 integrin) which reacted with both cell lines, although only data for 3T3-19 is shown (Figure 12). In contrast, adhesion of 3T3-19 fibroblasts to *C. albicans* hyphae was inhibited in a dose dependent manner by OKM1 and TS1/18. Maximum inhibition of 3T3-19 adhesion with the anti-CD11b mAb OKM1 was 67%, while TS1/18 maximum inhibition was 25%. However, the combination of 25 μg OKM1 + 25 μg TS1/18 had a very potent additive inhibitory effect with inhibition of 84%. Two other combinations of mAb also showed clear additive effects with 15 μg OKM1 + 15 μg TS1/18 inhibiting by 83% and 15 μg OKM1 + 15 μg M1/70 (anti-murine/human CD11b) blocking 3T3-19 transfectant adhesion to *C. albicans* hyphae by 59% (Figure 13). In a

fascinating development GRGDSPK and FBIP, two RGD-mimetic peptides which inhibited mIAL adhesion to hyphae, demonstrated no inhibitory effect on 3T3-19 adhesion when used alone. This raised the possibility that perhaps the transfected CD11b/CD18 was lacking the complete cytotoxic lymphocyte activation machinery present in mIAL. To address this question, small concentrations of anti-CD11b mAbs were added during preincubation with GRGDSPK (OKM1) and FBIP (M1/70) to facilitate cross-linking of the transfected CD11b/CD18 heterodimer in the hope of activating CD11b/CD18 for binding to the RGD-mimetic peptides. A synergistic effect was seen in which both GRGDSPK and FBIP inhibited adhesion of 3T3-19 transfectants in a concentration dependent manner (Figure 13). The data from these 3T3-19 transfectant mAb inhibition studies confirm that CD11b/CD18 expressed on mammalian cells is capable of mediating specific, mAb inhibitable adhesion to *C. albicans* hyphae. These data also confirm that the integrin inhibitable with RGD-mimetic peptides on YT lymphocytes, mIAL, and hIAL is indeed CD11b/CD18 and support a role for cross-linking in activation of Mac-1 adhesion to such peptides.

To confirm that Mac-1 has a primary physiological role during mIAL mediated growth inhibition of *C. albicans* hyphae, experiments were conducted to examine the effect of mAbs to CD11b/CD18 on mIAL anti-fungal activity (Figure 14). These experiments utilized an *in vitro* assay of *C. albicans* growth inhibition developed in this laboratory (Beno and Mathews, 1993). A concentration of 45 μg OKM1 which inhibited mIAL adhesion by 92% (Figure 10) completely eliminated mIAL growth inhibition of *C. albicans*. A concentration dependent effect for OKM1 inhibition was demonstrated by 25 μg OKM1 which allowed 43% inhibition of growth by mIAL. This effect was significantly different ($p < .05$; t-test) from 58% inhibition by mIAL alone, thus representing an intermediate level of inhibition relative to 45 μg OKM1. Similar results were obtained using mAb M/170 which eliminated mIAL

inhibition of growth at 45 μg and showed a significant ($p < .05$) concentration dependent effect, further demonstrating specificity as with OKM1. The anti-CD11b mAbs OKM1 and M1/70 demonstrated no inhibition of *C. albicans* growth when introduced alone without mIAL present. Additionally demonstrating specificity for *C. albicans* growth inhibition, mAbs to murine CD11a (M17/4.4) and murine CD11c (N418) demonstrated no effect on mIAL anti-candidal activity. This is particularly significant in light of many studies attributing a role for CD11a (LFA-1) in anti-tumor cytotoxicity (Anderson, 1994) and recent work showing LFA-1 cocapping with Mac-1 during cytotoxicity (Zhou *et al.*, 1993). These data confirm a principal role for CD11b/CD18 in mediating adhesion of murine IL-2 activated lymphocytes to *C. albicans* hyphae during mIAL mediated growth inhibition of the fungus (Beno and Mathews, 1992; Beno *et al.*, 1995).

The direct antimicrobial activity of T lymphocytes is an area of study that has only recently gained legitimacy in the mainstream of immunology (Levitz *et al.*, 1995). This laboratory has demonstrated previously that the populations of murine and human IAL mediating growth inhibition of *C. albicans* hyphae are $CD8^+$ T lymphocytes (Beno *et al.*, 1995). Two other laboratories have also demonstrated a direct antimicrobial effect of $CD8^+$ lymphocytes against *Cryptococcus neoformans* requiring direct contact but not opsonins (Murphy, 1991; Levitz and Dupont, 1994). Other groups have demonstrated $CD8^+$ T lymphocytes mediate defense against the fungus *Histoplasma capsulatum* (Deepe, 1994). As noted above, *H. capsulatum* was one of the earliest documented microbial ligands for Mac-1 (Bullock and Wright, 1987). $CD8^+$ lymphocytes have also been shown to directly kill *Schistosoma mansoni* (Ellner *et al.*, 1982), *Entamoeba histolytica* (Salata *et al.*, 1987), and bacteria including *Pseudomonas aeruginosa, Escherichia coli,* and *Staphylococcus aureus* (Levitz *et al.*, 1995). $CD8^+$ lymphocytes are a critical first line of protection against

Toxoplasma gondii (Shirahata *et al.*, 1994), and anti-murine Mac-1 blocks protection in *T. gondii* infection (Johnson *et al.*, 1996). $CD8^+CD11b^+$ T cells have been demonstrated to play a direct role in clearance of *Listeria* (Goossens *et al.*, 1995) and *Yersinia* (Autenrieth *et al.*, 1993). $CD8^+CD11b^+$ T cells have also been demonstrated to mediate the first line of defense in vivo against movement of intestinal microorganisms into the blood (Gautreaux *et al.*, 1994). This is especially relevant for *C. albicans*, since gastrointestinal colonization often precedes infection in neonates and AIDS patients (Van den Anker *et al.*, 1995).

It is clear that $CD8^+$ T lymphocytes have been demonstrated to exhibit broad anti-microbial activity. Important questions for the significance of this dissertation then become: on what $CD8^+$ lymphocytes is CD11b/CD18 expressed, and do these cells exist and exhibit a relevant non-MHC restricted cytotoxic phenotype, especially after IL-2 activation? The answers to these questions as they are known might seem surprising. Originally, CD11b/CD18 was identified as a marker of monocytes/macrophages and granulocytes (Springer *et al.*, 1979), and has since been identified in peripheral blood of normal mouse and human as comprising 60-100% of NK cells (Polli *et al.*, 1987; Robertson *et al.*, 1990; Triozzi *et al.*, 1992), 50-95% of LGL (Polli *et al.*, 1987; Timonen *et al.*, 1990; Triozzi *et al.*, 1992), and 20-30% of T lymphocytes (Dianzani *et al.*, 1989; Hoshino *et al.*, 1993; McFarland *et al.*, 1992; Nielsen *et al.*, 1994; Patel *et al.*, 1987; Razvi *et al.*, 1995). In 1983, CD11b/CD18 surface expression by a subset of human peripheral blood T cells ($15\% \pm 5\%$) was demonstrated with the monoclonal antibody OKM1 (Wilson *et al.*, 1983). Four years later a $CD8^+CD11b^+$ subset of human peripheral blood lymphocytes was demonstrated to be the precursor phenotype responsible for LAK generation (Patel *et al.*, 1989). All T lymphocyte clones generated in that study were $CD11b^+$. Those authors showed cytolytic activity of the T cell clones to be dependent on direct contact of the clones with tumor targets and they

concluded that T cells expressing CD11b correlate very strongly with the capacity to mediate MHC-unrestricted cytolytic activity. That work was expanded by a second group (Dianzani *et al.*, 1989) who showed that after separation of $CD3^+CD8^+$ peripheral blood lymphocytes into $CD11b^+$ and $CD11b^-$ cells only the $CD8^+CD11b^+$ subset produced LAK activity. The $CD8^+CD11b^+$ cells had an LGL morphology while the $CD8^+CD11b^-$ cells did not. These authors clearly demonstrated the $CD8^+CD11b^+$ population to be heterogeneous. This heterogeneity may explain the designation of the $CD8^+CD11b^+$ phenotype as characteristic of so-called suppressor cells by some authors (Hornquivst *et al.*, 1993; Lebeck *et al.*, 1993) and by others as cytolytic T lymphocytes (CTL) that exhibit antiviral activity (McFarland *et al.*, 1992; Razvi *et al.*, 1995) or the precursor and effector lymphocytes that mediate LAK activity following IL-2 stimulation in murine (McFarland *et al.*, 1992; Razvi *et al.*, 1995) and human (Dianzani *et al.*, 1989) peripheral blood lymphocytes.

CD11b/CD18 has been clearly shown to be expressed by activated murine lymphocytes in this research and by others (Timonen *et al.*, 1990; MacFarland *et al.*, 1992; Triozzi *et al.*, 1992; Brooks-Kaiser *et al.*, 1993; Gosselin *et al.*, 1995; Ikemoto *et al.*, 1995), and to facilitate the homing of $CD8^+$ lymphocytes to sites of inflammation in mice (Nielsen *et al.*, 1994). Of particular interest to this dissertation is the fact that a subset of peripheral blood T cells present in all healthy humans has been shown to constitutively express the phenotype: $CD3^+CD8^+CD11b^+CD16^+CD56^+$ (Zupo *et al.*, 1993) and the beta-chain of the IL-2 receptor. While these $CD56^+$ T cells are only about 10% of T cells in peripheral blood, they are 50% of those found in the liver and exhibit constitutive "LAK" ability to lyse NK-resistant targets (Garcia-Barcina *et al.*, 1994), and are often increased in patients with malignancy (Takii *et al.*, 1994). Upon stimulation with IL-2, the $CD3^+CD8^+CD11b^+CD16^+CD56^+$ clones demonstrate cytotoxicity against NK-sensitive and

NK-resistant tumor targets (Zupo *et al.*, 1993), similar to the murine and human IAL described by this laboratory (Beno and Mathews, 1992; Beno *et al.* 1995; Forsyth and Mathews, 1996). However, long-term culture of IAL results in the variable loss of $CD16^+$ and $CD56^+$ markers (Roussel *et al.*, 1990).

Other researchers have described distinct $CD11b^+$ subsets of IL-2 activated $CD56^+CD16^+$ "NK" cells, depending on the culture conditions with IL-2 (Vuianovic *et al.*, 1993). These data may explain why this laboratory and others (Zunino and Hudig, 1988; Beno and Mathews, 1992; Arancia *et al.*, 1995; Beno *et al.*, 1995) have described that NK cells bind but do not inhibit *C. albicans* growth, while others (Levitz and Dupont, 1994) show anti-cryptococcal activity in positively selected $CD56^+CD16^+$ "NK" or $CD4^+$ T cell IAL. In addition, a clearly defined but small subset of circulating $CD4^+$ T cells (Gane *et al.*, 1992; Hoshino *et al.*, 1993) are now known to express high levels of CD11b as well as the $\alpha_4\beta_1$ and $\alpha_5\beta_1$ integrins (the traditional fibronectin receptors). Perhaps these $CD4^+CD11b^+$ cells also have an anti-candidal role as defined for $CD4^+$ lymphocytes (Romani *et al.*, 1993; Romani and Howard; 1995).

Another intriguing alternative is that the recently described Ly-49 MHC class I receptors on murine NK lymphocytes, with homology to the C-type lectin superfamily to which the selectins also belong, interact with microbial polysaccharides or possibly CD11b/CD18 to deliver an inhibitory signal (Leibson, 1995). Also, the CD8 molecule could modulate cytotoxic cell signaling because it has been demonstrated to interact directly with a microbial ligand secreted by *Trypanosoma brucei* (Olsson *et al.*, 1993). Also, a number of T cell and NK cell specific signalling molecules associated with adhesion have been described which could explain these differences including the itk tyrosine kinase expressed in IL-2 stimulated T cells (Siliciano *et al.*, 1992), a 50 Kd integrin-associated protein (Reinhold *et al.*,

1995), and T cell specific G-proteins now known to transduce adhesion signals (Clark and Brugge, 1995). Mac-1 has been shown to be activated by signals from other ECM receptor integrins as well (Simms and D'Amico, 1995). Finally, Mac-1 has been shown to interact through the lectin domain with glycosylphosphatidylinositol-linked (GPI-linked) proteins in the plasma membrane. A recent review suggested this co-receptor modulation by Mac-1 may ultimately prove to be as important as adhesion mediated by Mac-1 (Stewart *et al.*, 1995). Receptors shown to associate with Mac-1 are FcγRIII (CD16)(Sehgal *et al.*, 1993), urokinase-type plasminogen activator (uPA) receptor, (Xue *et al.*, 1994), the fMLP receptor, CD14, and FcγRII (Petty and Todd, 1993), and possibly L-selectin (Simon *et al.*, 1995). Cross-linking of these receptors which bind to the Mac-1 lectin domain initiates signaling via the Mac-1 cytoplasmic domains (Zhou *et al.*, 1993; Simon *et al.*, 1995). In addition to transducing signals from these receptors, it has been proposed that Mac-1 regulates the activity of serine protease enzymes released during degranulation by cytotoxic cells (Altieri, 1995a; Altieri, 1995b). More detailed phenotypic/functional studies of $CD8^{-}CD11b^{+}$ lymphocytes are required to unravel the differences in Mac-1 signaling in different lymphocyte subsets (Gane *et al.*, 1992; Stewart *et al.*, 1995).

The Th_1 versus Th_2 characteristics of immune response have received much attention in recent years and have been highlighted by the progression of a Th_1 (CMI, IL-2, IL-12, IFN-γ; including $CD8^{+}$ cytotoxic T cells) to a Th_2 response (predominantly humoral immunity, IL-4, IL-10; including $CD8^{+}$ helper/suppressor T cells) in the progression of AIDS (Maggi *et al.*, 1994; Paganelli *et al.*, 1995). Such a Th_1 to Th_2 progression also characterizes the progression of infection with *C. albicans* (Romani *et al.*, 1993; Puccetti *et al.*, 1995), and treatment with anti-IL-4 cytokine reagents cures murine *C. albicans* infection (Puccetti *et al.*, 1994). Interestingly, a similar Th_1 to Th_2 progression and cure by anti-IL-4 characterizes

infection with *Leishmania major*, another CD11b/CD18 ligand (Kelso, 1995). Similarly, two recent studies show that treatment of $CD8^+$ lymphocytes from HIV^+ patients with Th_2 cytokines depressed anti-HIV activity, while treatment with the Th_1 cytokine IL-2 augmented anti-HIV activity (Barker *et al.*, 1995; Kinter *et al.*, 1995).

In fact, gastrointestinal colonization, usually as thrush, by *C. albicans* during HIV infection is a clinical hallmark of disease progression to acquired immune deficiency syndrome in over 70% of patients (Sternberg, 1994). Experimental application of corticosteroids to the oral mucosa is associated with depletion of intraepithelial lymphocytes and what the authors described as a "first line of defense" resulting in a four hundred-fold increase in oral thrush (Deslauriers *et al.*, 1995). Recently, a $CD8^+$ T cell population has been demonstrated as characterizing immediate protection in HIV infection by production of the chemokine MIP-1β. Chemokines have been shown to upregulate Mac-1 expression in lymphocytes. Perhaps this is a subset of the constitutively expressed $CD3^+CD8^+CD11b^+$ T cell population which exhibits a Th_1 type response to IL-2 (Dianzani *et al.*, 1989; McFarland *et al.*, 1992; Zupo *et al.*, 1993) shown by data from this laboratory (Beno and Mathews, 1992; Beno *et al.*, 1995; Forsyth and Mathews, 1996) and in this dissertation to have anti-*C. albicans* activity as well. An overlap in the subsets would explain susceptibility to opportunistic infection in AIDS patients. Besides probably functioning to eliminate both HIV and *C. albicans* in response to IL-2 therapy (Dianzani *et al.*, 1989; McFarland *et al.*, 1992). $CD8^+CD11b^+$ T cells also can be directly infected with HIV (Mercure *et al.*, 1994) which reduces their ability to kill fungi (Harrison *et al.*, 1995), and both unopsonized and opsonized HIV bind to Mac-1 (Thieblemont *et al.*, 1993). Perhaps HIV binds to Mac-1 without cross-linking in some way, and induces an anergic state in $CD8^+CD11b^+$ lymphocytes, resulting in the "default" Th_2 phenotype (Ausiello *et al.*, 1993). $CD8^+CD11b^+$ T cells have been shown

to be decreased in the blood of HIV-infected patients (Indraccolo *et al.*, 1995).

While $CD8^+$ lymphocytes have been implicated in many protective and harmful roles in disease processes (O'Rourke *et al.*, 1993), often the expression of CD11b/CD18 has not been assessed. Therefore, how central a role these lymphocytes play in the immune response needs to be much more rigorously evaluated. Patients with $CD8^+$ lymphocytosis syndrome who have a proliferation of $CD3^+CD8^+$ lymphocytes experience extremely low ($< 1\%$) incidence of opportunistic infection. $CD8^+CDIIb^+$ lymphocytes are absent in patients with leukocyte adhesion deficiency (LAD). This is a rare autosomal recessive disorder caused by the absent or severely reduced cell surface expression of β_2 integrins, including Mac-1. Patients often die in the first years of life of a severe bacterial or fungal infection which usually begin as skin or gastrointestinal infections (Paller *et al.*, 1994). There is strong evidence that $CD8^+$ cytotoxic cells become "converted " to $CD8^+$ T-helper lymphocytes during the Th_1 to Th_2 progression to full-blown AIDS accompanied by a syndrome of recurrent fungal infections (Maggi *et al.*, 1994; Paganelli, 1995). Th_2 $CD8^+CD11b^+$ T cells have been implicated as exacerbating herpes simplex virus infection by IL-4 production in mice (Ikemoto *et al.*, 1995), and this effect was reversed by injecting mice with a β-glucan derivative (Utsunomiya *et al.*, 1995) or a lipid-arabinomamman from *M. tuberculosis* (Kobayashi *et al.*, 1994).

Most significantly, in light of the identification of Mac-1 as the only β-glucan receptor (Thornton *et al.*, 1996), *C. albicans* cell wall derived mannan and β-glucan stimulate a Th_1 cytokine pattern (IL-1β, TNF-α, IL-6, and IL-2) in culture of peripheral blood mononuclear cells (but not IL-4 or IL-10)(Ausiello *et al.*, 1993). This response was severely depressed in mononuclear cells from AIDS patients. However, the protein synthesis inhibitor cycloheximide caused a superinduction of IL-4 and IL-10 production by these cells (Ausiello

et al., 1993). Similar results were obtained by stimulation of the cultures with IL-2 (Ausiello *et al.*, 1993). Similarly, injection of these mannan and β-glucan constituents results in removal of tumor in murine lymphoma (Cassone *et al.*, 1981), and intraperitoneal injection of heat killed *C. albicans* elicits LAK cells *in vivo* (Scaringi *et al.*, 1991). Integrins can deliver a co-stimulatory signal capable of activating cytolytic T cells by adhesion to the *Yersinia* invasin protein (Ennis *et al.*, 1993; Cronin *et al.*, 1994). This has been demonstrated for Mac-1 using a peptide fragment of ICAM-2 which activates killing (Li *et al.*, 1993; Li *et al.*, 1995). In fact, costimulation of cytotoxic T cells by β-glucan has been measured and is similar in magnitude to stimulation with IL-2 (Muto *et al.*, 1993). Perhaps these β-glucans and polysaccharides activate the $CD8^+CD11b^+$ T cells via cross-linking Mac-1 as suggested by others for macrophages (Thornton *et al.*, 1996), and as has been shown *in vitro* for T cells (Muto *et al.* 1993) and NK cells (Di Renzo *et al.*, 1991). The $CD8^+$-helper lymphocytes described in AIDS may very well be the $CD8^+CD11b^+$ "suppressor" cells of CMI which respond poorly to IL-2 described by others in mice (Brookes-Kaiser *et al.*, 1993; Hornqvist *et al.*, 1993; Ikemoto *et al.*, 1995) and humans (Lebeck *et al.*, 1992; Koyama and Fukao, 1994).

$CD3^+CD8^+CD11b^+$ lymphocytes have also been implicated as central to several autoimmune diseases including chronic fatigue syndrome (Tirelli *et al.*, 1994), early onset diabetes in humans (Hehmke *et al.*, 1995), islet cell destruction in the NOD mouse (Goldrath *et al.*, 1995), chronic progressive multiple sclerosis (Balashov *et al.*, 1995), and Whipple's disease (Marth *et al.*, 1994), as well as promoting bone marrow transplant survival (Lebeck *et al.*,1992; Dolstra *et al.*, 1995). Distinct subsets of $CD8^+CD11b^+$ T lymphocytes including "suppressor" subsets have been described in human bone marrow transplant recipients in which $CD3^+CD8^+CD11b^+$ lymphocytes may comprise as much as 80% of circulating T

lymphocytes (Lebeck *et al.*, 1992). There are more examples in addition to these. Much of the work investigating $CD8^+CD11b^+$ T lymphocytes has come in the last two years as investigators begin to appreciate the importance of this T cell subset in immunity which now includes anti-viral murine memory T cells (Razvi *et al.*, 1995). Quite clearly, the $CD8^+CD11b^+$ T cells are not a trivial population but in fact appear to play a variety of important roles in addition to anti-microbial defense. In fact, their involvement in both anti-microbial response and autoimmunity implies a central role in both processes. This is illustrated by the same $CD8^+CD11b^+$ T cells possibly mediating both epidermal anti-fungal defense and psoriasis (Rosenberg *et al.*, 1994).

The intracellular pathogen *Listeria* is also known to induce a strong $CD8^+$ T cell response, and one fascinating prospect for immunomodulation involves introducing specific recombinant antigens or cytokines expressed by *Listeria* to modulate the $CD8^+$ cell response (Goossens, *et al.*, 1995). Perhaps attenuated *Listeria*, HIV, or *C. albicans* could be used in such a way to modulate the $CD8^+CD11b^+$ T cell response to these or other diseases. In contrast to the activating effect of β-glucans, a class of compounds called leumedins have also been described which specifically inhibit Mac-1 function (Endeman *et al.*, 1996).

These data represent the first identification of the adhesion molecule on activated T lymphocytes which mediates binding to this or any opportunistic fungal pathogen. Such cell mediated immunity may be the predominant form of anti-*C. albicans* defense at the mucosal and epithelial sites that encounter *C. albicans* on a daily basis (Fidel and Sobel, 1994). Within an IL-2 activated population, $CD8^+$ T cells mediate anti-*C. albicans* effects (Beno *et al.*, 1995). In human peripheral blood, 20-30% of T cells are $CD11b^+$ (Hoshino *et al.*, 1993), and the $CD8^+$, IL-2-activated population which mediates MHC-unrestricted cytotoxicity are $CD8^+CD11b^+$ (Dianzani *et al.*, 1989). These are also the only peripheral

blood $CD8^+$ cells that express perforin (Nakata *et al.*, 1992). Mac-1 may represent a broadly specific receptor for MHC-unrestricted cytotoxicity of microorganisms by $CD8^+$ lymphocytes which may also facilitate lymphocyte homing to areas of fungal infection (Nielsen *et al.*, 1994). The novel $\alpha_d\beta_2$ is also expressed by a population of human PBL which are mostly $CD8^+$ including LGL (Danilenko *et al.*, 1995). How this population overlaps with the $CD11b^+CD8^+$ population will be an interesting area for future investigation. Verification of these data *in vivo* could have important implications for treatment of *C. albicans* infections.

In light of data from this dissertation, to the list of microbial-mammalian cell interactions should be added the RGD-mimetic inhibitable recognition of *C. albicans* by the Mac-1 integrin on the surface of murine and human IL-2 activated lymphocytes. This knowledge of the specific immune response to *C. albicans* should contribute to designing therapy for patients infected with this important pathogen and possibly other fungal pathogens which together are responsible for enormous morbidity and for 40% of deaths in hospital acquired infections each year and continue to increase.

CHAPTER 5.

CONCLUSIONS

The significant conclusions of this dissertation research are:

1. Adhesion of mIAL, hIAL, and YT lymphocytes to *C. albicans* hyphae is specific and therefore reproducibly quantifiable. This was demonstrated by the development and publication of an *in vitro* assay for quantifying mammalian cell adhesion to *C. albicans* hyphae and it's use in identifying the principal structure on mIAL which mediates adhesion to *C. albicans* hyphae (Forsyth and Mathews, 1993).

2. Certain extracellular matrix, blood proteins, and RGD-mimetic peptides inhibit adhesion of mIAL, hIAL, and YT lymphocytes as well as NIH-3T3 fibroblasts expressing recombinant human CD11b/CD18.

3. MAb blocking studies identify the principal molecule mediating adhesion of mIAL, hIAL, and YT lymphocytes to *C. albicans* hyphae is the integrin CD11b/CD18.

4. The identity of CD11b/CD18 as the receptor for *C. albicans* hyphae on mIAL, hIAL, and YT lymphocytes was confirmed using NIH-3T3 fibroblasts which demonstrated little adhesion to *C. albicans* hyphae while NIH-3T3 fibroblasts expressing CD11b/CD18

demonstrated specific adhesion to *C. albicans* hyphae inhibited with mAbs to CD11b/CD18.

5. The identity of CD11b/CD18 as the structure mediating adhesion of mIAL, hIAL, and YT lymphocytes to *C. albicans* was further confirmed by experiments in which mAbs to CD11b/CD18 eliminated mIAL mediated growth inhibition of *C. albicans* hyphae in a dose dependent manner.

In addition to these broad overall conclusions, certain aspects of the data using specific inhibitors of adhesion have resulted in a unique model being proposed by this investigator for the interaction of lymphocyte CD11b/CD18 with *C. albicans* hyphae and perhaps other microbial surfaces. The relevant aspects of the data are:

First, mAbs to the I domain and the lectin-like domain of CD11b blocked lymphocyte adhesion to hyphae demonstrating both of these functional domains of Mac-1 participate in lymphocyte adhesion to *C. albicans* hyphae.

Second, certain ECM, blood protein, and RGD-mimetic peptides were demonstrated to mimic mAb inhibition of lymphocyte adhesion to hyphae by interaction with an integrin, probably Mac-1 as confirmed by the CD11b/CD18 transfected fibroblast data, on lymphocytes and not a structure on *C. albicans* hyphae. Several of these inhibitory proteins and peptides are previously documented to interact as ligands of CD11b/CD18 only when this integrin is activated.

Third, participation of the Mac-1 lectin-like domain in lymphocyte adherence to hyphae was confirmed by a profile of inhibition by carbohydrates such as NADG which closely resembles that profile described for inhibition of Mac-1 mediated adherence of neutrophils to the yeast *S. cerevisiae*.

Finally, the model proposes that interaction of Mac-1 through the lectin-like domain with microbial polysaccharides (and/or hydrophobic ligands) such as β-glucan results in cross-linking of Mac-1 molecules and the development of an activated conformational state in which the Mac-1 I domain MIDAS motif can interact with RGD-mimetic ligands (Figure 17). This activation probably results from interactions of the Mac-1 cytoplasmic and transmembrane segments with other molecules as a consequence of the cross-linking of Mac-1 as demonstrated for β_1 integrins.

In the specific case of mIAL, the conformational changes in CD11b/CD18 and the interaction of ligand induced binding sites with RGD-mimetic ligands such as complement(C3) fragments or microbial ligands on the surface of the fungus then triggers degranulation by $CD8^+CD11b^+$ cytotoxic lymphocytes resulting in growth inhibition of *C. albicans* hyphae.

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The final copies have been examined by the director of the
dissertation and the signature which appears below verifies
the fact that any necessary changes have been incorporated and
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The dissertation is, therefore, accepted in partial
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