ANALYSIS OF A MURINE LYMPHOCYTE PROLIFERATION-ASSOCIATED ANTIGEN (MALA-2): THE MURINE HOMOLOG OF THE HUMAN ICAM-1 MOLECULE

BY

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B.Sc., University of British Columbia, 1985

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE STUDIES

(Department of Pathology)

We accept this thesis as conforming

to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

December, 1989

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ABSTRACT

MALA-2 (Murine Lymphocyte Activated Antigen-2) is a murine cell surface antigen that is detected at high concentration on activated, proliferating lymphocytes, but only weakly on resting lymphocytes. It is thought to play an important role in lymphocyte activation since the rat monoclonal antibody YN1/1.7.4 which recognizes MALA-2 is capable of inhibiting the mixed lymphocyte reaction. Considering the central role of lymphocyte activation to the generation and maintenance of the immune response, I undertook the purification and biochemical characterization of MALA-2. In these studies, MALA-2 was isolated and purified to homogeneity using immobilized YN1/1.7.4 monoclonal antibody and sodium docecylsulphate-polyacrylamide gel electrophoresis. Biochemical characterization studies revealed that MALA-2 is a M_r 95-100 kD glycoprotein containing a protein backbone of approximately 66 kD, and N-linked carbohydrate chains amounting to a M_r of approximately 35 kD. Two dimensional gel electrophoresis suggested that MALA-2 has an isoelectric point of 4.9. Although it was previously suspected that MALA-2 might be associated with the transferrin receptor on the cell surface, this was shown not to be the case on NS-1 cells. Additionally, ^{32}P-orthophosphate labelling of MALA-2 on NS-1 or MBL-2 cells could not be detected. Finally, the partial amino acid sequence of MALA-2 was determined by sequencing trypsin-generated peptides from purified MALA-2. Computer-assisted homology comparisons of the MALA-2 partial amino acid sequences with other known sequences showed that MALA-2 shared its most consistent homology with a class of proteins known as the immunoglobulin superfamily.

Subsequent to this study, the partial amino acid sequences obtained within this study were used to construct oligonucleotide probes. These probes were used for the screening of cDNA libraries, facilitating the successful cloning of the MALA-2 gene. This, in turn, resulted in the identification of MALA-2 as the murine counterpart of the human ICAM-1 molecule, a protein known to play a significant role in intercellular adhesion and lymphocyte activation within the immune system. Significantly, results obtained from the biochemical characterization of MALA-2 carried out in this thesis have been confirmed by the subsequent nucleotide sequence data from the cloning of MALA-2.

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LIST OF ABBREVIATIONS

APC	Antigen presenting cell
BAM	Binding assay medium
bis	N, N'-methylene-bis-acrylamide
BSA	Bovine serum albumin
C	Constant region
CD	Cluster of differentiation (or cluster determinant)
cDNA	Complementary DNA
Con A	Concanavalin A
CPM	Counts per minute
CTL	Cytotoxic T lymphocyte
ELAM-1	Endothelial-leukocyte adhesion molecule
FCS	Fetal calf serum
HEPES	N-2-hydroxyethylpiperazine
HEV	High endothelial venule
HPLC	High Performance Liquid Chromatography
ICAM-1	Intercellular adhesion molecule-1
IEF	Isoelectric focussing
Ig	Immunoglobulin
IL	Interleukin
kD	kilodalton
LFA	Lymphocyte (or leukocyte)-function-associated antigen
LGL	Large granular lymphocyte
LPS	Lipopolysacharride
LT	Lymphotoxin
MAb	Monoclonal antibody
MAG	Myelin-associated glycoprotein
MALA-2	Murine activated lymphocyte antigen-2
MaRIg	Mouse anti rat immunoglobulin antiserum
2 ME	beta-mercaptoethanol
MHC	Major histocompatibility complex
M_r	Relative molecular mass
MuLV	Murine leukemia virus
NBRF	National Biomedical Research Foundation
NCAM	Neural cell adhesion molecule
NK	Natural killer cell
PBS	Phosphate buffered saline
pI	Isoelectric point
PTH	Phenylthiohydantoin
RaMIg	Rabbit anti-mouse immunoglobulin antiserum
RaRIg	Rabbit anti-rat immunoglobulin antiserum
RPM	Revolutions per minute
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
T_H	T helper cell
T_C	T cytotoxic cell
TCR	T cell receptor
TFA	Triflouroacetic acid
TNF	Tumor necrosis factor
V	Variable region

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DEDICATION

This thesis is dedicated to the spirit of Howard Randall (Randy) Baker; my brother, friend, protector, and guide. As you perservered and followed your heart, so did I watch and learn.

ACKNOWLEDGEMENTS

I would like to thank my family, epecially my mother, for your everpresent support and encouragement. I would like to thank Georgia, for inspiring me, believing in me, and for giving me the reason to believe in commitment. I would like to thank all those involved with the Terry Fox Laboratory for making my time there enjoyable and educational. I would like to thank Dr. Fumio Takei for his supervision and and critical analysis, and Dr. Gerald Krystal, for critical reading of the thesis manuscript, and for final guidance "onto the runway". I would also like to thank the members of my thesis committee for your suggestions and guidance throughout this project. Lastly, I would like to thank Dr. Anne Autor, for without your energetic involvement and support, the completion of this project would have been impossible.

CHAPTER ONE

INTRODUCTION: OVERVIEW OF RELEVANT TOPICS

1.1 GENERAL CONCEPTS

1.1.1 The Immune System

The vertebrate immune system is a versatile organ system involved in the recognition of extracellular elements throughout the body; it is capable of recognizing and distinguishing between normal host tissues, and foreign agents which gain entry to the body. Agents recognized as abnormal or foreign are subject to any of a variety of destructive elements ranging from inflammation and humoral immunity to cell-mediated cytotoxicity. Just as importantly, normal host tissue must be recognized as normal to prevent autoimmune activities, and to allow processes such as lymphocyte trafficking, cell differentiation, and leukocyte interactions to take place.

Collectively, the immune system exists in both solid organ and fluid states. Lymphocytes are organized in a solid fashion during early cell development, enter the fluid circulation upon maturation, and utilize both solid organ and fluid organizational states upon contact with foreign antigens or abnormal tissues. Organization in lymph nodes, for example, allows a very high number of interactions between leukocytes. This enables an immune response to be mounted very quickly, and potentially through multiple immune effector mechanisms. The fluid environment of the circulation allows effector cells and other immunological components (i.e. immunoglobulins, complement) to reach specific sites and agents throughout the body. In the fluid environment, leukocytes are free to migrate throughout the circulatory system. These cells are also able to migrate out of the blood and into solid tissues at sites of injury and

inflammation, and in specific regions of specialized endothelium associated with lymphoid organs.

Lymphocytes compose a class of leukocytes which are agranular and mononucleate. They are derived from hematopoietic stem cells which originate in the bone marrow, and which may enter organs of maturation such as the thymus, fetal liver, gut-associated lymphoid tissue, or the bone marrow itself. Those that enter the thymus are called "thymus-dependent", or T-lymphocytes, while those that mature outside of the thymus are called "thymus-independent", or B-lymphocytes (in reference to the Bursa of Fabricus, the organ of B-lymphocyte maturation in birds). Maturation is the process in which lymphocytes differentiate into immunocompetent cells which express specific receptors for antigens. Having completed the maturation process, mature virgin or "resting" lymphocytes migrate from their organ of maturation and form the lymphocyte fraction of peripheral blood leukocytes.

T-lymphocytes constitute approximately 70-80% of peripheral blood lymphocytes, while B lymphocytes constitute approximately 10-20%. Secondary lymphoid organs such as lymph nodes are the main sites of initiation of immune responses, in part due to the great concentration of lymphocytes and antigen presenting cells in these locations. Within the lymph nodes, lymphocytes undergo a myriad of interactions, allowing activation and proliferation of lymphocytes which specifically recognize stimulating antigens. Thus, events leading to T-lymphocyte dependent humoral resonses, and cell mediated cytotoxicity are initiated through an efficient communication focus.

Activated T-lymphocytes mediate the proliferation of B-lymphocytes and their differentiation to antibody-secreting plasma

cells. This subset of T-cells is additionally capable of inducing proliferation of other activated T-lymphocytes, and so are called "helper" T-lymphocytes. T-lymphocytes are further able to regulate immune responses by limiting immune responses through another subset of cells called "suppressor" T-lymphocytes. Finally, T-lymphocytes can differentiate into cytotoxic, or "killer" T-lymphocytes, and are able to induce cytotoxic activities in macrophages through lymphokine stimulation. Functional subsets of T-lymphocytes can be discriminated through characteristic cell surface phenotypes.

1.1.2 Monoclonal Antibodies

Collectively, microbial organisms compose an extrememly large variety of biochemical structures, and utilize many pathogenic routes for entry and replication in hosts. The vertebrate immune system has developed the ability to recognize a virtually unlimited number of chemical configurations through a humoral response to antigen. This is accomplished through rearrangement of B-cell DNA encoding various regions of the immunoglobulin molecule, and through somatic mutation mechanisms which increase the structural diversity of the immunoglobulin idiotype (Hood et al., 1984). Further, there are several different classes of antibody which can be synthesized, each having its own special properties. IgM is known to have the greatest affinity for complement; IgG is capable of crossing the placenta, and is known to be especially effective against viral and bacterial agents; and secretory IgA is present in mucous secretions, thereby providing immunity to the respiratory and gastrointestinal tracts. Monoclonal antibodies (MAbs) differ from

antibodies produced in a polyclonal response in that they are identical, both with regard to class, and with regard to the idiotypic recognition site (the polyclonal recognition of a single antigen reflects the response of a heterogeneous group of cells, secreting antibodies of distinct classes and idiotypes). Thus, whereas a humoral response reflects the involvement of multiple gene rearrangements, MAbs are the product of a single gene rearrangement, propagated indefinitely. Monoclonal antibodies are secreted by hybridomas, a fusion of immune spleen cells with an immortal (reproduces itself indefinitely) cell line such as a myeloma cell line. This technology was pioneered by Kohler and Milstein (1975) and results in an immortal hybrid cell line which is capable of secreting large amounts of specific, identical MAbs. The production of human MAbs has been a far mor arduous task than the production of murine MAbs; chromosome instability and limited sources of appropriate cells are two of the most significant problems. Recently, human MAb technology has become more feasible, and prospects for more extensive use in the future are becoming better (for review, see Thompson, 1988).

Murine MAbs have been invaluable to the isolation and identification of many cell surface antigens, and have been very useful in the exploration of biological function and molecular interaction. Murine MAbs are also of importance to human diagnostics and therapeutics. Because of problems inherent in the development of reliable sources of human MAbs, murine MAbs have played important roles in cancer therapy (for review, see Catane & Longo, 1988), renal translantation (for review, see Norman, 1988), radioimmunodetection of cancer (for review, see Murray & Unger, 1988), and immunocytochemistry (for review, see Bosman, 1988).

MAbs are useful because of their homogeneity in recognition of a

single epitope (specific antigenic determinant). Although the degree of specificity of this recognition is functionally very high, there are two types of cross-reactivity which have been observed (Yelton et al., 1980). One form is due to the ability of antibody to recognize similar or identical epitopes on distinct molecules. A second form of cross-reactivity can result from heterogeneity in the antibody culture supernatent. This is caused by the secretion of both antibody identical to that from the parental speen cell, and secretion of hybrid antibody molecules from the fusion of parental spleen cell and myeloma cell components. Thus, although MAbs can be used to isolate a particular cell surface molecule through immunoprecipitation, they can not be depended upon to deliver antigen in pure form; the possibility of some degree of specific coprecipitation always exists. Although one must be aware of these properties when using MAbs, they do not detract significantly from their usefulness.

Hybridomas are capable of secreting 10-50 ug of MAb per ml of culture supernatent, and 1-10 mg per ml of ascites fluid (Kohler et al., 1986). Thus, they are capable of supplying a virtually limitless supply of uniform, specific recognition elements. Murine MAbs have been extremely valuable tools in the recognition and isolation of cell surface antigens, exploration of biological functions, and human diagnostics and therapeutics. One further advantage remains; murine MAbs can be used to explore the <u>in vivo</u> functions of the antigens which they recognize in appropriate animal models, a function which is difficult to accomplish with MAbs recognizing human antigens, for ethical reasons.

1.2 THE IMMUNOGLOBULIN SUPERFAMILY AND T-CELL ACTIVATION

1.2.1 Molecules with Immunoglobulin Homology

Molecules with sequence homology to the variable and/or constant regions of immunoglobulins (Igs) comprise a group called the Ig superfamily. Criteria for superfamily membership also stipulates that each member has at least one region of conserved Ig-like tertiary protein structure, called a homology unit. The homology unit consists of approximately 110 ammino acids which form two sheets of antiparallel beta-strands (for review, see Williams and Barclay, 1988).

Members of the Ig superfamily include the immunoglobulins (Edelman, 1970), MHC molecules (Kaufman et al, 1984; Lew et al. 1986), B_2 microglobulin (Becker & Reeke, 1985), the TCR alpha/beta (Hood et al., 1985; Kronenberg et al, 1986), the TCR delta/gamma (Hata et al, 1987), CD3 gamma, delta, epsilon, and zeta subunits (Gold et al, 1986, 1987), CD2, CD4, and CD8 (Maddon et al., 1985; Sewell et al., 1986; Clark et al., 1987; Littman et al., 1987a; Johnson et al, 1987), CD28 (Aruffo & Seed, 1987), ICAM-1 (Simmons et al., 1988; Staunton et al., 1988) expressed on lymphocytes, and Thy-1 (Williams & Gagnon, 1982b; Seki et al., 1985) expressed on both T lymphocytes and neural cells. Members of this family which are not expressed on lymphocytes but are instead expressed on neural cells include NCAM (Cunningham et al., 1987), myelin-associated glycoprotein (MAG) (Arquint et al., 1987; Salzer et al., 1987; Lai et al., 1987), and the peripheral myelin glycoprotein, Po (Lemke & Axel, 1985; Lai et al., 1987). Other members include the poly Ig receptor (Mostov et al., 1984), Fc receptor (Ravetch et al., 1986), receptors for growth factors including platelet derived

growth factor (PDGF-R) (Yarden et al., 1986), colony stimulating factor-1 (CSF-1R) (Sherr et al., 1985), and the c-fms oncogene (Coussens et al., 1986; Lai et al., 1987).

The functions of this class of immunoglobulin-like molecules are similar; they are involved in recognition interactions. The immunoglobulin-like domains that these molecules have in common provide a framework in which variations in the loops of sequence between folds can potentially lead to differences in recognition specificity (Williams, 1984). This is true for Igs and the clonotypic TCR (Novotny et al., 1986), and may also play a role in other Ig-related recognition molecules (Williams, 1985).

1.2.2 Activation through the T Lymphocyte Receptor Complex

Specific recognition of an antigen, in combination with recognition of autologous major histocompatibility complex antigens, or simple recognition of allogenic MHC molecules, activates T lymphocytes through the T cell receptor/CD3 complex. Foreign antigen is recognized by the clonotypic T cell receptor, while MHC molecules are recognized concurrently by the clonotypic T cell receptor, and either CD4 or CD8. It has been demonstrated that in CD8+ T-cells, recognition of MHC results in optimal activation only when CD8 participates with the T-cell receptor complex, and that CD8 involvement is necessary for I1-2 production (Samstag et al., 1988); further, it is suggested that the MHC class I molecule acts as the physiological cross-linking ligand for CD8 and the T-cell receptor. Emmrich et al. (1986, 1987) have shown that antibody cross-linking of either CD4 or CD8 with the TCR stimulates IL-2 receptor (IL-2R) expression on T-cells. Kupfer and Singer (1987) and

Kupfer et al. (1988) have shown that CD4 and the TCR cocluster (cap) upon interaction with an antigen presenting cell (APC), that this cocapping occurs in the region of cell-cell contact, and that it is dependent on the presence of both appropriate antigen, and the appropriate MHC class II molecules on the APC. (CD4 and the TCR are independent of each other in the resting T-cell membrane.) Signal transduction is also a function of the CD4 receptor, as antibody directed against CD4 inhibits the mobilization of cytoplasmic free calcium, which occurs in response to CD3 cross-linking. In contrast, when anti-CD3 and anti-CD4 are cross-linked together, the degree of calcium mobilization is substantially increased over that of CD3 cross-linking alone (Ledbetter et al., 1988). Further, it has been shown that human T4 cells can be activated by cross linking of class I MHC molecules alone (Geppert et al., 1988), clearly demonstrating the ability of these molecules to transduce signals across the membrane. Similarly, CD8 is also thought to be capable of signal transduction, sending a signal which contributes to IL-2 production and responsiveness to IL-2 (Samstag, 1988). The recognition of foreign antigens in the context of MHC molecules, as described above, is referred to as "MHC restriction" (Klein, 1975).

The TCR is a heterodimer, made up of either an alpha/beta subunit combination, or a gamma/delta subunit combination. The alpha/beta TCR, like immunoglobulin, has a variable antigen-recognizing region, a constant region, and a small hydrophobic region which enables it to anchor within the membrane (Williams, 1985). Variability in the antigen recognition site arises from rearrangement of a large number of variable (V), diversity (D), and joining (J) segments, similar to the

rearrangement of immunoglobulin genes. However, unlike the generation of variability in the antigen-recognition region of Ig, the T cell receptor genes do not gain variability from point mutations (Collins & Owen, 1985). Both the alpha/beta (Reinherz et al., 1982; Meuer et al., 1983, Reinherz et al., 1983a; Kaye & Janeway, 1984; Weiss & Stobo, 1984; Brenner et al, 1985) and gamma/delta TCRs (Borst et al., 1987; Ferrini et al., 1987; Pantaleo et al., 1987a; Faure et al., 1988) are non-covalently associated with the CD3 complex. The murine alpha and beta subunits of the TCR have a similar M_r of 45-50 kD, while the gamma and delta TCR subunits have M_r of 35 kD and 45 kD respectively. CD3 complex consists of gamma (M_r21=kD), delta (M_r=26kD), epsilon (M_r=25 kD) zeta (homodimer of two M_r=16 kD chains), and p21 (M_r=21 kD, present in mice, not present in human CD3 complex.) (Samelson et al., 1985a; Oettgen et al., 1986). The CD3 complex chains contain long cytoplasmic tails, while the alpha and beta subunits of the TCR only have very short intracellular regions; for this reason, CD3 is thought to effect signal transduction upon stimulation of the TCR/CD3 complex. This is supported by the fact that signal transduction is triggered by antibodies directed against the CD3 complex in the absence of accessory cells (Tsoukas et al., 1985; Geppert & Lisky, 1987; Ledbetter et al., 1988). GTP dependence of the transduction of the CD3 mediated signal infers involvement of G-protein activity (Mustelin, 1987). Recent evidence has shown that a GTP binding protein regulates the phosphorylation of the CD3 complex in human T lymphocytes (Davies et al. 1988), which occurs in response to lymphocyte activation (Davies et al, 1987). This phosphorylation has been shown to involve protein kinase C (Friedrich et al, 1988), and is associated with down-regulation of the

T3/TCR complex (Cantrell et al., 1985). Only those T3/TCR complexes which are phosphorylated are endocytosed (Krangel, 1987).

1.2.3 The Alternative Pathway of Activation: The CD2 Molecule

CD2 is a glycoprotein with a M_r of 50 kD. CD2 has also been referred to as T11 (Meuer et al., 1984b), LFA-2 (Sanchez-Madrid et al., 1982), and the sheep erythrocyte receptor (Kamoun et al., 1981). CD2 is expressed on all T lymphocytes, large granular lymphocytes (LGLs) with NK activity, and thymocytes (Sanchez-Madrid et al., 1982; Krensky et al., 1983). CD2 is involved in both adhesion (Takai et al., 1987) and activation of T cells (Kamoun et al., 1981; Sanchez-Madrid et al., 1982; Krensky et al., 1983; Meuer et al., 1984; Yang et al., 1986; Springer et al., 1986). The ligand to which CD2 binds is LFA-3, which has a very wide tissue distribution on both hematopoietic and non-hematopoietic cells (Krensky et al., 1983; Plunkett et al., 1987; Selvaraj et al., 1987; Takai et al., 1987). The binding of CD2 to LFA-3 is independent of divalent cations (Spits et al., 1986), and contributes to antigen-independent T cell conjugate formation (Shaw et al., 1986). The CD2-mediated pathway of T cell activation requires the presence of a functional CD3 complex in the T cell leukemic line Jurkat (Bockenstedt et al., 1988; Alcover et al., 1988), but interestingly is independent of cell surface expression of CD3 in LGLs (June et al., 1986). Binding of LFA-3 to CD2 leads to an increase in the intracellular concentration of free Ca^+ ions through a mechanism involving the hydrolysis of membrane phosphoinositides. The hydrolysis of phosphatidylinositol biphosphate (PIP_2) results in the formation of inositol triphosphate (IP_3) which is involved in the release of Ca^+ from internal stores, and 1,2

diacylglycerol (DAG) which is the physiological activator of protein kinase C (Pantaleo et al., 1987). Interestingly, it has been shown that protein kinase C has different regulatory roles with respect to CD3 and CD2. While activation of protein kinase C down-regulates the expression of CD3, it stimulates increased expression of CD2. Prolonged stimulation of protein kinase C inhibits cell activation via CD3, and promotes activation via CD2 (Cantrell et al., 1988). Thus, it seems reasonable to assume that upon recognition of antigen presented by an APC, activation of the T lymphocyte is enhanced by concurrent binding of CD2 to its ligand LFA-3, thus contributing stability to the antigen-TCR complex interaction (Shaw et al., 1986; Spits et al., 1986), and setting in motion the synergistic activation pathways of CD3 and CD2 (Yang et al, 1986, 1988). Activation through the CD2 pathway is dependent on an intact CD2 cytoplasmic domain, inferring that this domain is involved in the genesis of a stimulatory signal (Bierer et al., 1988). Cross-linking of CD3 to CD2 results in a marked enhancement of T cell activation (Andereson et al., 1988), suggesting that CD2 plays an important role with CD3 in the activation of T cells, in addition to its role in cellular adherence.

1.2.4 The Major Histocompatibility Complex

The major histocompatibility complex (MHC) genes encode cell surface proteins which fall into two classes. Class I MHC antigens are found on the surface of virtually all cell types, and constitute what are normally considered to be "transplantation antigens". The expression of class II MHC antigens is primarily restricted to cells within the lymphoid system, such as macrophages and other

antigen-presenting cells (APC), including Langerhans cells of the skin, B-cells, and some activated T-cells. However, under special circumstances, another cell type can be recruited to an APC phenotype; gamma interferon induction of class II MHC in endothelial cells (Pober et al., 1983; Geppert & Lipsky, 1987), signifies a unique, inducible role of endothelium in antigen presentation. Products of the MHC serve to bind processed foreign antigens to be recognized by the clonotypic T-lymphocyte receptor complex, while the MHC molecules themselves are recognized by specific T-lymphocyte recognition molecules known as CD4 and CD8. CD4 is known to bind directly to class II molecules (Doyle & Strominger, 1987; Gay et al, 1988), and CD8 is known to bind directly to class I molecules (Norment et al., 1988; Rosenstein et al., 1989) (both even in the absence of T-lymphocyte receptor-antigen interactions). Because distribution of CD4 and CD8 are mutually exclusive on mature T-lymphocytes, two functional subsets of T-lymphocytes are distinguished on the basis of their recognition of MHC molecules (CD4+ & CD8+ subsets of T-cells). More generally, CD4+ cells are helper or delayed hypersensitivity associated, whereas CD8+ cells are suppressor or cytotoxic activity associated.

Murine class I antigens span the membrane, and comprise a glycoslylated heavy chain of 44-47 kD, that is non-covalently linked to B_2 microglobulin (11.5 kD), (not encoded by the MHC genes) (Steinmeitz, 1984). Like class I molecules, class II molecules are encoded by the MHC genes. Class II molecules also span the membrane, and are composed of two glycosylated proteins, which are strongly associated to form a two-chain structure. The larger chain has a M_r of 32-36 kD and is termed alpha, while the smaller chain has a M_r of

25-30 kD and is termed beta. Both class I (Townsend et al., 1985, 1986) and class II molecules (Babbit et al., 1985) have been shown to have affinity for peptide antigens, and it has been shown that T cells recognize a complex of MHC class II/antigen. (Ashwell et al., 1986; Watts et al., 1986). Both MHC class I and II are highly polymorphic, with 50 different alleles identified in class I K and D loci alone (Klein, 1979).

1.2.5 Other activation antigens within the immunoglobulin superfamily

In addition to MHC class I and II, CD4, CD8, the TCR/CD3 complex, and CD2, there are other molecules within the immunoglobulin superfamily which are involved in the activation or interactions of T lymphocytes. These include murine Thy-1, human CD28, and ICAM-1. Thy-1 is a membrane glycoprotein of M_r 18-25 kD. Thy-1 is expressed on both lymphoid lineages and neural cells, and is attached to the membrane through a phosphatidyl inositol moiety (Low & Kincade, 1985). As the hydrolysis of phosphatiyl inositol is involved in several physiological processes, it is possible that this anchor confers special properties to those proteins which are anchored by it in the membrane (for review, see Low and Saltiel, 1988). Thy-1 has been shown to function as a signal transduction molecule in both T cells and transfected B cells (Kroczek et al., 1986), and like CD2, appears to require the coexpression of the CD3/Ti complex in order to influence activation of T cells (Gunter et al., 1987).

CD28 is a homodimer of two glycoproteins of M_r 44 kD (also known as T44). The homodimer is disulphide-linked, as it has an apparent M_r of 80-85 kD under non-reducing conditions (Moretta et al.,

TABLE I

LYMPHOID MOLECULES OF THE IMMUNOGLOBULIN SUPERFAMILY

Antigen	Distribution	Functions
Immunoglobulin	B cells, plasma cells.	Antigen recognition.
MHC Class I	Most vertebrate cells.	Transplantation antigens, corecognition marker on virally-infected cells for activation of T_C cells.
MHC Class II	Thymic epithelium, macrophages, dendritic cells, inducible on endothelium, B cells, some activated T cells.	Corecognition marker for activation of T_H cells and some T_C cells.
TCR alpha/beta	Most mature T cells.	Recognition of antigens in context of MHC.
TCR gamma/delta	CD3+ alpha/beta- T cells Thy-1+ dendritic cells, CD3+ LGL's.	Antigen recognition on some T_C cells, possible regulation of MHC-non-restricted cytotoxicity.
CD3 gamma, delta, epsilon, zeta subunits.	Most T cells, some NK cells.	Involved in signal transduction through the TCR/CD3 complex, down-regulated by protein kinase C.
CD2	All thymocytes, peripheral T cells, some LGL's.	Antigen-independent activation molecule. Expression increased by protein kinase C, ligand is LFA-3.

TABLE I (cont.'d)

LYMPHOID MOLECULES OF THE IMMUNOGLOBULIN SUPERFAMILY

Antigen	Distribution	Functions
LFA-3	Lymhoid and non-lymphoid cells.	Ligand for CD2, capable of triggering antigen-independent activation of T cells through CD2.
CD4	Most helper/inducer & suppressor/inducer T cells.	Recognition of MHC class II molecules. Capable of signal transduction.
CD8	Most cytotoxic and suppressor T cells.	Recognition of MHC class I molecules. Capable of signal transduction.
CD28	Majority of peripheral T cells, some thymocytes, plasma cells.	T cell activation in the presence of phorbol ester (PMA) or accessory cells, synergism with anti-CD3.
Thy-1	Neural cells, T-cells	Involved in signal transduction
ICAM-1	Epithelial cells, macrophages, dendritic cells, lymphocytes (esp. activated lymphocytes), inducible on fibroblasts and endothelium.	Ligand for LFA-1; adhesion molecule involved in lymphoid interactions and esp. inflammation.

1985; Martin et al., 1986). It is expressed in humans on resting and activated T lymphocytes, some T cell leukemias, and a small population of thymocytes (Hansen et al., 1980). Antibodies directed against CD28 are capable of enhancing and sustaining CD3 activated cells for extended periods (Ledbetter et al., 1985), without involving inositol lipid metabolism and resultant mobilization of Ca^{+} from intracellular stores (Pantaleo et al., 1986). Recent evidence suggests that the signal transduced through CD28 may play a very important role in CD3/TCR mediated T-cell responses (Damle at al., 1988).

The last molecule of this immunoglobulin-related family is also the most recently discovered, ICAM-1; this molecule shall be examined in detail in Section 1.3.5.

1.2.6 Neural Molecules with Immunoglobulin Homology

Interestingly, within the immunoglobulin superfamily are several molecules expressed on cells of neural origin. These molecules include the previously mentioned Thy-1 molecule, which is also expressed on T lymphocytes, the myelin-associated glycoprotein (MAG) (Salzer et al., 1987), and NCAM (Barthels et al., 1987; Cunningham et al., 1987; Matsunaga & Mori, 1987; Williams, 1987). Of significance to this thesis is the relationship between MAG, NCAM, and ICAM-1, which share a high degree of homology (Simmons et al., 1988). ICAM-1 and NCAM share homology between all five of their extracellular domains (Ibid). NCAM shares a similarly homologous relationship with CD2, yet ICAM-1 and CD2 are not nearly as extensively related (Ibid). Thus, it has been speculated that some precursor of NCAM has given rise to both CD2 and ICAM-1 molecules of the immune system (Ibid).

This is one of many relationships which unites the immune system with the nervous system. As a discussion of this topic is beyond the scope of this thesis, the following references are recommended for further information regarding this relationship: (Roszman & Brooks, 1988; Benveniste, 1988; Bost, 1988; Blacock, 1988; Fontana et al., 1987).

1.3 ADHESION MOLECULES WITHIN THE IMMUNE SYSTEM

1.3.1 The Role of Adhesion

Cellular adhesion is a necessary attribute of the cells involved in immune and inflammatory processes. Adhesion is requisite to leukocyte differentiation, trafficking, activation, and proliferation. Examples of immunological mechanisms requiring adhesion are (1) the extravasation of lymphocytes through post capillary high endothelial venules (HEV) enabling the recirculation of these cells between the blood and lymphoid organs (Rasmussen et al., 1985), (2) extravasation of leukocytes at sites of inflammation (Freemont & Ford, 1985), (3) interactions between effector and regulatory T cells (Inaba and Steinman, 1985), (4) interactions between T cells and antigen presenting cells (APC), whether they be macrophages, B cells, dendritic cells, or endothelial cells (Hirschberg et al., 1980; Chesnut et al., 1982; Inaba et al., 1984), (5) T cell help for B cells (Tedder et al., 1985), and (6) cell-mediated lysis of virus-infected or tumor target cells (Martz, 1977).

Several molecules have been defined which make specific contributions to cell adhesion in the immune system, and which influence the functions of lymphocytes and other leukocytes. The molecules LFA-1, ICAM-1, CD2, LFA-3, CD4 and CD8 are all known to enhance antigen specific functions (Springer et al., 1987), while LFA-1, ICAM-1, CD2, and LFA-3 are known to contribute additionally to antigen-independent T-lymphocyte adherence. These molecules act as receptor-ligand pairs. The ligands for CD4 and CD8 are MHC class II and class I molecules respectively. The ligand for CD2 is LFA-3, and a ligand for LFA-1 is

ICAM-1. All of these molecules except LFA-1 are members of the immunoglobulin superfamily, and three of these four pairs of receptor-ligands represent adhesive interactions between members of this superfamily. LFA-1 and ICAM-1 represent the first known example of natural receptor-ligand adhesive interactions between a member of the integrin superfamily (LFA-1) and a member of the immunoglobulin superfamily (ICAM-1) (Staunton et al., 1988). Although ICAM-1 does not contain the classic integrin recognition sequence (Simmons et al., 1988) ARG-GLY-ASP (RGD) (Rouslahti & Pierschbacher, 1986), its interaction with LFA-1 may be by virtue of a portion of its sequence which is very similar in sequence and in biochemical properties, ARG-GLY-GLU (RGE) (Horley et al., 1987).

1.3.2 Endothelial Cells

The endothelium is now being recognized as playing a very interactive role in the immune system. Pober (1988) has reintroduced the term "endothelial activation", defining it similarly to macrophage activation (Adams & Hamilton, 1984), as "quantitative changes in the level of expression of specific gene products (i.e., proteins) that, in turn, endow endothelial cells with new capacities that cumulatively allow endothelial cells to perform new functions." Endothelial cells thus become activated upon stimulation by cytokines, especially IL-1 (interleukin-1) alpha and beta, TNF (tumor necrosis factor/cachectin), LT (lymphotoxin), and IFN-gamma (interferon-gamma). The cytokine-mediated activation of endothelial cells includes the increased expression of the normal endothelial surface MHC class I molecule and the induced expression of MHC class II molecules (Pober et al., 1983,

Collins et al., 1984); reorganization of cytoskeletal elements in vitro (Stolphen et al., 1986); increased endothelial adhesiveness in vitro (Yu et al., 1985; Masuyama et al, 1986); facilitation of antigen-presentation in vitro (Geppert & Lipsky, 1985); induction of the transient expression of ELAM-1 (endothelial-leukocyte adhesion molecule-1) (Bevilacqua et al., 1987); and induction of prolonged and stable expression of ICAM-1 (Pober et al., 1987).

It is beyond the scope of this thesis to cover the range of this topic even briefly; the above description is meant to increase awareness that the endothelium is not an inert permeability barrier, but is instead actively involved in many physiological processes including adhesion of leukocytes, trafficking and homing of lymphocytes, and acute and chronic inflammation.

1.3.3 Lymphocyte Homing

It has been postulated that cells will migrate to places favorable for their adhesion, the operating principle being that migration is facilitated by adhesive interactions which do not immobilize the cell, while immobilization occurs only at sites where very strong adhesive interactions (Rouslahti & Pierschbacher, 1987) take place. One of the most important functions of cell migration in vertebrates is the process of lymphocyte homing and recirculation. Lymphocyte homing refers to the organ- or region-specific adhesion to endothelium by lymphocytes. This facilitates lymphocyte escape from the vascular compartment (extravasation) which is known to be involved in the normal process of lymphocyte recirculation or trafficking. Recirculation of lymphocytes is initiated in specific regions of

endothelium associated with lymphoid organs called post capillary high endothelial venules (HEV). The microscopic appearance of HEV were apparently described as early as 1898 by Thome. The endothelium of HEV are modified, composed of polygonal-columnar or -cuboidal cells: "high" in comparison to the pavemented endothelium characteristic of most vascular endothelia. Large numbers of lymphocytes can be seen in the lumen and in the various layers of the venule walls. Cahill and coworkers (1976) estimated that 10^4 lymphocytes leave the bloodstream in a single lymph node every second, and may increase by an order of magnitude upon antigen stimulation.

The phenomenon of lymphocyte recirculation was first recognized by Gowans in 1964, and has since been shown to be extremely important in the maintenance of immune surveillance. Lymphocyte extravasation is promoted by adhesion to endothelia, and this adhesion is mediated by cell surface determinants on both the lymphocyte and the endothelial surface. HEV are normal tissue components of lymphoid organs, but a similar morphology is also induced at sites of chronic inflammation. Coupled with other information, this suggests that the HEV morpholgy is a product of immune cell activity. First, it has been shown that rat lymph node HEV revert from the cuboid form to the flat endothelial form when deprived of afferent lymphatics (Hendricks et al., 1980, Hendricks & Eestermans, 1983). Second, lymphocytes are capable of extravasation at sites of acute inflammation outside of lymhoid tissue, before the HEV-like morphology has developed (Freemont & Ford, 1985). Third, the elaborated products of activated lymphocytes, such as interferon-gamma, have been shown to induce the expression of an HEV-specific antigen (Duijvestij et al., 1986). It has thus been postulated that the

development of the HEV morphology may be linked to persistent stimulation by immune cells (Pals et al., 1989).

Adhesive interactions between lymphocyes and lymphoid tissue HEV have been shown to be organ-selective; although all HEV are capable of promoting extravasation of lymphocytes, different lymphocytes exhibit distinct preferences for adhesion to, and migration through the HEV of particular types of tissues. This selectivity exists with regard to at least three different tissues including peripheral lymph nodes, Peyers's patches, and the synovium of inflamed joints (Butcher et al., 1980; Jalkanen et al., 1986a; and Chin et al., 1988). Selectivity for specific tissues allows lymphocytes to become segregated on the basis of their tissue preference into distinct populations, and helps to explain the prevelence of B cells in Peyer's patches, T cells in peripheral lymph nodes, and region-specific localization of antigen-stimulated effector cells (for review, see Berg et al., 1989).

Tissue specificity of lymphocyte migration through HEV suggests that HEV express a class of lymphocyte-specific adhesion molecules which are not expressed in typical endothelia, and which vary between the HEV of different lymphoid or inflammatory tissues. Indeed, this is the case; the term "vascular addressins" has been coined (Butcher et al., 1987) to represent tissue-specific endothelial molecules, while the lymphocyte molecules which recognize these endothelial markers are called "homing receptors". A recently described example of an addressin is the mucosal HEV antigen defined by the MECA-367 and MECA-89 MAbs (Streeter et al. 1988). These antibodies inhibit the binding of lymphocytes to mucosal lymphoid tissue HEV, but not non-mucosal lymphoid tissue HEV. Known lymphocyte homing receptors are defined by the

antibodies anti-gp90, Hermes-1, -2, -3, (in humans) and MEL-14 (in mice). These antibodies recognize glycoproteins with a M_r of 90 kD, and a common acidic isoelectric point (pI=4.2)(Gallatin et al., 1983; Jalkanen et al., 1986, 1987). Hermes-1 likely recognizes a constitutive portion of gp90 homing receptor family, in that it is capable of blocking lymphocyte binding in HEV of all tested human tissues. MEL-14 blocks binding of lymphocytes to murine HEV, and cross reacts with Hermes-1, yet is only capable of blocking adhesion to lymph node HEV, and not mucosal HEV in humans.

The cycle of recirculation of lymphocytes ceases when the lymphocyte becomes activated by specific antigen. Lymphocytes then lose expression of functional homing receptors (Reichert et al., 1983), and regain the ability to remain fixed within the lymphoid tissue. Thus, it seems likely that the function of the MEL-14/Hermes-1 antigens is to direct the recirculation of mature, unstimulated lymphocytes.

Another lymphocyte molecule implicated as a homing receptor is the integrin LFA-1. Although LFA-1 is known to be involved in the binding of lymphocytes to endothelium (Mentzer et al., 1986) and lymphocyte homing (Pals et al., 1988; Hamann et al., 1988), its role in homing is likely to be an accessory one since inhibition of lymphocyte binding to HEV by anti-LFA-1 is not tissue-specific, and effects are not as pronounced as the MEL-14/Hermes group. Since LFA-1 expression is constitutive and widespread on leukocytes, it seems more likely that LFA-1 and the other integrins play an important role in leukocyte migration, and contribute accessory adhesive forces to that of the tissue-specific homing receptors.

1.3.4 The Integrins

The integrin superfamily consists of a group of alpha/beta, non-covalently associating hetero-dimeric cell-surface proteins which function as receptors for ligands which (usually) contain an RGD recognition sequence (Ruoslahti & Pierschbacher, 1986; Hynes, 1987). Within the integrins, three groups of molecules can be discerned on the basis of their beta subunits: The very late activation (VLA) antigen cluster, chicken integrin complex, and fibronectin receptor share a common $beta_1$ subunit; LFA-1, Mac-1, and p150,95 share a common $beta_2$ subunit, while the vitronectin receptor and platelet glycoprotein IIb/IIIa share a common $beta_3$ subunit (Hynes, 1987). Within each group, the shared beta subunit is paired with various alpha subunits which confer unique characteristics to each member of the family. The $beta_1$ subgroup are receptors for extracellular substrates, and have acidic isoelectric points; LFA-1 of the $beta_2$ subgroup binds to ICAM-1 and may have other ligands as well, while Mac-1 and p150/95 bind the C3bi component of complement; and in the $beta_3$ subgroup, the vitronectin receptor binds vitronectin, while the platelet glycoprotein IIb/IIIa binds fibronectin, fibrinogen, vitronectin, von Willebrand factor, and possibly thrombospodin (for review, see Hemler, 1987).

As LFA-1 is the receptor for ICAM-1, this section has its main emphasis on this molecule. LFA-1, as mentioned above, is most closely related to Mac-1 and p150/95, on the basis of sharing identical beta subunits which have a M_r of 95 kD, collectively referred to as CD18. LFA-1 is differentiated from Mac-1 and p150/95 by the fact that each of these molecules has a unique alpha subunit: the alpha subunit of LFA-1

is referred to as CD11a, and has a M_r of 180 kD; the alpha subunit of Mac-1 is referred to as CD11b, and has a M_r of 170 kD; and the alpha subunit of p150/95 is referred to as CD11c, and has a M_r of 150 kD (Sanchez-Madrid et al., 1983). The importance of this subgroup of the integrins is made very apparent by the presence of a genetically linked immunodeficiency disease which results from the inability or decreased abilty to synthesize the beta chain common to these three molecules. This disease, leukocyte adhesion deficiency (LAD), is "characterized by recurrent bacterial infections, impaired pus formation and wound healing, and abnormalities in a wide spectrum of adherence-dependent functions of granulocytes, monocytes, and lymphoid cells." (Anderson & Springer, 1987). Mac-1 and p150,95 are shown to play very important functional roles in granulocytes and monocytes (Springer et al., 1984, 1986; Springer & Anderson, 1986) as LAD patients have deficent chemotaxis and phagocytosis of C3bi opsonized particles (Bowen et al., 1982). Recurrent bacterial infection in the absence of recurrent viral or fungal infections suggests that monocytes and granulocytes are most greatly affected by the loss of the CD18 (common beta subunit) molecule. It has been hypothesized that lymphocytes are less drastically affected by virtue of the fact that they only express, LFA-1, while granulocytes and monocytes express Mac-1, p150/95, and LFA-1 (Sanchez-Madrid et al, 1983; Krensky et al, 1983). This may also be due to other compensatory mechanisms expressed in lymphocytes, although the relative difference in effect is abrogated if the LFA-1 deficiency is too severe. In this case, CTL- and NK-mediated cytolysis is greatly depressed (Krensky et al, 1985).

The beta chain of the LFA-1 subgroup shares significant homology

with the beta chains of the other integrins (Kishimoto et al., 1987). As previously mentioned, the integrins as a group recognize their ligands at regions which contain the sequence RGD (Hynes, 1987), although the only described ligand of LFA-1 in the human system does not contain this sequence. Binding of LFA-1 to ICAM-1 may be due to the presence of a similar sequence (RGE) (Horley et al., 1989), or may be due to immunoglobulin-like adhesive properties of ICAM-1 (Simmons et al, 1988).

While the upregulation of expression of Mac-1 and p150/95 are thought to play an important role in their functions (Miller et al., 1987), LFA-1 expression tends to be far less variable. However, in some cases, LFA-1 expression does change. This has been documented through the increased expression of LFA-1 seen on human memory lymphocytes, which also exhibit an enhanced ability to produce interferon-gamma (Sanders et al., 1988), and by the demonstration that LFA-1^+ circulating monocytes can give rise to LFA-1^- macrophages after emigration into tissues (Kurzinger et al., 1982; Strassman et al., 1985), while stimulation of macrophages with interferon-gamma leads to reexpression of LFA-1 (Strassman et al., 1985).

Homotypic aggregation of resting lymphoid cell types has been shown to be LFA-1 dependent (Rothlein & Springer, 1986; Mazerolles et al., 1985), and independent of the other antigen-independent adhesion pathway mediated by CD2 and LFA-3, which requires activation of T lymphocytes before it becomes involved (Mazerolles et al., 1985). As previously mentioned (Sec. 1.2.3), the CD2/LFA-3 adhesion pathway contributes to activation of T-cells, since MAb to CD2 can activate T cells (Meuer et al., 1984), and LFA-3 binding to CD2 can contribute to

this activation pathway (Bierer et al., 1987; Hunig et al., 1987). The fact that the contribution of CD2 to T cell activation is lost without an intact CD2 cytoplasmic domain strongly implicates this molecule as being capable of signal transduction, as well as adherence (Bierer et al., 1988). The lymphocyte recognition elements CD4 (Ledbetter et al., 1988) and CD8 (Samstag et al, 1988) are also thought to be capable of transducing signals across the cell membrane. Recently, evidence provided by Pircher (Pircher et al, 1986) showed that a MAb to LFA-1 was capable of inducing murine T cell proliferation and interferon-gamma production. This has been supported by evidence that MAb to the alpha subunit of LFA-1 is capable of strongly enhancing the T cell proliferative response in CD3 stimulated cells (van Noesel et al., 1988; Carrera et al., 1988). Thus, like the other lymphocyte-function associated adhesion molecules, LFA-1 seems capable of delivering a significant transmembrane signal.

LFA-1 is involved in a myriad of immunological interactions. For example, T cell-mediated cytotoxicity depends on three sequential steps, starting with intercellular conjugate formation between the T effector cell and the target cell (Martz, 1977). It has often been supposed that antigen-specific recognition by the TCR initiated this contact, and that accessory adhesion molecules strengthen the bond, but it now seems likely that bond formation is dependent upon antigen-independent conjugate formation (Shaw & Ginther Luce, 1987; Blanchard et al., 1987), and that the involvement of the antigen-specific TCR is related to the delivery of the lethal hit (Blanchard et al., 1987). Both of these studies implicated LFA-1 in the first step, antigen-independent conjugate formation.

Similarly, the conjugation of helper T cells with B cells to provide B cells with proliferative signals was once thought to be mediated by the T4 (CD4) antigen, but has since been shown to be independent of T4, and instead dependent on the presence of LFA-1 on the T cell (Tedder et al., 1986; Howard et al., 1986). Homotypic adhesive interactions between B cells have also been shown to be mediated by the LFA-1 molecule (Mentzer et al, 1985) and LFA-1 is even thought to play a role in the discrimination between self and non-self (Benjamin et al., 1988). Antibody to LFA-1 can induce tolerance (Springer et al., 1987), patients with LAD accept HLA-mismatched bone marrow grafts from their parents, and anti-LFA-1 antibodies have been used successfully to mimic this condition, and have resulted in acceptance of of HLA-mismatched bone marrow grafts (Fischer et al, 1986). More recently, anti-LFA-1 antibody has been used to prevent, and even reverse graft-vs-host disease (GVHD) in mice with skin grafts (Shiohara et al., 1988).

Cellular interactions between T cells and monocytes have been found to depend on LFA-1, with no dependence on p150/95 or Mac-1, although all three of these molecules exist on the monocyte surface. The T-cell/monocyte interaction could be inhibited by pretreatment of either monocytes or T cells with anti-LFA-1, showing that LFA-1 on both cells is involved in the binding of these cell types (Dougherty & Hogg, 1987). Monoclonal antibodies directed against LFA-1 have also been found to inhibit the mixed lymphocyte reaction (MLR) (Davignon et al, 1981; Pierres et al., 1982). It has been suggested that "LFA-1 mediated contact is an essential step in cellular interactions in the immune system in general." (Hamann et al., 1986).

The interaction between LFA-1 and its ligand has been

shown to be heterophilic, since EBV-transformed cell lines derived from patients deficient in LFA-1 fail to self-aggregate, yet can aggregate with LFA-1^+ cells. Monoclonal antibodies were thus developed against LFA-1^- cells, and were screened for ability to inhibit aggregation of LFA-1^+ cells. The result was MAb RR1/1, which recognizes the first known ligand of LFA-1, intercellular adhesion molecule-1 (ICAM-1) (Rothlein et al., 1986).

1.3.5 The intercellular adhesion molecule (ICAM-1)

The human intercellular adhesion molecule (ICAM-1) (Rothlein et al. 1986), was first discriminated through antibodies raised against LFA-1 deficient cells, and screened for the abilty to inhibit phorbol ester induced aggregation of the LFA-1^+ EBV-transformed cell line JY. This cell surface protein was shown to be a single peptide chain with M_r of 90 kD. It has a 55 kD protein backbone (Dustin et al., 1986), and N-glycosylation sites are predicted by its primary sequence (Staunton et al., 1988). Also known as CD54 (Knapp et al., 1989), ICAM-1 has been shown to be a ligand of LFA-1 (Marlin & Springer, 1987; Simmons et al., 1988). Although the hallmark of the integrin superfamily is the binding of ligands which contain the RGD sequence (Hynes, 1987), ICAM-1 does not contain this sequence. Instead, the ICAM-1 molecule contains a similar sequence in which the last residue, aspartic acid, is substituted for by glutamic acid, an amino acid which bears the closest resemblence to aspartic acid in terms of structure and biochemical properties. Thus, the RGE sequence found at position 152 in the amino acid sequence of ICAM-1 (Simmons et al, 1988) may be the basis of recognition of ICAM-1 by LFA-1. The binding of

been shown to be energy-dependent (binding is inhibited by the addition of sodium azide plus 2-deoxy-D-glucose), temperature-dependent (reduced binding at 14 degrees centigrade, loss of binding at 4 degrees centigrade), and requires the presence of an intact cytoskeleton (binding inhibited by cytochalasin B) (Marlin & Springer, 1987).

The ICAM-1 molecule has been shown to be extremely important in its interaction with LFA-1 in a variety of leukocyte adhesion functions including T cell/T cell, T cell/B cell, T cell/monocyte, B cell/B cell, and T cell/endothelial cell interactions (Boyd et al., 1988; Makgoba et al., 1988; Dougherty et al., 1988; Dustin et al., 1988) involved in regulation of the humoral and cell-mediated immune responses, and in the process of inflammation. Although it is not the only ligand for LFA-1 (Dustin & Springer, 1988; Staunton et al., 1989), its widespread involvement in immunological interactions and the process of inflammation underscore the importance of this particular ligand of LFA-1. The second known ligand of LFA-1 is ICAM-2 (Staunton et al., 1989), a molecule with a predicted M_r of 46 kD. ICAM-2 contains two extracellular domains, which are 34% homologous to the two N-terminal-most domains of ICAM-1 (Ibid). Significantly, these two domains are responsible for the binding properties of ICAM-1 (Ibid). The primary structures of ICAM-1 and ICAM-2 show that they are members of the immunoglobulin superfamily; ICAM-1 having five extracellular immunoglobulin-like domains (Staunton et al., 1988), and ICAM-2 having two immunoglobulin-like domains (Staunton et al., 1989).

Recently, three classes of immunoglobulin-like domains have been distinguished: V, C_1, and C_2 (Williams & Barclay, 1988). ICAM-1 belongs to the C_2 class which corresponds to proteins involved in cell

adhesion (Staunton et al., 1988), members of which include CD2, LFA-3, MAG, and NCAM. ICAM-1 also contains conserved amino acid residues characteristic of the C_1 class, which corresponds to proteins involved in antigen recognition, but overall homology is most significant with the C_2 immunoglobulin-like domain class (Ibid), and the neural cell adhesion molecule (NCAM) (Simmons et al., 1988). The homology between NCAM and ICAM-1 allows alignment between all five extracellular immunoglobulin-related domains (Ibid) and the binding of NCAM is similarly dependent on divalent cations, although its binding is homophilic (Brakenbury et al., 1977). Significantly, CD2 has been shown to bear a similar resemblance to NCAM as does ICAM-1, yet CD2 and ICAM-1 exhibit only weak homology. Thus it seems that a precursor to NCAM has served as ancestor to both CD2 and ICAM-1 (Simmons et al., 1988). Other similarities exist between ICAM-1, NCAM, and the other members of the immunoglobulin superfamily (for reviews, refer to Sec. 1.1.5). This similarity, as well as many other known relationships between the immune and nervous systems, is unveiling a very complex, yet precisely structured system of what is now becoming referred to as the neuroendocrine-immune network (Roszman & Brooks, 1988).

It has been suggested that ICAM-1 plays a unique and essential role in inflammation (Boyd et al. 1989) since, although its expression is low on a wide variety of hematopoietic and non-hematopoietic tissues, its expression is high on tissues in areas of inflammation, or in lymph nodes which drain regions of inflammation (Dustin et al., 1986; Cotran et al., 1987). The wide distribution of ICAM-1, even at low levels, is very significant because ICAM-1 expression is inducible by inflammatory cytokines (see below). In contrast, ICAM-2 has not been found to be

inducible on any tissues tested thus far (Staunton et al., 1989). ICAM-1 expression can be dramatically increased through stimulation by inflammatory cytokines such as interferon-gamma, IL-1, TNF, or lymphotoxin (Dustin et al., 1986; Pober et al., 1987; Dustin & Springer, 1988). Endothelial cells can be rapidly induced to express, and sustain expression, of high levels of ICAM-1 by IL-1, TNF, and LT (Pober et al., 1987). In cultured umbilical vein and saphenous vein endothelial cell cultures, ICAM-1 expression was increased rapidly (over a period of one to eight hours), and then more slowly for a period of days, in agreement with the results of Pober's group (see above). This increased expression was maintained up to seven days, as long as IL-1 or TNF were present, but returned to basal levels of expression upon removal of inflammatory cytokines. Basal expression on these cells was 5-10 X 10^4 sites/cell, but after stimulation with TNF, its density increased to 3.5 X 10^6 sites/cell (Dustin & Springer, 1988). Experiments performed with dermal fibroblasts and with HL-60 cells suggest that upregulation of ICAM-1 in response to inflammatory cytokines occurs at the level of transcription (Dustin et al., 1986; Simmons et al., 1988). It is suggested that this expression promotes homing to and extravasation from the endothelium in inflammatory regions, where these cytokines are released. Activated lymphocytes in this area releasing interferon-gamma are then capable of enhancing ICAM-1 expression on the endothelium even further (Dustin et al., 1986). In fact, it may be that the role of ICAM-1 is not only to promote cell migration into areas of acute inflammation, it may also play an important part in antigen-presentation to those cells which invade the tissue as part of the inflammatory response. "It is significant that expression of ICAM-1

appears to be a general response of all tissues to cytokine stimulation." (Boyd et al., 1989).

ICAM-1 is expressed on non-hematopoietic cells such as vascular endothelium (especially HEV), mucosal epithelial cells, dendritic cells, and lymphoid tissues with high T lymphocyte populations (Dustin et al., 1986). These cells, including endothelial cells (Geppert & Lipsky) are known to be capable of antigen presentation, while mucosal cells are significant targets for invading microorganisms. ICAM-1 is also expressed on the keratinocytes of a variety of benign cutaneous skin lesions such as allergic contact eczema, and the extent of ICAM-1 expression has been positively correlated with the extent of mononuclear cell infiltration of the area (Wantzin et al., 1988). Contrastingly, there was no expression of ICAM-1 on normal keratinocytes, or the small number of mononuclear cells which exist in normal skin. ICAM-1 has been found to be the primary cell surface molecule on epidermal keratinocytes mediating the adhesion of these cells to T lymphoblasts, and its expression is induced most significantly by interferon-gamma (25-fold increase) and TNF (8-fold increase) which are capable of synergy (Dustin et al., 1988). This study also clarified the role of MHC class II expression induced in the same environment by interferon-gamma. It has been speculated that MHC class II is involved in allograft rejection (Lampert et al., 1982), however HLA-DR was shown to play no part in cellular contact, although it is still likely to play a role in antigen presentation.

Expression of ICAM-1 is highly regulated, being inducible in a wide variety of tissues. It can be induced in myelomonocytic cell lines by treatment of the cells with phorbol esters, and expression is

concurrent with adoption of the mature macrophage phenotype (Dustin et al., 1986). The majority of human monocytes tested have been shown to express high levels of cytoplasmic ICAM-1, and to express variable amounts of ICAM-1 on their surface, whereas the surface of resting T cells was ICAM-1^- (Dougherty et al., 1988). It is suggested that the intracellular store of ICAM-1 may allow its rapid recruitment to the cell surface of monocytes, without the delay involved with protein synthesis (Ibid). It has previoiusly been found that LFA-1 on the surface of both T lymphocytes and monocytes contribute to the adhesive interactions between these two cell types (Dougherty & Hogg, 1987). Combining these results, Dougherty et al. have suggested that induction of monocyte ICAM-1 expression is likely to be a significant initial event. "The physiological signals responsible for such induction remain to be determined, but include adherence to fibronectin. Interaction between T cell LFA-1 and monocyte ICAM-1 could enable these cells to adhere together and allow delivery of activation signals to the T cell and the induction of ICAM-1 expression on the T cell surface." (Ibid). Expression of ICAM-1 on the activated T cell surface may then be involved in interactions between the activated T cell, and other LFA-1^+ cells such as other T cells and B cells (Boyd et al., 1988). Anti-ICAM-1 antibodies have been shown to inhibit the homotypic binding of activated T cells, B cells, aggregation of mixed T cell/B cell populations, the mixed lymphocyte reaction, and T cell-mediated B-cell activation (Ibid).

The ICAM-1/LFA-1 interaction is central to immunological function, and is involved in most cell contact-mediated interactions. It is involved in the events which lead to both humoral and cell

mediated immunity (Boyd et al., 1988; Dougherty et al., 1988), and inflammation (Boyd et al., 1989). Considering its widespread involvement in immunity, it seems likely that abnormalities in ICAM-1 would be involved in some pathological states. Not suprisingly, the degree of expression of ICAM-1 on B cell tumors has been found to be correlated with the adhesiveness of these cells. Those B cell tumors with strong ICAM-1 expression formed large, solitary masses, mediated by a high degree of homotypic adhesions; those with low ICAM-1 expression exhibited diffuse, widespread distribution (Boyd et al., 1989). Because of the relatively consistent expression of LFA-1 in most stages of leukocyte development, and the highly inducible nature of ICAM-1, it is also suggested that ICAM-1 expression is the controlling factor in ICAM-1/LFA-1 interactions involved with homotypic adhesion, and that expression of ICAM-1 is increased on activated cells (Ibid). Also with regard to B cell tumors, it has been suggested that down-regulation of ICAM-1 and LFA-3 is responsible for this tumor's ability to evade T cell surveillance (Gregory et al., 1988). Thus, on hematopietic cells (which normally express LFA-1), increased expression of ICAM-1 can lead to enhancement of homotypic aggregation, while decreased expression is correlated with escape from T cell surveillance.

The use of anti-LFA-1 antibodies has been shown to be an effective therapy in promoting tolerance of bone marrow grafts in humans (Fischer et al., 1986), and both prevention and reversal of graft-vs-host disease in murine skin grafts (Shiohara et al., 1988). Since ICAM-1 is strongly expressed on activated endothelial cells and other APCs (which are involved in the initial stages of the immune response), and since it is an important ligand of LFA-1, it has been

suggested that ICAM-1 may be an appropriate antigen to direct therapy toward in the blocking of graft rejection (Boyd et al., 1989). There is potential that this and other immunological therapies are likely to be based upon the moderation of ICAM-1 in the future, but there is much still to be resolved regarding the in vivo function of ICAM-1. The use of animal models and antibodies specific for LFA-1 and ICAM-1 in vivo has been suggested as the only practical way to assess the physiological roles that these molecules play (Arfors et al., 1987).

1.4 THESIS OBJECTIVES

The objective of my thesis was to biochemically characterize a novel murine cell surface molecule, detected mainly on the surface of activated lymphocytes. This molecule, MALA-2 (Murine Activated Lymphocyte Antigen-2), is recognized by the monoclonal antibody YN1/1.7.4. MALA-2 is a single peptide chain, with a M_r of 95-100 kD under both reducing and non-reducing conditions. Expression of MALA-2 is highest on activated lymphocytes, but has been detected at low levels on non-activated lymphocytes and some non-lymphoid cells. Since the YN1/1.7.4 MAb has been shown to be capable of inhibiting the mixed lymphocyte reaction, it was thought to be involved in lymphocyte activation.

The objectives of this research were (1) to purify MALA-2 to homogeneity, (2) to determine the density of MALA-2 on lymphoid cell lines, (3) to determine the isoelectric point of MALA-2, (4) to determine if MALA-2 is a glycoprotein, (5) to examine a possible realtionship between MALA-2 and the transferrin receptor, (6) to

determine the partial amino acid sequence of MALA-2, and (7) to compare this data with existing information to gain insight into the functional identity of MALA-2. The objectives of this thesis have been achieved, and results presented herein have facilitated further research into MALA-2.

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CHAPTER TWO

MATERIALS AND METHODS

2.1 SOURCES OF MATERIALS

2.1.1 Animals

Fisher 344 rats were purchased from Charles River Canada Ltd. (St. Constant, Quebec, Canada).

2.1.2 Cell Lines

The NS-1 cell line is a BALB/c myeloma. The T lymphoma cell lines EL-4 and MBL-2 are of C57BL/6 mouse origin. EL-4 cells were chemically induced while MBL-2 cells were induced by Moloney leukemia virus (Mo-MuLV). All three of these cell lines were obtained from Dr. E.S. Lennox (MRC Laboratory of Molecular Biology, Cambridge, U.K.) These cell lines were grown in Dulbecco's modified minimum essential medium containing 5% fetal calf serum (FCS), 50 U/ml penicillin, and 50 ug/ml streptomycin.

2.1.3 Monoclonal Antibodies

The YN1/1.7.4 rat monoclonal antibody was generated in our laboratory from a fusion between rat myeloma Y3 and Fisher 344 rat spleen cells immunized with NS-1 cells, and boosted with additional NS-1 cells four days before sacrifice. The YE1 series of rat monoclonal antibodies was also generated in our laboratory from a fusion between the rat myeloma Y3 and Fisher 344 rat spleen cells immunized with ECA17.9.8, a mouse T cell hybrid of EL-4BU and Con A activated AKR spleen cells (Takei and Horton, 1981). All hybridomas were cloned twice. YE1/48.10.6 recognizes a murine T lymphocyte surface dimer called YE1/48 antigen. YE1/9.9.3 recognizes the

transferrin receptor in all proliferating mouse cells (Takei, 1983). YE1/21.2.1 recognizes the CD45 (T200) antigen (Trowbridge, 1978). YE1/30.4.1 recognizes the Thy-1 molecule (Williams and Gagnon, 1982). YE6/26.1.1 was similarly generated, using the MBL-2 cell line as immunizing cells (Takei, 1987), and recognizes the Moloney MuLV envelope protein gp70 (Nowinski et al., 1972).

2.1.4 Xenoantisera

Polyclonal antisera containing rabbit anti-rat Ig (RaRIg), rabbit anti-mouse Ig (RaMIg), and mouse anti-rat Ig (MaRIg) antibodies were separately developed and affinity purified in our laboratory. A rat immune antiserum was also developed by immunizing a Fisher 344 rat with purified MALA-2 (see 2.3.4 below).

2.2 BIOCHEMICAL TECHNIQUES

2.2.1 Cell Surface Labelling (Iodination)

Lymphocyte surface proteins were radiolabelled by the iodogen method (Markwell, 1978). In brief, 2-3 X 10^7 cultured cells were agitated in an iodogen coated (100 ug) vial in 0.5 ml PBS containing 0.5 mCi ^{125}I (Amersham Corporation, Arlington Height, IL) for one hour at 20 C (room temperature). Radiolabelled cells were then washed four times in PBS to remove residual unreacted ^{125}I.

2.2.2 Immunoprecipitation

Radiolabelled cells were lysed in 2-3 ml of 50 mM Tris-HCl buffer (pH 7.5) containing 1% (w/v) Triton X-100, 0.5% (w/v) bovine

serum albumin (BSA), 0.15 M NaCl, and 0.01% (w/v) NaN_3. Nuclei and insoluble materials were removed by microfuging for 10 minutes at 4 C. 30 ul of hybridoma culture supernatent was then added to the lysates, which were then incubated on ice for one hour. Agarose beads (30-50 ul of 50% suspension) coupled with RaRIg antibodies (2-4 mg/ml) were then added to the mixture for another two hour incubation at 4 C, and mixed slowly on a rotary mixer. After this incubation, the beads were washed with the same lysis buffer without BSA, and the bound immune complex was eluted from the beads by boiling in 50 ul SDS-PAGE sample buffer (with or without 2% 2-ME) for 5 minutes.

2.2.3 SDS-PAGE Analysis

Sodium dodecylsulphate (SDS) polyacrylamide gel electrophoresis (PAGE) analysis (Laemmli, 1970) was performed either using a Protean apparatus (16cm X 18cm X 1.2 mm slab gel, BIO-RAD Laboratories, Richmond, CA) or Mini-Slab apparatus (8 cm X 10 cm X 1mm, Idea Scientific, Corvallis, OR). Reduced protein markers were obtained from BIO-RAD Laboratories and include myosin (200 kD M_r), beta-galactosidase (116.2 kD M_r), phosphorylase B (92.5 kD M_r), BSA (66.2 kD M_r), ovalbumin (45.0 kD M_r), and carbonic anhydrase (31.0 kD M_r). These molecular weight standards were visualized by staining with Coomassie blue, or Silver staining. Specific radiolabelled antigens were detected by autoradiography on KODAK XAR films with Dupont Cronex intensifying screens (DuPont, Wilmington, DE).

2.2.4 Direct Binding Assay

Cells were first washed twice in binding assay medium (B.A.M.) consisting of Earls medium, 0.5% BSA, 10 mM HEPES, and 0.5% NaN_3. 2 10^5 cells were dispensed per well, and centrifuged at 1800 RPM in a Beckman TJ-6 centrifuge. 25 ul of non-radiolabelled (blocking) antibody was used to resuspend the cell pellets of control wells, while 25 ul of straight media was used to resuspend pellets of all other wells. To both test and control wells, 25 ul of ^{125}I-labelled antibody was added from a serially diluted range of 4 X 10^6 CPM/25 ul to 4 X 10^4 CPM/25 ul. This method was based on determination of the saturating level of the YN1/1.7.4 antibody on test cells. After adding the radiolabelled antibody, the wells were incubated for one hr. at 4 C. Cells were then washed three times in B.A.M., transferred to gamma counter tubes, and levels of radioactivity determined.

2.2.5 Chloramine T Iodination of Protein

10 ul of 1 M Tris HCl (pH 7.4), followed by 10 ul of 1.0 mg/ml of chloramine T solution was added to 20-50 ug of antibody (5-100 ul) sample. 5-10 ul of ^{125}I were then added, and the mixture held at room temperature for 15 min. At this time, 20 ul of 2 mg/ml sodium metabisulphite (NaS_2O_5) was added, followed by 10 ul of 0.1 M KI. This was held at room temperature for 2 min., then loaded onto a P30 (BIO-RAD) column, with a 1.0 ml bed volume. The column was run with PBS, fractions (7 drops/gamma tube) were collected by hand in a ventilated fume hood, and the radioactive fractions were pooled.

2.2.6 Two-Dimensional Gel Analysis (Isoelectric Focussing/SDS-PAGE)

Two dimensional gel analysis (IEF versus SDS-PAGE) was performed according to the method of O'Farrell (1975) using ampholines of isoelectric points (pI) 3.5-10.0 (LKB, Bromma, Sweden), an improvised IEF apparatus for tube gels (13 cm long), and the Protean Slab Gel apparatus (BIO-RAD). IEF tube gels were pre-focussed for 1-2 hours before running the gel. A mixture of proteins with known pI's (BSA, pI=4.9; carbonic anhydrase II, pI=5.9; horse myoglobin, pI 6.8, 7.2) were included in each sample along with an aliquot of ^{125}I-labelled purified MALA-2, and the tube gels were run at 500 V for 22 hrs. Tube gels were loaded onto the SDS-PAGE second dimension by inserting the tube gels horizontally within a long well along the top of the SDS-PAGE gel, and were set in place with agarose. The SDS-PAGE second dimension was run at 200 V until the dye front reached the bottom of the gel. IEF markers were visualized by Coomassie staining, while radioactively labelled MALA-2 was visualized by autoradiography.

2.2.7 Metabolic Labelling

3 X 10^7 NS-1 cells were washed twice in methionine-free media. Cells were then resuspended in 3 ml of methionine-free medium, with 10% dialyzed FCS. 1 mCi of ^{35}S-methionine was added, and the cells were incubated at 37 C for 4 hrs. Cells were then washed 3 times with ice cold PBS containing 5% FCS and 0.1% NaN_3. Cells were then lysed, and the lysate subjected to immunoprecipitation.

2.2.8 Phosphorylation Study

3 X 10^7 NS-1 cells were washed twice in phosphate-free medium. Cells were then resuspended in 3 ml of phosphate-free DMEM with 10% dialyzed FCS. 1 mCi of carrier-free ^{32}P-orthophosphate was added, and the cells were incubated at 37 C for 4 hrs. Cells were washed 3 times with ice cold PBS containing 5% FCS and 0.1% NaN_3. Cells were then lysed, and the lysate subjected to immunoprecipitation.

2.2.9 Generation of Polyclonal Antiserum

A Fisher 344 rat was immunized repeatedly at two week intervals with 5 ug of purified MALA-2 suspended vigorously in Freund's complete adjuvant. The rat was bled from its tail, and serum was tested for immune reactivity by western blot.

2.2.10 Western Blot

Analyzed material consisted of either an SDS-PAGE separated immunoprecipitation, or SDS-PAGE separated membrane preparation when testing for initial development of polyclonal immune serum. Membrane preparation is described below; immunoprecipitation has been described previously (Sec. 2.2.2). For the membrane preparation, 2 X 10^7 cells were washed three times with PBS, and resuspended in 10 mM Tris-HCl (pH 8.0). Cells were then sheared by force through a 26-gauge needle. Disrupted cells were then centrifuged at 2000 RPM for 5 minutes in a Beckman TJ-6 centrifuge to remove nuclei. Supernatent was recovered, and centrifuged in a 15 ml corex tube for one hour at 18000 RPM in a Beckman fixed angle (JA-20) rotor. The

pellet was then recovered, and resuspended in 200 ul of non-reducing sample buffer. The sample was boiled for 5 minutes, and microfuged at 15000 RPM for 15 min. This yielded approximately 260 ul of dissolved membrane in sample buffer. Blotting consisted of running 10-20 ul of membrane sample per lane on an SDS-PAGE minigel system, along with prestained molecular weight standards. The proteins separated on the minigel from either the membrane preparation or a specific immunoprecipitation were blotted onto nitrocellulose using a transblot (BIO-RAD) apparatus (200 mA overnight, or 220 mA for a minimum of 3 hours). The blotted nitrocellulose filter was then transferred to a petri dish containing NET buffer (50 mM Tris-HCl, pH 7.5, 5mM EDTA, 150 mM NaCl, 0.05% NP-40 [Sigma, St. Louis, MO], 5% gelatin, 0.5% BSA, 0.01% NaN_3). The nitrocellulose filter was stained by incubating the filter with hybridoma culture supernatent diluted 1:5 in NET buffer or 1:50 similarly diluted immune serum. 10 mls of this primary antibody dilution and the nitrocellulose filter blot were gently shaken in a petri dish for 1 hr. at room temperature. The blot was then washed four times in NET buffer, for 10, 20, 30, and 40 min. respectively. The washed blot was incubated with gentle shaking in 10 ml of radiolabelled MaRIg (second antibody) undergoing gamma-decay at 2 X 10^6 CPM/ml, washed again four times with NET buffer, and dried. The blot was then wrapped in Saran wrap, and was used to expose KODAK XAR films with Dupont Cronex intensifying screens (DuPont, Wilmington, DE).

2.2.11 Endoglycosidase F Analysis

Endoglycosidase F digestion of MALA-2 was carried out

according to the procedure of McIntyre and Allison (1984). 100 pg (5 ul of 20 pg/ul) of ^{125}I-labelled (3.2 X 10^5 CPM/ul) purified MALA-2 was incubated with various concentrations of endoglycosidase F (New England Nuclear, Boston, MA) in 0.1 M sodium phosphate buffer (pH 6.1) containing 50 mM EDTA, 1% (v/v) Nonidet P-40, and 0.1% SDS. Incubation was carried out at 37 C for various lengths of time up to 12 hrs. The digestion was terminated by transferring the samples to -20 C. Digestion samples were combined with SDS-PAGE sample buffer, and analyzed by SDS-PAGE.

2.3 PURIFICATION OF THE MALA-2 MOLECULE

2.3.1 Large scale preparation of cell lysate

Spinner flasks were seeded with NS-1 cells at a density of not less than 1 X 10^5 cell/ml, and were not filled past 650 mls. These large scale quantities of NS-1 cells were maintained in exponential growth in 1 litre spinner flasks, and cells were harvested at a density of less than 2 X 10^6/ml. Total harvested cell number in large scale preparations averaged 1 X 10^{10}. These cells were harvested by centrifugation, washed three times in PBS, and lysed in filtered lysis buffer containing 1% Triton X-100, 10mM Tris-HCl (pH 7.5), 0.85% NaCl, and 0.01% NaN_3 for 15 min. at 4 C. Typically, 800 ml of lysis buffer were used for 5 X 10^9 cells. The lysate was then centrifuged at 18000 RPM for 75 min. in a Beckman JA-10 rotor. The supernatent was recovered and lysate from 3 X 10^7 ^{125}I-labelled NS-1 cells (prepared as above) was added to the batch lysate at this time, allowing MALA-2 to be tracked in subsequent isolation and purification steps.

2.3.2 Affinity Chromatography

YN1/1.7.4 MAb was purified from ascites fluid by $(NH_4)_2SO_4$ precipitation (50% saturation) followed by DEAE Affi-gel blue chromatography (BIO-RAD). The column fractions were analyzed for IgG purity by SDS-PAGE. Purified antibody fractions were pooled, dialysed against 0.1 M $NaHCO_3$ (pH 8.0) and coupled to Affi-gel 10 agarose beads (BIO-RAD) at 2-4 mg per ml of packed beads. After coupling the beads were thoroughly washed with Earl's balanced salt solution containing 0.5% BSA, 10 mM HEPES (pH 7.2) and 0.01% NaN_3 to saturate the uncoupled sites. The beads were extensively washed with elution buffer followed by lysis buffer before each use.

The large scale NS-1 cell lysate was incubated with approximately 3 ml of YN1/1.7.4 MAb-coupled beads on ice for 4 hrs. with constant agitation. The beads were then collected by centrifugation at 2000 RPM for 10 min. in a Beckman TJ-6 centrifuge, and were packed into a column. The column was thoroughly washed overnight with 10mM Tris-HCl buffer (pH 7.5) containing 1% Triton X-100, 0.8% NaCl, and 0.01% NaN_3. The bound material was then eluted with 100 mM glycine-HCl buffer (pH 2.9) containing 0.05% Triton X-100, 0.8% NaCl, and 0.01% NaN_3. 1 ml fractions were collected, immediately neutralized with 0.5 ml of 1 M Tris-HCl buffer (pH 7.5), and radioactive fractions were pooled and concentrated in a Centricon 30 microconcentrator (Amicon Corp, Danvers, MS).

2.3.3 Preparative SDS-PAGE

Preparative SDS-PAGE was performed using the Protean apparatus from BIO-RAD. Precautions were taken to minimize the destruction of

amino acid residues on the purified protein within the gel. The acrylamide gels were polymerized overnight and were pre-run extensively with a pre-clearing protein of low molecular weight (i.e. lysozyme). Sodium glycothiolate was added at 0.1 mM in the cathode buffer before electrophoresis of the real sample.

Affinity isolated MALA-2 was concentrated by ultrafiltration in a Centricon 30 microconcentrator (Amicon Corp., Danvers, MS) at 20 C. An equal volume of non-reducing sample buffer was added to the concentrated MALA-2, which was then denatured at 55 C to minimize protein aggregation (Hunkapillar et al., 1983). The MALA-2 preparation was then separated on the 7.5% pre-run, pre-cleared preparative SDS-PAGE gel. After electrophoresis was complete, the gel was sandwiched between two layers of dialysis membrane and dried by heat and negative pressure. Autoradiography revealed the region of MALA-2 migration. This band was cut out, reconstituted in 0.5 X filtered SDS-PAGE running buffer (25 mM glycine, 12.5 mM Tris-HCl, and 0.05% SDS). MALA-2 was then eluted from the gel slice electrophoretically into a closed dialysis tube. The eluant was then concentrated in a Centricon 30 microconcentrator, and resuspended in an equal volume of 0.2 M Tris-HCl (pH 8.0), 4% SDS, and 100 mM dithiothreitol (DTT). The MALA-2 preparation was then heated in a boiling water bath for 10 minutes. Iodoacetamide was added to a concentration of 50 mM subsequent to heating, and was then stored in the dark at 37 C for 1 hr. The MALA-2 preparation was then run on a second SDS-PAGE preparative gel, similar to initial procedure, except that the sample was reduced and akylated (see above). The purified MALA-2 was eluted electrophoretically from this second gel, and

dialysed. The sample was then concentrated in a Centricon 30 concentrator, and stored at -20 C.

2.3.4 Assessment of Purity and Yield

A small quantity of purified MALA-2 was run on a 10% SDS-PAGE gel. The gel was stained by the silver staining method using silver nitrate (AnalaR, BDH, Toronto, ON). Development of the gel was arrested by apiration of the developing solution and replacement of the solution with water. The intensities of the resultant silver stained bands were compared with a range of (0.04, 0.1, 0.2, 0.5, and 1.0 ug) BSA quantitative standards stained in the same gel. Typical purified MALA-2 yield from a large batch of 1 X 10^{10} cells was approximately 80 ug. Considering the density of MALA-2 on NS-1 cells this amount represents a recovery of approximately 37% (80 ug from 300 ug projected maximum yield) in purified form.

2.4 PARTIAL AMINO ACID SEQUENCE DETERMINATION OF MALA-2

2.4.1 Tryptic Digestion of Purified MALA-2

Methanol precipitation and digestion of the precipitated MALA-2 sample was carried out according to the method used by Stearne et al. (1985). Between 50-80 ug (500-800 pmol) of purified MALA-2 was transferred to a siliconized Corex tube to which 9 volumes of high pressure liquid chromatography (HPLC) grade methanol (BDH, Toronto, ON) was added. The methanol had been precooled to -20 C. Addition of methanol was immediately followed by addition of TPCK-treated trypsin (Sigma, St. Louis, MO) at 1% of the weight of the

MALA-2 sample. The mixture was held overnight at -20 C, and then centrifuged for 45 minutes at 16000 X G and -5 C in a Beckman JA-20 rotor (and Beckman J2-21 centrifuge). The supernatent was carefully aspirated, and the sediment (precipitated MALA-2 and 1% trypsin) was vacuum-dried. The dried sediment was resuspended in 100ul of 0.1 M NH_4HCO_3 (pH 8.0) with 2 mM $CaCl_2$ and another 1% TPCK-treated trypsin, for a final w/w ratio of MALA-2:trypsin of 50:1. The digestion sample was then incubated at 37 C for 24 hrs.

2.4.2 HPLC Separation of Trysin-Generated Peptides of MALA-2

Following the trypsin digestion of MALA-2, the sample was made up to 3 M Guanidinium HCl (ARISTAR, BDH, Poole, England)/0.2% TFA by the addition of an equal volume of 6 M Guanidinium HCL/0.2% TFA. This mixture was incubated at 37 C for 30 min., prefiltered, and then fractionated on a BONDAPAK C_{18} reverse phase HPLC column (Waters Associates, Milford, MS; 3.9 mm X 30 cm). The column was pre-equilibrated in 0.1% TFA. The injected sample was separated on the basis of a gradient of prefiltered 0-60% acetonitrile (v/v) in 0.1% TFA, forming the mobile phase of the column. The gradient was run over a period of 105 minutes at a flow rate of 1 ml/minute. The HPLC system (Waters) utilized two model 510 pumps, a 660 automated gradient controller, a 490 programmable multi-wavelength detector, and a U6K manual injector. Absorbance set at 215 nm was recorded on an SE 120 recorder at a chart speed of 0.5 cm/min, and an absorbance range of 0.0 to 0.2 absorbance units. Fractions of the eluent were collected by hand in a manner correlating with observed absorbance peaks, and were stored at -20 C.

2.4.3 Microsequencing of Trypsin-Generated Peptides

Selected fractions corresponding to the sharpest, most symmetrical absorbance peaks were selected for amino acid sequencing, in an effort to select fractions containing only a single peptide. These fractions were sequenced (according to the method of Hunkapillar et al., 1983) in an automated gas phase protein sequenator (Model 470, Applied Biosystems) equipped with an on-line PTH (phenylthiohydantoin) analyzer. Sequencing was performed at the Tripartite Microsequencing Centre (University of Victoria, Victoria, B.C.).

2.5 COMPUTER-ASSISTED HOMOLOGY STUDIES

2.5.1 Searching the NBRF Protein Sequence Database with Partial Amino Acid Sequences of MALA-2 and the WORDSEARCH and FASTA Programs

Each of the trypsin-generated MALA-2 peptides was compared with the list of amino acid sequences in the NBRF (National Biomedical Research Foundation) database (release 21.0). Amino acid sequence comparisons were carried out with the WORDSEARCH program and with the FASTA program University of Wisconsin, Genetics Computer Group, Madison, WI) based on the algorithms of Wilbur and Lipman (1983), and of Pearson and Lipman (1988) respectively.

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CHAPTER THREE

RESULTS

3.1 PURIFICATION OF MALA-2

3.1.1 Density of MALA-2 on Lymphoid Cell Lines

The density of MALA-2 on three cell lines was examined using the direct binding assay. A known quantity of the YN1/1.7.4 MAb was radioactively labelled by protein iodination, and the level of radioactivity per ug of antibody was determined. Incubation of a known quantity of cells with the labelled antibodies facilitated a sensitive quantification of specifically bound antibodies. A serial range of labelled antibody was used to determine the level at which all YN1/1.7.4 binding sites were saturated on the various cell types. The level at which cell-bound radioactivity could not be increased by increasing concentrations of antibody was considered the level of saturation. The highest expressing cell line was the murine myeloma cell line NS-1. (See Table II for results).

3.1.2 Specific Immunoprecipitation of MALA-2 from the NS-1 Cell Line

MALA-2 was immunoprecipitated from the surface of ^{125}I-labelled NS-1 cells, using the YN1/1.7.4 antibody. Immunoprecipitations were carried out coccurrently with YE1/9.9.3 (positive control) specific for the transferrin receptor, and YE1/48 (negative control) specific for the YE1/48 antigen, not expressed on NS-1. Immunoprecipitated material was separated by SDS-PAGE under non-reducing conditions, and visualization was achieved by autoradiography (refer to FIG. 1). Two specific bands could be visualized, a sharply defined band detecting the transferrin receptor (lane a) at approximately 200 kD (M_r), and a diffuse band detecting

TABLE II

DENSITY OF MALA-2 ON LYMPHOID CELL LINES

Cell line	Origin	Density
NS-1	BALB/c myeloma	1.8×10^5/cell
EL-4	C57BL/6 T lymphoma	3.9×10^4/cell
MBL-2	C57BL/6 T lymphoma	4.4×10^4/cell

NOTE: A known quantity of cells of each cell line was washed and distributed among a series of wells. Control well cells were resuspended using 25 ul of culture supernatent of non-radiolabelled antibodies (blocking antibodies), while test cells were resuspended with 25 ul of straight media. Each of the wells then received 25 ul of radiolabelled antibody from a serially diluted range (4×10^6 CPM/25 ul to 4×10^4 CPM/25 ul). After incubation, the cells were washed, and levels of bound radioactivity measured. Control wells showed that bound radioactivity on the cells was specific, and could be abrogated by the blocking antibody. The level of saturation of antibody binding to cells was determined, and the total number of antibody binding sites calculated. For example, with the level of saturation of binding sites on NS-1 cells being 1.4×10^5 CPM, the calculation was performed in the following manner:

$$\frac{1.4 \times 10^5 \text{ CPM}/10^5 \text{ cells}}{3.2 \times 10^7 \text{ CPM/ug Ab}} = 4.4 \times 10^{-3} \text{ ug Ab}/10^5 \text{ cells}$$

$$\frac{6.02 \times 10^{23} \text{ Ab}}{1.5 \times 10^{11} \text{ ug}} \times 4.4 \times 10^{-3} \text{ ug Ab}/10^5 \text{ cells} = 1.8 \times 10^5 \text{ antibodies/cell}$$

MALA-2 at approximately 95-105 kD (M_r). The appearance of the band detecting MALA-2 is characteristic of immunoprecipitations of this molecule.

3.1.3 Isolation of MALA-2

Large scale NS-1 cell-lysate was incubated with YN1/1.7.4 MAb-coupled agarose beads. The beads were then thoroughly washed, and bound materials were eluted with low pH buffer (see Sec. 2.3.2, Materials and Methods). This resulted in the isolation of MALA-2 from the lysate, although the preparation at this point was still crude. FIG. 2. shows the degree of purity of the MALA-2 preparation, visualized in this SDS-PAGE gel (non-reducing conditions) by Coomassie blue staining on the left side of the gel, and by silver staining on the right side of the gel. This latter method is approximately 100 times more sensitive in detecting the presence of protein. Lanes h,i,j show that, although the most prominent band exists in the region corresponding to the M_r of MALA-2, there are many other contaminating proteins present, including likely transferrin receptor contamination at approximately 200 kD. Lanes h and i contain 5 ul of MALA-2 crude preparation (out of a total sample size of approximately 1800 ul), while lanes f and j contain 3 ul of crude MALA-2 preparation. Lanes f,g are stained with Coomassie brilliant blue which also detects the presence of proteins, but at a much less sensitive level. Although none of the contaminating proteins are visualized by Coomassie staining, this method does indicate that the major band of protein is localized within an area which is discrete relative to the corresponding area in the silver stain. Other lanes

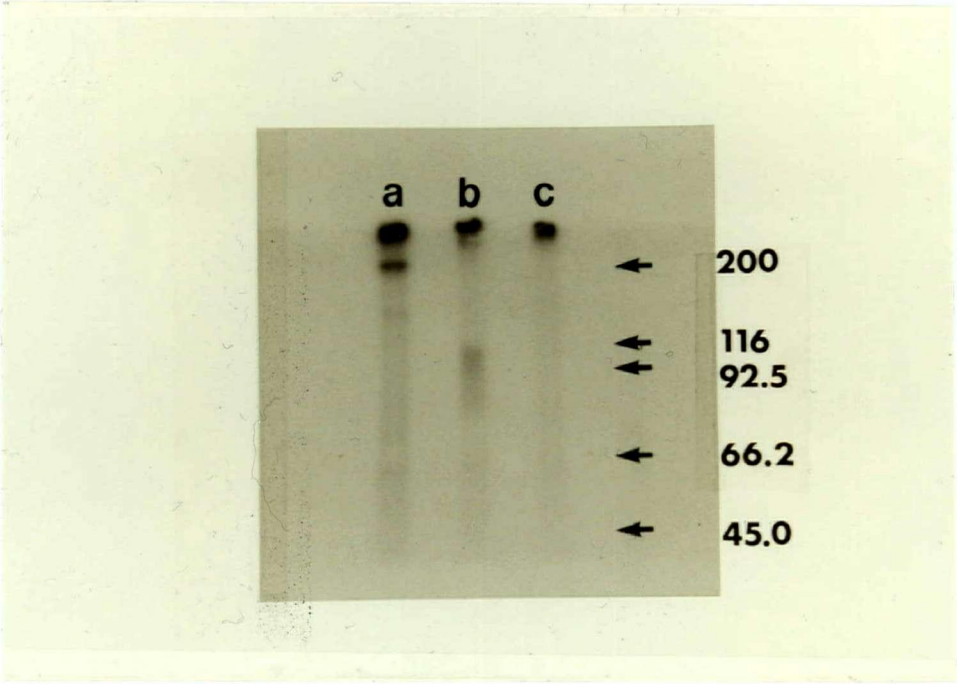

FIG. 1. **Specific immunoprecipitaion of MALA-2 from cell line NS-1.** NS-1 cells were cell-surface iodinated with ^{125}I, and then subjected to immunoprecipitation by the following monoclonal antibodies: (a) YE1/9.9.3, specific for the transferrin receptor, (b) YN1/1.7.4, specific for MALA-2, and (c) YE1/48, specific for the antigen of the same name, not expressed on this cell line. Immunoprecipitated proteins were analyzed by SDS-PAGE and autoradiography. The arrows and corresponding numbers indicate the positions of protein standards (visualized at an earlier stage by Coomassie blue staining of the gel itself). M_r of the protein standards is as follows: myosin (200 kD), beta-galactosidase (116 kD), phosphorylase B (92.5 kD), BSA (66.2 kD), and ovalbumin (45.0 kD). A diffuse band in the 90-115 kD range in lane b is representative of MALA-2 in typical immunoprecipitations.

correspond to the high molecular weight standards (lane a) or various known concentrations of BSA (b-e, k-n) used comparatively to determine the relative quantity of protein present in the test lanes (f-j).

3.1.4 Purification of MALA-2

MALA-2 was purified from the crude preparation by two consecutive steps of SDS-PAGE. The first preparative gel was run under non-reducing conditions to ensure separation of the transferrin receptor and any other contaminating proteins having a M_r distinct from MALA-2 under non-reducing conditions. The band corresponding to MALA-2 was then cut from the gel, eluted, concentrated, and run on a second preparative gel under reducing conditions. This second purification step increased purity of the MALA-2 preparation by removing proteins which migrate similarly to MALA-2 under non-reducing conditions, but which may migrate differentially under reducing conditions. The band corresponding to MALA-2 was cut from this second gel, and eluted. FIG. 3. shows the purity of the MALA-2 preparation subsequent to these purification procedures, run under reducing conditions and on an SDS-PAGE gel (10%). In this gel, the left side was stained by Coomassie blue, while the right side was stained with Silver stain. Lanes e,f,i, and j correspond to the purified MALA-2 preparation, and indicate a high degree of purity. BSA quantitative standards are represented by lanes a-d and k-o, while lane h represents high molecular weight standards. Considering the density of MALA-2 on NS-1 cells determined by direct binding assay in this thesis study (1.8×10^5/cell), the calculated theoretical yield of MALA-2 from 1×10^{10} NS-1 cells is 300 ug. After purification,

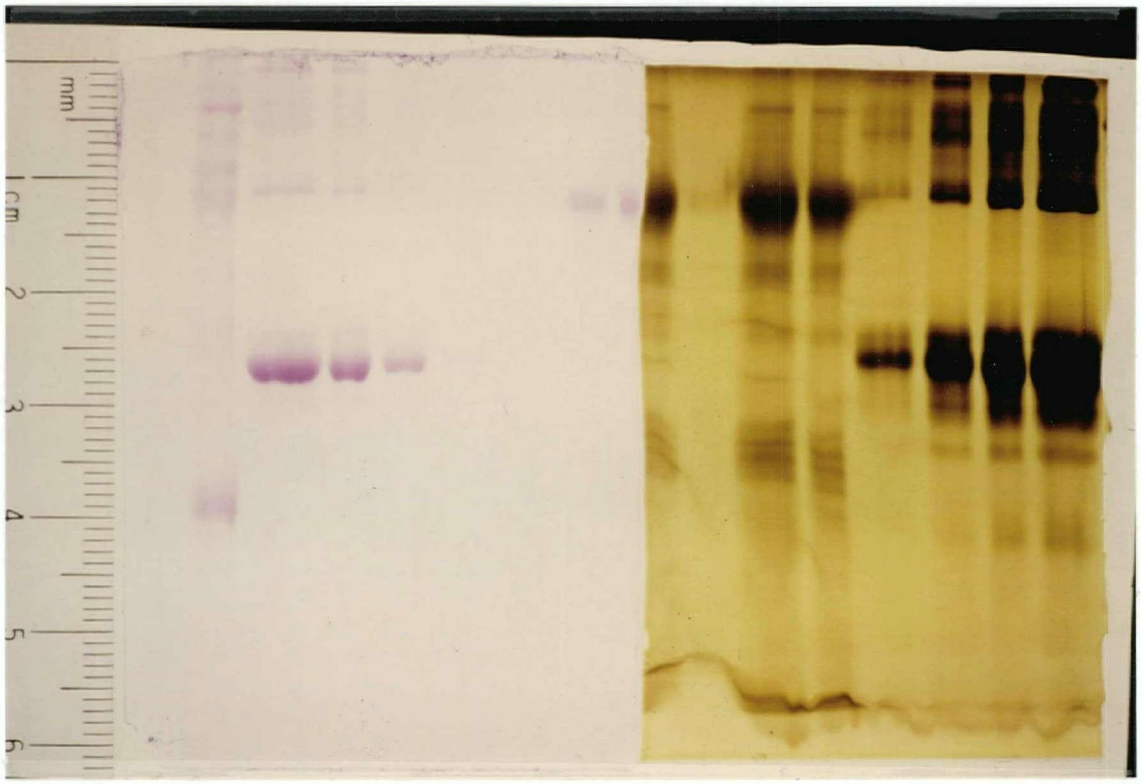

FIG. 2. **Degree of purity of MALA-2 after specific elution from antibody affinity column (and comparison of the relative sensitivities of Coomassie blue staining and Silver staining of proteins).** Gel is stained with Coomassie brilliant blue (left side of gel) and Silver stain (right side of gel). (lane a) High M_r protein standards: myosin (200 kD), beta-galactosidase (116 kD), phosphorylase B (92.5 kD), BSA (66.2 kD), and ovalbumin (45.0 kD). (lanes b,c,d,e) BSA quantitative standards; 1.0, 0.5, 0.2, 0.1 ug respectively; (lanes f,g,h,i,j) MALA-2 isolated via antibody-affinity column, lanes f and j contain 3 ul, and lanes g/h and i contain 5 ul of MALA-2 crude preparation (after affinity column isolation, and before additional purification steps). MALA-2 is seen as a wide band migrating to approximately 90-95 kD, while several contaminating proteins can also be seen in the Silver stain. (lanes k,l,m,n) BSA quantitative standards, 0.1, 0.2, 0.5, and 1.0 ug respectively.

the total yield was found to be approximately 80 ug, or 37% of the total (theoretical) yield. The pure preparation of MALA-2 was used for several subsequent experiments.

3.2 CHARACTERIZATION OF THE BIOCHEMICAL PROPERTIES OF MALA-2

3.2.1 MALA-2 is a Glycoprotein

Purified MALA-2 was iodinated using the Chloramine T method (see Sec. 2.2.5, Materials and Methods), and was incubated with various concentrations of endoglycosidase F (FIG. 4, lanes A-D), an enzyme which is capable of cleaving N-linked carbohydrate chains. At each concentration of enzyme, the preparation was incubated for three different time periods, ranging from 2 hours to 12 hours (lanes 1-3 under each letter corresponding to an enzyme concentration). The three lanes grouped under letter A were devoid of enzyme, and represent the normal state of the purified MALA-2 after iodination. The groups of lanes under B-D show a steadily decreasing M_r, indicating the cleavage of N-linked carbohydrate chains (this procedure does not remove O-linked carbohydrate chains). The lowest M_r species exhibits a M_r of approximately 66 kD, indicating that the peptide backbone of MALA-2 is approximately 66 kD M_r. In each group of lanes, lane 3 ran poorly, and seemed to be aggregated near the top of the gel.

3.2.2 Isoelectric Point of MALA-2

Purified MALA-2 was iodinated using the chloramine T method, and was used to determine the isoelectric point (pI) of MALA-2. In

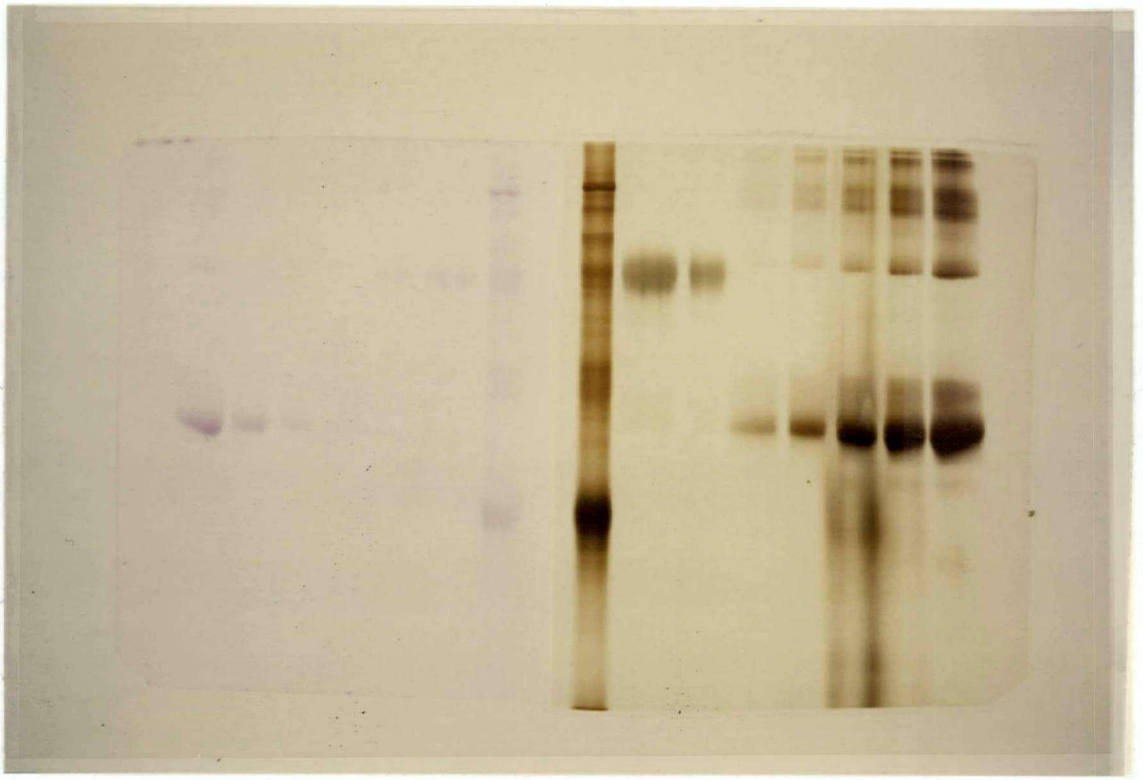

FIG. 3. **Degree of purity of MALA-2 after two consecutive purification steps (consecutive NR and R SDS-PAGE).** The gel is stained with Silver stain on the right, and with Coomassie brilliant blue on the left. (lanes a-d) BSA quantitative standards; 1.0, 0.5, 0.2, 0.1 ug respectively; (lanes e,f) purified MALA-2; (lanes g,h) high M_r protein standards; (lanes i,j) purified MALA-2; (lanes k-o) BSA quantitative standards, 0.04, 0.1. 0.2, 0.5, 1.0 ug respectively. Lanes f and i contain 2 ul of pure MALA-2 preparation, and lanes e and j contain 1 ul of pure MALA-2 preparation (from a total volume of approximately 800 ul). This gel shows the MALA-2 preparation to be highly purified.

this experiment, MALA-2 was subjected to two-dimensional gel electrophoresis. MALA-2 was first loaded onto an IEF tube-gel along with non-radioactively labelled IEF protein standards (BSA pI=4.9, M_r=66.2 kD; carbonic anhydrase II pI=5.9, M_r=30.0 kD; horse myoglobin pI=6.8, 7.2, M_r=17.5 kD). The IEF tube gel was pre-focussed, thereby setting up a charge gradient within the gel. Running this gel allowed MALA-2 and the IEF protein standards to migrate to their isoelectric points within a pre-focussed isoelectric gradient (referred to as the first dimension). The tube gel was then placed along the top of an SDS-PAGE gel (the second dimension) facilitating separation of MALA-2 from the IEF standards based on size. FIG. 5. shows the first dimension represented on the horizontal axis, and the second dimension represented on the vertical axis. The spot on the autoradiograph represents the radiolabelled MALA-2, and corresponds to an acidic isoelectric point of 4.9.

3.2.3 Metabolic Labelling of MALA-2

In this experiment, MALA-2 was immunoprecipitated from the surface of NS-1 cells which had not been cell-surface iodinated. Instead, these cells were incubated with ^{35}S-labelled methionine, which can be used as a metabolic substrate. FIG. 6. is a gel (run in reducing conditions) showing that the labelled methionine was used as an anabolic substrate in the synthesis of both the transferrin receptor (lane D), and MALA-2 (lane A), while no specific bands could be visualized for either the YE1/48 antigen or T200 (lanes B and C respectively). The transferrin receptor has migrated to approximately 95 kD, which is normal for this disulphide-bonded protein under

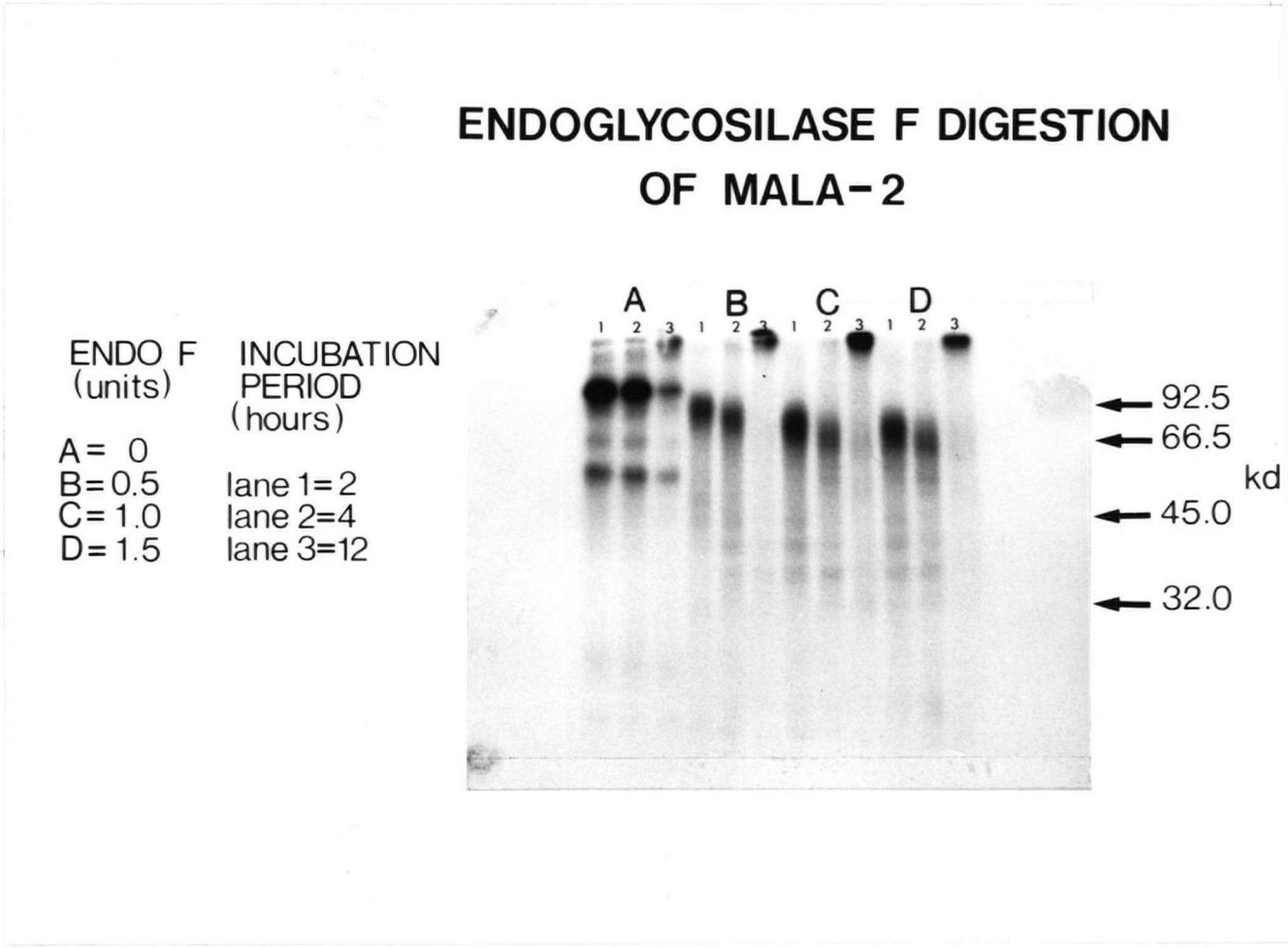

FIG. 4. **Glycoprotein nature of MALA-2; digestion with endoglycosidase-F.** Purified MALA-2 was ^{125}I-labelled by the choramine T method. 100 pg (5 ul of 20 pg/ul) of labelled MALA-2 was incubated with 0, 0.5, 1.0, and 1.5 units of endoglycosidase F (represented by the groups of three lanes labelled A-D respectively). Incubation took place at 37 C for 2 hrs (lanes labelled 1), 4 hrs (lanes labelled 2) and 12 hrs (lanes labelled 3). Numbers and arrows correspond to the following M_r proteins standards: phosphorylase B (92.5 kD), BSA (66.2 kD), ovalbumin (45.0 kD), and carbonic anhydrase (31.0 kD), visualized by Coomassie blue staining of the gel at an earlier stage. The most completely digested specie of MALA-2 is seen in lane D2, and corresponds roughly to a M_r of 66 kD. This figure is an autoradiograph exposed by an SDS-PAGE gel on which the digestion samples were analyzed.

reducing conditions. Lane A shows a specific band representing MALA-2 which is characteristic of this molecule under both reducing and non-reducing conditions. The difference in intensity of these bands, detected by autoradiography, may be a reflection of either a difference in the number of methionine residues, or a more substantial rate of synthesis of the transferrin receptor compared to MALA-2.

3.2.4 Development of Polyclonal Serum Directed Against MALA-2

In order to obtain immune serum capable of staining MALA-2 in western blots, polyclonal antiserum raised against MALA-2 was developed by administering intraperitoneal immunizations to a rat. The injected material consisted of purified MALA-2 combined with Freund's complete adjuvant. FIG. 7. shows the results of examination of the immunized rat's serum by western blot. Cell membrane isolated from NS-1 cells was run on a gel, transferred to nitrocellulose, and the nitrocellulose stained by normal rat serum (lanes a,b,c) and by immunized rat serum (lanes d,e,f). The serum-stained nitrocellulose was then incubated with a radioactive second antibody (mouse anti-rat Ig) to trace the regions of specific antibody staining. This figure shows that the immunized rat serum could specifically recognize MALA-2 (band at approximately 95 kD M_r) and another membrane component with a M_r of approximately 50 kD. This dual specificity of immune serum recognition may be a result of either cross-reactivity of the serum with a lower molecular weight molecule, or may be due to contamination of the injected material with another protein, resulting in an immune response to both MALA-2 and the putative contaminant. The polyclonal immune serum was subsequently used to examine a possible relationship between MALA-2 and the transferrin receptor.

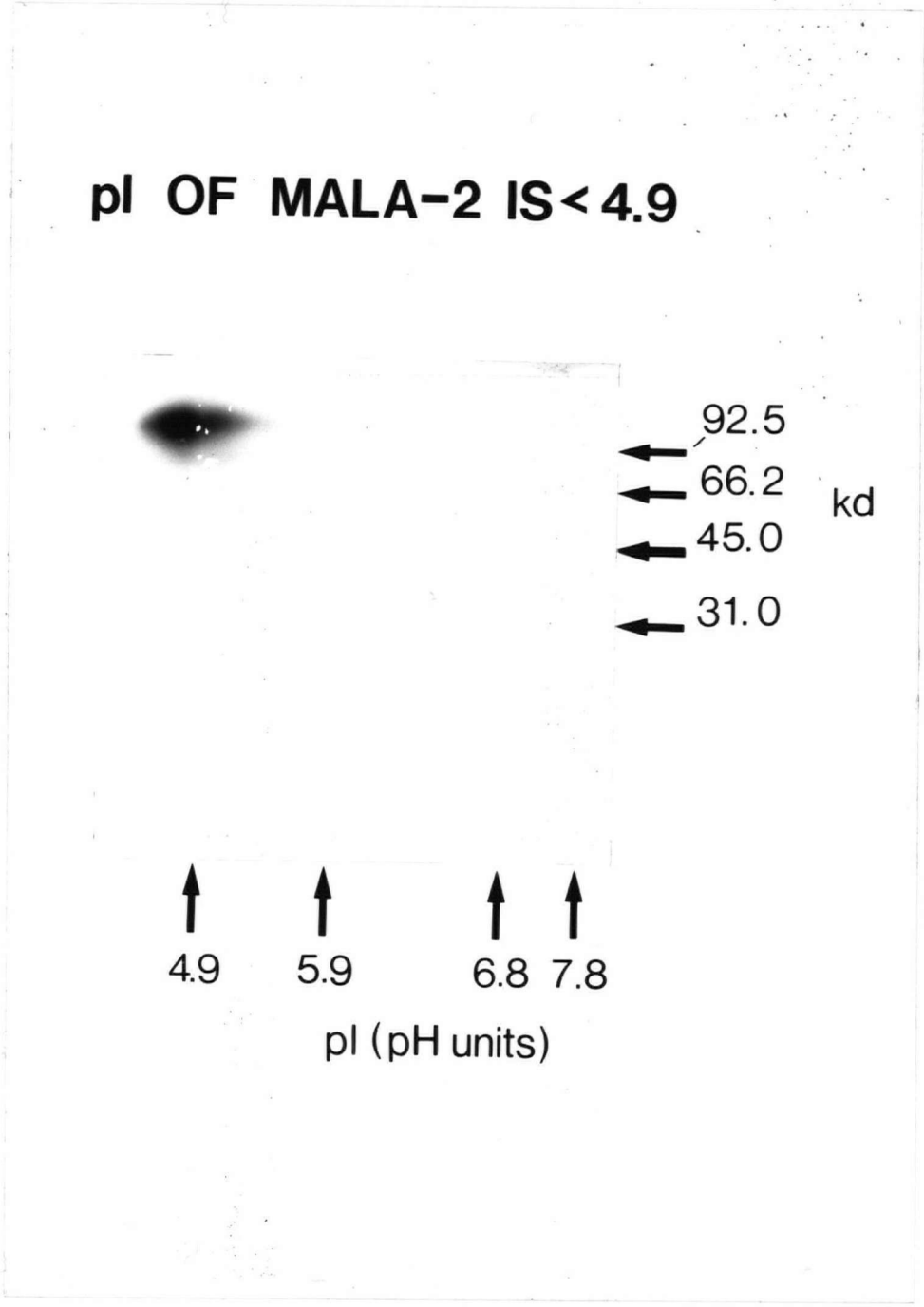

FIG. 5. **Two-dimensional gel electrophoresis analysis of purified MALA-2.** Purified MALA-2 was ^{125}I-labelled by the chloramine T method. The MALA-2 sample was analyzed in the first dimension by isoelectric focussing (horizontal axis), and in the second dimension by SDS-PAGE (vertical axis). The following protein standards were used as markers for isoelectric focussing: BSA (pI=4.9), carbonic anhydrase II (pI=5.9), and horse myoglobin (pI=6.8 and 7.2). The following protein standards were used as M_r markers for SDS-PAGE: phosphorlyase B (92.5 kD), BSA (66.2 kD), ovalbumin (45.0 kD), and carbonic anhydrase (31.0 kD). All standards were visualized by Coomassie blue staining. The 2-dimensional gel was subjected to autoradiography to detect MALA-2, seen in this figure to localize to a pI of just less than 4.9.

3.2.5 MALA-2 Is Not Associated with the Transferrin Receptor

Considering the similar pattern of expression of MALA-2 and the transferrin receptor on lymphoid cell types, that antibodies against both the MALA-2 and the transferrin receptor are capable of inhibiting mitogen stimulation and the MLR, and that transferrin receptor immunoprecipitations coprecipitate a band of approximately M_r 95 kD, it was suspected that a physical relationship existed between the transferrin receptor and MALA-2. Investigation of this relationship was facilitated by the generation of polyclonal antiserum against MALA-2, capable of staining MALA-2 on western blots. The transferrin receptor was specifically immunoprecipitated and run out on an SDS-PAGE gel. The contents of the entire lane (which would include any coprecipitating materials) was transferred to nitrocellulose, and stained with the MALA-2 polyclonal antiserum. FIG. 8. shows that MALA-2 was not coprecipitated along with the transferrin receptor, as there was no specific band detected by autoradiography in the approriate M_r range. Similarly, MALA-2 did not coprecipitate with any of the other control immunoprecipitations.

3.2.6 Phosphorylation Study of MALA-2

The possibility that MALA-2 may be phosphorylated was investigated, as there were some indications that peptide fragments of MALA-2 shared slight homology to a group of proteins known as tyrosine kinases, as well as homology to the immunoglobulin superfamily (see section 3.4). In this study, NS-1 and MBL-2 cells were incubated with ^{32}P-orthophosphate. The cells were subsequently lysed, and

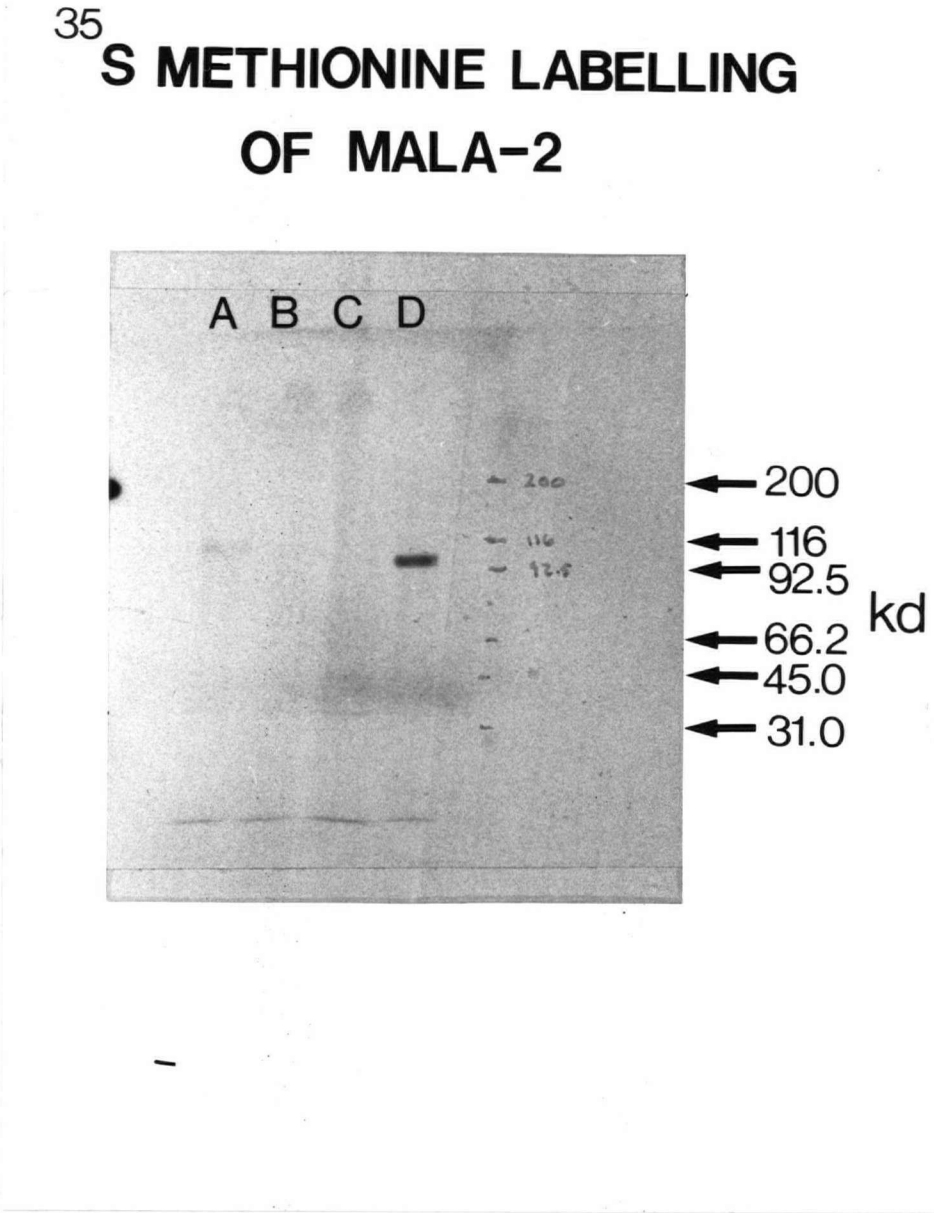

FIG. 6. **Metabolic labelling of MALA-2.** NS-1 cells were incubated in methionine-free media, supplemented with ^{35}S-labelled methionine, for 4 hrs. Cells were then lysed and subjected to immunoprecipitation. Immunoprecipitated samples were subjected to SDS-PAGE analysis, and the gel subjected to autoradiography. Arrows and numbers refer to M_r standards. Immunoprecipitations were carried out by specific monoclonal antibodies. (lane A) YN1/1.7.4, specific for MALA-2; (lane B) YE1/48, specific for the cell-surface molecule of the same name; (lane C) YE1/21, specific for T200 (CD45); and (lane D) YE1/9.9.3, specific for the transferrin receptor. This gel was run under reducing conditions. Lane A shows a faint band (indicated by single arrow) corresponding to MALA-2, while lane D shows a band corresponding to the reduced form of the transferrin receptor.

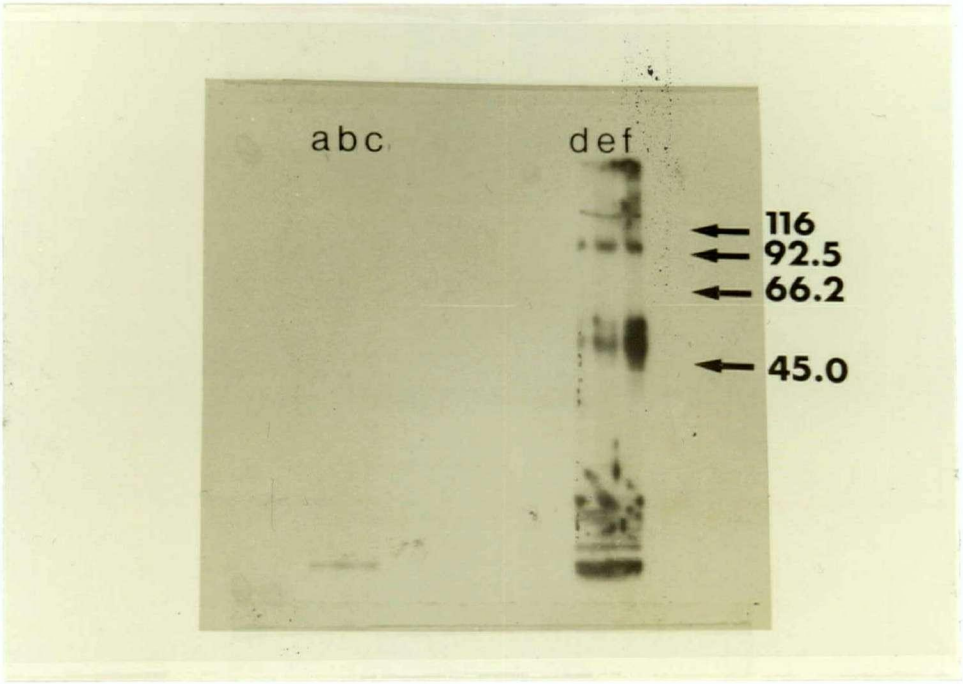

FIG. 7. **Generation of polyclonal serum against MALA-2.** A Fisher 344 rat was immunized with MALA-2 suspended in Freund's complete adjuvant. Rat serum was then tested for reactivity against MALA-2 by western blot. Membrane was prepared from 2 X 10^7 NS-1 cells, separated by SDS-PAGE, and blotted onto nitrocellulose, which was then incubated with serum from the immunized rat, or from a normal rat. The antibody stained blots were then incubated with radioactively labelled secondary antibodies (MaRIg) to detect the presence of specifically bound primary antibodies in the rats' serum. The blot was then dried and subjected to autoradiography. The arrows and numbers correspond to M_r protein standards. Lanes a,b,c are stained with normal rat serum as source of primary antibody, while lanes d,e,f are stained with immunized rat serum. Lanes a,b,d and e were run with 10 ul of membrane preparation, while lanes c and f were run with 15 ul of membrane preparation. Lanes d-f show the immunized rat serum to contain antibodies to MALA-2 and also to a lower M_r molecule. Normal rat serum showed no specific antibody staining.

appropriate cell surface antigens were immunoprecipitated and analyzed on SDS-PAGE. FIG. 9. shows that a phosphorylated species of MALA-2 could not be detected in either of these cell lines (lanes 1a, 2a), although phosphorylation of the transferrin receptor could be detected in MBL-2 cells (lane 1d). Neither of the negative controls (lanes 1b,1c, 2b,2c) exhibited specific banding.

3.3 DETERMINATION OF THE PARTIAL AMINO ACID SEQUENCE OF MALA-2

3.3.1 Trypsin Digestion of MALA-2

Digestion of purified MALA-2 was necessary to determine the partial amino acid sequence of this molecule, since an attempt to determine the amino terminal sequence was impossible due to N-terminal blockage. MALA-2 was consequently incubated with trypsin (which specifically cleaves proteins after arginine and lysine residues), in an attempt to determine whether peptide fragments of MALA-2 could be generated by this method. FIG. 10 shows the result of having digested purified, chloramine T-iodinated MALA-2. Autoradiography showed that undigested MALA-2 exhibited characteristic migratory properties (lane A), while MALA-2 incubated with trypsin showed a distinct banding pattern which was indicative of significant digestion (lane B). Lane B shows that two major fragments of MALA-2 were present, migrating to regions corresponding to M_r's of 60 kD and 37 kD respectively, and likely representing incompletely digested fragments. The majority of radioactively labelled MALA-2 was seen to migrate to the bottom of the gel, indicating that a large number of relatively short peptides were present.

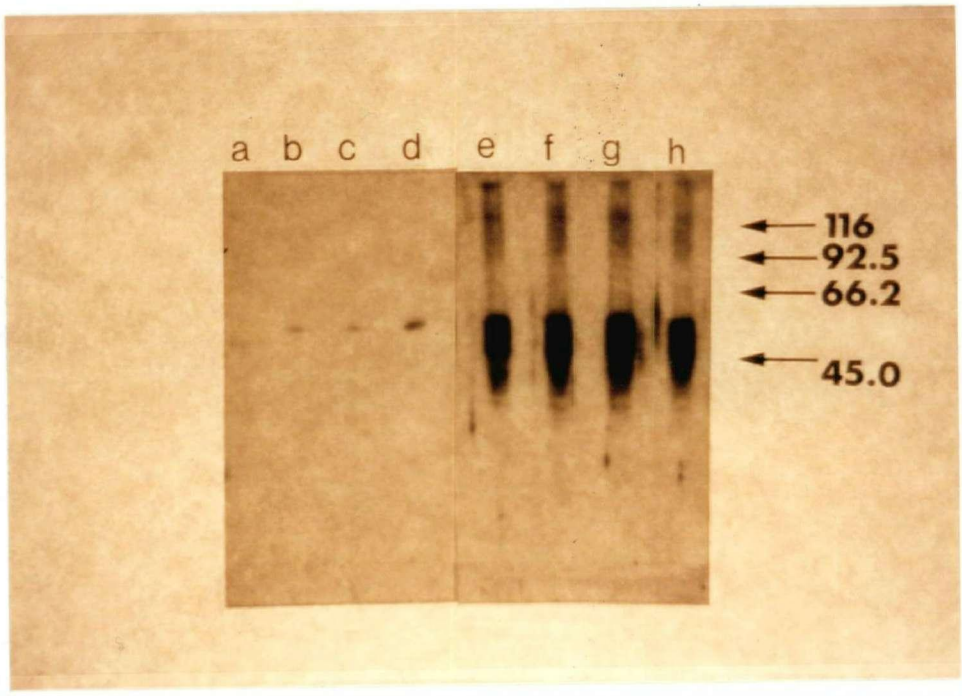

FIG. 8. Western blot of transferrin receptor immunoprecipitation with anti-MALA-2 polyclonal immune serum. Specific immunoprecipitations were run on SDS-PAGE, blotted to nitrocellulose, and incubated with normal rat serum (lanes a-d) or immune rat serum (lanes e-h). Immunoprecipitations were carried out by YE1/21 (lanes a,e) specific for T200; YE6/6 (lanes b,f); YE1/9 (lanes c,g) specific for the transferrin receptor; YE6/26 (lanes d,h) specific for Moloney MuLV envelope protein gp70. Nitrocellulose blots were stained with radioactively labelled second antibody (MaRIg), dried, and subjected to autoradiography. No specific antibody staining of MALA-2 could be detected in any of the lanes.

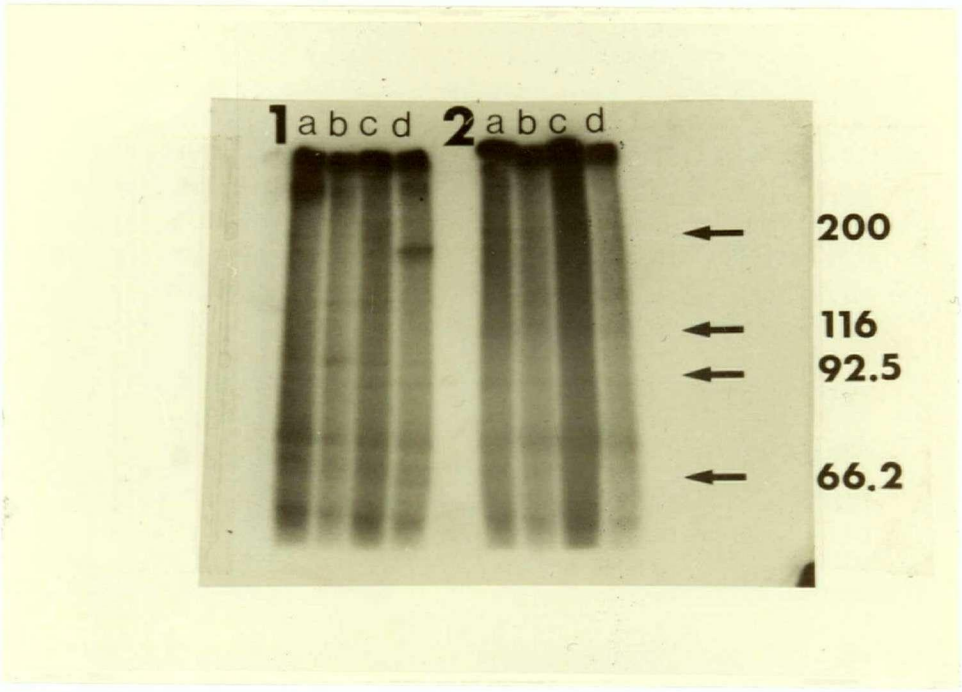

FIG. 9. **Phosphorylation study of MALA-2.** NS-1 (lanes 1a-d) and MBL-2 cells (lanes 2a-d) were incubated in phosphate-free medium supplemented with ^{32}P-orthophosphate for 4 hrs at 37 C. Cells were washed and lysed, and the lysate subjected to specific immunoprecipitations. (lanes 1a, 2a) YN1/1.7.4; (lanes 1b, 2b) YE1/30.4.1 specific for the Thy-1 molecule; (lanes 1c, 2c) YE1/48; (lanes 1d, 2d) YE1/9.9.3. Immunoprecipitations were analyzed by SDS-PAGE and subjected to autoradiography. Lane 1d shows phosphorylation of the transferrin receptor, but no phosphorylation of MALA-2 can be detected.

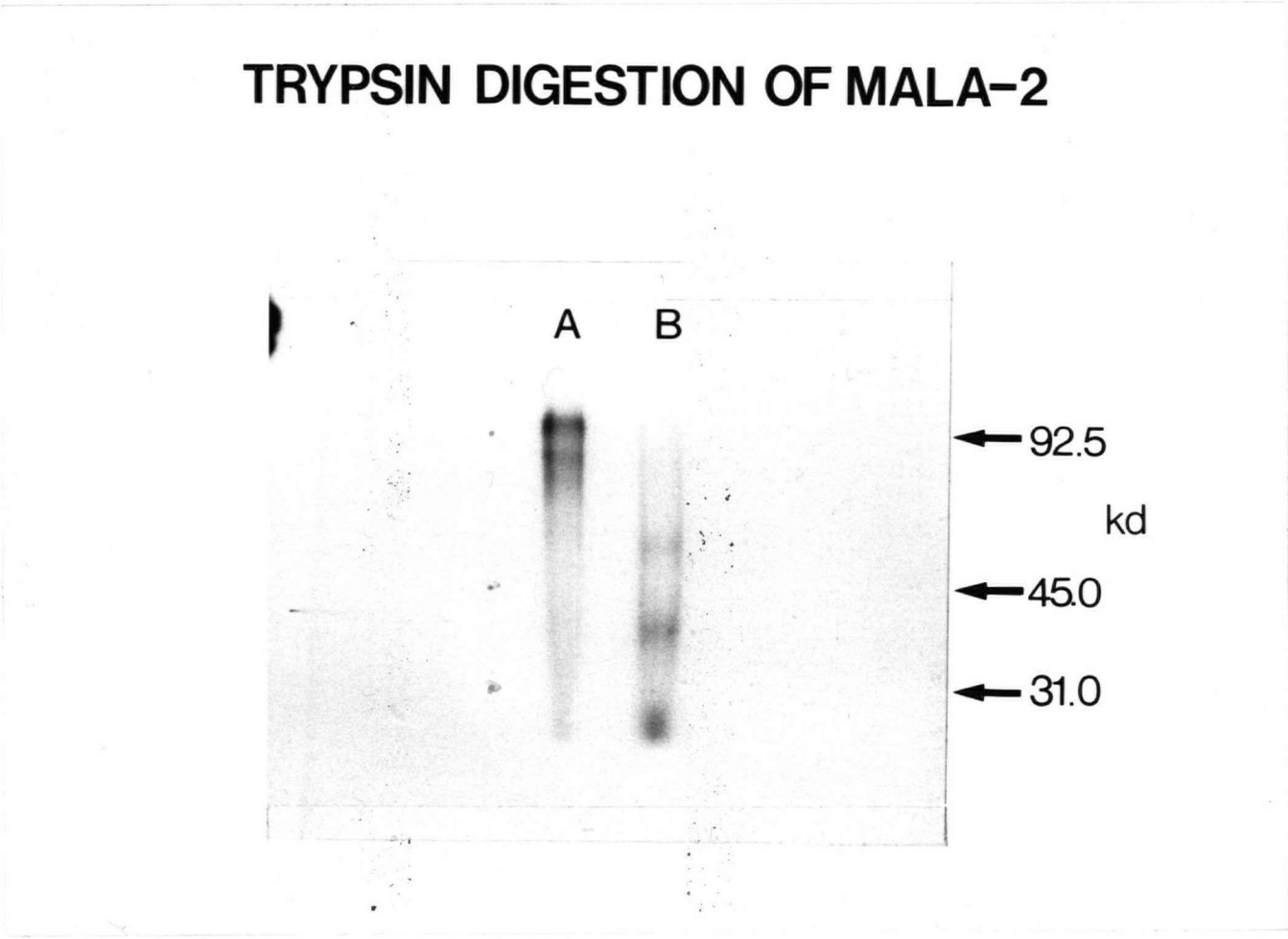

FIG. 10. **Trypsin digestion of MALA-2.** Purified MALA-2 was ^{125}I-labelled by the chloramine T method, and incubated at 37 C for 12 hrs without trypsin (lane A) or with trypsin (lane B). Arrows and numbers refer to M_r protein standards. Samples were analyzed by SDS-PAGE, and the gel subjected to autoradiography. Lane B shows that tryptic digestion of MALA-2 took place.

3.3.2 Separation and Sequencing of Tryptic Peptides

Tryptic peptides generated from purified MALA-2 were separated by using a C-18 reverse phase column in conjunction with high pressure liquid chromatography. The stationary phase of this column is hydrophobic, while the mobile phase consisted of an acetonitrile gradient (0-60% acetonitrile in ddH_2O, 0.1 % TFA) progressing from hydrophilic to hydrophobic conditions. Since the mobile phase was initiated with 0% acetonitrile in ddH_2O, 0.1 % TFA, and was gradually increased to 60% acetonitrile, relatively hydrophilic peptides were the first to be eluted from the column, followed by peptides of increasing hydrophobicity. Absorbance of the eluent was monitored by a spectrophtometer set to detect absorbance at 215 nm, a wavelength known to be absorbed by peptide bonds. Fractions of the eluent were collected by hand corresponding to the observed spectrophotometric peaks.

Typical quantities of peptides present ranged from 75-100 pmol, from a starting quantity of approximately 800 pmol in the tryptic digestion mixture. Trial runs with radioactively labelled MALA-2 show that approximately 50% of the starting material is lost in the methanol precipitation step, while further loss takes place after removal of the resuspended digestion mixture from its original container, during a pre-filtering step in preparation for HPLC separation, and during the actual loading of the material onto the column. Overall yield of tryptic peptides, therefore, was approximately 10% of the total starting material. 40% of each purified tryptic peptide sample was used for sequencing, resulting in a peptide quantity of 30-40 pmol being sequenced. Since it is

possible to identify up to 10 consecutive amino acids from just 1 pmol of protein, 30-40 pmol was sufficient for identification of the MALA-2 tryptic peptide sequences (each peptide was fully sequenced, the largest peptide being 15 amino acids long).

The separation of the tryptic peptides generated by the first digestion of MALA-2 is represented by the spectrophotometric profile in FIG. 11. Peaks of absorbance with the least apparent contamination (by co-eluting peptides) were selected for sequencing. Of several fractions selected from this first HPLC separation, only fraction 7 yielded a sequence: QPVGGHPK. Several of the fractions selected for sequencing did not yield sequence. It was suspected that this initial digestion of MALA-2 did not contain a high enough quantity of starting material to generate peptides in detectable amounts, or that absence of a reduction and alkylation step resulted in presence of disulphide bonded peptides, which may have perturbed the sequencing process. Thus, a second digestion was performed after purifying another large batch of MALA-2. At this time it became apparent that an uncalculated length of tubing carrying HPLC eluent was responsible for the problems experienced with the first separation, resulting in an asynchronous collection of the original HPLC fractions. The asynchronously collected fractions (from the first tryptic digestion of MALA-2) were pooled together, and reseparated by C-18 reverse phase column and HPLC. This resulted in the separation of peptide fragments represented by the spectrophotometric profile in FIG. 12. Four of these fractions were judged to be relatively pure, and were selected for sequencing. Fraction 14r yielded TLNASSADHK, fraction 18r yielded GQTLELH, fraction 25r yielded DELESGPNWK, and fraction 38r yielded TFDLPATIPK.

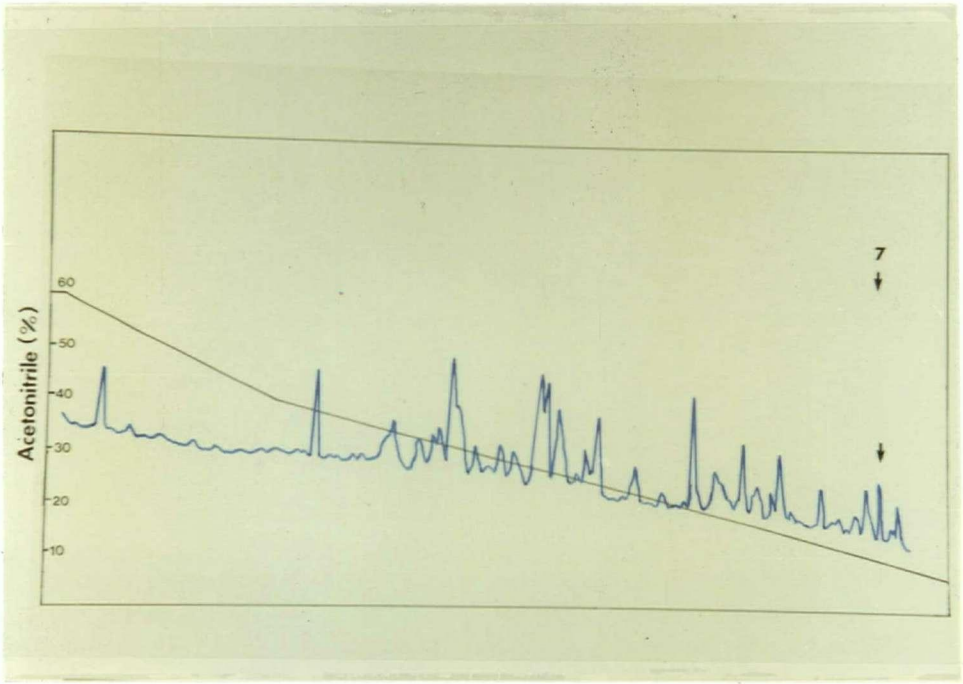

FIG. 11. **Spectrophotometric profile of the separation of tryptic peptide fragments of MALA-2 by HPLC.** A large quantity (80 ug) of purified MALA-2 was subjected to tryptic digestion. The digestion products were separated by HPLC using a C18 reverse phase column. Absorbance was set at 215 nm to detect peptide bonds. Acetonitrile gradient was effected by mixing acetonitrile with ddH_2O/0.1% TFA, and is represented by the line tangent from the horizontal axis. Fraction 7 yielded an amino acid sequence upon microsequencing.

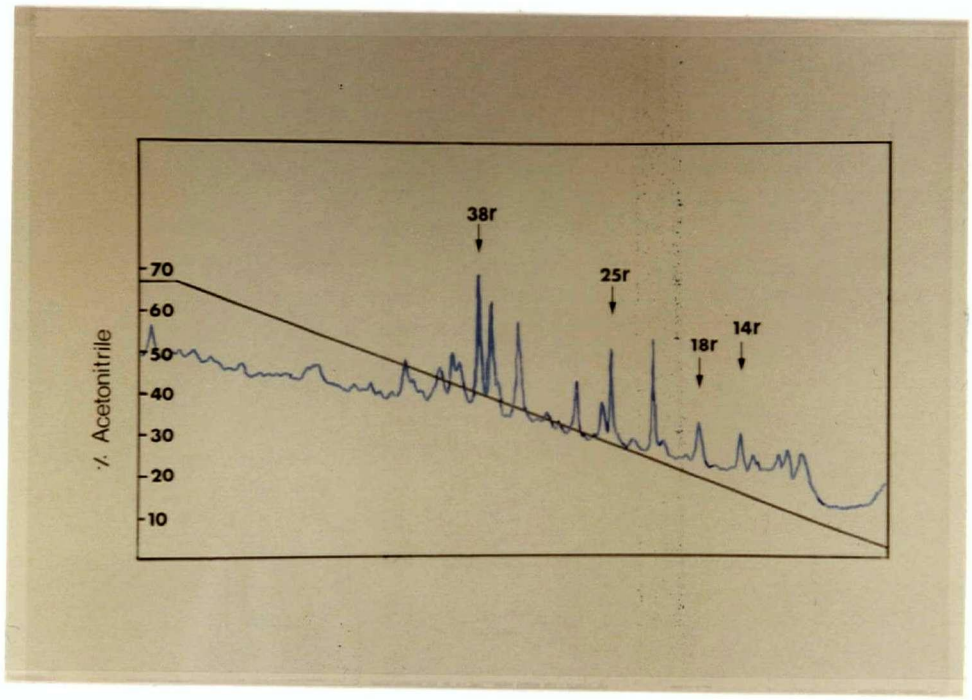

FIG. 12. **Spectrophotmetric profile of the separation of re-pooled tryptic peptide fragments of MALA-2 by HPLC.** (See FIG. 11 for origional separation of fragments) Asynchronously collected fractions of the first tryptic peptide separation were repooled and subjected to a second separation by HPLC (using the C18 reverse phase column). Absorbance was set at 215 nm to detect peptide bonds. Acetonitrile gradient is represented by the line tangent from the horizontal axis. Fractions 14r, 18r, 25r, and 38r yielded amino acid sequences upon microsequencing.

FIG. 13. is the spectrophotometric profile of the separation of peptides generated by the second tryptic digestion of MALA-2. Seven fractions selected for sequencing yielded amino acid sequences. Fraction 18 yielded TLPLR, fraction 20 yielded DQAEGNPSYQG, fraction 28 yielded QMPTQEST, fraction 33 yielded two distinct sequences distinguished quantitatively: 33a yielded ALVEVTEEFDR (approximately 40 pmol level), 33b yielded ETLGAQMPTQEST (approximately 10 pmol level), fraction 49 yielded LPESLEGLFPASEAR, and fraction 62 yielded LELADQILETQ.

FIG. 14. is the spectrophotometric profile of a separation of peptide fragments generated from the second MALA-2 trypsin digestion. Fractions 1-15 were pooled, and re-run on the C-18 reverse phase column using HPLC. A greater degree of discrimination was achieved by slowing the progression of the gradient. Fraction 15 yielded two sequences which were resolved quantitatively; 15a yielded GDHQANFSCR (approximately 25 pmol level), while 15b yielded LKEGLAK (approximately 15 pmol level). Fraction 11r yielded QPVGGHPK.

Notable characteristics of these tryptic peptide sequences are several. First, of the 128 amino acids of total distinct amino acid sequence obtained, 23 residues are acidic, while 7 are basic. Although these partial MALA-2 sequences are not necessarily representative of the entire molecule, they do agree with the acidic pI of MALA-2 observed within this thesis study. Secondly, three potential N-linked glycosylation sites (N-X-S, or N-X-T) are present within the tryptic peptides, being found in sequences 14r, 15a, and 20. Each of these potential glycosylation sites are the N-X-S type. This finding is in agreement with the endoglycosidase F study

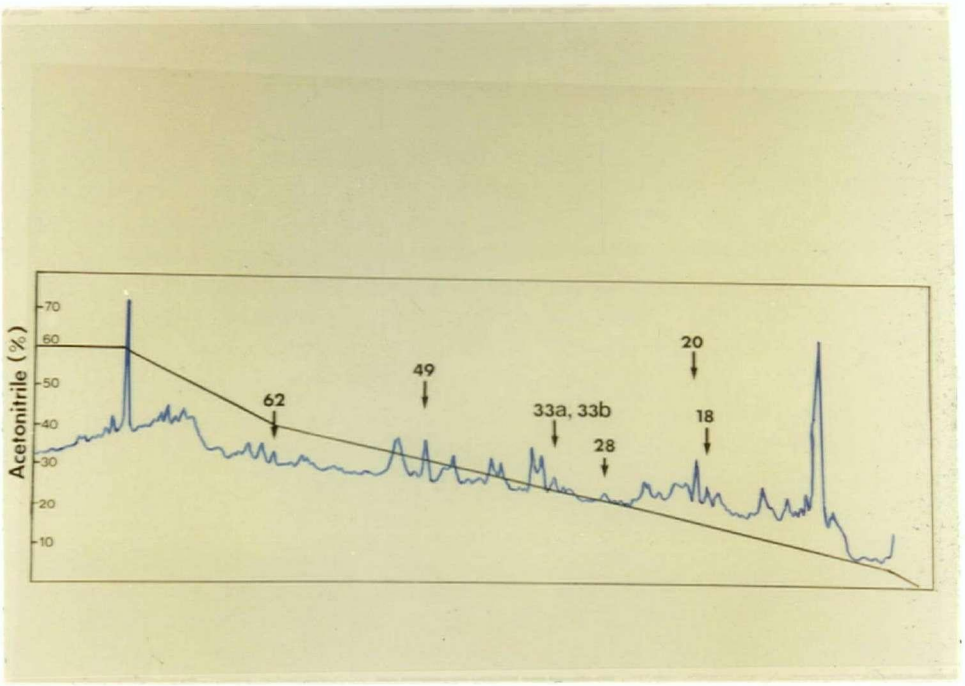

FIG. 13. **Spectrophotometric profile of the separation of tryptic peptide fragments of MALA-2 from a second digestion experiment.** A second large scale tryptic digestion of MALA-2 was performed. Digestion products were separated by HPLC using a C18 reverse phase column. Absorbance was set at 215 nm. Acetonitrile/ddH_2O, 0.1% TFA gradient is represented by the line tangent to the horizontal axis. Fractions 18, 29, 28, 33, 49, and 62 yielded amino acid sequences.

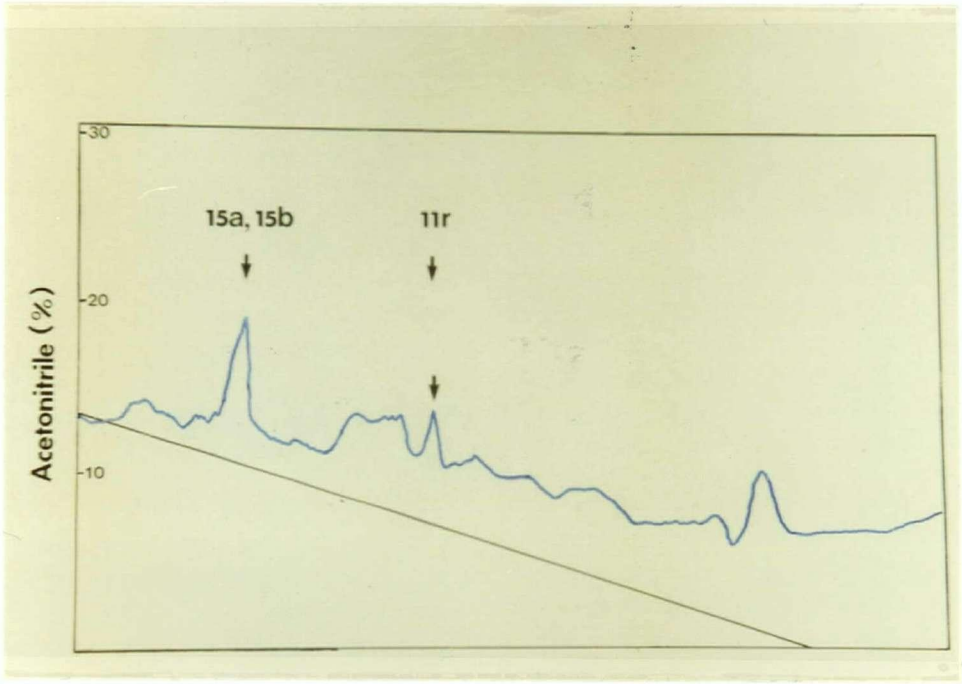

FIG. 14. **Spectrophotometric profile of the HPLC separation of repooled tryptic peptide fragments of MALA-2 (2nd digestion).** (See FIG. 13 for profile of tryptic digestion-2) Fractions 1-17 from the second tryptic digestion were repooled and separated by HPLC using a C18 reverse phase column. Absorbance was set at 215 nm. The acetonitrile gradient was extended to achieve a higher resolution of the eluting peptides. The gradient is represented by the line tangent to the horizontal axis. Fractions 11r and 15 yielded amino acid sequences.

performed in this thesis study, which shows MALA-2 to be a glycoprotein. Thirdly, peptide sequence 15a contains a cysteine residue, which is potentially involved in disulphide bonding within the MALA-2 molecule. Additionally, since the first two amino acids of peptide 15a are GD, and since trypsin is known to cleave after either arginine (R) or lysine (K), there is a possibility that these first two amino acids represent the latter portion of an RGD sequence, known to be the classical binding site of the integrins (Hynes, 1987). Table III lists the tryptic peptides of MALA-2.

3.4 HOMOLOGY COMPARISONS USING PARTIAL AMINO ACID SEQUENCES OF MALA-2

3.4.1. Comparisons of Partial Amino Acid Sequences within the NBRF Database: Limited Homologies with Immunoglobulins and Kinases

Using the NBRF protein sequence library and the wordsearch comparison function, partial amino acid sequences of MALA-2 were compared with all reported protein sequences in the database. As the resulting individual homologies were not convincing, an additional search parameter was added. The additional parameter was a comparison as to which class(es) of proteins in the data base were consistently showing limited homology to the partial MALA-2 sequences. Table III lists the peptide sequences, and shows the two classes of proteins which most consistently exhibited homology: the immunoglobulin superfamily, and tyrosine kinases. The relationship to the immunoglobulin superfamily can be seen to be the most consistent, being listed as homologous to 11 of 14 partial MALA-2 sequences, while tyrosine kinases are listed as homologous to 7 of 14 partial MALA-2

TABLE III

TWO CLASSES OF PROTEINS MOST CONSISTENTLY EXHIBITING HOMOLOGY TO PARTIAL AMINO ACID SEQUENCES OF MALA-2

Partial Amino Acid Sequences	Homology to Immunoglobulin Superfamily Members	Homology to Kinases
7,11r/ GPVGGHPK	-	+
14r / TLNASSADHK	+	-
15a / GDHQANFSCR	+	+
15b / LKEGLAK	+	+
18 / TLPLR	+	+
18r / GQTLELH	-	-
20 / DQAEGNPSYQG	+	-
25r / DELESGPNWK	+	+
28 / QMPTQEST	+	+
33a / ALVEVTEEFDR	+	-
33b / ETLGAQMPTQEST	-	-
38r / TFDLPATIPK	+	+
49 / LPESLEGLFPASEAR	+	-
62 / LELADQILETQ	+	-

- : No homologous proteins of this class are present within the 50 most related sequences in the database.
+ : Homologous proteins of this class are present within the 50 most related sequences in the NBRF database.

sequences. Figure 15 exemplifies homologies seen between the MALA-2 peptide fragments and members of the immunoglobulin superfamily, tyrosine kinase-related proteins, and the endothelial cell glycoprotein IIIa, which is known to exhibit strong homology with the fibronectin receptor, an integrin (Fitzgerald et al., 1987). Although the extent of direct homologies ranged from 30-71%, few NBRF proteins showed homology to more than one of the MALA-2 peptides. An interesting example of a single protein showing homology to multiple peptides of MALA-2 is the endothelial cell glycoprotein IIIa, which exhibits homology to peptides 7 (38%), 20 (36%), and 49 (67%). Peptides 7 and 20 align in regions of internal repeat, thought to be important in the adhesive capacities of this protein (Ibid), and which contain an RGE sequence.

FIGURE 15

EXAMPLES OF HOMOLOGY DEMONSTRATED BETWEEN PARTIAL AMINO ACID SEQUENCES OF MALA-2 AND PROTEINS IN THE NBRF DATABASE

MALA-2 peptide (top sequence)	NBRF protein (bottom sequence)	Comparison	% Homology
14r	TCR alpha chain V region[1]	TLNASSADHK ! !!:!:! 37 LLLKSSTDNK	50
15a	Ig kappa chain V region[2]	GDHQANFSCR ::!!::!!! 17 LGDQASISCR	50
15b	Ig epsilon chain C region[3]	LKEGLAK ::!:!:! 400 IHEALQK	43
18	TCR alpha chain V region[4]	TLPLR !!: 8 GLPVM	40
28	Ig kappa chain V-I region[5]	QMPTQEST !!: !! 3 QMTQSPST	50
7	Transforming protein (ras)[6]	QPVGGHPK !!! 7 VIVGGGGV	38
15b	Kinase related transforming protein (yes)[7]	LKEGLAK !!!! :! 350 LKEGEGK	71
25r	Kinase related transforming protein (hck)[8]	DELESGPNWK !!:: :! 85 VVLEESGEWW	30
28	Kinase related transforming protein (ros)[9]	QMPTQEST :!!!! 73 TLPTQEEI	50
49	Endothelial cell glycoprotein IIIa[10]	LPESLEGLFPASEAR !!!:! !!:!!!! 50 APESIE--FPVSEAR	67

(see over)

FIGURE 15 (cont'd)

NOTE: All sequences were aligned using the FASTA program based on the aligorithm of Pearson and Lipman (1988). A word size of 1 was used to maximize accuracy.

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1 Becker et al., 1985
2 Caton et al., 1986
3 Ishida et al., 1982
4 Becker et al., 1985
5 Shinoda, 1975
6 Reymond et al., 1984
7 Kitamura et al., 1982
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9 Birchmeier et al., 1986
10 Fitzgerald et al., 1987

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CHAPTER FOUR

DISCUSSION

4.1 HOMOLOGY OF MALA-2 TO ICAM-1

4.1.1 Homology of MALA-2 Partial Amino Acid Sequences to ICAM-1

The purpose of this thesis has been to characterize the murine activated lymphocyte antigen-2 (MALA-2). Since MAb specific for MALA-2 has been shown to inhibit the mixed lymphocyte reaction, it has been suggested that MALA-2 plays a direct role in the activation of T cells (Takei, 1985). Research detailed within this thesis has included the investigation of a series of biochemical properties of MALA-2, culminating in the determination of its partial amino acid sequence. Data generated subsequent to this thesis study in our laboratory has shown the entire amino acid sequence of MALA-2 to be 512 amino acids long. Thus, having obtained a total of 128 amino acids in distinct partial amino acid sequences, fully one quarter of the entire sequence of this molecule has been revealed in this thesis study.

Comparison of the partial amino acid sequences of MALA-2 within the NBRF database revealed only limited homologies. No single protein could be revealed as being homologous to the majority of the partial amino acid sequences of MALA-2. The significance of homology studies was recognized as being very limited, due to limited length of the MALA-2 peptides, the absence of overlap between the MALA-2 peptides (disallowing combination of peptide sequences), and the lack of strong homology between any MALA-2 peptide and the proteins in the NBRF database. At this point, an additional search parameter was applied. This involved searching for homology between the partial amino acid sequences of MALA-2 and any single class of proteins.

Interestingly, this strategy revealed limited, but somewhat consistent homology to only two separate classes of proteins, each of which could be seen as being potentially appropriate when compared to the known functional properties of MALA-2. This "class" homology was exhibited to both the immunoglobulin superfamily, and to the tyrosine kinases, although the relationship to the immunoglobulin superfamily was more consistent. Subsequent to this thesis study, the primary sequence for the human ICAM-1 molecule was published, and was seen to be the one single protein to which the majority of partial sequences of MALA-2 were homologous. The majority of MALA-2 partial sequences shared direct amino acid homology to ICAM-1 of 35-70%. Since ICAM-1 has been shown to be a member of the immunoglobulin superfamily containing five extracellular immunoglobulin-like domains (Staunton et al., 1988; Simmons et al., 1988), indications that MALA-2 was most closely related to the immunoglobulin superfamily were confirmed. Thus, this research has shown that even short amino acid sequences (eight to fifteen amino acid length) can be useful in exposing homologous relationships if all sequences are used in conjunction to find the one class of proteins which bears greatest resemblance to the group of short peptides. Studies in our laboratory subsequent to this thesis have identified cDNA clones encoding the MALA-2 molecule, and these studies further confirm the high degree of homology between MALA-2 and ICAM-1 (approximately 50% direct homology) (Horley et al., 1989).

Data relevant to the homology between MALA-2 peptides and ICAM-1 are seen in Table IV and Figures 16 & 17. Of the 14 partial MALA-2 sequences, 11 showed over 35% identity with ICAM-1 based on direct matches, the most stringent possible parameter for comparison.

Of these, 5 sequences were over 50% identical, 4 were over 60% identical, and 2 were 70% identical to the ICAM-1 primary sequence (see Table IV). Sequences were seen as exhibiting even further homology considering additional biochemical similarities between amino acids at positions where direct matches were not present (see FIG. 16).

Interestingly, peptide 15a can be seen to contain an N-linked glycosylation site conserved between ICAM-1 and MALA-2 (N-X-S sequence), and additionally contains a cyteine residue followed by an arginine residue, which is also conserved between MALA-2 and ICAM-1. The positions of this cysteine and the immediately following arginine (positions 159,160 in ICAM-1) have been shown to be conserved between ICAM-1 and the T cell receptor alpha subunit V domain (Staunton et al., 1988), while the position of the cysteine residue alone is additionally conserved in NCAM (Simmons et al., 1988; Staunton et al., 1988). Similarly, four amino acids conserved between ICAM-1 and the constant domain of the heavy chain of IgM (positions 212 [L], 214 [G], 219 [S], and 221 [A]) (Staunton et al., 1988), are also conserved in a spatially identical manner in MALA-2, as seen in peptide 49. Another example of conserved amino acid residues between MALA-2, ICAM-1, and other members of the immunoglobulin superfamily include the glutamine residue (position 168 in ICAM-1) conserved between ICAM-1, NCAM, and the variable region of the T cell receptor alpha chain (Ibid); this residue is conserved as well in MALA-2, as seen in peptide 18r.

Finally, three partial sequences of MALA-2 exhibit regions of conservation between ICAM-1, MAG, and immunoglobulin-like domain-1 of the T cell receptor. Over a region of 29 amino acids in ICAM-1, the

TABLE IV

PARTIAL AMINO ACID SEQUENCES OF MALA-2 AND DEGREE OF HOMOLOGY TO ICAM-1

Tryptic Digestion	Fraction #	Peptide Sequence	Homology to ICAM-1
1	7	QPVGGHPK	+
2 (re-run)	11r	QPVGGHPK	+
1 (re-run)	14r	TLNASSADHK	++
2 (re-run)	15a	GDHQANFSCR	+++++
2 (re-run)	15b	LKEGLAK	++
2	18	TLPLR	++++
1 (re-run)	18r	GQTLELH	+++
2	20	DQAEGNPSYQG	-
1 (re-run)	25r	DELESGPNWK	++
2	28	QMPTQEST	-
2	33a	ALVEVTEEFDR	-
2	33b	ETLGAQMPTQEST	-
1 (re-run)	38r	TFDLPATIPK	+++++
2	49	LPESLEGLFPASEAR	++++
2	62	LELADQILETQ	+

Total number of distinct amino acids sequenced = 128.

Homology has been determined by the percent of direct amino acid matches:

\+ : 35-39%
++ : 40-49%
+++ : 50-59%
++++ : 60-69%
+++++ : 70%

FIGURE 16

HOMOLOGY BETWEEN ICAM-1 AND PARTIAL AMINO ACID SEQUENCES OF MALA-2

```
 1 (MAPSSPRPAL PALLVLLGAL FPGPGNA)QTS VSPSKVILPR GGSVLVTCST

24  SCDQPKLLGI ETPLPKKELL LPGNNRKVYE LSNVQEDSQP MCYSNCPDGQ
                       : !   ! !:!
                  (25r) DEL-ESGPNWK

74  STAKTFLTVY WTPERVELAP LPSWQPVGKN LTLRCQVEGG APRANLTVVL
                                              !!  !
                                          LKEGL-AK  (15b)

124 LRGEKELKRE PAVGEPAEVT TTVLVRRDHH GANFSCRTEL DLRPQGLELF
                                    ! :!!!!!!       !:!!!
                           (15a) GDH-QANFSCR (18r) GQTLELH

174 ENTSAPYQLQ TFVLPATPPQ LVSPRVLEVD TQGTVVCSLD GLFPVSEAQV
              !! !!!!:!                  :: !!: !!!!:!!!
        (38r) TFDLPATIPK          (49) LPESLE-GLFPASEAR

224 HLALGDQRLN PTVTYGNDSF SAKASVSVTA EDEGTQRLTC AVILGNQSQE

274 TLQTVTIYSF PAPNVILTKP EVSEGTEVTV KCEAHPRAKV TLNGVPAQPL
                                                      !!:
                                                 (7) QPV-

324 GPRAQLLLKA TPEDNGRSFS CSATLEVAGQ LIHKNQTREL RVLYGPRLDE
    ! ::   ! !   !!:        ! :!! :: :
    GGHPK TLNA-SSEDHK (33a) ALVEVTEE-FDR
          (14r)

374 RDCPGNWTWP ENSQQTPMCQ AWGNPLPELK CLKDGTFPLP IGESVTVTRD
                                          !:!!
                                     (18) TLPLR

424 LEGTYLCRAR STQGEVTREV TVNVLSPRYE IVIITVVAAA VIMGTAGLST

474 YLYNRQRKIK KYRLQQAQKG TPMKPNTQAT PP
```

: = similar amino acid properties
! = direct amino acid match
(Brackets enclose putative hydrophobic signal peptide; ICAM-1 sequence from Staunton et al., 1988).

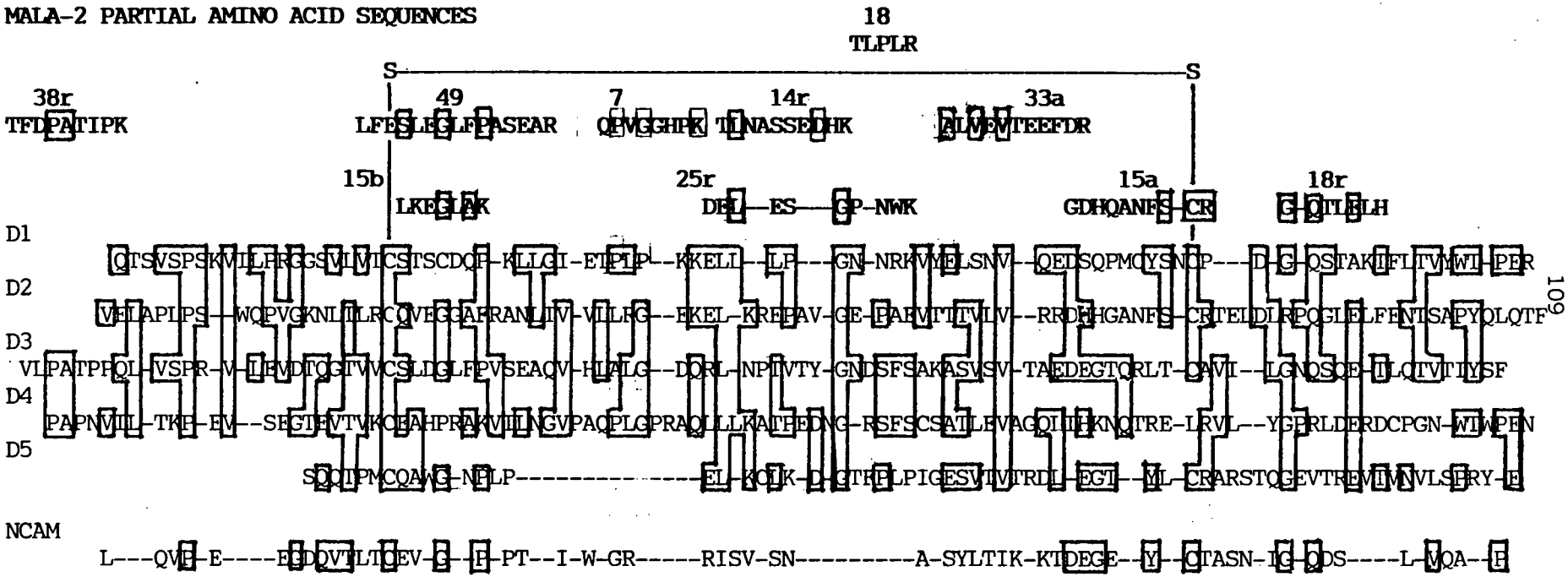
MALA-2 partial sequence peptides 7, 14r, and 33a, aligned as shown in FIG. 16, contain 8 of 19 amino acids conserved between ICAM-1, MAG, and the TCR. These conserved residues are at the following positions in ICAM-1: 321 [Q], 322 [P], 324 [G], 333 [A], 336 [E], 337 [D], 346 [A], and 349 [E] (Ibid).

The consistent homology exhibited between ICAM-1 and the partial amino acid sequences of MALA-2, even in regions of similarity between ICAM-1 and other immunoglobulin superfamily members, is strongly suggestive that MALA-2 is a member of the immunoglobulin superfamily, and likely the murine homolog of ICAM-1. This is supported by the fact that each of the ten partial sequences seen in FIG. 16 contains amino acid residues which are conserved between the immunoglobulin domains of ICAM-1, including one of the conserved cysteine residues which participates in the disulphide bond forming the characteristic loop of immunoglobulin domains, in domain 1 of ICAM-1 (see FIG. 17). The partial amino acid sequences of ICAM-1, seen in FIG. 17, have been aligned with the appropriate sequence corresponding to FIG. 16. Spaces inserted into the MALA-2 sequences correspond to spaces inserted in the exact corresponding sequence of ICAM-1 as seen in FIG. 15, with the exception of one space inserted in peptide 18r. The conserved cysteine residue in peptide 15a is conserved through four of the five immunoglobulin-like domains of ICAM-1.

Thus, a relationship is seen to exist between all ten of the homologous peptides, and the regions of internal repeat within the ICAM-1 molecule (FIG. 17). Significantly, one of the peptide sequences (15a) also contains the last two amino acids of an RGD

FIGURE 17

COMPARISON OF THE PARTIAL SEQUENCES OF MALA-2 WITH REGIONS OF CONSERVATION IN THE IMMUNOGLOBULIN-LIKE DOMAINS OF ICAM-1



D1-5 refers to domains 1-5 of ICAM-1; figure adapted from Staunton et al., 1988)

NOTE: S———S refers to disulphide bond formation between conserved cysteine residues in each of the immunoglobulin-like domains.

sequence (15a starts with GD..., and trypsin is known to cleave after R or K). The nucleotide sequence of MALA-2 determined subsequent to this thesis study has since confirmed that peptide 15a does indeed contain the last two residues of an RGD sequence, known to be essential to the binding site recognized by the integrins (Hynes, 1987). Further, the positions of two glycosylation sites are also conserved, as evidenced by the N-X-S sequences seen in peptide sequences 14r and 15a (the glycosylation site can also be N-X-T, as seen in the ICAM-1 glycosylation site corresponding to the similar site in 14r). Also likely to be significant are the amino acids which can be seen to be conserved between MALA-2 peptide sequences when aligned in this consistent fashion. A serine and a leucine residue are conserved between peptide sequences 14r and 25r, and a consecutive sequence of three amino acids (glutamic acid, glycine, leucine) is seen to be conserved between peptides 49 and 15b. Thus, this evidence suggests that MALA-2 may be made up of repeating sequences, similar to ICAM-1. This suggestion has been confirmed by subsequent nucleotide sequence information.

Collectively, this data suggests that MALA-2 is a member of the immunoglobulin superfamily; the degree of direct homology between the partial sequences of MALA-2 and ICAM-1 further suggests that MALA-2 may be the murine homolog of ICAM-1. Biochemical evidence presented in this thesis also supports the similarity between MALA-2 and ICAM-1.

4.1.2 Additional Similarities Between MALA-2 and ICAM-1

Complimentary to the sequence homology between partial

sequences of MALA-2 and ICAM-1, several other findings exposed in this thesis study reflect similarities between ICAM-1 and MALA-2 (Only peptide 15a showed homology to the predicted primary sequence of ICAM-2). These similarities start with the density of expression on activated and non-activated cells. Basal expression of ICAM-1 on non-activated endothelial cells was seen to be 5-10 X 10^4/cell, while activated endothelial cells (endothelial cells stimulated with TNF) were seen to express 3.5 X 10^6/cell (Dustin & Springer, 1988). Similarly, two cell lines displaying a non-activated lymphocyte phenotype displayed MALA-2 at a density of approximately 4 X 10^4/cell (MBL-2, EL-4), while another lymphoid cell line expressing an activated phenotype (NS-1) expressed MALA-2 at approximately 2 X 10^5/cell. Thus, an increased density of both MALA-2 and ICAM-1 is seen to occur in activated cells. This increased expression of MALA-2 on activated cells has been previously documented (Takei, 1985).

Intimately related to the increased expression of both ICAM-1 and MALA-2 on the surface of activated cells is the mechanism of upregulation. ICAM-1 expression can be rapidly and dramatically induced by INF-gamma, IL-1, TNF, or lymphotoxin (Dustin et al., 1986; Pober et al., 1987; Dustin & Springer, 1988). Experiments with dermal fibroblasts and HL-60 cells suggest that upregulation of ICAM-1 occurs at the level of transcription (Dustin et al., 1986; Simmons et al., 1988). Similarly, it was found through metabolic labelling with ^{35}S-methionine that expression of MALA-2 on the surface of NS-1 cells was dependent upon new protein synthesis. This finding suggests that, in NS-1 cells (which diplay an activated phenotype), the expression of MALA-2 involves ongoing transcription. The predicted

amino acid sequence of MALA-2 from cDNA data show the presence of only four methionine residues in the native protein, which helps to explain the faintness of the ^{35}S-methionine labelled MALA-2 band in FIG. 6.

More significantly, MALA-2 and ICAM-1 share almost identical M_rs, single peptide structure, and similar heterogeneity in migration in SDS-PAGE, resulting in a characteristic "range" of both molecules of between 90-115 kD M_r (this thesis; Makgoba et al., 1988), the ICAM-1 molecule has been shown to be a glycoprotein with a M_r 55 kD protein backbone (Dustin et al., 1986). Again, the biochemical properties of MALA-2 parallel those of ICAM-1. Endoglycosidase F digestion of MALA-2, performed in this thesis study have shown that MALA-2 is a glycoprotein, containing N-linked carbohydrate moieties. The most highly digested species of MALA-2 migrates to a position corresponding to a M_r of approximately 66 kD. Considering that cDNA information has shown MALA-2 to be 512 amino acids long, and using 120 kD as the average M_r of typical amino acids, the calculated M_r of MALA-2 is 61 kD. Thus, the deglycosylation study performed in this thesis was reasonably accurate in its assessment of the size of the MALA-2 protein backbone. Discrepancies may have arisen from either incomplete digestion of some of the carbohydrate chains, or from factors inherent in SDS-PAGE analysis of M_r. ICAM-1 and MALA-2 are glycoproteins of similar M_r, and exhibit a relatively similar degree of glycosylation.

Finally, in this thesis study, MALA-2 was shown to be neither associated with the transferrin receptor, nor was it apparently phosphorylated. There has been no mention in the literature that ICAM-1 is phosphorylated, nor has there been any detection of its association with the transferrin receptor.

The association between LFA-1 and ICAM-1 is the first known example a receptor-ligand relationship between a member of the integrin superfamily and the immunoglobulin superfamily. Two aspects of MALA-2 exposed in this thesis may help to shed light on this interaction. MALA-2 has been shown in this research to have an acidic isoelectric point. Thus, its structure, even though related to immunoglobulins, may share certain qualities similar to integrins, whose isoelectric points have been shown to be acidic (Hemler, 1988), as have those of another group of molecules involved in adhesion, the Hermes/MEL-14 group of homing receptors (common pI of 4.2) (Gallatin et al., 1983; Jalkanen et al., 1986, 1987). In addition to the highly acidic nature of MALA-2, three of the partial sequences of MALA-2 have been shown to exhibit homology to the endothelial glycoprotein IIIa. This molecule has been shown to exhibit homology to the fibronectin receptor, which belongs to the integrin superfamily. Two of the three peptides align in regions thought to be important in the adhesive properties of this molecule. Again, MALA-2 exhibits a similarity to adhesion molecules.

An unusual aspect of the relationship between ICAM-1 and LFA-1 is that ICAM-1 does not contain the classic integrin recognition sequence, RGD. Instead, it contains a similar sequence, RGE, which has been suggested as an alternative binding site for LFA-1 (Horley et al., 1989). Interestingly, MALA-2 also contains an RGE sequence (Ibid), and also interestingly, endothelial glycoprotein IIIa contains an RGE sequence, in the region thought to be involved in its adhesive interactions (Fitzgerald et al., 1987).

4.1.3 Comparison with Data Derived from the Nucleotide Sequence

All of the tryptic peptides can be found within the predicted amino acid sequence, with only seven predicted residues differing from the tryptic peptides, out of the 128 amino acids obtained from the tryptic peptide sequencing (5% discrepancy). Discrepant residues include the following tryptic peptide residues as compared to predicted sequence derived from the cloned nucleotide sequence. Peptide 15a, Q instead of (/) G; peptide 49, E/C; peptide 20, D/C, G/W, Y/P, Q/K, and G/M. Twice, cysteine residues were mistaken for acidic residues. Thus, all of the partial sequences of MALA-2 have been confirmed as accurate, with the exception of the latter portion of peptide 20. Reciprocally, the partial sequences confirm that the cloned gene is indeed for MALA-2.

The nucleotide sequence data predicts the presence of a potential nine glycosylation sites, thereby confirming research in this thesis that multiple N-glycosylation sites exist, and that the protein backbone of MALA-2 is approximately 60 kD M_r. Also explained is the N-terminal blockage of MALA-2, which likely took place at the N-terminal glutamine residue, which is capable of cyclysing (thus, the N-terminus may be correspond to position 2 of the sequence shown in Horley et al., 1989). Comparison of the nucleotide and predicted amino acid sequence data from MALA-2 with GenBank, and with the NBRF database did not reveal significant homology with any one single protein, but did reveal striking homology to the recently published nucleotide and amino acid sequences of ICAM-1. The amino acid homology to ICAM-1 was found to be 50%, which correlates well with the average homology of the partial amino acid sequences to

ICAM-1. Further experiments demonstrated that MALA-2 is capable of binding to LFA-1.

Thus, the evidence in this thesis supports the identification of MALA-2 as the murine homolog of ICAM-1. Subsequent confirmation through cloning of the gene for MALA-2, was facilitated by the partial amino acid sequences revealed in this thesis, which have served as templates in the construction of cDNA probes. The cDNA probe which facilitated cloning was based on the amino acid sequence of peptide 15a.

4.2 ROLE OF THE YN1/1.7.4 ANTIBODY IN IMMUNOLOGICAL RESEARCH

4.2.1 The Role of MALA-2 and ICAM-1 in the Immune System

The human ICAM-1 molecule has been far more intensively studied to date than the corresponding murine homolog, MALA-2. MALA-2 was known prior to this thesis study to be involved in some manner in lymphocyte proliferation (Takei, 1985). Since MALA-2 has been shown to be a murine ligand for LFA-1, it would seem that the ability of the YN1/1.7.4 antibody to block lymphocyte proliferation was the result of this antibody's ability to block lymphocyte adhesive interactions, as has been shown for ICAM-1 (Boyd et al., 1988; Makgoba et al., 1988; Dougherty et al., 1988; Dustin et al., 1988). This role in lymphocyte intercellular adhesion is antigen-independent, and is critical to the generation of an immune response, both humoral and cellular (Boyd et al., 1988; Dougherty et al., 1988). The receptor for ICAM-1 (and MALA-2) is LFA-1, a molecule which has been studied intensively over the past five years. The central significance of LFA-1 has been

vividly demonstrated by the ability of anti-LFA-1 antibodies to induce tolerance (Springer et al., 1987), and to promote acceptance of HLA-mismatched bone marrow grafts (Fischer et al., 1987). The role of ICAM-1 (and MALA-2) as a ligand of LFA-1 is thus of great importance, although it must be recognized that ICAM-1 is not the only ligand for LFA-1 (Rothlein et al., 1986; Makgoba et al., 1988; Dustin et al., 1988).

In addition to mediating antigen-independent cellular adherence in the immune system, ICAM-1 has been suggested to be the controlling factor in its adhesive interactions with LFA-1 since its expression is much more highly regulated than LFA-1 (Boyd et al.,1989). Because the expression of ICAM-1 is strongly influenced on a wide variety of cells by inflammatory cytokines, it has further been suggested that ICAM-1 plays an essential and regulatory role in inflammation reactions (Ibid).

Thus, as the murine homolog of ICAM-1, it is likely that MALA-2 functions in a similar manner, although this has yet to be shown. Further research will determine the in vivo functions of MALA-2, which can then be compared to the known properties of ICAM-1, and which may contribute to our knowledge of this LFA-1 ligand in general.

4.2.2 YN1/1.7.4 Monoclonal Antibody as an Investigative Tool

The monoclonal antibody YN1/1.7.4 developed in our laboratory specifically recognizes the murine activated lymphocyte antigen-2 (MALA-2). Since this molecule has been shown to be homologous to the human ICAM-1 molecule, this antibody serves as an important tool in

the investigation of its function. It has been suggested that the use of animal models and MAbs is the only practical way to assess the physiological roles of LFA-1 and ICAM-1 *in vivo* (Arfros et al., 1987). Since the YN1/1.7.4 antibody is the only MAb shown to recognize an animal homolog of the ICAM-1 molecule, its use in this manner may be invaluable in the exploration of the *in vivo* significance of the LFA-1/MALA-2 (ICAM-1) interaction.

4.3 SUMMARY

In this thesis study, the murine lymphocyte activation antigen MALA-2 has been purified to homogeneity, biochemically characterized, and its partial amino acid sequence determined. MALA-2 was shown to share its most consistent homology with the immunoglobulin superfamily, to have an acidic isoelectric point, and to be a glycoprotein with a protein backbone of not more than 66 kD M_r. Subsequent to this study, the partial amino acid sequences of MALA-2 have facilitated the cloning of the gene for MALA-2. The resulting cDNA information has confirmed the research detailed within this thesis, and has identified MALA-2 as the murine homolog of the human ICAM-1 molecule. The YN1/1.7.4 MAb which recognizes MALA-2 should prove to be a significant tool in investigations of the *in vivo* functions of MALA-2 and ICAM-1.

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Appendix 120

The EMBO Journal vol.8 no.10 pp.2889–2896, 1989

Molecular cloning of murine intercellular adhesion molecule (ICAM-1)

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Communicated by A.Williams

We have previously reported a murine lymphocyte surface antigen MALA-2 of ~95 000 M_r which is expressed mainly on activated lymphocytes. The rat monoclonal antibody YN1/1 that detects this antigen profoundly inhibits mixed lymphocyte response. We have now purified MALA-2 and determined its partial amino acid sequence. By using non-redundant synthetic oligonucleotides as probes, based on the amino acid sequence, we have isolated two full length cDNA clones encoding MALA-2. The two clones are identical except for the 5′ end sequence. Expression of MALA-2 on transfected COS cells is only achieved with one of the two cDNA clones. The nucleotide sequence as well as the deduced amino acid sequence of MALA-2 display striking homology with those of the recently reported human intercellular adhesion molecule ICAM-1. All the unique features of the human ICAM-1, including its homology with the neural adhesion molecule NCAM, its internal repeat structure and the immunoglobulin-like structure, are found in MALA-2. Furthermore, purified MALA-2 crosslinked to a solid support binds Con A blasts that express LFA-1, the putative receptor for ICAM-1, and the binding can be blocked by YN1/1 antibody or anti-murine LFA-1 antibody indicating a direct interaction of these molecules in cell adhesion. Therefore, we consider MALA-2 to be the murine homolog of human ICAM-1. Since ICAM-1 is known to be of primary importance in immune responses and inflammatory reactions, having a monoclonal antibody and a mouse model will provide the opportunity to study the functional role of ICAM-1 *in vivo*.

Key words: adhesion molecule/lymphocyte adhesion/ immunoglobulin supergene family

Introduction

Specific cell–cell interactions in embryogenesis, histogenesis, immune responses and inflammatory reactions are thought to be mediated by adhesion molecules expressed on the cell surface. In the immune system adhesion molecules have been shown to be vital proteins in cell–cell interactions essential for a wide range of immune responses. In particular, the lymphocyte-function associated molecule (LFA-1) is involved in many adherence dependent cell functions including T lymphocyte mediated cytolysis and proliferation (Krensky *et al.*, 1983), homotypic aggregation of lymphocytes (Mentzer *et al.*, 1985; Rothlein and Springer, 1986) and antigen non-specific natural killing (Hildreth *et al.*, 1983). LFA-1 has a broad distribution within the hemopoietic cell types including lymphocytes, natural killer cells, monocytes, macrophages and granulocytes (Springer *et al.*, 1987). It is a heterodimer ($\alpha\beta$) sharing its common β chain with the receptor for complement component iC3b (Mac1) and the p150/95 protein (Sanchez-Madrid *et al.*, 1983). These antigens are restricted to leukocytes and constitute a subfamily of integrins involved in cell–cell and cell–matrix interactions (Hynes, 1987).

The intercellular adhesion molecule (ICAM-1) has recently been identified as the ligand for LFA-1 (Rothlein *et al.*, 1986; Marlin and Springer, 1987; Makgoba *et al.*, 1988a). It has been characterized in the human system as a 90–115 kd glycoprotein, most prominently expressed on activated lymphocytes and at sites of inflammation (Dustin *et al.*, 1986; Simmons *et al.*, 1988). It is a member of the immunoglobulin (Ig) supergene family displaying highest homology with two other cell adhesion molecules, namely neural cell adhesion molecule (NCAM) and myelin associated glycoprotein (MAG) (Simmons *et al.*, 1988; Staunton *et al.*, 1988). The murine homolog of ICAM-1 has not yet been reported.

A specific receptor–ligand relationship exists between LFA-1 and ICAM-1 (Marlin and Springer, 1987; Makgoba *et al.*, 1988a). This is the first example of a member of the integrin family interacting with a member of the Ig supergene family (Dustin *et al.*, 1988). Monoclonal antibodies (MAb) to either ICAM-1 or LFA-1 inhibit several T cell dependent responses *in vitro*, indicating the importance of these molecules in immune responses. However, it has been postulated that ICAM-1 may belong to a family of ligands which bind LFA-1 since some LFA-1 dependent aggregation is unaffected by ICAM-1 MAb (Makgoba *et al.*, 1988b). ICAM-2 has recently been characterized as a second ligand for LFA-1 (Staunton *et al.*, 1989). Its distribution and size is different from ICAM-1, and may account for the ICAM-1 independent binding observed.

We have previously described a murine antigen, MALA-2, primarily expressed on activated lymphocytes (Takei, 1985). The MAb YN1/1 that detected MALA-2 profoundly inhibits mixed lymphoycte response, suggesting that MALA-2 is involved in lymphocyte activation. We now report the isolation and sequencing of full length cDNA clones encoding MALA-2 and expression of one of these clones in transfected COS cells. The deduced amino acid sequence of MALA-2 shows significant homology with that of the human ICAM-1 sequence (Simmons *et al.*, 1988; Staunton *et al.*, 1988). Furthermore, purified MALA-2 binds to LFA-1^+ cells in a MALA-2/LFA-1 dependent manner supporting the notion that MALA-2 may be the murine homolog of ICAM-1. Although the role of ICAM-1 in immune responses *in vitro* has been well studied, its *in vivo*

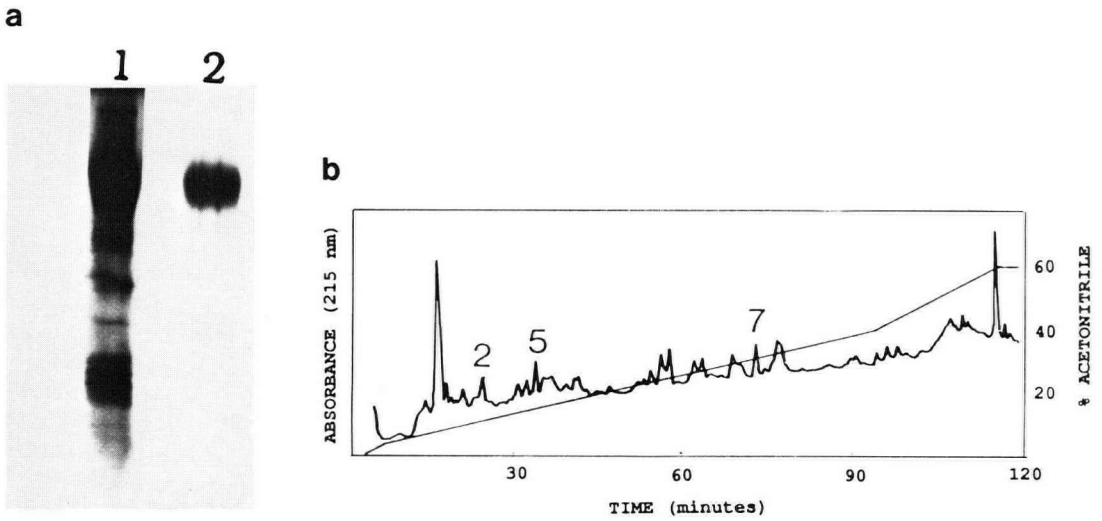


Fig. 1. Purification of MALA-2 and separation of tryptic peptides. (**a**) MALA-2 was purified from NS-1 cells by the combination of YN1/1 antibody affinity chromatography and preparative SDS−PAGE. The purified protein was analyzed by SDS−PAGE and silver staining. Lane 1 shows the fraction recovered from the antibody affinity column. Lane 2 is the fraction eluted from the preparative non-reducing SDS−PAGE gel. (**b**) Separation of MALA-2 tryptic peptides on C18 reverse phase HPLC. The numbers on the peaks indicate the three peptides whose sequences were used to synthesize the oligonucleotides for the library screening.

role is yet to be established. The monoclonal antibody as well as the cDNA clones will be invaluable tools in the elucidation of the functional significance of this adhesion molecule *in vivo*.

Results

Purification and amino acid sequence

The MALA-2 protein was purified from NS-1 cells by the combination of YN1/1 antibody affinity chromatography and preparative non-reducing SDS−PAGE. The protein eluted from the gel was re-analyzed by reducing SDS−PAGE. The gel showed the isolated protein to be essentially pure (Figure 1a). Approximately 50 pmol of the purified protein was subjected to N-terminal sequencing. However, no detectable signal was obtained, suggesting that the purified protein was N-terminal blocked. Therefore, ~200 pmol of the purified protein was reduced, alkylated and digested with trypsin. The resulting tryptic peptides were separated by C18 reverse phase HPLC (Figure 1b) and sequenced. From two purification and sequencing experiments, a total of 14 peptides were sequenced, four of which were sequenced twice (Table I).

Table I. MALA-2 tryptic peptides

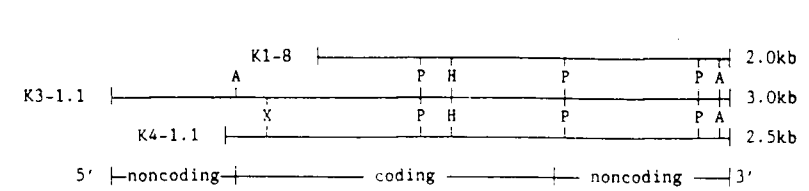
Fraction	Peptide sequence	Confirmed
1	QPVGGHPK	+
2[a]	SDHQANFSCR	−
3	TLPLR	−
4	DQAEGNPSYQG	−
5[b]	QMPTQEST	+
6	ALVEVTEEFDR	−
7[c]	LFESLEGLFPASEAR	+
8	LELADQILETQ	+
9	TFDLPATIPK	−
10	DELESGPNWK	−
11	GQTLELH	−
12	TLNASSEDHK	−
13	AQEEAIK	−
14	FLFK	−

[a]Peptide sequences used for construction of nonredundant probes.
[b]Peptide sequence used to construct redundant probe (17mer mixture).
[c]Peptide sequences duplicated from independent tryptic digestions.

Isolation and analysis of MALA-2 cDNA

Three tryptic peptide sequences confirmed by repeated purification and amino acid sequencing experiments were used to synthesize oligonucleotides. Due to high redundancies, two non-redundant oligonucleotides were constructed based on the preferred codon usage table (Lathe, 1985). The third oligonucleotide had a redundancy of 64.

A λgt10 cDNA library constructed from NS-1 mRNA was initially screened with the short non-redundant probe at low stringency (1×SSC, 30°C) allowing 25% mismatch. From 10^5 plaques, 45 positive phage clones were isolated and screened with the other two probes. One phage clone, K1-8, hybridized with all three probes. The 2.0 kbp cDNA insert was subcloned into pTZ19R plasmid vector and sequenced. The sequence had a long open reading frame (ORF) and polyadenylation signal but lacked an initiation codon (Figure 2a). Northern blot analysis of NS-1 RNA detected a transcript of >2.4 kbp. Therefore, the K1-8 cDNA insert was considered incomplete and the library was rescreened, initially using K1-8 cDNA insert deleted of the poly A tail (*Pvu*II digestion) and subsequently the 5′ *Hin*dIII fragment of K1-8 as probes. The screening identified 41 additional phage clones (16 positive with the 5′ *Hin*dIII fragment), all with inserts of 2.2−3.0 kbp. Two phage clones, K3-1.1 and K4-1.1 were selected for their long cDNA insert (3.0 and 2.5 kbp, respectively). They were subcloned into plasmids and sequenced. K3-1.1 clone is 3031 bp long and has a long ORF of 1593 nucleotides, a 5′ untranslated sequence of 552 nucleotides and a 3′ untranslated region of 851 nucleotides (Figure 2a). The 3′ untranslated region contains a poly-adenylation signal and a poly A tail. The K4-1.1 clone is 2525 bp long with only 29 bp 5′ untranslated sequence and



b K4-1.1

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CGCTACCTGCACTTTGCCCTGGCCCTGCAATGGCTTCAACCCGTGCCAAGCCCACGCTACCTCTGCTCCTGGCCCTGGTCACCGTTGTGATCCCTGGGCCTGGTGATGCTCAGGTA  116
                                MetAlaSerThrArgAlaLysProThrLeuProLeuLeuLeuAlaLeuValThrValValIleProGlyProGlyAspAlaGlnVal    4
                              -25                                                                  +1
TCCATCCATCCCAGAGAAGCCTTCCTGCCCCAGGGTGGGTCCGTGCAGGTGAACTGTTCTTCCTCATGCAAGGAGGACCTCAGCCTGGGCTTGGAGACTCAGTGGCTGAAAGATGAGCTC  236
SerIleHisProArgGluAlaPheLeuProGlnGlySerValGlnValAsnCysSerSerSerCysLysGluAspLeuSerLeuGlyLeuGluThrGlnTrpLeuLysAspGluLeu   44
                                                    ---CHO---
GAGAGTGGACCCAACTGGAAGCTGTTTGAGCTGAGCGAGATCGGGGAGGACAGCAGTCCGCTGTGCTTTGAGAACTGTGGCACCGTGCAGTCGTCGGCTTCCGCTACCATCACCGTGTAT  356
GluSerGlyProAsnTrpLysLeuPheGluLeuSerGluIleGlyGluAspSerSerProLeuCysPheGluAsnCysGlyThrValGlnSerSerAlaSerAlaThrIleThrValTyr   84

TCGTTTCCGGAGAGTGTGGAGCTGAGACCTCTGCCAGCCTGGCAGCAAGTAGGCAAGGACCTCACCCTGCGCTGCCACGTGGATGGTGGAGCACCGCGGACCCAGCTCTCAGCAGTGCTG  476
SerPheProGluSerValGluLeuArgProLeuProAlaTrpGlnGlnValGlyLysAspLeuThrLeuArgCysHisValAspGlyGlyAlaProArgThrGlnLeuSerAlaValLeu  124

CTCCGTGGGGAGGAGATACTGAGCCGCCAGCCAGTGGGTGGGCACCCCAAGGACCCCAAGGAGATCACATTCACGGTGCTGGCTAGCAGAGGGGACCACGGAGCCAATTTCTCATGCCGC  596
LeuArgGlyGluGluIleLeuSerArgGlnProValGlyGlyHisProLysAspProLysGluIleThrPheThrValLeuAlaSerArgGlyAspHisGlyAlaAsnPheSerCysArg  164
   R  G  E                                                                                R  G  D   ***   ---CHO---
ACAGAACTGGATCTCAGGCCGCAAGGGCTGGCATTGTTCTCTAATGTCTCCGAGGCCAGGAGCCTCCGGACTTTCGATCTTCCAGCTACCATCCCAAAGCTCGACACCCCTGACCTCCTG  716
ThrGluLeuAspLeuArgProGlnGlyLeuAlaLeuPheSerAsnValSerGluAlaArgSerLeuArgThrPheAspLeuProAlaThrIleProLysLeuAspThrProAspLeuLeu  204
                                    ---CHO---
GAGGTGGGCACCCAGCAGAAGTTGTTTTGCTCCCTGGAAGGCCTGTTTCCTGCCTCTGAAGCTCGGATATACCTGGAGCTGGGAGGCCAGATGCCGACCCAGGAGAGCACAAACAGCAGT  836
GluValGlyThrGlnGlnLysLeuPheCysSerLeuGluGlyLeuProAlaSerGluAlaArgIleTyrLeuGluLeuGlyGlyGlnMetProThrGlnGluSerThrAsnSerSer  244
                  ***                                                                                          ---CHO---
GACTCTGTGTCAGCCACTGCCTTGGTAGAGGTGACTGAGGAGTTCGACAGAACCCTGCCGCTGCGCTGCGTTTTGGAGCTAGCGGACCAGATCCTGGAGACGCAGAGGACCTTAACAGTC  956
AspSerValSerAlaThrAlaLeuValGluValThrGluGluPheAspArgThrLeuProLeuArgCysValLeuGluLeuAlaAspGlnIleLeuGluThrGlnArgThrLeuThrVal  284

TACAACTTTTCAGCTCCGGTCCTGACCCTGAGCCAGCTGGAGGTCTCGGAAGGGAGCCAAGTAACTGTGAAGTGTGAAGCCCACAGTGGGTCGAAGGTGGTTCTTCTGAGCGGCGTCGAG 1076
TyrAsnPheSerAlaProValLeuThrLeuSerGlnLeuGluValSerGluGlySerGlnValThrValLysCysGluAlaHisSerGlySerLysValValLeuLeuSerGlyValGlu  324
   ---CHO---
CCTAGGCCACCCACCCCGCAGGTCCAATTCACACTGAATGCCAGCTCGGAGGATCACAAACCAAGCTTCTTTTGCTCTGCCGCTCTGGAGGTGGCGGGAAAGTTCCTGTTTAAAAACCAG 1196
ProArgProProThrProGlnValGlnPheThrLeuAsnAlaSerSerGluAspHisLysProSerPhePheCysSerAlaAlaLeuGluValAlaGlyLysPheLeuPheLysAsnGln  364
                              ---CHO---                                                                          ***
ACCCTGGAACTGCACGTGCTGTATGGTCCTCGGCTGGACGAGACGGACTGCTTGGGGAACTGGACCTGGCAAGAGGGGTCTCAGCAGACTCTGAAATGCCAGGCCTGGGGGAACCCATCT 1316
ThrLeuGluLeuHisValLeuTyrGlyProArgLeuAspGluThrAspCysLeuGlyAsnTrpThrTrpGlnGluGlySerGlnGlnThrLeuLysCysGlnAlaTrpGlyAsnProSer  404
                                                    ---CHO---                    ***
CCTAAGATGACCTGCAGACGGAAGGCAGATGGTGCCCTGCTGCCCATCGGGGTGGTGAAGTCTGTCAAACAGGAGATGAATGGTACATACGTGTGCCATGCCTTTAGCTCCCATGGGAAT 1436
ProLysMetThrCysArgArgLysAlaAspGlyAlaLeuLeuProIleGlyValValLysSerValLysGlnGluMetAsnGlyThrTyrValCysHisAlaPheSerSerHisGlyAsn  444
*********                                                                    ---CHO---                    ---
GTCACCAGGAATGTGTACCTGACAGTACTGTACCACTCTCAAAATAACTGGACTATAATCATTCTGGTGCCAGTACTGCTGGTCATTGTGGGCCTCGTGATGGCAGCCTCTTATGTTTAT 1556
ValThrArgAsnValTyrLeuThrValLeuTyrHisSerGlnAsnAsnTrpThrIleIleIleLeuValProValLeuLeuValIleValGlyLeuValMetAlaAlaSerTyrValTyr  484
CHO---
AACCGCCAGAGAAAGATCAGGATATACAAGTTACAGAAGGCTCAGGAGGAGGCCATAAAACTCAAGGGAACAGCCCCACCTCCCTGAGCCTGCTGGATGAGACTCCTGCTGGACCCCCTG 1676
AsnArgGlnArgLysIleArgIleTyrLysLeuGlnLysAlaGlnGluGluAlaIleLysLeuLysGlyThrAlaProProPro                                    512

CAGGCAACAGCTGCTGCTGCTTTTGAACAGAATGGTAGACAGCATTTACCCTCAGCCACTTCCTCTGGCTGTCCACAGAACAGGATGGTGGCCTGGGGGATGCACACTTGTAGCCTCAGA 1796
GCTAAGAGGACTCGGTGGATGGAGCAAGACTGTGAACACGTGTGACCCGGACCCACCTACAGCCCGGTGGACCTTCAGCCAAGAAACGCTGACTTCGTTCTCTATTGCCCCTGCTGAGGG 1916
GTCCTGCTAAGGAAGACATGATATCCAGTAGACACAAGCAAGAAGACCACACTTCCCCCCGACAAGGAAAGCTGAGACATTGTCCCCATCTCTTCTTGATGTATTTATTAATTTAGAGTT 2036
TTACCAGCTATTTATTGAGTACCCTGTATATAGTAGATCAGTGAGGAGGTGAATGTATAAGTTATGGCCTGGACCCTGCTGCAGATGCTGTGAGAGTCTGGGGAAAGATCACATGGGTCG 2156
ACGGTTTCTCTACTGGTCAGGATGCTTTTCTCATAAGGGTCGACTTTTTTCACCAGTCACATAAACACTATGTGGACTGGCAGTGGTTCTCTGCTCCTCCACATCCTGGAGCGTCCCAGC 2276
ACCTCCCCACCTACTTTGTTCCCAATGTCAGCCACCATGCCTTAGCAGCTGAACAATCGAGCCTCATGCTCATGAAATCATGGTCCCAGGCGGCTCCACCTCAAAGAGAAAGCCTGGAA 2396
GGAAATGTTCCAACTCCTTAGAAGGGTCGTGCAAGCTGCTGTGGGAGGGTAAGCACCCCTCCCAGCACAGAAACCTTTCCTTTGAATCAATAAAGTTTTATGTCGGCTGAAAAAAAAAAA 2516
AAAAAAAAA                                                                                                              2525
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c K3-1.1

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AATTCCTTTCACGATGGCAAATATTAGGAATTTATTAAAACCACTGTCTCCAACGGTACAAAAATTAAATATTAAGGCATCTTATACTCTCATCCTGACCTATGCAGCAAAGAGAATTATC 121
TTAAATAAGAGCTAGCACTTATTCAAATGCTTTTCTAACGTATTTGGCAAATTTGTTGTTTTTGTTTTGTTCGGTGTACGGTCATGCATGCAAGTGTACCTGGTATTTGTATGTGTGTGC  241
AAACGGGTGTATGCACATGTGCTTGCCTGTTGAGGCCAGAGATTTATGTTCTACATCTTCCTCTATTGCTCTCTACCTGAACCAGGTGCTCAGTGACTCAGCCAGACTGTCAGGGCAGCA  361
AGCTGGGGAATCCTCCTGTCTCTGCCTCCCCAGCTCTGCGTCTCAGGTCATGCCCCGCTTTTTATGTGGCTGTTGGGGATCTGAACTCAGGACCTCAGGCTTGCACAGCAAACCTTTACT  481
GACTGAGCCACTTCTCCGGCCCCTCACTTTTCTTCTTATATTCTTTTATTATTCACATAACTGAAAGCATTCATGATCACACACCGGCATCCAGTCCGAGAGAAAAGCATAAACAGTTAT  601
                                                                                                    MetIleThrHisArgHisProValArgGluLysSerIleAsnSerTyr

CAATTTATTAAGGAGAAGCAGTTTCCTGCTGAAAATGAAGCCTTCCTGCCCCAGGGTGGGTCCGTGCAGGTGAACTGTTCTTCCTCATGCAAGGAGGACCTCAGCCTGGGCTTGGAGACT  721
GlnPheIleLysGluLysGlnPheProAlaGluAsnGluAlaPheLeuProGlnGlyGlySerValGlnValAsnCysSerSerSerCysLysGluAspLeuSerLeuGlyLeuGluThr
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Fig. 2. Sequence of MALA-2 cDNA. (**a**) A partial restriction map of the MALA-2 cDNA clones is shown. A—*Asp*700I, P—*Pvu*II, H—*Hin*dIII, X—*Xho*I. The cDNA clone K1-8 was initially isolated. Subsequently, the full length cDNA clones K3-1.1 and K4-1.1 were isolated and sequenced. (**b**) Complete nucleotide sequence of MALA-2 cDNA (K4-1.1) and predicted amino acid sequence are shown. Cysteine residues are in bold letters, and the potential glycosylation sites are marked by −CHO−. The transmembrane domain is underlined by a bold line. The sequences identified by the tryptic peptide sequencing are underlined, and discrepant amino acid residues are marked by asterisks. The polyadenylation signal sequence in the 3′ untranslated segment is also underlined. The amino acid sequence is numbered from the predicted cleavage site of the signal peptide. (**c**) The nucleotide sequence of K3-1.1 cDNA and the deduced amino acid sequence is shown. Only the 5′ end of the sequence is shown. The sequence that is identical to those of the K4-1.1 clone are in bold and underlined. ATG codons are also underlined.

a 1611 bp ORF. The two clones have an identical sequence with the exception of their 5′ ends. The K4-1.1 clone encodes a typical type I transmembrane protein with a highly hydrophobic N-terminal amino acid sequence which probably functions as a leader sequence, and a hydrophobic region of 23 residues typical of transmembrane domains (underlined by a bold line in Figure 2b), followed by a cluster of highly charged amino acids. In contrast, the K3-1.1 clone has a long 5′ untranslated region (552 nucleotides) with multiple ATG codons (underlined in Figure 2c). The codon at position 14 best corresponds to the consensus sequence proposed by Kozak *et al.* (1986), but the ORF terminates at position 134. Two other codons (positions 204 and 411) also partially satisfy the criteria; however, these lack ORFs. The amino acid sequence immediately following the initiating codon contains mainly charged or polar amino acids lacking

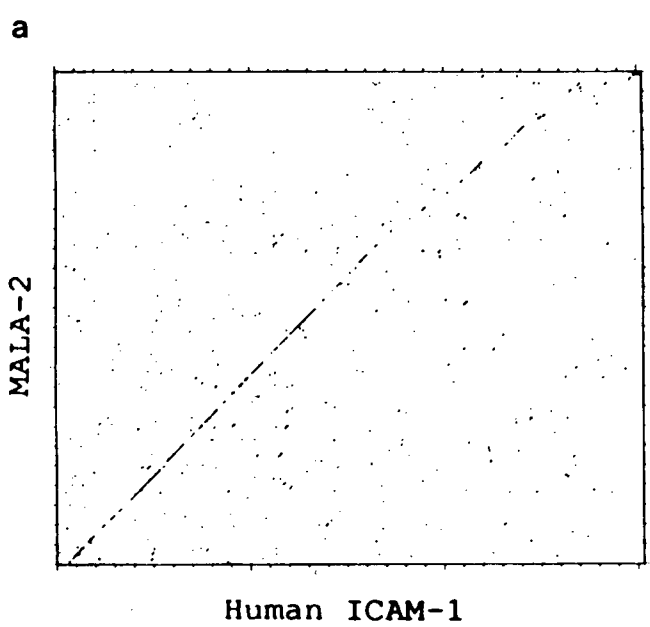

Fig. 3. Homology of MALA-2 sequence with ICAM-1 sequence. (**a**) The nucleotide sequence of MALA-2 is compared with the human ICAM-1 cDNA sequence (Simmons *et al.*, 1988) by diagonal dot-matrix comparison. Where 14 within a stretch of 21 nucleotides are the same between the MALA-2 and the ICAM-1 sequences, a dot is plotted. (**b**) The amino acid sequence of MALA-2 is aligned with that of the human ICAM-1. The third line shows the amino acid residue shared by the two sequences. Conserved cysteine residues are underlined.

hydrophobicity commonly found in leader sequences of type 1 transmembrane proteins. Therefore, the K4-1.1 clone is considered to code for a functional transmembrane protein, whereas the K3-1.1 clone probably represents an alternative splicing product, whose function is unknown. Thus, MALA-2 is a type I transmembrane protein with an extracellular domain of 461 amino acids and cytoplasmic domain of 28 amino acids. It has nine potential N-linked glycosylation sites and both an RGD and an RGE sequence which are found with the extracellular domain of the protein. All of the tryptic peptide sequences are accounted for within the deduced amino acid sequence (underlined in Figure 2b) although a few discrepant residues were noted (marked by asterisks in Figure 2b). None of the discrepancies can be explained by a single base pair change in the cDNA sequence. In light of the low signals of these amino acid residues in the amino acid sequencing experiments (40 pmol and below) we consider the cDNA-deduced sequence more reliable than those determined by peptide sequencing. Comparison of the cDNA sequence with the non-redundant probes based on tryptic peptides 2 and 7 (using a preferred codon usage criteria) revealed a 78% (peptide 2) and 69% (peptide 7) identity.

Homology studies

Searches of GenBank data base (Release 56.0) and the NBRF protein data base (Release 16.0) did not reveal any significant similarities to other proteins. However, comparison with recently published lymphocyte surface proteins revealed a striking similarity between MALA-2 and the human adhesion molecule ICAM-1. The similarity was evident both at the nucleotide level (Figure 3a) as well as the protein level (Figure 3b). The overall amino acid identity with the human ICAM-1 is 50%. Furthermore, the overall protein structure of MALA-2 is quite similar to that of ICAM-1. All the cysteine residues are conserved, and the internal repeat motif of ICAM-1 is also found in the MALA-2 sequence indicating

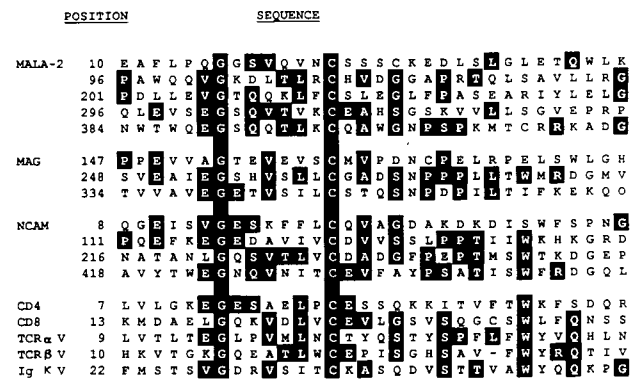

Fig. 4. Repeated sequences of MALA-2 and homology with other members of the immunoglobulin superfamily. Sequences were aligned using the Genetic Computer Group (University of Wisconsin) sequence analysis programs and by eye. No gaps were introduced except the one gap in the TCRβV sequence. Only sections of five internal repeats of MALA-2 are shown and compared with the internal repeats of myelin associated glycoprotein (MAG), neural cell adhesion molecule (NCAM), murine CD4 (Tourvieille *et al.*, 1986), CD8 (Nakauchi *et al.*, 1985), and the variable region of the T cell receptor α (Saito *et al.*, 1984), β (Morinaga *et al.*, 1985), and Igκ (Kelley *et al.*, 1982) chains. The residues in the MALA-2 sequence conserved in at least five sequences were boxed with exceptions of those found in four sequences in three different proteins. The tryptophan residues highly conserved among the members of the immunoglobulin superfamily are also boxed.

that MALA-2 is a murine homolog of human ICAM-1. MALA-2 was also compared with human ICAM-2 and found to have a 21% amino acid identity. Comparison of MALA-2 to members of the Ig supergene family (Figure 4) revealed homology in specific segments of amino acid sequence ~100 residues in length. This indicates a repeating motif within the MALA-2 protein representing Ig-like domains. In the relatively short segments surrounding the cysteine residues the sequence similarity with the other members of the Ig

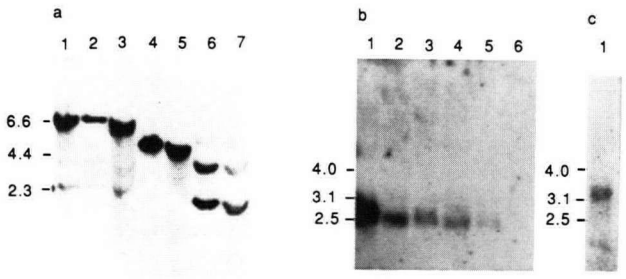

Fig. 5. Southern and Northern Blot Analysis. (**a**) Genomic DNA was isolated from NS-1 cells (lane 1) and BALB/c (lanes 2, 4 and 6) and C57BL/6 (lanes 3, 5 and 7) spleen cells. Approximately 10 μg was digested with *Eco*RI (lanes 1, 2 and 3), *Bam*HI (lanes 4 and 5) and *Dra*I (lanes 6 and 7), and Southern blots were prepared and probed as described in Materials and methods. (**b**) Total RNA was isolated from NS-1 cells (lane 1), LPS activated spleen cells (lane 2), Con A activated spleen cells (lane 3), spleen cells (lane 4), thymocytes (lane 5) and BALB/c bone marrow cells (lane 6). Approximately 10 μg of total RNA was run on formaldehyde gel and probed with K4-1.1 cDNA insert. (**c**) Total RNA from NS-1 cells was probed with the 5′ *Asp*700I fragment of K3-1.1 clone.

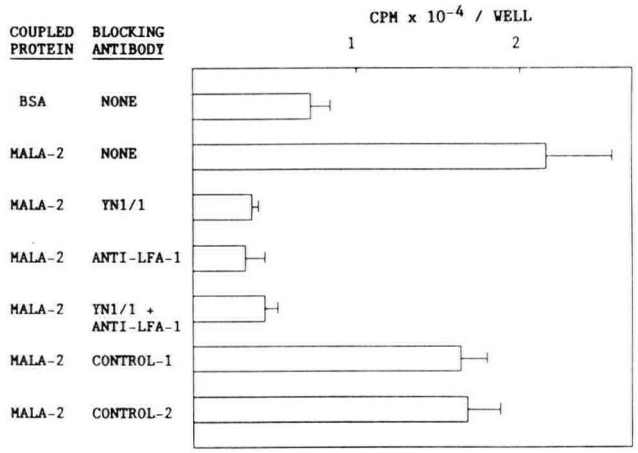

Fig. 6. Binding of Con A stimulated spleen cells to purified MALA-2. MALA-2 was purified from NS-1 cells and coupled to microculture wells as described in Materials and methods. To control wells, BSA was coupled in place of MALA-2. ^{51}Cr-labelled Con A stimulated spleen cells were incubated in the wells in the presence of blocking antibodies, and the binding of the cells were estimated by the radioactivity bound to the wells. The results shown are means of triplicate tests (± SD). Two control antibodies are rat monoclonal antibodies of the same isotype (IgG 2b) and they bind to unrelated surface antigens expressed on Con A activated spleen cells.

family is quite evident. Figure 4 shows the first 37 amino acids within the 100 residue segments. The $G-X_6-C$ motif is found in all of the sequences listed including two other adhesion molecules, the neural cell adhesion molecule (NCAM) and myelin associated glycoprotein (MAG). The residues between the cysteine and glycine residues as well as those on the C-terminal side of the cysteine are relatively conserved; however, the residues on the N-terminal side of the glycine residue are less conserved. the tryptophan residues boxed in Figure 4 are highly conserved among most members of the Ig superfamily, but it is not found in any of the Ig-like repeated segments of MALA-2. Considering the homology of MALA-2 with members of the Ig supergene family, disulfide loops of the Ig-like domains are probably formed between pairs of cysteine residues at the positions 23 and 70, 109 and 163, 214 and 267, 309 and 349, 381 and 436.

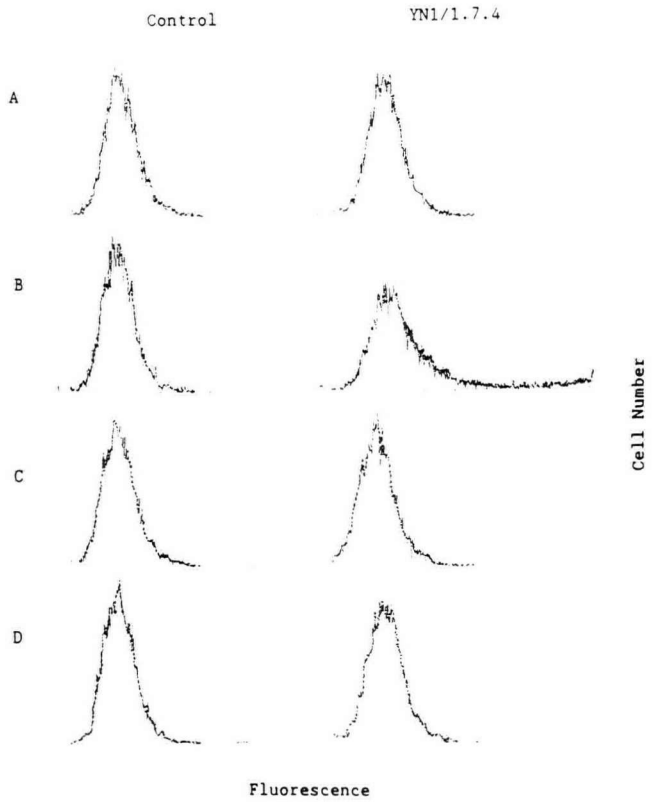

Fig. 7. COS cells were transfected with K3-1.1 cDNA in pAX82 (**A**), K4-1.1 cDNA in pAX82 (**B**) pAX82 alone (**C**) or mock transfected (**D**). MALA-2 expression on cell surface was analyzed with YN1/1 antibody and goat anti-rat immunoglobulin-FITC by flow cytometry using a FACStar Plus. As controls, cells were incubated with the second antibody alone. Data are plotted with fluorescence intensity versus cell number.

Southern and Northern blot analysis

In genomic southern blot analysis, C57BL/6 and BALB/c murine DNA digested with *Bam*HI or *Eco*RI resulted in the detection of a single band (Figure 5a). The *Dra*I digestion displayed two bands, but due to the presence of a *Dra*I site within the cDNA insert this result was expected. Therefore, MALA-2 appears to be a single copy gene.

Analysis of the transcripts in normal lymphatic tissues revealed that the expression of MALA-2 in the bone marrow and thymus is very low, while the spleen exhibits readily detectable levels and activated lymphocytes have higher levels. NS-1 cells express the highest level of the transcripts. This is consistent with our previous study demonstrating the expression of MALA-2 protein primarily on activated lymphocytes and lymphoid cell lines (Takei, 1985). Primary transcripts of 2.5 kb were detected by K4-1.1 cDNA; however, another transcript of ~3.1 kb was also observed. This 3.1 kb transcript, but not the 2.5 kb transcript, hybridized with the *Asp*700I fragment of K3-1.1, consisting of the 5′ end 548 bp. Therefore, the K3-1.1 cDNA clone probably represents the 3.1 kb mRNA.

Binding assay

To determine if MALA-2, like human ICAM-1, could act as a ligand for LFA-1, purified MALA-2 was crosslinked to microwells and the binding of LFA-1$^+$ cells to the immobilized MALA-2 was tested. Con A stimulated spleen cells used as LFA-1$^+$ cells bound to the MALA-2 coupled wells but not to BSA coupled control wells (Figure 6). The

binding of Con A blasts to MALA-2 was inhibited by YN1/1 antibody, anti-LFA-1 antibody or both together. Two control antibodies with the same isotype (IgG 2b), YE3/19 (Takei, 1984) and YE6/6 (Takei, 1987) reactive with unrelated antigens expressed on Con A stimulated spleen cells, did not significantly inhibit the binding. These results demonstrate that MALA-2 has a similar function as human ICAM-1 and does act as a ligand for murine LFA-1.

MALA-2 expression in COS1 cells

COS1 cells were transfected with the transient expression vector pAX82 (R.Kay, manuscript in preparation) construct containing either the K3-1.1 or K4-1.1 clone (Figure 7). Of the cells transfected with the K4-1.1 clone, 23% expressed MALA-2 on their surface whereas cells transfected with the K3-1.1 clone were negative for MALA-2 expression. Thus the K3-1.1 clone does not seem to be efficiently processed for cell surface expression. COS cells transfected with the K3-1.1 clone lacking the 5′ untranslated region stain negative for surface expression of MALA-2 (data not shown). Mock transfected cells and cells transfected with pAX82 alone were also MALA-2 negative.

Discussion

MALA-2 is a 95 kd monomer antigen expressed on murine activated lymphocytes and lymphoid cell lines (Takei, 1985). The MAb YN1/1 that detects this antigen inhibits mixed lymphocyte reaction suggesting that it is involved in the activation of T cells. In this study, we have determined partial amino acid sequences of MALA-2, isolated and characterized cDNA clones encoding MALA-2, and expressed MALA-2 in COS cells by transfection. The amino acid sequence deduced from the cDNA clones contains all the tryptic peptide sequences generated from the purified MALA-2 indicating that the isolated cDNA clones indeed code for MALA-2. The nucleotide as well as the deduced amino acid sequence have striking similarities with those of human ICAM-1. The similarity between MALA-2 and ICAM-1 is particularly evident in the overall structures of these two proteins. Both consist of five similar segments, each having a size of ~100 amino acid residues and also exhibiting homology with members of the Ig supergene family. The amino acid sequence flanking the first cysteine residue in each repeated segment is particularly well conserved among some members of the Ig supergene family, including murine CD4 (Tourvieille *et al.*, 1986). CD8 (Nakauchi *et al.*, 1985), T cell receptor Vα (Saito *et al.*, 1984) and Vβ (Morinaga *et al.*, 1985), and Ig V$\varkappa$ (Kelley *et al.*, 1982). The MALA-2 structure is also similar to two other cell adhesion molecules, MAG and NCAM. They all consist of multiple repeats, each having homology with other segments. MALA-2 also demonstrates functional similarity with ICAM-1. ICAM-1 has been shown to bind another cell surface molecule, LFA-1 (Marlin and Springer, 1987). In this study, we have also demonstrated that purified MALA-2 immobilized on microtiter plates binds Con A stimulated spleen cells and binding is specifically inhibited by antibody to MALA-2 or murine LFA-1. Thus, both structurally and functionally, MALA-2 is similar to human ICAM-1. The size and distribution of the two proteins is virtually identical (Takei, 1985; Prieto *et al.*, 1989). Although ICAM-2 has recently been identified as another ligand for LFA-1, it is only slightly homologous (21%) to MALA-2, and has a different size and tissue expression from MALA-2. The summation of this evidence strongly supports MALA-2 as the murine homolog of human ICAM-1.

We have isolated two full length cDNA clones encoding MALA-2. One (K4-1.1) codes for a typical type I transmembrane protein whereas the other (K3-1.1) encodes a protein that has an atypical N-terminal sequence for a type 1 transmembrane protein. Its N-terminal sequence lacks obvious hydrophobicity of a leader sequence. The K3-1.1 clone probably represents the mRNA of ~3.1 kb detected by Northern blot analysis using K4-1.1 as a probe. The 3.1 kb mRNA is also detected using the 5′ *Asp*700I fragment of K3-1.1 as a probe, thus proving this 5′ region is not a cloning artifact. It is likely that the two transcripts are generated by differential splicing of the same gene. Expression of MALA-2 in COS cells is only detected in those transfected with K4-1.1 clone (Figure 7). Thus, it seems the K3-1.1 clone is not processed to a detectable level of expression in COS cells. Whether it is translated and the protein product is expressed in some cells is currently under investigation.

The expression of MALA-2 mRNA in different lymphocyte populations closely parallels our previous studies on cell surface expression of MALA-2 by flow cytometric analysis. Although MALA-2 mRNA and its protein product are primarily expressed in activated T and B cells, they are also detected in spleen cells. The majority of spleen cells are MALA-2$^-$ but a significant portion of them are MALA-2$^+$ (Takei, 1985). It is not known at this time whether the MALA-2$^+$ spleen cells represent functionally distinct lymphocytes such as previously activated memory cells as opposed to virgin lymphocytes. It is also unknown whether enhanced expression of MALA-2 on activated lymphocytes is functionally significant. LFA-1 is constitutively expressed on lymphocytes at a high level, while ICAM-1 expression on resting cells is quite low. However, ICAM-1 is readily detected on activated accessory cells (Dougherty *et al.*, 1988). Therefore, in the course of T cell activation, accessory cells are thought to express ICAM-1 first which interacts with LFA-1 on T cells, thus stabilizing the interaction of T cell−accessory cell contact. Once T cells are activated, this cell−cell contact does not seem to be required for the further proliferation and differentiation of T cells. Nevertheless, activated T cells express high levels of ICAM-1. Whether ICAM-1 is required for T cell−T cell or T cell−B cell interactions are important questions yet to be solved.

Our previous studies have shown that YN1/1 antibody almost completely inhibits mixed lymphocyte reaction. However, the antibody only partially inhibits Con A stimulation of spleen cells (up to 35%) and it does not inhibit LPS stimulation of spleen cells (Takei, 1985). In light of the present finding that MALA-2 is a cell adhesion molecule and functions as a ligand for LFA-1, the differences in the degree of sensitivities to YN1/1 antibody in different stimulation pathways may be due to differences in the requirement of cell−cell contact. The activation of T cells by specific antigens is known to require accessory cells that present antigens to T cells, and that process requires direct contact between T cells and macrophages. Although stimulation of T cells by mitogens also requires the presence of accessory cells (Williams *et al.*, 1984), direct T

cell–accessory cell contact may not be crucial. Further studies are needed to clarify the precise role of accessory cells and adhesion molecule mediated cell–cell contact in different activation pathways.

LFA-1 is a member of the integrin family of proteins and most integrin ligands have a core sequence Arg–Gly–Asp (RGD) which act as a binding domain for their receptors (Hynes, 1987; Ruoslahti and Pierschbacher, 1986). Human ICAM-1 lacks an RGD sequence but has an RGE sequence located within the second domain (Staunton *et al.*, 1988). MALA-2 contains an RGE as well as an RGD sequence. The conservation of the RGE between human ICAM-1 and our postulated murine ICAM-1 suggests that it may be important in the folding of the protein or as a binding site for LFA-1. Further studies deleting or modifying these sequences by site directed mutagenesis will give indication of their importance in maintaining the proper protein configuration and acting as binding sites for LFA-1.

The importance of the interaction between ICAM-1 and LFA-1 in immune responses *in vitro* has been well established by the profound inhibitory effects of MAb to these antigens. However, its functional significance in immune responses *in vivo* is yet to be established. The cDNA and the monoclonal antibody to murine ICAM-1 will be invaluable in the studies of the functional roles of the molecular interaction between ICAM-1 and LFA-1 in immune responses *in vivo*.

Materials and methods

Cells and antibodies

NS-1 cells (BALB/c myeloma) were maintained in tissue culture in Dulbecco's modified minimum essential media (DMEM) containing 5% fetal calf serum (FCS) and antibiotics. Spleen cells were stimulated with either Con A or Lipopolysaccharide (LPS) as described (Takei, 1985). The rat MAb YN1/1 that recognizes the murine lymphocyte antigen MALA-2, was purified from ascites fluid by $(NH_4)_2SO_4$ precipitation (50% saturation) followed by DEAE affi-gel blue (BioRad, Richmond, CA) chromatography. The rat hybridoma line FD441.8 (T1B 213) producing anti-mouse LFA-1 antibody was obtained from the American Type Culture Collection (Rockville, MD).

Purification of MALA-2 and amino acid sequencing

The plasma membrane fraction of NS-1 cells was solubilized in 1% Triton X-100, 10 mM Tris–HCl, 0.5 M NaCl, 0.01% NaN_3, and MALA-2 was purified by affinity chromatography with YN1/1 coupled to Affi-gel 10 (Bio-Rad). In brief, the membrane lysate combined with a cell lysate of 10^7 iodinated cells was incubated with YN1/1 Mab-coupled agarose beads on ice for 4 h with constant agitation. Beads were washed thoroughly overnight with 10 mM Tris–HCl buffer (pH 7.5) containing 1% Triton X-100, 0.5 M NaCl, 0.01% NaN_3 until no radioactivity was detected in the flow through. The column was briefly washed with the same buffer containing 0.1% Triton X-100, and MALA-2 was eluted with 0.05 M glycine–HCl (pH 2.9) buffer containing 0.05% Triton X-100, 0.15 M NaCl and 0.01% NaN_3. Radioactive fractions were pooled, neutralized, concentrated and loaded on non-reducing preparative SDS–PAGE gels. The major band of 95 kd relative molecular mass (M_r) was electroeluted as described (Chan and Takei, 1988). The purity of the eluted protein was assessed by SDS–PAGE followed by silver staining of the gels. The purified MALA-2 was reduced, alkylated and digested with TPCK treated trypsin, and the resultant peptides were separated on C18 reversed-phase HPLC as described (Chan and Takei, 1988a). The peptides were sequenced by a gas phase sequencer at the Tripartite Microsequencing Center (University of Victoria, BC).

Cloning and sequencing of MALA-2 cDNA

A cDNA library was constructed according to the method of Gubler and Hoffman (1983) using poly A^+ RNA from NS-1 cells. Based on tryptic peptide sequences (Table I), three oligonucleotides (antisense) were synthesized in Dr Mike Smith's laboratory (Department of Biochemistry, University of British Columbia). Two non-redundant oligonucleotides (probe 1: 5′GCGGCAGGAGAAGTTGGCCTGGTGGTC3′; probe 2: 5′GCGGGCCTCAGAGGCAGGGAACAGGCCCTCCAGGGACTCGAA3′) were based on preferred codon usage table (Lathe, 1985), while the third probe (17mer mix) (5′TCYTGNGTNGGCATYTG3′) (Y represents T or C; N represents A, T, G or C) had a redundancy of 64. All of the probes were 5′ end-labelled and used to screen the λgt10 library. Positive phages were purified and the cDNA inserts were subcloned into plasmid vectors pTZ19R, pTZ18R or pUC19 (United States Biochemical Corporation, Cleveland, OH). The cDNA inserts were sequenced by the dideoxynucleotide chain termination method (Sanger *et al.*, 1977). Initially, the ends of the cDNA inserts were sequenced and then sequential deletion clones were generated using exonuclease III and both strands were fully sequenced.

Southern and Northern blot analyses

Genomic DNA was isolated from normal spleen and NS-1 cells. Approximately 10 μg of DNA was digested with various restriction enzymes, run on 0.8% agarose gel, alkaline blotted onto nylon membranes (BIO-RAD) and probed with random primer labelled cDNA insert as described (Chan and Takei, 1989). the stringency wash was at 0.1 × SSC, 65°C.

Total RNA was isolated from various lymphocytes by the acid–phenol extraction method (Chomczynski and Sacchi, 1987). Approximately 10 μg of RNA was run on a 1.0% agarose gel containing formaldehyde, blotted onto nylon membranes (BIO-RAD), and probed with the MALA-2 cDNA insert or the appropriate fragment. The stringency wash was at 0.1 × SSC, 65°C.

Binding assay

MALA-2 was purified from the membrane fraction of NS-1 cells by three cycles of antibody affinity chromatography. The first cycle was identical to that described for MALA-2 purification in the previous section. The bound fraction was eluted from the YN1/1 column, applied to the second column of an unrelated antibody (YE1/48) (Chan and Takei, 1988) and the unbound fraction was collected. This unbound fraction was applied to the YN1/1 column again and the MALA-2 fraction was eluted. Purified protein was coupled to microwell plates as described by Magkoba *et al.* (1988a) with modifications. Wells of micro plates (Falcon 3072, Becton-Dickinson, Oxnard, CA) were treated with 100 μl of 0.2% glutaraldehyde in 0.1 M sodium carbonate–HCl (pH 9.0) for 1 h at room temperature. The wells were then washed twice and 50 μl of poly-L-lysine (50 μg/ml) was added. The plates were incubated for 2 h at room temperature and washed. To each well, 50 μl of 0.2% glutaraldehyde in 0.05 M sodium carbonate buffer (pH 9.0) was added and the plates were incubated for 1 h at room temperature. The wells were washed three times and 25 μl (0.25 μg) of purified MALA-2 in 0.1 M sodium carbonate buffer (pH 9.0) was added to each well. Control wells received 1% bovine serum albumin (BSA). After a 1 h incubation at room temperature, the wells were washed and the residual free glutaraldehyde groups were reacted with 1% BSA. Target cells (Con A blasts) were labelled by ^{51}Cr, resuspended in RPMI-1640 medium containing 5% FCS and dispensed into the microwells (10^5 cells/well). Blocking antibodies were added immediately before the addition of the ^{51}Cr-labelled cells. The plates were centrifuged at 300 *g* for 1 min and then incubated for 20 min at 37°C. After the incubation, the wells were filled with the medium and the plates were inverted for 5 min at room temperature to allow the detachment of unbound cells from the wells. The plates were then flicked to remove the unbound cells. The bound cells were lysed with 100 μl of 2% Triton X-100 and counted on a γ-counter.

COS1 cell transfection and FACS analysis

COS1 cell expression vector pAX82 (R.Kay, manuscript in preparation) was used. It is similar to the previously described CDM8 (Seed and Aruffo, 1987). *Escherichia coli* strain MC1061/p3 (Yamasaki *et al.*, 1988) was used for transformation. COS1 cells were transfected with plasmid DNA by DEAE–dextran (Hammarskjold *et al.*, 1986), incubated for 70 h in the presence of DMEM with 5% FCS and harvested with phosphate buffered saline (PBS) containing 2.5 mM EDTA. The cells were then stained with YN1/1 hybridoma supernatant and goat anti-rat Ig-FITC as a second antibody (Cooper Biomedical, Malvern, PA). Analysis of the COS1 cells was carried out on FACStar Plus (Becton-Dickinson).

Acknowledgements

This study was supported by a grant from the Medical Research Council of Canada with core support from the Cancer Control Agency of British Columbia and British Columbia Cancer Foundation. K.J.H. and C.C. are recipients of studentships from the National Science and Engineering

Research Council (Canada) and the Medical Research Council (Canada), respectively. We thank Dr R.Kay for providing the pAX82 vector as well as his technical advice, Wieslawa Dragowska for FACStar analysis, and Lesley Reynolds for secretarial assistance over this manuscript.

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Received on March 9, 1989; revised on June 7, 1989