CHARACTERIZATION AND cDNA CLONING OF A NOVEL MURINE T CELL SURFACE ANTIGEN YE1/48

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ABSTRACT

T cell surface antigens are thought to play significant roles in immunological functions. They are involved in cellular interactions and T cell activation and proliferation. Characterization of T cell antigens is important in understanding the molecular machanisms underlying immune responses. The subject of this thesis is to characterize a novel murine T cell surface antigen called YE1/48.

YE1/48, defined by two rat monoclonal antibodies YE1/48.10.6 and YE1/32.8.5, is a dimeric glycoprotein with molecular size and charge resembling the murine T cell antigen receptor α/β. It was initially detected at high levels on two T cell lymphomas, EL-4 and MBL-2. In my thesis studies, the YE1/48 antigen was characterized biochemically, a cDNA clone was isolated, and its expression in lymphoid cell populations was determined. The YE1/48 antigen was found to be distinct from the T cell receptor based on direct comparisons of their primary sequences as well as immunological analyses. It is likely a homodimer with similar or identical subunits. No homology with any known proteins could be detected, including the human T cell activation antigen CD28 (T44) which also has a similar dimeric structure as YE1/48. No function of the YE1/48 antigen could be derived from its primary sequence or with the use of the two monoclonal antibodies because the antibodies do not appear to bind to the surface of intact normal T lymphocytes.

Some intriguing characteristics of the YE1/48 antigen were observed in the current studies. The YE1/48 antigen belongs to a rare group of type II membrane proteins with orientation of the amino-terminus inside the cell and the carboxy-terminus outside. The YE1/48 gene may have two alleles among different mouse strains and may belong to a multigene family. YE1/48 is expressed at low levels on a wide range of T cells with no restriction to

their differentiation stages, and on spleen B cells as well as bone marrow cells. Its expression on lymphocytes is not related to activation or proliferation. However, YE1/48 expression appears to be induced at high levels by Abelson Murine Leukemia Virus-transformation of pre-B cells. Moreover, the epitopes defined by the YE1/48.10.6 and YE1.32.8.5 antibodies seem to be exposed only on three T lymphomas but not on normal T cells. It is thus tantalizing to speculate a correlation of the high level expression of YE1/48 antigen and its epitope exposure on transformed lymphocytes with cellular transformation.

In summary, YE1/48 was found to be a novel T cell surface antigen which has similar dimeric structure as the murine T cell receptor α/β and human CD28 (T44). It has now been characterized biochemically, molecularly cloned, and its expression on lymphoid cells has been determined. Although the function of YE1/48 antigen remains unknown, a number of intriguing characteristics observed in the current studies have certainly called for further studies on the antigen and the determination of its function.

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LIST OF ABBREVIATIONS

ALL	Acute lymphoblastoid leukemia
ATL	Adult T cell leukemia
BL	Burkitt lymphoma
bp	Base pairs
BSA	Bovine serum albumin
CALLA	Common acute lymphoblastoid leukemia antigen
CD	Cluster of differentiation (antigen)
cDNA	Complementary DNA
Con A	Concanavalin A
cpm	Counts per minute
CTL	Cytotoxic T cell
EBV	Epstein-Barr virus
EGF-R	Epidermal growth factor receptor
FcR	Fc receptor
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
GaRIg	Goat anti-rat immunoglobulin antiserum
GPI	Glycosyl-phosphatidylinositol
HEBF	High endothelial binding factor
$HEBF_{LN}$	Peripheral lymph node-specific HEBF
$HEBF_{PP}$	Peyer's patches-specific HEBF
HEPES	N-2-hydroxyethylpiperazine
HEV	High endothelial venules
HEV_{LN}	Peripheral lymph node-specific HEV
HEV_{PP}	Peyer's patches-specific HEV
HEV_{SN}	Synovial-specific HEV
HPLC	High performance liquid chromatography
HTLV-I	Human T lymphocyte virus type I
ICAM-1	Intercellular adhesion molecule-1
IEF	Isoelectric focussing
IFN	Interferon
Ig	Immunoglobulin
IL-	Interleukin-
In-R	Insulin receptor
kb	Kilobases
LFA	Lymphocyte-function-associated antigen
LCA	Leukocyte common antigen
LGL	Large granular lymphocyte
LMP	Latent infection membrane protein
LPS	Bacterial lipopolysaccarides
MAb	Monoclonal antibody
MAG	Myelin-associated glycoprotein
MaRIg	Mouse anti-rat immunoglobulin antiserum
$MaRIg_{\kappa}$	Mouse anti-rat immunoglobulin κ light chain antiserum
2ME	β-mercaptoethanol
MHC	Major histocompatibility complex
M_r	Relative molecular mass
mRNA	Messenger RNA
MuLV	Murine leukemia virus
MW	Molecular weight
NBRF	National Biomedical Research Foundation
NCAM	Neural cell adhesion molecule
NK	Natural killer cell
PBS	Phosphate buffered saline
PDGF-R	Platelet-derived growth factor receptor

LIST OF ABBREVIATIONS (CONT'D)

pfu	Plaque forming units
pI	Isoelectric point
PNA	Peanut agglutinin
PTH	Phenylthiohydantoin
R	Receptor
RaMIg	Rabbit anti-mouse immunoglobulin antiserum
RaRIG	Rabbit anti-rat immunoglobulin antiserum
rpm	Revolutions per minute
SDS-PAGE	Sodium dodecylsulphate-polyacrylamide gel electrophoresis
sIg	Surface immunoglobulin
TAP	T cell activating protein
TCR	T cell receptor
TDL	Thoracic duct lymphocytes
TFA	Trifluoroacetic acid
TGF-β	Transformating growth factor-β
Th	T helper cell subset
TL	Thymic leukemia antigen
TNF	Tumor necrosis factor
TSTA	Tumor-specific transplantation antigen

CHAPTER ONE

OVERVIEW: THE T CELL SURFACE ANTIGENS

In the vertebrate immune system, diverse immunological functions are initiated by events at the cell surface of lymphocytes. These events include 1) the transmission of positional information by cell-cell recognition, as in the homing of leukocytes to specific lymph organs; and 2) the triggering of cellular differentiation and proliferation by recognition of specific cognate antigens and soluble polypeptide factors, as in the maturation of B lymphocytes into antibody-producing plasma cells and T lymphocytes into cytolytic effectors, and in the clonal expansion of antigen-specific T and B cells. These events are mediated by recognition elements anchored in the plasma membrane which are capable of transducing the external stimuli, either in the form of cell-bound or soluble ligands, into appropriate cellular responses.

This chapter reviews the properties of various recognition proteins on the plasma membrane of T lymphocytes and their roles in eliciting immunological functions.

1.1 GENERAL CONCEPTS

1.1.1 The Cell Surface Proteins

The plasma membrane separates cells from their environment and from each other. Although it is relatively permeable to water and small hydrophobic molecules, it is impermeable to macromolecules and polar molecules. Membrane proteins mediate the active and passive transport of ions and metabolites into and out of the cell. In addition, membrane proteins mediate the transfer of information from the exterior of cells by first recognizing the external molecular ligands, then transducing the signal across the cell membrane, and finally initiating a response inside the cell. Hormones and neurotransmitters are examples of circulating extracellular signals. Cell adhesion proteins

such as extracellular matrix proteins and tissue-homing ligands are examples of signals localized on the surface of other cells. To selectively elicit a cellular response, the recognition of extracellular signals by the membrane protein receptors must be highly specific and the expression of these receptors must be restricted to the responding cell types. Moreover, these receptors must be as diverse as the population of external signals the cells respond to. Very often, the combination of membrane receptors defines the functions and tissue types of the cells bearing them (Hood et al., 1984).

From the few receptor proteins characterized, some general properties can be derived. Most receptor proteins are integral membrane proteins that span the plasma membrane. They have an extracellular recognition domain which specifically interacts with the external molecular signal. The interaction likely depends on the complementarity of the binding sites on the receptor and ligand pair. It can occur between identical receptors on two different cells. An example of such homophilic interactions is the interaction of nerve cells via neural cell adhesion molecules (NCAM) (Hoffman et al., 1983). The complementary interaction can also be heterophilic and occurs between two different molecules. The interaction of hormones and growth factors with their receptors are well known examples. In addition, heterophilic interactions can involve two cells bearing different receptors with complementary binding sites. Examples are the antigen receptor on T lymphocytes recognizing foreign antigen in association with the major histocompatibility complex (MHC) antigen on antigen-presenting cells (Marrack and Kappler, 1986), and the lymphocyte-function-associated antigen 1 (LFA-1) on leukocytes interacting with the intercellular adhesion molecule 1 (ICAM-1) on various tissue cells (Marlin and Springer, 1987; Makgoba et al., 1988). A special case of heterophilic interaction may involve identical receptors that recognize the same multivalent linker macromolecule. Fibronectin, a large extracellular matrix protein, is an example of linker molecules (Hynes, 1986).

Upon interaction with the recognition domain, the external binding signal is transduced across the plasma membrane, which may involve conformational changes in a transducer domain or ligand-mediated aggregation of identical membrane receptors (Carpenter, 1987). These changes can in turn stimulate a number of intracellular effector pathways via some functional sites in the cytoplasmic domain of the receptor. In some cases where the cytoplasmic domain is very small, the signal may be transduced to another membrane protein or membrane-associated cytosolic protein which is capable of initiating the cellular response. A putative example is the T cell antigen receptor (TCR), which induces T cell activation mediated by a non-covalently associated integral membrane protein CD3 (CD = cluster of differentiation) (Brenner et al., 1985; Oettgen et al., 1985). Intracellular effector pathways may include 1) the reorganization of the cytoskeletal elements to modify cell movement, 2) the activation of nucleotide cyclases to produce second messengers such as cyclic AMP (cAMP) or cyclic GMP (cGMP) which in turn modulate the activities of other enzymatic or regulatory proteins, 3) the activation of enzymatic properties inherent to the receptor molecules, such as tyrosine kinase activities in many polypeptide growth factor receptors, and 4) the opening or closing of specific ion gates and the activation or deactivation of specific ionic pumps to change the intracellular ionic environment or ionic potential.

Another group of membrane proteins capable of external ligand recognition and signal transduction are proteins anchored to the plasma membrane via a glycosyl-phosphatidylinositol (GPI) moiety (Ferguson and Williams, 1988; Low and Saltiel, 1988). These receptors lack a transmembrane polypeptide domain. The carboxyl(C)-terminus is covalently linked to ethanolamine at the glycosyl-end of GPI by an amide bond whereas the fatty acid residue at the phosphatidylinositol-end is embedded into the plasma membrane lipid bilayer. The GPI anchorage allows a higher lateral diffusion mobility of the membrane proteins than the conventional polypeptide-anchored integral proteins do

(Ishihara et al., 1987) and, at least *in vitro*, allows receptor removal from the cell surface upon cleavage by GPI-specific phospholipase C. Examples of GPI-anchored proteins capable of eliciting signal transduction include Thy-1 antigen on murine T lymphocytes (Gunter et al., 1984; Low and Kincade, 1985; Tse et al., 1985; Kroczek et al., 1986), Ly-6-related T cell activating protein (TAP) on mature murine T lymphocytes (Reiser et al., 1986a, 1986b; Rock et al., 1986), and lymphocyte-function-associated antigen 3 (LFA-3) on human thymic epithelial cells and monocytes (Krensky et al., 1983; Dustin et al., 1987b; Le et al., 1987). Thy-1 and TAP are involved in T lymphocyte activation whereas LFA-3 can induce cytokine production in thymic epithelial cells and monocytes *in vitro* (Le et al., 1987). Their mechanisms of signal transduction are unknown. The association of these GPI-anchored proteins with other membrane proteins has not been described. In Thy-1 and TAP-mediated responses, cross-linking of the antigens and the expression of T cell antigen receptor (TCR) on the cell surface are prerequisites for their activities (Yeh et al., 1986a; Gunter et al., 1987; Schmitt-Verhulst et al., 1987).

1.1.2 Differentiation Antigens

The diverse cell types with unique biochemical and morphological characteristics of a multicellular organism are the result of cell differentiation. Cell differentiation is the manifestation of qualitative and quantitative changes in gene expression, in a developmentally programmed manner as well as in response to extracellular signals. The efficient functioning of the organism depends on the coordinate interaction of these cells specialized for different tasks. The modification of cell surface antigens during differentiation has been known for decades. They are designated "differentiation antigens" because they define stages of differentiation. Many of them are thought to perform functions relevant to

the external signals in the microenvironment even though their functions are not yet well characterized.

The term "cell differentiation" is sometimes used to refer to the irreversible specialization of cells. This may imply loss of genetic materials, gene translocation, or irreversible changes in gene regulatory mechanisms. It is becoming clear that, in many cases, cell differentiation is reversible as the pattern of gene expression, at least in vitro, can be modified in response to environmental stimuli or by a disruption of the regulatory circuits between nucleus and cytoplasm (Nover, 1980; Blau et al., 1985). For instance, some neoplastic cells express oncofetal antigens that are normally only expressed during certain stages of embryogenesis (Shively, 1985). Similarly, the term "differentiation antigens" is often used to refer to protein markers of different cell lineages or specialized differentiated cell types, but recent studies have revealed that many of these antigens are shared by different cell types or subsets. This distribution of differentiation antigens among different cell populations is apprehensible considering that cell differentiation is a coordinate response to multiple external stimuli and involves complicated functional pathways. Some of these external stimuli are shared in different differentiation microenvironments. Thus, it is the coordinate expression of differentiation antigens that determines the differentiation pathways and specialization of cells.

1.1.3 Monoclonal Antibodies

Monoclonal antibodies (MAb's) have been a basic tool in the identification and studies of cell surface antigens. They are derived from antibody-producing hybridomas devised by Kohler and Milstein (1975). The hybridomas are generated by fusion of a myeloma cell line with spleen cells from an animal, mouse or rat, which has been immunized with the antigen or

cells expressing the antigen of interest. The fusion hybrids are immortal and they secrete antibodies characteristic of the parental antibody-secreting spleen B lymphocytes. Thus, the antibody secreted by each hybridoma is monoclonal in origin as it is the product of a single immunoglobulin (Ig) gene rearrangement. It has the property of monospecific recognition of an antigenic determinant (epitope) on the immunizing antigen, in contrast to the polyclonal antiserum which carries antibodies of multispecificities. However, two types of irrelevant cross-reactivities of MAb's are sometimes observed (Yelton et al., 1980). The first type of cross-reactivity, intrinsic to any single antibody, refers to the same antibody binding site recognizing similar epitopes on more than one molecular species. The second type is due to antibody heterogeneity in the hybridoma culture supernatant. Other than the MAb derived from the parental spleen cell, the culture supernatant contains covalently-linked hybrid antibody molecules carrying either the light or heavy Ig polypeptide derived from the parental myeloma, depending on the variant myeloma line used. These multispecific recognition properties usually do not present serious problems in the identification of new surface antigens.

In addition to the high precision recognition specificity of MAb's, hybridoma technology has also provided an unlimited supply of antibodies. Hybridomas secrete 10-50 μg MAb per ml of culture supernatant, and upon injection into the peritoneal cavity of mice, can produce up to 1-10 mg MAb per ml of ascites fluid (Köhler, 1986). With these two advantages, MAb's have been replacing conventional polyclonal antisera. They have proven to be extremely useful in identifying antigens on bacteria, viruses, cells and antigenic determinants on other biological materials. They are powerful tools in the definition and separation of cell subpopulations, in the discrimination of different stages in ontogeny, in the purification of antigens for structural characterization, and in the studies of functions of cell surface antigens by mimicking their natural ligands and promoting antigen cross-

linkage. Recently, they have also been used in the targeting of drugs or toxins to the cells bearing particular cell surface antigens for therapeutic purposes.

1.1.4 The T Lymphocytes

Thymus-dependent lymphocytes (T lymphocytes) are derived from hemopoietic stem cells in the bone marrow. T lymphocyte precursors enter the thymus where they differentiate and mature into immunocompetent cells capable of responding to foreign antigens. Upon completion of their intrathymic maturation, mature virgin T cells migrate from the thymus and form the peripheral pool of T lymphocytes. They recirculate via the blood stream and lymph between the body tissues and various peripheral lymph nodes which drain foreign antigens such as viruses and bacteria from the lymph circulation. It is in the lymph nodes that immune responses are usually initiated. Foreign antigens are rapidly phagocytosed, processed and presented by macrophages which are abundant at the sites of entry in the nodes. Upon encountering cognate antigens on the macrophages, the antigen-specific T lymphocytes become activated. They proliferate and differentiate into mature functional T cells. Some activated T lymphocytes interact with B lymphocytes which also recirculate through the lymph nodes, and they initiate a T cell-dependent humoral response by inducing B cell proliferation and differentiation into antibody-secreting plasma cells. The mature T cells eventually reenter the general circulation. The shuffling of T lymphocytes through the lymph nodes allows the full repertoire of lymphocyte specificities to be available throughout the body while also facilitating the cell-cell interactions necessary for the generation and regulation of T cell-dependent immune responses.

T lymphocytes mediate two general types of immunological functions: effector and regulatory. Effector functions include the induction of

cytotoxicity for allografts carrying foreign transplantation antigens (alloantigens), virus-infected cells and some tumor cells, and the induction of delayed hypersensitivity against virus- or microorganism-infected cells by lymphokine-mediated activation of macrophages. The regulatory functions refer to the capability of T lymphocytes to cooperate with B lymphocytes in the stimulation of the latter to proliferate and differentiate into antibody-secreting plasma cells, and also to their capability to induce the stimulation of other T cells. Some of these effector and regulatory functions are dependent on the direct cell-cell interactions of T lymphocytes with other cells like antigen-presenting accessory cells, B lymphocytes, and target cells. Some other functions, on the other hand, rely on the secretion of lymphokines from activated T lymphocytes to enhance the cytolytic activity of macrophages, as well as to promote the proliferation and differentiation of B cells and other T cells. Another mode of immunoregulation by T lymphocytes is the modulation of T cell functions in a negative manner by active suppressive influences.

The diverse T cell functions had long suggested that T cells consist of specialized subpopulations. T cell heterogeneity was well appreciated about two decades ago by the identification of differentiation antigens on the surface of murine T cells using alloantisera and xenoantisera. Lyt-1, Lyt-2 and Lyt-3 are among the first surface antigens described. It was subsequently found that T cells with "helper" functions (i.e. cooperation with B cells) and "inducer" functions (i.e. secretion of lymphokines) express high levels of Lyt-1 antigen, whereas T cells with "cytotoxic" functions and "suppressor" functions and their precursors express Lyt-2 and Lyt-3. These differentiation antigens have been useful markers for the separation of T cell subpopulatons with distinct functional potentials. The identification of many more differentiation antigens have accelerated since the advent of MAb technology. With the high specificity of MAb reactivities, many differentiation antigens

have been found to be important mediators of lymphocyte cell-cell interactions and receptors for soluble regulatory factors. Perturbations of these antigens (such as cross-linking) by MAb's on the cell surface induce enhancing or inhibitory effects on specific T cell functions and have given insight into the biological processes in which the antigens participate. However, in many cases, the precise functions of these surface antigens remain obscure. In the following section (1.2) of this chapter, selected cell surface antigens important in T cell activation, migration, and response to soluble regulatory factors are reviewed.

1.2 INTERACTIONS OF T LYMPHOCYTES WITH THE ENVIRONMENT

1.2.1 Molecules Involved In T Cell Activation

A) The T Cell Receptor/CD3 Complex

T lymphocytes are activated upon recognition of foreign antigens in the context of autologous major histocompatibility complex (MHC) antigens on the surface of antigen-presenting cells, or upon recognition of alloantigens on target cells. Antigen/MHC corecognition is mediated by the clonotypic T cell antigen receptor (TCR) (for reviews, see Reinherz et al., 1984; Allison et al., 1984). Two categories of TCR have been identified (see Table I). TCR-α/β (Haskins et al., 1983; Kappler et al., 1983b; Marrack et al., 1983a, 1983b; Meuer et al., 1983; Kaye and Janeway, 1984) is expressed on helper/inducer T cells, cytotoxic T cells (CTL) and some suppressor T cells (Bensussan et al., 1984; Yoshikai et al., 1984; Hedrick et al., 1985; Kronenberg et al., 1985; Modlin et al., 1987). It consists of two disulphide-linked glycoproteins with variable and constant domains encoded by genes that rearrange specifically in T cells (Acuto et al., 1983; Kappler et al., 1983a; McIntyre and Allison, 1983; Chien et al., 1984; Hedrick et al., 1984; Saito et

TABLE I

THE T CELL RECEPTOR/CD3 COMPLEXES

Antigen		M_r Mouse	Human	Distributions	Functions
TCR-α/β	α	45-50K	49K	Most mature T cells	Recognition of antigens in the context of MHC
	β	45-50K	43K		
	(Disulphide-linked)				
TCR-γ/δ	γ	35K	45K	$CD3^+\alpha/\beta^-$ T cells;	Ligand unknown;
	δ	45K	43K	$Thy\text{-}1^+$ dendritic	may recognize MHC
	(Disulphide-linked or unlinked)			epidermal cells; $CD3^+$ LGL's	determinants
CD3	γ	21K	25K	Most T cells;	Possible signal
	δ	26K	20K	Some NK cells	transduction upon
	ε	25K	20K		perturbation of TCR-α/β
	ζ	2x16K	2x16K		and TCR-γ/δ
	p21	21K			

al., 1984a, 1984b; Siu et al., 1984; Yanagi et al., 1984). The α and β gene rearrangements are the basis of the diverse repertoire of antigen recognition specificities in T cells, as analogous to that derived by the immunoglobulin (Ig) gene rearrangements in B lymphocytes. In mouse, the α and β subunits have similar relative molecular mass (M_r) of 45-50,000, whereas in human, the α subunit is 49,000 M_r and the β subunit is 43,000 M_r. The α subunits are more acidic than the β subunits. The α/β heterodimer is non-covalently associated with the CD3 protein complex (Reinherz et al., 1982; Meuer et al., 1983; Reinherz et al., 1983a; Kaye and Janeway, 1984; Weiss and Stobo, 1984; Brenner et al., 1985) consisting of several invariant subunits, γ(21,000 M_r in mouse and 25,000 M_r in human), δ(26,000 M_r in mouse and 20,000 M_r in human), ε(25,000 M_r in mouse and 20,000 M_r in human), p21(21,000 M_r in mouse only) and ζ(homodimer of 16,000 M_r chains in both mouse and human) (Borst et al., 1983a, 1983b; Samelson et al., 1985a; Oettgen et al., 1986; Weissman et al., 1986). Expression of the α/β dimer requires a coexpression of CD3 molecules on the cell surface (Weiss and Stobo, 1984; Saito et al., 1987).

Perturbations of the α/β/CD3 complex by antigen/MHC, alloantigens, or MAb's result in a cascade of early metabolic events that lead to T cell activation. The primary metabolic events largely stem from an increase in intracellular Ca^{2+}, and the enhancement of the turnover of phosphoinositides mediated by guanine nucleotide binding regulatory proteins (G proteins) and phosphatidylinositol-specific phospholipase C, which leads to the activation of protein kinase C (Imboden and Stobo, 1985a; Imboden et al., 1985b; Nisbet-Brown et al., 1985; Gray et al., 1987; Imboden et al., 1987; Kuno and Gardner, 1987; Mustelin, 1987; Pecht et al., 1987; Treves et al., 1987; Imboden, 1988). Since the cytoplasmic tails of the TCR-α/β polypeptides are very short and the CD3 subunits contain longer intracellular domains (van de Elsen et al., 1984; van de Elsen et al., 1985; Rabbitts et al., 1985; Gold et al., 1986; Krissansen et al., 1986; Haser et al., 1987; Weissmen et al., 1988), it is

thought that CD3 mediates signal transduction upon antigen/MHC or alloantigen recognition by the α/β dimer. However, none of the CD3 genes cloned so far contains kinase domains or GTP binding sites. Some CD3 polypeptides are phosphorylated upon mitogenic stimulation which appears to mediate the down-regulation of the α/β/CD3 complex (Cantrell et al., 1985; Samelson et al., 1985b; Oettgen et al., 1986; Samelson et al., 1986b; Cantrell et al., 1987; Samelson et al., 1987). In abnormal lymphocytes of autoimmune mice (gld and lpr), constitutive tyrosine phosphorylation of the CD3-p21 subuit may be related to the decreased efficacy in signal transduction via α/β/CD3 (Samelson et al., 1986a). Since isolated α/β/CD3 complex does not have any kinase activity, a yet unknown kinase component may be involved in signal transduction.

The other TCR, γ/δ heterodimer, has recently been identified on $\alpha/\beta^{-}CD3^{+}$ immature thymocytes and peripheral T lymphocytes largely of $CD4^{-}CD8^{-}$ phenotype (Moingeon et al., 1986; Lew et al., 1986; Borst et al., 1987; Brenner et al., 1987; Ioannides et al., 1987; Moingeon et al., 1987; Nakanishi et al., 1987), on $Thy\text{-}1^{+}CD3^{+}CD4^{-}CD8^{-}$ dendritic epidermal cells (Koning et al., 1987), as well as $CD3^{+}$ large granular lymphocytes (LGL's) displaying natural killer (NK) activities (Ang et al., 1987; Colamonici et al., 1988). Recently, using MAb's reactive with the native γ/δ dimer, TCR-γ/δ is also detected on a small subset of human $CD4^{-}CD8^{+}$ peripheral blood T cells (Jitsukawa et al., 1987; Borst et al., 1988). Like TCR-α/β, the γ (35,000 M_r in mouse and 45,000 M_r in human) and the δ (45,000 M_r in mouse and 43,000 M_r in human) subunits are variant glycoproteins encoded by rearranged variable and constant genes (Saito et al., 1984a, 1984b; Lefranc and Rabbits, 1985; Murre et al., 1985). The γ/δ heterodimers occur in disulphide-linked and unlinked forms depending on the γ constant region gene used (Krangel et al., 1987; Lanier et al., 1987; Littman et al., 1987b; Pardoll et al., 1987). The γ/δ dimer is non-covalently associated with CD3 proteins and is functional, as anti-CD3 or anti-γ MAb's

stimulate an increase in intracellular Ca^{2+} via the phosphatidylinositol pathway, induce the production of interleukin-2 (IL-2) in $CD3^+\gamma/\delta^+$ thymocytes and peripheral lymphocytes, and stimulate or inhibit MHC non-restricted NK-like activity in $CD3^+\gamma/\delta^+$ clones derived from peripheral blood lymphocytes (Borst et al., 1987; Brenner et al., 1987; Ferrini et al., 1987; Moingeon et al., 1987; Pantaleo et al., 1987a; Colamonici et al., 1988; Faure and Anderson, 1988; Marusic-Galesic et al., 1988). The MHC non-restricted cytotoxicity of the TCR-γ/δ dimer appears to be induced by IL-2 in the culture as it is lost upon factor depletion, similar to that observed in some TCR-α/β^+ clones (van de Griend et al., 1984; Phillips et al., 1987; Borst et al., 1988). The dimer therefore is probably not involved in target cell recognition in such MHC non-restricted cytotoxicity. The nature of their physiological ligands has not been defined yet, although it has recently been shown that MHC molecules may be the ligand for TCR-γ/δ (Matis et al., 1987).

In vitro studies of helper/inducer T cell clones and CTL clones have revealed that at least two signals are required to induce T cell activation via TCR-α/β: 1) perturbation of the α/β/CD3 complex by antigen or MAb, in a manner that cross-linkage of the α/β/CD3 complex is induced by MAb coupled to sepharose beads, or by the presence of accessory cells capable of interacting with MAb through their surface Fc receptor (FcR); 2) influences from accessory cells such as the secretion of interleukin-1 (IL-1) (Kaye et al., 1983; Meuer et al., 1984a; Hara and Fu, 1985a; Schwab et al., 1985; Williams et al., 1985). Signal (1) is generally referred as the primary signal and signal (2) as the secondary signal. These signals can be replaced by calcium ionophores and phorbol esters which activate cytosolic protein kinase C (Truneh et al., 1985), as well as by lectins such as concanavalin A (Con A) and phytohaemagglutinin (PHA) which probably cross-link various surface glycoproteins involved in T cell activation by binding to their carbohydrate side chains (Taylor et al., 1984; Gelfand et al., 1985; Leca et al., 1986).

Two steps can be distinguished and dissociated in the activation of T cells. The first step induces the expression of Ia (MHC class II) antigens and the receptor for interleukin-2 (IL-2R). The second step drives these activated cells into autocrine proliferation by stimulating IL-2 secretion (Cantrell and Smith, 1984; Klaus and Hawrylowicz, 1984). The molecular events following the binding of IL-2 to IL-2R are described in section 1.2.3 B.

B) Accessory Molecules

Although the interaction of TCR-α/β dimers with antigen/MHC or alloantigens alone can trigger the initiation of an antigen-specific T cell immune response, several "accessory molecules" on T cells are found to participate in this reaction by enhancing the conjugate formation between T cells and antigen-presenting cells or target cells. They include CD4 (also known as L3T4 in mouse and T4 in human), CD8 (also known as Lyt-2/Lyt-3 in mouse and T8 in human), LFA-1 (also known as CD11a), and CD2 (also known as LFA-2, T11 and sheep erythrocyte receptor) (see Table II). They all have adhesive properties for complementary surface structures on the antigen-presenting cells or target cells.

The expression of CD4 and CD8 is mutually exclusive on mature resting T lymphocytes such that CD4 is expressed on MHC class II-restricted cells and CD8 on class I-restricted cells (Meuer et al., 1982; Swain, 1983). It is thought that CD4 and CD8 antigens interact with the MHC class II and class I molecules respectively on antigen-presenting cells and target cells (Biddison et al., 1982; Demic et al., 1987; Doyle and Strominger, 1987; Gabert et al., 1987; Gay et al., 1987; Ratnofsky et al., 1987). CD4 is a 52,000 M_r glycoprotein found on MHC class II-restricted helper/inducer T cells and suppressor inducer T cells (Reinherz et al., 1979a; Ledbetter et al., 1981a; Dialynas et al., 1983). It is also expressed on brain cells, macrophages (in rat and human but not in mouse), neutrophils and lymphoblastoid B cell lines

TABLE II

ACCESSORY MOLECULES IN T CELL ACTIVATION

Antigen	M_r	Distribution	Functions
CD4	52K	Most helper/inducer and suppressor inducer T cells	Recognition of MHC II determinants
CD8	(Murine) (Human) α 34K 2x32K α′ 38K β 30K (Disulphide-linked dimers and multimers)	Most cytotoxic and suppressor T cells	Recognition of MHC I determinants
LFA-1	α 180K β 95K (Non-covalently linked dimer	All leukocytes; some other hemopoietic cells	Conjugate formation and antigen-independent cellular interactions; ICAM-1 is a LFA-1 ligand
CD2	50K	All thymocytes; peripheral T cells; some LGL's	Antigen-independent interactions between cytotoxic T cells and target cells as well as antigen-presenting cells; also rosetting of sheep erythrocytes; LFA-3 is the CD2 ligand

(Crocker et al., 1987; Littman, 1987a). CD8 is expressed mostly on MHC class I-restricted T cells with cytotoxic or suppressor activities (Reinherz et al., 1979a). It is a family of disulphide-linked heterodimers in mouse composed of α (34,000 M_r), α′ (38,000 M_r), and β (30,000 M_r) glycopolypeptide subunits (Ledbetter et al., 1981b; Walker et al., 1984). Both α and α′ polypeptides, which differ in the length of their cytoplasmic tails, are Lyt-2 gene products derived by differential mRNA splicing (Zamoyska et al., 1985). The β polypeptide is encoded by the Lyt-3 gene closely linked to Lyt-2 (Gorman et al., 1988) on the chromosome. Three dimeric combinations of α/β, α′/β and α/α′ have been detected. Multimeric forms such as tetramers and hexamers have also been described on thymocytes (Ledbetter et al., 1981b). On peripheral T cells and some T lymphomas, a small fraction of CD8 exists as α/α homodimers (Nakauchi et al., 1985). In human, CD8 is a disulphide-linked homodimer of two 32,000 M_r glycopolypeptides. It has also been found to be disulphide-linked to CD1 (MHC class I-like molecule of 43-49,000 M_r) on thymocytes (Snow et al., 1983; Snow et al., 1985).

LFA-1 is a non-disulphide-linked dimer of α (180,000 M_r) and β (95,000 M_r) glycoprotein subunits. It is classified as a member of the integrin family because of high sequence homology and structural similarities. The integrins are cell surface integral proteins with adhesive properties to extracellular matrix substrates and other cell surface components (Hynes, 1987). They constitute a versatile recognition system providing cells with anchorage, traction for migration, and signals for polarity, position, differentiation, and possibly growth (Rouslathti and Pierschbacher, 1987). Integrins that bind to extracellular matrix include fibronectin receptor and vitronectin receptor on various cell types, and Glycoprotein IIb/IIIa on platelets. LFA-1, Mac-1 and p150,95 on leukocytes are a subfamily of integrins that bind to specific cell surface components. They share the β subunit which contains several tandem repeats of high cysteine content in the

extracellular domain (Kishimoto et al., 1987b; Law et al., 1987). Their α subunits are homologous to each other but not to the β subunit, and contain a Ca^{2+}/Mg^{2+} binding site as well as an additional sequence not found in other integrin members (Corbi et al., 1987; Corbi et al., 1988). LFA-1 is widely distributed on all leukocytes. It mediates conjugate formation and antigen-independent cellular interactions (reviewed in Springer et al., 1987). Mac-1 and p150,95 are expressed on monocytes, macrophages and granulocytes which have been indicated in various adhesive cellular interactions. They are important in promoting adhesiveness to endothelial cells at sites of inflammation (reviewed in Anderson and Springer, 1987). They also function as receptors to the C3bi fragment of complement. Leukocyte adhesion deficiency is a heritable disease caused by genetic defects in the common β subunit shared by LFA-1, Mac-1 and p150,95, and is characterized by recurrent bacterial infections and impaired wound healing (Anderson and Springer, 1987; Kishimoto et al., 1987a), implicating the importance of these molecules in immune responses in vivo. The intercellular adhesion molecule-1 (ICAM-1, 90,000 M_r) has recently been identified as a LFA-1 ligand on a variety of human tissue cells (Marlin and Springer, 1987; Boyd et al., 1988; Makogoba et al., 1988). Its sequence is highly homologous to NCAM and myelin associated glycoprotein (MAG) which are intercellular adhesive molecules in the nervous system (Simmons et al., 1988; Stauton et al., 1988). The expression of ICAM-1 is strongest upon cellular stimulation by inflammatory mediators like gamma-interferon (IFN-γ) (Dustin et al., 1986; Dustin et al., 1988). The interaction of LFA-1 and ICAM-1 is an active process requiring incubation at 37°C in the presence of Mg^{2+} (Marlin and Springer, 1987; Makogoba et al., 1988). However, since anti-ICAM-1 does not inhibit all LFA-1-mediated adhesions (Rothlein et al., 1986), other LFA-1 ligands likely exist and remain to be identified. The alternative LFA-1 ligands may provide discrimination of the LFA-1 interactions with different cell types.

CD2 is a 50,000 M_r glycoprotein which was initially identified as a receptor mediating the specific rosetting of sheep erythrocytes around human T lymphocytes (Kamoun et al., 1981). It is expressed on all thymocytes, peripheral T lymphocytes, and some LGL's with NK activity (Krensky et al., 1983). CD2 specifically binds to the LFA-3 antigen which is widely expressed on most hemopoietic cells including erythrocytes, and on thymic epithelium as well as fibroblasts (Krensky et al., 1983; Dustin et al., 1987a, Plunkett et al., 1987; Selvaraj et al., 1987; Takai et al., 1987).

Recent studies using human CTL clones and appropriate target cells have shown that antigen-independent conjugate formation may occur concurrently with or precede antigen recognition, thus providing a stabilizing environment to enhance the interaction between the TCR-α/β dimer and the alloantigen (Shaw et al., 1986; Spits et al., 1986). This phenomenon probably occurs in helper/inducer T cells as well. Addition of MAb's against any of the accessory molecules mentioned above can partially inhibit TCR-α/β-mediated T cell activation (Golde et al., 1985; Hoffman et al., 1985; Shaw et al., 1986; Geppert and Lipsky, 1987; Leo et al., 1987b). For each accessory molecule, the level of inhibition of activation by MAb's is inversely correlated to the affinity of the TCR-α/β dimer for the antigen/MHC or alloantigen (MacDonald et al., 1982; Marrack et al., 1983a; Reinherz et al., 1983b; Golde et al., 1986).

In addition to promoting the interaction of TCR-α/β with antigen, CD4 and CD8 are also involved in antigen-mediated T cell activation. In vitro studies have shown that the cross-linkage of TCR-α/β/CD3 complex to CD4 or CD8, using heteroantibodies or second antibodies, appears to deliver an antigen-independent activation signal that synergizes with the signal triggered by anti-TCR-α/β/CD3 alone (Emmrich et al., 1986; Anderson et al., 1987; Ledbetter et al., 1987; Owens et al., 1987). The cross-linkage phenomenon can be considered analogous to the simultaneous binding of TCR-α/β and CD4 or CD8 to antigen/MHC epitopes during antigen-mediated T cell activation (Kupfer et al.,

1987; Takada and Engelman, 1987; Weyland et al., 1987). However, under other experimental conditions, perturbations of CD4 and CD8 may deliver negative signals inhibiting T cell activation (Band and Chess, 1985; Wassmer et al., 1985). Their exact roles in cellular activation remain to be fully elucidated.

Like CD4 and CD8, some MAb's to CD2 can synergize with anti-TCR-α/β/CD3 to induce T cell responses (Yang et al., 1986, 1988). In these experiments, anti-CD2 and anti-α/β/CD3 can be used with or without promoting cross-linkage of the two MAb molecules. When anti-CD2 is used without anti-α/β/CD3, combinations of two anti-CD2 MAb's, one of which is reactive with a neo-epitope induced by binding of the other MAb, are generally needed. The MAb combinations can induce both T helper activity for antibody response and antigen-independent killing by CTL clones (Meuer et al., 1984b; Brottier et al., 1985; Siliciano et al., 1985). Perturbation of CD2 by MAb's induces an increase in intracellular Ca^{2+} (Alcover et al., 1986; O'Flynn et al., 1986) and stimulates the phosphatidylinositol pathway (Pantaleo et al., 1987b). In transfection studies, the interaction of CD2 with its natural ligand LFA-3 on antigen-presenting cells can significantly enhance the antigen-dependent response (Bierer et al., 1988). In contrast, antigen-independent, CD2- and LFA-3-dependent conjugation between CTL's and target cells does not result in increased Ca^{2+} levels. Therefore, the CD2 and LFA-3 interaction appears to merely enhance the avidity of antigen recognition by the TCR-α/β dimer on CTL's (Springer et al., 1987). A recent report has suggested that, in the helper cell system, LFA-3 can stimulate production of IL-1 by monocytes, thymic epithelium and skin keratinocytes upon binding to CD2 (Le et al., 1987). IL-1 may in turn promote the secretion of lymphokines by activated T cells and thus stimulate their autocrine proliferation.

C) T Cell Activation Antigens

In addition to the CD4, CD8 and CD2 molecules which synergize with the TCR-α/β/CD3 complex in T cell activation, several T cell surface antigens have been identified to mediate T cell activation in the presence or absence of other supplementary signals. They include murine Thy-1, human CD28 (also known as T44), T cell activating protein (TAP) in mouse, CD45 (also known as leukocyte common antigen (LCA) and T200), and CD5 (also known as Lyt-1 in mouse and T1 in human) (see Table III).

Thy-1 is a 18-25,000 M_r GPI-anchored membrane glycoprotein expressed on all T lymphocytes, neurons, hemopoietic stem cells, and some dendritic epidermal cells in mouse, but only on neurons and a small subpopulation of thymocytes in human (Williams, 1982a; Williams and Gagnon, 1982b; Low and Kincade, 1985; Tse et al., 1985). In mouse, some anti-Thy-1 antibodies alone can induce the activation of resting T cells, but some require the addition of phorbol esters or the presence of FcR^+ accessory cells (Jones and Janeway, 1981; Konaka et al., 1981; Gunter et al., 1984; MacDonald et al., 1985). IL-2 production induced by Thy-1 appears to require the expression of TCR-α/β/CD3 complex on the cell surface (Gunter et al., 1987; Schmitt-Verhulst et al., 1987; Sussman et al., 1988), although Thy-1 alone is capable of signal transduction leading to an increase in intracellular Ca^{2+} in transfected B cells and transfected TCR-α/β variant clones (Kroczek et al., 1986; Gunter et al., 1987). Hence, Thy-1-mediated T cell activation may depend on elements associated with the TCR-α/β/CD3 pathway.

CD28, so far identified only in humans, is a homodimer of 44,000 M_r glycopolypeptides expressed on a small subpopulation of thymocytes and the majority of peripheral T lymphocytes including helper/inducer T cells, CTL's, but not suppressor cells (Lum et al., 1982; Hara et al., 1985b; Martin et al., 1986). It is also highly expressed on plasmacytomas and appears to be induced during B cell differentiation into plasma cells (Kozbor et al., 1987).

TABLE III

T CELL ACTIVATION ANTIGENS

Antigen	M_r	Distributions	Functional Properties
CD4 CD8	As in Table II		Involved in Antigen-mediated T cell activation
CD2	As in Table II		Synergism with anti-TCR/CD3; Two MAb's can stimulate alone
Thy-1	18-25K (GPI-anchored)	All T cells, neurons, hemopoietic stem cells, and some dendritic epidermal cells in mice; only neurons and some thymocytes in human	T cell activation in the presence or absence of phorbol esters or accessory cells
CD28	2x44K (Homodimer)	Majority of peripheral T cells; some thymocytes; plasma cells	T cell activation in the presence of accessory cells or phorbol esters; synergism with anti-TCR/CD3
CD5	67K	All mature T cells; some B cells	Replaces macrophages in T cell activation
CD45	180K; 190K; 200K; 220K; 240K (Isoforms)	All lymphoid and myeloid cells	similar to CD5
TAP	12K (GPI-anchored)	Majority of peripheral T cells; some thymocytes	T cell activation in the presence of accessory cells or IL-1

Anti-CD28 stimulates resting T cells in the presence of accessory cells or phorbol esters. It can also augment anti-CD3-induced T cell responses by increasing IL-2R expression and IL-2 production (Hara et al., 1985b; Moretta et al., 1985; Martin et al., 1986; Weiss et al., 1986; Damle et al., 1988). It has been found that the expression of CD28 is confined to $CD3^+TCR\text{-}\alpha/\beta^+$ peripheral blood T cells (Poggi et al., 1987). However, unlike the TCR-α/β/CD3-mediated pathway which involves an increase in cAMP levels, a release of Ca^{2+} from internal storage, as well as an influx of extracellular Ca^{2+}, CD28 appears to trigger only the influx of extracellular Ca^{2+} (Pantaleo et al., 1986) and an increase in cGMP levels without affecting cAMP levels (Ledbetter et al., 1986).

CD5 is a 67,000 M_r glycoprotein expressed on all mature T cells and some B cells (Reinherz et al., 1979b; Antin et al., 1986). Anti-CD5 appears to provide a secondary signal for the activation of resting T cells stimulated by sepharose-bound anti-CD3, by enhancing IL-2R expression and IL-2 production (Ledbetter et al., 1985a; Ceuppens and Baroja, 1986). Similar to anti-CD28, anti-CD5 induces an increase in cGMP levels and a rise in intracellular Ca^{2+} only via an ion influx from the extracellular environment (Ledbetter et al., 1986; June et al., 1987). The depletion of protein kinase C seems to uncouple signal transduction between CD5 and the calcium channel. The CD5 response may be dependent on the expression of CD3 on the cell surface (June et al., 1987).

CD45 is a 180-220,000 M_r glycoprotein expressed on all lymphoid and myeloid cells. CD45 molecules (180,000 M_r, 190,000 M_r, 200,000 M_r and 220,000 M_r) on T cells (Tung et al., 1981; Sarmiento et al., 1982; Woollett et al., 1985) and B cells (240,000 M_r, also called B220) (Coffman and Weissman, 1981) consist of distinct isoforms derived from the alternative use of 5′ exons of the gene (Saga et al., 1987; Strueli et al., 1987; Thomas et al., 1987). Thymocytes express only the 180,000 M_r isoform of CD45 whereas different functional supopulations of mature peripheral T cells express combinations of

the four isoforms. Similar to anti-CD5, some anti-CD45 can replace macrophages when peripheral resting T cells are stimulated by sepharose-bound anti-CD3, by inducing IL-2R expression and IL-2 production (Ledbetter et al., 1985b; Martorell et al., 1987). CD45-mediated activation is restricted to $CD4^+$ T cells, whereas some anti-CD45 antibodies inhibit cytolytic activities by CTL's and NK cells (Newman et al., 1983; Lefrancois and Bevan, 1985).

TAP is a 12,000 M_r GPI-anchored glycoprotein encoded by a gene mapped to the Ly-6 locus in mouse (Reiser et al., 1986a, 1986b). It is expressed on a small subpopulation of thymocytes and the majority of peripheral T cells (Yeh et al., 1986a, 1986b). Some anti-TAP antibodies are mitogenic to resting T cells in the presence of accessory cells or IL-1 (Rock et al., 1986; Yeh et al., 1987). Adult thymocytes can also be activated through the TAP molecule (Yeh et al., 1986a) and the activation appears to depend on the expression of the TCR-α/β/CD3 complex (Sussman et al., 1988). There has been evidence suggesting that TCR-α/β^+TAP^- cortical thymocytes are not immunocompetent and that TAP expression is neccessory for the Con A responsiveness of total thymocytes (Yeh et al., 1986a). It implicates that TAP may be important in defining the immunocompetent thymocyte compartment. Other Ly-6-encoded antigens have also been demonstrated to participate in T cell activation (Malek et al., 1986; Leo et al., 1987a).

All the above surface antigens, upon perturbation by MAb's, are capable of triggering an increase in intracellular Ca^{2+}. The increase in intracellular Ca^{2+} is known to enhance the activation of protein kinase C, a biochemical pathway shared by the stimulation via the TCR-α/β/CD3 complex. This may underlie the mechanisms by which these surface antigens mediate or enhance T cell activation. Alternatively, protein kinase C-independent mechanisms may be involved. The physiological ligands of these activation antigens have yet to be identified and their roles in the T cell-mediated response remain obscure. It is tantalizing to postulate that some of them may

represent receptors to external activation signals such as growth factors, or to surface molecules involved in the interactions with membrane-bound ligands on antigen-presenting cells or target cells like CD2 does. Further studies have recently identified many other T cell antigens which may participate in T cell activation. They include Tp90 (90,000 M_r) (Carrel et al., 1987b), Tp45 (45,000 M_r) (Carrel et al., 1987a), and 2H1 (140,000 M_r and 105,000 M_r) (Morimoto et al., 1988) in human. Considering the complexity of events involved in cellular activation, the list of such surface antigens will likely continue to expand.

D) Members Of The Immunoglobulin Superfamily

The immunoglobulin (Ig) superfamily is a group of molecules with sequence homology to the variable and/or constant domains of Ig. Other than primary sequence homology, criteria for superfamily membership include a conserved Ig-like tertiary protein structure such that each member has one or more Ig-like homology unit. Each homolgy unit consists of approximately 110 amino acids which fold into two sheets of antiparallel beta-strands. Some homology units have a conserved disulphide bond between two beta-strands but it is not an invariant characteristic of all members (reviewed in Williams and Barclay, 1988).

Initial members of the superfamily, including MHC class I (both heavy chain and β_2 microglobulin), class II (both α and β chains), poly-Ig receptor and Thy-1, have led to the concept that Ig-like structures are membrane molecules that play a role in cell surface recognition (Williams and Gagnon, 1982b). In the last few years, with many more members added to the superfamily (a total of 25), this concept is greatly strengthened. The majority of the Ig-like molecules are surface antigens on lymphocytes involved in antigen recognition and cell-cell interactions, and are capable of triggering subsequent events at the cell surface related to a proliferative or

differentiation response. They include TCR-α/β (Kronenberg et al., 1986), TCR-γ/δ (Hata et al., 1987), CD3-$\gamma,\delta,\varepsilon$ subunits (Gold et al., 1987), CD4 (Clark et al., 1987; Littman, 1987a), CD8 (Both α and β subunits in mouse) (Johnson, 1987; Littman, 1987a), CD2 (Sewell et al., 1986; Sayre et al., 1987), CD28, (Aruffo and Seed, 1987) and Thy-1 (Williams and Gagnon, 1982b) on T cells. Other members not found on lymphocytes have a diversity of functions and tissue distribution. They include poly-Ig receptor (Mostov et al., 1984), FcR for IgG_{2b} and IgG_1 (Lewis et al., 1986), receptors for platelet-derived growth factor (PDGF-R) and colony stimulating factor-1 (CSF-1R) (Sherr et al., 1985; Yarden et al., 1986; Williams and Barclay, 1988), and three neural adhesion antigens, NCAM (Cunningham et al., 1987), MAG (Salzer et al., 1987), and P_0 myelin protein (Lemke and Axel, 1985). Most of them are capable of mediating cell surface recognition, conforming with the functions of other members.

Ig-homology units are found on the extracellular portion of all members of the superfamily. The transmembrane sequences and cytoplasmic domains show great diversity and vary in length. For example, IgM has only three amino acids in the cytoplasm (Kelry et al., 1980), and PDGF-R has a 543 amino acid cytoplasmic domain with tyrosine kinase activity (Yarden et al., 1986). Moreover, a few members do not have transmembrane and cytoplasmic domains but are anchored to the membrane via GPI. They include Thy-1 (Low and Kincade, 1985; Tse et al., 1985), LFA-3 (Dustin et al., 1987b; Seed, 1987), Qa-2 (one of the MHC class I antigens) (Stiernberg et al., 1987; Stroynowski et al., 1987), and an isoform of NCAM (Barthels et al., 1987; Cunningham et al., 1987).

Some members of the Ig superfamily are known to interact with each other on opposed cell surfaces. Examples of heterophilic recognition of superfamily members are poly-Ig receptor and FcR with soluble Ig (Fruitiger et al., 1986; Ravetch et al., 1986), CD2 with LFA-3 (Dustin et al., 1987a; Plunkett et al.,

1987; Selvaraj et al., 1987), TCR-α/β with polymorphic epitopes on MHC antigens in conjunction with antigen (Allison et al., 1984; Reinherz et al., 1984), and probably CD4 with MHC class II as well as CD8 with MHC class I (Dembic et al., 1987; Doyle and Strominger, 1987; Gabert et al., 1987; Gay et al., 1987; Ratnofsky et al., 1987). Some other members, NCAM and P_0 myelin protein, are thought to exhibit homophilic interactions (Hoffman and Edelman, 1983; Lemke and Axel, 1985). The interactions between superfamily members may be a common phenomenon considering that pairs of Ig homology units can form unique domain structures within the same molecule. For instance, a variable domain in the Ig molecule is made up of one homology unit from the light chain and one from the heavy chain. Similar domains composed of two homology units may also be found in dimeric members like TCR-α/β, MHC class I, class II and CD8. These intramolecular and intermolecular interactions of Ig homology units have led to the hypothesis that heterophilic receptor pairs may have evolved from a homophilic interaction system (Matsunaga, 1985). In analogy to the Ig molecule, the Ig-like domains may provide a stable structure allowing the presentation of unique determinants for recognition via sequence variation at the bends of the beta-strands (Williams and Barclay, 1988).

1.2.2 Molecules Involved In T Cell Migration

Cell migration primarily occurs in two stages of T cell development. Prothymocytes, which are hemopoietic progenitors committed for differentiation in the T lymphoid lineage, migrate from the bone marrow to the thymus and are capable of restoring the thymic population of a lethally irradiated host (Ezine et al., 1984; Spangrude et al., 1988). After development in the thymus, emigrant T lymhocytes recirculate in the blood stream and lymphoid tissues and throughout the body until they either encounter foreign antigens, specific proliferative stimuli, or die (Weissman, 1967). There has been

evidence suggesting that prothymocytes respond to chemotactic substances secreted by thymic epithelium and exhibit directional locomotion in a Zigmond migration chamber (Ben Slimane et al., 1983; Champion et al., 1986). However, polypeptide chemotactic factors and cell surface receptors have not been identified. On the other hand, substantial evidence has shown that migration of recirculating T lymphocytes is mediated by cell-cell adhesion involving specific homing receptors on T cells and surface components on specialized microvascular structures, high endothelial cell venules (HEV), which are located throughout peripheral lymph nodes, mucosal lymphoid organs (Peyer's patches and appendix), and inflamed synovium (joint tissue) in patients with rheumatoid arthritis (Jalkanen et al., 1986c; reviewed in Gallatin et al., 1986; Jalkanen et al., 1987; and Woodruff et al., 1988). Peripheral blood lymphocytes extravasate by first binding to HEV and then migrating between the endothelial cells, crossing the HEV. No HEV are present in the thymus and non-lymphoid organs.

A) Homing Receptors For Lymphoid Tissues

The interaction of lymhocytes with HEV was first demonstrated in vivo by short-term localization ("homing") of intravenously injected lymphocytes, and in vitro by cell adhesion assays of viable lymphocytes on frozen sections of lymphoid tissues (Jalkanen et al., 1986b). It was found that some lymphocyte clones can discriminate between HEV cells in the lymph nodes (HEV_{LN}) and in the mucosal lymphoid organs (HEV_{PP}), whereas most normal virgin T and B lymphocytes bind to both classes of HEV. It suggests that at least two classes of homing receptors exist on lymphocytes, one specific for HEV_{LN} and the other for HEV_{PP}. Most normal virgin T and B cells express both receptors (Jalkanen et al., 1986b). The existence of distinct homing receptors on lymphocyte surfaces was first confirmed by a rat MAb MEL-14 which blocks the binding of normal and transformed T and B lymphocyte populations to HEV_{LN} but

fails to block their binding to HEV_{PP} in mouse (Gallatin et al., 1983). The MEL-14 antigen is a glycoprotein of 90,000 M_r and consists of a branching polyubiquitin near its amino-terminus which is important in defining the MEL-14 epitope (St. John et al., 1986; Siegelman et al., 1986). Ubiquitin is known in other systems to be required for intracellular protein degradation (Hershko, 1983). It has been suggested that the ubiquitination of MEL-14 may lead to its rapid internalization and degradation so that the lymphocyte entry into lymph nodes is directional and irreversible (Siegelman et al., 1986). A similar homing receptor (Hermes-1) has recently been identified in human by the Hermes-1 and Hermes-3 MAb's (Jalkanen et al., 1986a, 1986b; Jalkanen et al., 1987). Hermes-1 MAb stains both HEV_{LN}- and HEV_{PP}-specific lymphoid cell lines without blocking their binding to HEV, suggesting that it identifies a common determinant shared by both of these homing receptor classes. Hermes-3 MAb blocks lymphocyte binding to HEV_{PP} only. A polyclonal antiserum against the Hermes-1 antigen from a HEV_{PP}-specific cell line blocks lymphocyte binding to HEV_{LN}, HEV_{PP} and synovial HEV (HEV_{SN}). It implicates the presence of a class of 90,000 M_r homing receptors on human lymphocytes. MEL-14 is likely the murine homologue to this family as the MEL-14 MAb cross-reacts with the Hermes-1 antigen and blocks the binding of human lymphocytes to HEV_{LN} (Jalkanen et al., 1987) (see Table IV).

Another group of lymphocyte homing receptors called "high endothelial binding factors" (HEBF) have also been described in rat (Woodruff et al., 1988). They were identified by MAb's that were raised against shed molecules in the supernatants of cultured rat thoracic duct lymphocytes (TDL) and were able to inhibit the adhesion of TDL to HEV. Anti-$HEBF_{LN}$ blocks lymphocyte binding to HEV_{LN} and detects a surface protein of 80,000 M_r. Anti-$HEBF_{PP}$ blocks lymphocyte binding to HEV_{PP} and immunoprecipitates three polypeptides of 135,000 M_r, 60,000 M_r and 40,000 M_r (Chin et al., 1980, 1984). These homing receptors are also detected on a large proportion of rat peripheral

TABLE IV

LYMPHOCYTE HOMING RECEPTORS TO LYMPHOID TISSUES

Antigen	M_r	HEV as target of interaction
MEL-14 (Murine)	90K	MEL-14 MAb blocks the binding to HEV_{LN}[a]
Hermes-1 (Human)	90K	Hermes-1 MAb stains HEV_{LN}- and HEV_{PP}[b]-specific lymphocytes; Hermes-3 MAb blocks the binding to HEV_{PP}; Anti-Hermes-1 antiserum blocks the binding to HEV_{LN}, HEV_{PP} and HEV_{SN}[c]
$HEBF_{LN}$ (Rat)	80K	$HEBF_{LN}$ MAb blocks binding to HEV_{LN}
$HEBF_{PP}$ (Rat)	135K; 60K; 40K	$HEBF_{PP}$ MAb blocks binding to HEV_{PP}

[a] LN = Peripheral lymph nodes
[b] PP = Mucosal lymphoid tissues such as the Peyer's patches
[c] SN = Inflamed synovium

lymphcytes. Moreover, antigen similar to the rat $HEBF_{LN}$ has been detected on 60-70% human peripheral blood lymphocytes (Woodruff et al., 1987).

No physiological ligands to either mouse MEL-14, human Hermes-1, rat or human $HEBF_{LN}$ and $HEBF_{PP}$ have so far been identified. It has been suggested that the MEL-14 antigen may interact with specific oligosaccharide structures on HEV_{LN} because mannose-6-phosphate-rich polysaccharides can block the binding of mouse lymphocytes to HEV_{LN} via the MEL-14 antigen (Yednock et al., 1987). However, it remains possible that the actual endothelial cell ligand, although mimicked by mannose-6-phosphate, may in fact have an unrelated molecular structure. Recently, a murine endothelial cell surface molecule (58-60,000 M_r), defined by MECA-367 MAb and selectively expressed on mucosal lymphoid organs, has been shown to be required for lymphocyte homing (Streeter et al., 1988). It is tentatively designated as a member of "vascular addressins", which possibly represent a family of endothelial cell surface proteins providing positional information for circulating blood cells. The MECA-367 antigen is a putative ligand for a HEV_{PP}-specific lymphocyte homing receptor, and its selective expression on mucosal lymphoid organs has suggested the presence of additional tissue-specific "vascular addressins" in other sites to direct lymphocyte extravasation.

In young mice, T lymphocytes preferentially bind to HEV_{LN} whereas B lymphocytes preferentially bind to HEV_{PP} (Stevens et al., 1982). Similar binding differences have also been demonstrated between the $CD8^+$ and $CD8^-$ T cell subpopulations (Kraal et al., 1983). These findings suggest that selective lymphocyte migration may help control the relative availability of functionally distinct virgin lymphocyte populations in mucosal versus non-mucosal lymphoid organs. Upon antigen stimulation, lymphocytes are sequestered in the lymphoid tissues, where they undergo localized clonal expansion and differentiation. In the mouse, submitogenic stimuli up-regulate and mitogenic stimuli down-regulate the MEL-14 antigen expression on the

majority of lymphocytes, implicating a complex regulation of homing receptor expression (Hamman et al., 1988b). These regulatory phenomena may reflect the requirements for increased migration to lymphoid tissues by lymphocytes experiencing low level stimuli, and for the sequestration of actively dividing lymphocytes to interact with the local lymphoid microenvironment. Other homing receptors may be regulated appropriately too. Following the sessile differentiation in the lymphoid tissues, stimulated lymphocytes appear to reexpress only a single homing receptor that leads to their selective migration to the sites of immunization (Jalkanen et al., 1986b). Likewise, ligands to homing receptors on HEV cells may also be regulated by local factors associated with immunological activities. For instance, the murine HEV-specific endothelial antigen, defined by MECA-325 MAb, can be specifically induced by IFN-γ in cultured endothelial cells from diverse sources, which is accompanied by the differentiation of endothelial cells to attain HEV-like morphological characteristics (cuboidal shape) (Duijvestijn et al., 1986). This may bear significance in the control of lymphocyte traffic to lymphoid tissues and to sites of inflammation in vivo.

B) Accessory Molecules Involved In Lymphocyte Homing

Recently, in vitro studies have demonstrated that LFA-1 serves as an organ non-specific adhesion molecule in lymphocyte-HEV interactions, analogous to its accessory role in strengthening weak interactions between TCR-α/β on T lymphocytes and antigen/MHC on antigen-presenting cells (Hamman et al., 1988a; Pals et al., 1988). MEL-14^{high} cells which bind well to HEV_{LN} cells are less susceptible to inhibition by anti-LFA-1 than poor binders (MEL-14^{low}) are (Hamman et al., 1988a). In vivo, anti-LFA-1 reduces lymphocyte migration to both peripheral lymph nodes and mucosal lymphoid tissues (Hamman et al., 1988a). Other studies have suggested that another adhesive molecule on T lymphocytes, CD2 (Plunkett et al., 1987), may also play an accessory role in

lymphocyte migration. Finally, specific cell-cell interactions between lymphocytes and HEV of various lymphoid tissues have so far indicated a group of distinct adhesive molecules on lymphocytes. In light of the complexity of lymphocyte recirculation through the blood stream and body tissues, it is likely that more homing receptors will be identified in the future.

1.2.2 Receptors To Soluble Regulatory Factors

T lymphocytes are responsive to a variety of regulatory polypeptide factors at different stages of development. These factors include 1) thymic hormones like thymopoietin, thymulin and thymosin α, that appear to influence the differentiation of T lymphocytes (reviewed in Low and Goldstein, 1985); 2) other hormones like insulin and some neuropeptides that directly or indirectly modulate T cell proliferation and functions (reviewed in Plaut, 1987); 3) hormone-like cytokines such as the interleukins and interferons that are secreted in the course of immunologic and inflammatory reactions, and serve as endogenous second signals in conjunction with antigenic stimuli to trigger proliferative responses as well as to stimulate functional differentiation (see below). The above hormones and factors interact with specific receptors on the cell surface to elicit their activities. The receptor specificities of many of them, however, have not been fully characterized. This section reviews only the effects of cytokines on T cell proliferation and differentiation.

A) Cytokines Involved In T Cell Growth

Cytokines are soluble intercellular polypeptide mediators that regulate local and sometimes systemic inflammatory responses by modulating proliferation, mobility and differentiation of leukocytes as well as non-leukocytic cells. Cytokines produced by T and B cells are called

"lymphokines" and those produced by monocytes and macrophages are called "monokines". Cytokines were initially described by their biological activities that induce growth and differentiation of specific cell types, and were often prepared as unpurified or fractionated culture supernatants of the producer cells. Recently, MAb's to individual cytokine factors have facilitated their purification and have improved the specificity of bioassays. The molecular cloning of some of them have subsequently allowed sequence comparisons and have concluded that some factors defined by different bioassays are indeed identical. The availability of recombinant cytokines has also been invaluable in establishing their individual activities. In many cases, it has been shown that a single cytokine can act on more than one cell type (being pleiotropic) and can induce similar or different responses in each target cell (being multifunctional). Thus, a cytokine may bear several different names, each reflecting the biological activity through which the protein has been independently discovered. Many cytokines are now renamed interleukins (proteins that carry messages between leukocytes) (O'Garra et al., 1988; reviewed in Sporn and Roberts, 1988). Given the complexity of requirements for proliferative and differentiational responses in the diverse leukocyte populations, more mediators likely remain to be discovered. Interleukins acting on T cells are described as follows (also see Table V):

1) Interleukin-1 (IL-1), with former names like lymphocyte activating factor (LAF) and monocyte cell factor (MCF), is secreted by monocytes and macrophages as well as virtually all nucleated cell types when stimulated by a variety of agents. There are two major stable forms of IL-1 with similar M_r of 17,000, called IL-1α and IL-1β (reviewed in Oppenheim et al., 1986, 1987). They have different isoelectric points and are encoded by two distinct genes bearing only low sequence homology with each other. IL-1β is more abundantly secreted than IL-1α from monocytes stimulated by bacterial lipopolysaccharides (LPS). The two IL-1 forms appear to have similar biological activities which include

TABLE V

CYTOKINES AND THEIR RECEPTORS INVOLVED IN T CELL GROWTH

Cytokine (M_r)	Receptor (M_r)	Distributions	Effects on T cells
IL-1 α (17K) β (17K)	IL-1R (80K)	All cell types; $CD4^+$ subset only; up-regulated in activated T cells	Augments IL-2, IL-4 and IFN-γ production by T cells; costimulates thymocytes with lectins
IL-2 (15K)	IL-2R α/β (55K/75K)	β subunits on resting T, B cells, and LGL's; α/β dimer only on activated T cells	Autocrine growth of $CD4^+2H4^-4B4^+$ Th1 helper cells
IL-4 (20K)	IL-4R (55-60K)	Many hemopoietic and non-hemopoietic cells; up-regulated in activated T and B cells	Autocrine growth of $CD4^+2H4^+4B4^-$ Th2 helper cells
IL-5 (36-62K)			Induces CTL generation from thymocytes in the presence of IL-2
IL-6 (25-30K)			Similar to IL-1 and IL-5
TNF (24K)	TNF-R (138K; 90K; 75K; 54K)	All cell types; 138K polypeptide only on cell lines highly sensitive to TNF cytotoxicity	Induces IL-6 production; IL-1-like effects
TGF-β (25K)	TGF-βR (360K as 2x180K dimer)	All cell types	Inhibition of T cell proliferation without effects on IL-2 and IL-2R expression
IFN-γ (20-25K)	IFN-γR (140K on hemopoietic cells; 95K on other cells)	All cell types	Generation of CTL activity in mixed lymphocyte reactions

augmenting the production of lymphokines such as IL-2, IL-4 and IFN-γ (see below) from T cells, comitogenic effects on thymocytes with lectins, promoting T cell and B cell proliferation, and enhancing differentiation and antibody production by B cells. They also promote the growth or functional activities of almost all non-lymphocytic cell types, accounting for the diverse manifestations such as leukocyte infiltration of inflammatory sites, fever, and bone and cartilage resorption. The cell surface receptors (80,000 M_r) for IL-1α and IL-1β (IL-1R) are identical on both fibroblasts and T lymphocytes. (Bird & Saklatvala, 1986; Dower et al., 1986a, 1986b). Activated T cells express about three times more IL-1R than resting T cells do. The IL-1R expression on mature T cells appears to be restricted to the $CD4^+$ subset (Lowthenthal and MacDonald, 1987).

2) Interleukin-2 (IL-2), formerly known as T cell growth factor (TCGF), is a 15,000 M_r glycoprotein lymphokine mainly produced by the $CD4^+$ helper subset of T cells upon mitogenic stimulation. It is the first autocrine growth factor described for T cells and is thought to be the major factor that drives T cells into proliferation (reviewed in Cantrell and Smith, 1984). It also induces the production of other lymphokines by T cells. Recently, it has been demonstrated that only the human $CD4^+2H4^-4B4^+$ Th1 helper subset secretes IL-2 and uses it as the autocrine growth factor (Kurt-Jones et al., 1987; Boom et al., 1988; Greenbaum et al., 1988), whereas the $CD4^+2H4^+4B4^-$ Th2 helper subset secretes IL-4 and uses it as the autocrine growth factor (see below). It has also been shown in mice that only the Th1 helper subset mediates delayed hypersensitivity activity and the Th2 subset is primarily responsible for delivering help to B cells (Mosmann et al., 1986; Mosmann and Coffman, 1987). IL-2 also interacts with non-T cells, leading to the proliferation and enhanced NK activity of LGL's (Trinchieri et al., 1984), the proliferation and antibody production of activated B cells (Mingari et al., 1984; Ralph et al., 1984), and the cytocidal induction of activated macrophages (Malkovsky et al.,

1987). The cell surface receptor for IL-2 (IL-2R) has been partially characterized (see below in part B of this section).

3) Interleukin-4 (IL-4), also known as B cell stimulating factor 1 (BSF1), is a 20,000 M_r lymphokine produced by $CD4^+2H4^+$ Th2 helper T cells. In earlier studies, it was found to costimulate with anti-Ig MAb the proliferation of resting B cells and their subsequent antibody production (reviewed in Sideras et al., 1988). More recent studies using recombinant IL-4 have shown that it also stimulates the proliferation of $CD3^+$ NK cells (Spits et al., 1987), thymocytes (Palacios et al., 1987; Zlotnik et al., 1987; Carding and Bottomly, 1988; Lowenthal et al., 1988b) and mature T cells (Hu-Li et al., 1985; Grabstein et al., 1987; Spits et al., 1987), as well as the generation of CTL's (Pfeifer et al., 1987; Widmer et al., 1987a, 1987b). It is the sole autocrine growth factor for $CD4^+2H4^+$ Th2 helper T cells like IL-2 is for the $CD4^+2H4^-$ Th1 subset (Ferandez-Botran et al., 1986; Kurt-Jones et al., 1987; Lichtman et al., 1987; Greenbaum et al., 1988). The cell surface receptor for IL-4 (IL-4R) is widely distributed in hemopoietic and non-hemopoietic cells (Ohara and Paul, 1987; Park et al., 1987; Lowenthal et al., 1988a). The expression of IL-4R on resting T and B cells is low but increases by 5-10 fold upon mitogenic stimulation (Ohara and Paul, 1987; Park et al., 1987). Affinity cross-linking studies, in which radiolabeled IL-4 is chemically cross-linked to IL-4R on target cells and the ligand/receptor complex is visualized by SDS-PAGE analysis, have identified a putative IL-4R as a 55,000-60,000 M_r protein (Ohara and Paul, 1987; Park et al., 1987).

4) Interlerkin-5 (IL-5) is a 46,000 M_r T cell lymphokine also known as T cell replacing factor (TRF), B cell growth factor II (BCGF II) and eosinophil differentiation factor (EDF). Its functions include the stimulation of mitogen-activated B cells to proliferate, the induction of terminal differentiation of late-developing B cells to Ig-secreting B cells, as well as the induction of the differentiatin of eosinophils (reviewed in Sideras et

al., 1988). It has recently been shown that IL-5 can also induce CTL generation from immature thymocytes in the presence of IL-2, probably via the enhancement of IL-2R expression (Takatsu et al., 1987). The cell surface receptor for IL-5 (IL-5R) has not been described so far.

5) Interleukin-6 (IL-6) is also known as T cell activating factor (TAF), interferon β_2 (IFN-β_2), hybridoma growth factor (HGF), plasmacytoma growth factor, and B cell stimulatory factor 2 (BSF2). It is a 25-30,000 M_r cytokine which, like IL-1, is produced by mitogen-stimulated leukocytes and a variety of cell types which are responsive to the factor (reviewed in Sehgal et al., 1987). IL-6 has IL-1-like activities in promoting T cell proliferation. For instance, it stimulates IL-2 production and hence the proliferation of peripheral blood T cells and mature thymocytes in conjunction with a submitogenic dose of lectins (Garmen et al., 1987; Lotz et al., 1988; Uyttenhore et al., 1988). IL-6 also induces CTL generation from thymocytes in the presence of IL-2 and is needed for the production of serine esterase in CTL granules (Takai et al., 1988). The cell surface receptor for IL-6 (IL-6R) has yet to be reported.

Other than the interleukins mentioned above, additional cytokines which may have biological activities on T cells include tumor necrosis factor (TNF or TNF-α), transforming growth factor-β (TGF-β), and interferons (IFN). TNF is a 24,000 Mr polypeptide mainly secreted by activated macrophages along with IL-1. TNF, in addition to causing tumor destruction by inducing hemorrhagic necrosis, also has IL-1-like biological effects such as T cell stimulation and other events leading to the manifestation of fever, perhaps partly via the induction of IL-6 production (Ranges et al., 1988; and reviewed in Old, 1988). TNF shares the same receptor with lymphotoxin (LT; also known as TNF-β) which is 50% homologous to TNF (Aggarwal et al., 1985). Affinity cross-linking studies using radiolabeled TNF have identified the TNF receptor (TNF-R) as three or four non-covalently linked polypeptides (138,000 M_r, 90,000 M_r,

75,000 M_r, and 54,000 M_r). The 138,000 M_r polypeptide is least abundant or even absent on TNF-binding cells. It is expressed only on a cell line (MCF-7) highly sensitive to the cytotoxic action of TNF, and not on the TNF-resistant MCF-7 variant. The exact association of the four polypeptides is as yet uncertain (Creasy et al., 1987).

TGF-β is a 25,000 M_r disulphide-linked homodimeric protein produced by platelets as well as activated monocytes and lymphocytes. It is initially described as a factor that enhances the growth and induces the phenotypic transformation of normal cells in vitro (Roberts et al., 1981; Roberts et al., 1983). Recently, it has been found to have multipotent immunoregulatory functions including chemoattractant activity for peripheral blood monocytes (Wahl et al., 1987), induction of IL-1 secretion by activated monocytes (Wahl et al., 1987), and inhibition of T (Kehrl et al., 1986b, Ristow, 1986) and B cell proliferation (Kehrl et al., 1986a). The inhibitory effects of TGF-β on T lymphocytes occur late in the proliferative response as the IL-1-induced expression of IL-2R, IL-2 and the transferrin receptor is not blocked. Hence, TGF-β appears to serve as a negative feedback mechanism to limit T cell clonal expansion, yet allowing the modulation of tissue repair mediated by IL-1 and other monokines (Wahl et al., 1988). The receptor for TGF-β is a disulphide-linked dimeric glycoprotein of approximately 360,000 M_r with subunits of similar size (Massague, 1985; Fanger et al., 1986). Preliminary studies show that the binding of TGF-β does not trigger clustering or autophosphorylation of its receptor (Fanger et al., 1986).

IFN's were initially identified by their antiviral activities. IFN-α (18-20,000 M_r) is produced primarily by leukocytes whereas IFN-β (23,000 M_r) is produced by cells of solid tissues such as fibroblasts upon viral induction. IFN-γ (20-25,000 M_r) is produced by T cells upon mitogenic activation. IFN-γ can either augment or suppress cellular and humoral immunity in vivo depending on the dose and time of administration. The

mechanism of its variable activities is not well understood. IFN-γ may play a role in the generation of CTL activity in mixed lymphocyte reactions. It also increases the expression of MHC class I and II molecules on many cell types, thus making them better targets for CTL killing (reviewed in Oppenheim, 1987). IFN-α and IFN-β share the same receptor whereas the receptor for IFN-γ (IFN-γR) is distinct. IFN receptors are expressed on most cells at various levels. IFN-γR appears to exist in two molecular forms in human: 140,000 M_r on peripheral blood monocytes and several cell lines of hemopoietic origin, and 95,000 M_r on other cell types. The monocyte receptor is down-regulated upon IFN-γ binding while the other receptor is not. The different molecular forms and their different regulation may correlate with the different responses of various cells to IFN-γ (reviewed in Rubinstein, 1987).

B) The Interkeukin-2 Receptor

The expression of various cytokine receptors is important in rendering T cells responsive to the secondary signals necessary for promoting or inhibiting their proliferation and differentiation. Among the interleukin receptors expressed on T cells, the IL-2R has been best characterized. Its α subunit has been molecularly cloned (Leonard et al., 1984; Nikaido et al., 1984) and its ligand/receptor interaction has been characterized. Putative IL-1R and IL-4R have only been described by affinity cross-linking studies, which have not revealed definitive information on their protein structures. Both IL-1R and IL-4R are expressed at low levels on resting T cells but are up-regulated upon mitogenic activation (Lowenthal and MacDonald, 1987; Ohara and Paul,1987; Park et al., 1987), whereas the functional IL-2R is only expressed upon activation.

The functional IL-2R is a non-covalently associated complex of at least two subunits (α 55,000 M_r; β 75,000 M_r). Each subunit alone binds IL-2 with low affinity but together they exhibit a high affinitiy for IL-2 binding

(reviewed in Smith, 1987, 1988). Only the high affinity receptor (α/β) is functional. The β subunit is constitutively expressed at low levels on T cells and LGL's (Dukovich et al., 1987; Sharon et al., 1988), whereas the α subunit is induced when T cells are stimulated by antigen/MHC or lectins (Leonard et al., 1984; Durand et al. 1987). Thus, the expression of the functional IL-2R-α/β dimer is dependent on the induction of the α subunit which associates with the preexisting β subunit (Durand et al., 1987). Interaction of the high affinity IL-2R with IL-2 further up-regulates the expression of the α subunit while down-regulating the β subunit, leading to an overall decrease of the high affinity receptor and thus a transient loss of IL-2 responsiveness (Smith and Cantrell, 1985). It constitutes an important feedback mechanism to dampen further proliferative response to IL-2 after the initial mitogenic stimulation.

The mechanism of signal transduction upon IL-2/IL-2R interaction has not been fully elucidated. The interaction of IL-2 with IL-2R results in the progression into the S phase of the cell cycle and sometimes the production of IFN-γ. Like many other cell surface receptors for growth factors in non-lymphoid systems, such as the epidermal growth factor receptor (EGF-R), PDGF-R and the insulin receptor (In-R) (reviewed in Sibley et al., 1987), IL-2R is also phosphorylated (Gaulton and Eardley, 1986). The α subunit is phosphorylated equally in the presence or absence of IL-2 whereas the β subunit is phosphorylated only upon interaction with its ligand (Benedict et al., 1987). EGF-R, PDGF-R and In-R are tyrosine kinases that autophosphorylate. Their phosphorylation has been shown to regulate receptor functions. For instance, the phosphorylation of EGF-R and In-R at Ser/Thr residues reduces their affinities for ligand binding and their tyrosine kinase activities, and is essential for the internalization of the ligand/receptor complex as well as for signal transduction (Sibley et al., 1987). In contrast, the cytoplasmic domain of the α subunit of the IL-2R, as deduced

from cDNA sequences, is too small (13 amino acids) to mediate any enzymatic activity (Leonard et al., 1984; Nikaido et al., 1984). Studies of mutant α subunits of the IL-2R have shown that the potential cytoplasmic phosphorylation sites are not essential for signal transduction or regulation of the IL-2R (Hetakeyama et al., 1986). Considering the inducible phosphorylation of the β subunit upon IL-2 binding, it seems likely that the β subunit is the moiety functioning in signal transduction and the α subunit merely serves to increase the ligand-binding affinity. This hypothesis is further supported by the observations that some circulating T cells and LGL's bearing only the β subunit can proliferate and exhibit NK and lymphokine-activated killer (LAK) activities in response to exogenous IL-2, leading to the subsequent induction of the α subunit and the expression of the high affinity α/β receptor (Harbel-Bellan et al., 1986; Siegel et al., 1987; Tsudo et al., 1987). It has been reported that IL-2 binding can activate protein kinase C (Farrar and Anderson, 1985). However, additional mechanisms are likely needed as phorbol esters alone cannot stimulate the proliferation of IL-2-dependent cell lines (Albert et al., 1985; Koyasu et al., 1987). It is possible that, like other receptors for polypeptide factors, signal transduction via IL-2R may involve tyrosine kinase activities on the β subunit or on an associated molecule (Saltman et al., 1988).

1.3 CELL SURFACE ANTIGENS RELATED TO NEOPLASTIC TRANSFORMATION OF LYMPHOCYTES

The transformation of a normal cell into a neoplastic one is primarily marked by the acquisition of the potential for autonomous growth with no absolute requirements of exogenous growth signals, and the failure to respond to regulatory signals responsible for normal growth. When these transformed cells are introduced into a nude (athymic) mouse or an irradiated syngeneic animal, they form tumorous growths. Cell surface changes exhibited by

transformed cells of various sources generally include alterations in the membrane glycoprotein compositions, the complexity of membrane glycolipids, the level of sialylation of surface components, and the membrane microviscosity probably resulted from a less organized arrangemment of submembrane cytoskeleton (Nilsson, 1981; Hakomori, 1986; Watson et al., 1987). These membrane changes are intriguing as they may influence the neoplastic behavior of the transformed cells by modulating cellular interactions, interactions with the extracellular matrix, and cellular responses to external growth factors. Much effort has been made to identify glycoprotein and glycolipid components whose expression or structural alterations may be associated with the neoplastic phenotypes of various cell types, in search for diagnostic tools for human malignancies, as well as a better understanding of the molecular mechanisms underlying oncogenesis and the basic control of cell proliferation.

The existence of cell surface antigens or determinants unique to tumor cells but not found on normal cells is classically demonstrated by the rejection of tumors when they are transplanted into a syngeneic animal previously immunized with the same tumors. These molecules are generally called tumor antigens, and their identification relies upon their ability to elicit an immune response in the host. Recent studies however have largely made use of MAb's generated against individual tumors which specifically react with the same tumors but not with normal untransformed cells. Tumor antigens found only on tumor cells but not on normal cells are considered tumor-specific. Other tumor antigens which are found on tumor cells as well as on some normal cells, with the qualitative or quantitative differences in their expression permitting the tumor cells to be distinguished from the normal cells, are considered tumor-associated. At present, most tumor antigens identified are tumor-associated but not tumor-specific. They are mostly differentiation antigens found on normal cells at much lower levels, or on

cells of different differentiation stages and lineages, or in slightly altered forms. They are aberrantly expressed probably due to a general loss of stringent control of gene regulation in the transformed cells, but may also represent selective deregulation of gene expression that underlies the loss of growth control and the progression of tumor development. A notable example is the preferential down-regulation of the MHC class I antigens, which appears to be an important property that allows transformed cells to evade detection by the immune system (reviewed in Tanaka et al., 1988). Little is known about the identity of tumor-specific antigens. There has been a great deal of skepticism about their existence because antigens identified as tumor-specific may represent very rare normal determinants amplified at high quantity on expanded clones of transformed cells. Perhaps the likely candidates for tumor-specific antigens are those identified on chemically-induced neoplasms. Chemically-induced tumors generally possess tumor-specific transplantation antigens (TSTA's) that do not cross-react with TSTA's expressed on other tumors induced by the same chemical carcinogen. Although the identity of these TSTA's are still unclarified, they may be mutated cellular gene products, endogenous viral proteins, or products of gene conversions (de Plaen et al., 1988).

1.3.1 Murine leukemias/lymphomas

Most of the lymphocytic leukemias/lymphomas in mice studied so far are induced by exogenous murine leukemia retroviruses (MuLV) in the laboratory, or have arisen spontaneously. Moloney MuLV and Radiation MuLV cause T cell leukemias/lymphomas, whereas Abelson MuLV predominantly causes B cell leukemias/lymphomas. They seem to transform early hemopoietic progenitors committed to the lymphoid differentiation pathways (reviewed in Hehlmann et al., 1984 and Teich et al., 1984). MuLV-induced leukemias/lymphomas typically

express on the cell surface viral products, gp70 of the envelope (env) gene and glycosylated precursor proteins of the internal structure (gag) gene (reviewed in Dickson et al., 1984). In fact, spontaneous leukemias/lymphomas, like those derived from AKR mice, also express similar antigens of endogenous viral origins (reviewed in Risser and Horowitz, 1983). Moreover, gp70 is produced in some normal virus-negative mouse tissues. It is the major envelope glycoprotein of 70,000 M_r and is the principal antigen against which virus-neutralizing antibodies are generated. It carries antigenic determinants that are unique or shared by different MuLV. The cell surface gp70 molecules contain fewer terminal sialic acid residues than those incorporated into virions produced by the same cell. It is associated with another env hydrophobic polypeptide p15 (15,000 M_r), which is not radiolabeled on the cell surface, either by disulphide bonds or by weaker non-covalent bonds. This association is thought to help anchor gp70 in the membrane. The normal gag products are not glycosylated and are structural constituents of the virion capsid, the membrane and the internal ribonucleoprotein complex. The glycosylated precursor polypeptides $gP95^{gag}$ (95,000 M_r) and $gP85^{gag}$ (85,000 M_r) are however universally found on the surface of MuLV-infected cells.

Derepression of differentiation antigens is observed in some murine leukemias/lymphomas. Thymic leukemia (TL) antigen (45,000 M_r) is a MHC class I heavy chain glycoprotein encoded by the Tla locus which is expressed on immature thymocytes in certain mouse strains. It has been detected on some thymic lymphomas from mouse strains that do not normally express it (Old et al., 1963; Rothenberg, 1980). The activation of the Tla locus in the thymus shortly after the irradiation treatment of some mice has suggested that TL may be a marker for preleukemic changes in the thymus (Stockert and Old, 1977). Non-viral antigens distinct from other known cellular differentiation antigens have also been detected in some leukemias/lymphomas induced by MuLV. Examples

include the MCSA antigen (52,000 M_r, 92,000 M_r and 180-190,000 M_r) in a Moloney MuLV-induced lymphoma (Troy et al., 1977), and the 6C3 antigen (125,000 M_r and 160,000 M_r) in all Abelson MuLV-induced pre-B cell lymphomas (Pillemer et al., 1984).

1.3.2 Human leukemias/lymphomas

Aberrant expression of differentiation antigens is commonly found on human lymphocytic leukemias/lymhomas. For instance, some differentiation antigens normally expressed on T lymphocytes are expressed on transformed B cells and other hemopoietic cells, and vice versa (McCulloch, 1983; Smith et al., 1983). This "lineage infidelity" of antigen expression may represent a specific pattern for leukemic differentiation, or may represent an early stage of normal differentiation when cells not completely differentiated along a specific pathway can still express antigens common to cells of another lineage. The latter possibility conforms to the hypothesis that leukemias/lymphomas are resulted from clonal expansions of phenotypically normal hemopoietic cells which have become arrested at a certain stage of hemopoiesis or lymphopoiesis.

A well characterized leukemia/lymphoma-associated antigen is the common acute lymphoblastic leukemia antigen (CALLA, also called CD10) in human. It is a membrane glycoprotein of 100,000 M_r highly expressed on lymphoblasts from patients with acute lymphoblastic leukemia (ALL), some lymphomas, and some non-hemopoietic tumors, but also at very low levels on some lymphoid progenitor cells in normal bone marrow and thymus, and other normal non-hemopoietic cells (Newman et al., 1981; Pesando et al., 1983). The CALLA antigen is actively released from the cell surface of some lymphoblastoid cell lines, which is associated with cell growth (Komada et al., 1986). No defined functions of CALLA have yet been described on normal or malignant cells.

Despite its leukemia non-specific expression, its presentation common to all ALL diseases has rendered it a useful leukemia marker in clinical diagnosis. Additional leukemia/lymphoma-associated surface antigens identified in human include a 150,000 M_r glycoprotein by SN1 MAb, called TALLA (Seon et al., 1983). It appears to be specifically expressed on T cell ALL. No reactivity of the SN1 MAb with various normal tissues has been detected (Matsuzaki and Seon, 1987).

The human T lymphocyte virus type-I (HTLV-I) is thought to be an important causative agent of adult T cell leukemia (ATL). The HTLV-I provirus is found in all some of ATL and its genes are expressed in cultured cell lines (Franchini et al., 1984). Antisera from ATL patients have identified on the ATL cell surface viral envelope glycoproteins of 21,000 M_r and 46,000 M_r as well as their precursor of 65,000 M_r in size (Schupbach et al., 1984; Sugamura et al., 1984; Matsushita et al., 1986). However, consistent viral expression is not needed to maintain the neoplastic state, as no viral transcription is detected in some fresh leukemia cells (Franchini et al., 1984) and the viral envelope proteins are not present on HTLV-I-transformed non-producer cells (Schupbach et al., 1984). ATL cells appear to be restricted to the $CD4^+$ T cell subset (Hattori et al., 1981). A notable characteristic of ATL cells and in vitro HTLV-I-transformed cells is the constitutively high expression of the functional IL-2R on the cell surface (Depper et al., 1984; Gazzolo et al., 1987) and, in some cases, the production of IL-2 (Gootenberg et al., 1981; Inoue et al., 1986; Gazzolo et al., 1987). It has been shown that the HTLV-I genome contains a unique locus that produces a trans-activating factor tat-I (also known as x-lor or $p40^x$), which activates transcription from the promotors of IL-2 and IL-2R α genes (Cross et al., 1987; Maruyama et al., 1987; Siekevitz et al., 1987). The induced IL-2R α subunit then associates with the constitutively expressed IL-2R β subunit to form the functional IL-2R. The coexpression of IL-2 and IL-2R has implicated an aberrant IL-2

autocrine stimulation loop, which constitutes to the initial continual proliferation of HTLV-I-infected cells (Arima et al., 1986; Maruyama et al., 1987; Yamada et al., 1987), and may predispose them for the induction of chromosomal abnormalities characteristic of most tumorigenic ATL cells (Sanada et al., 1985).

Epstein-Barr virus (EBV) is another human virus that is believed to be involved in the development of leukemias/lymhomas. It is a herpesvirus that causes infectious mononucleosis as a primary disease and remains latent in human B lymphocytes, transforming them into lymphoblastoid cells (Henle et al., 1967). It is frequently found in African Burkitt lymphoma (BL), a B cell neoplasia, as episomes or integrated into the host genome. In vitro, EBV infection of human lymphocytes rapidly induces sustained cell proliferation (Pope et al., 1968). In EBV^+ BL cells and in vitro-transformed cells, the EBV genome encodes a latent infection membrane protein (LMP) (60,000 M_r) which consists of six hydrophobic transmembrane domains and a large (200 amino acids) cytoplasmic carboxy-terminus (Fennewald et al., 1984). Recently, it has been shown that the LMP antigen patches on the cell surface with strong association to the submembrane cytoskeletal protein vimentin, abruptly altering the distribution of vimentin which is normally organized into filaments (Liebowitz et al., 1987). The functional implication of such cytoskeletal interaction is still unknown. However, the significance of LMP in transformation has been suggested by transfection studies in which the LMP expression in fibroblast cell lines NIH3T3 and Rat-1 has led to the loss of contact inhibition of growth, the reduction in serum requirement, and anchorage independence (Wang et al., 1985). Nonetheless, the role of EBV and LMP in the generation of BL is still obscure because not all BL cells carry the EBV genome. The chromosomal translocation of c-myc oncogene in juxtaposition to the highly active enhancer of the Ig locus is more commonly observed (Zech et al., 1976).

1.4 THESIS OBJECTIVES

T lymphocytes are central in maintaining a fully responsive immune system. The heterogeneity of T cells and the functional specialization of each subpopulation underlie the basis of their diverse immunological functions. Many of the T cell functions, regulatory or effector, are known to involve cellular interactions, mediated either by direct cell contact or by response to soluble regulatory factors. T cell surface antigens are thought to play significant roles in these interactions. In the past, many T cell surface molecules have been identified by the use of MAb's and have been found useful in studying the functions and properties of different T cell subpopulations. They include antigens with important biological properties such as receptors for antigens, soluble regulatory factors, homing ligands, and other cell surface ligands in cell-cell interactions. Yet, the precise functions of many other antigens remain to be elucidated.

The objective of my thesis is to characterize a novel T cell surface antigen, called YE1/48. YE1/48 is defined by two rat MAb's, YE1/48.10.6 and YE1/32.8.5, which were generated against mitogen-activated murine T lymphocytes. It is a disulphide-linked dimeric molecule of 90-95,000 M_r, composed of 45-50,000 M_r subunits with distinct isoelectric points. The molecular size and the apparent heterodimeric structure of the YE1/48 antigen resemble properties of the murine TCR-α/β dimer. By flow cytometric analysis, the two anti-YE1/48 MAb's react with only two T lymphoma cell lines EL-4 and MBL-2(4.1), but not with normal resting or proliferating lymphocytes. This specific reactivity of the MAb's with only two T cell lines compares closely to the initial identification of the TCR-α/β by clonotypic MAb's on antigen-specific T cell clones. Since no other dimeric molecules of similar size had been identified at that time, it appeared that YE1/48 might be the authentic murine TCR-α/β.

The first phase of my research (described in Chapter Three) involved the biochemical analysis of the YE1/48 antigen and the determination of its expression in normal lymphoid cell populations by immunoprecipitation. Both the biochemical data and the antigen distribution revealed some similarities as well as differences between YE1/48 and murine TCR-α/β.

In the second phase of my research (described in Chapter Four), I attempted to determine if the YE1/48 antigen was the authentic murine TCR-α/β. Since the YE1/48.10.6 and YE1/32.8.5 MAb's do not show detectable binding on intact normal cell surface, no biological effects on T cell activation could be derived by perturbation of the YE1/48 antigen. Two approaches were used to directly compare YE1/48 with TCR-α/β. The first approach employed immunological methods such as sequential immunoprecipitation of YE1/48 and TCR-α/β from the EL-4 cell line. The second approach was to compare the partial amino acid sequences of YE1/48 to the published TCR sequences. These experiments together concluded that YE1/48 is distinct from TCR-α/β or TCR-γ/δ.

In the third phase of my research (described in Chapter Five), I undertook the molecular cloning of YE1/48 in an attempt to elucidate its possible function and correlation with other known antigens. Although the sequence of the YE1/48 cDNA clone isolated has not revealed definitive conclusions on the function of the antigen and its correlation with other proteins, genetic analyses using the cDNA clone as a probe have demonstrated some intriguing properties of the YE1/48 antigens.

In summary, this thesis will describe the detailed characterization of a novel T cell surface antigen, YE1/48, encompassing its initial identification, its discrimination from TCR-α/β, and the isolation of a cDNA clone. Although the function of the YE1/48 antigen remains unknown, the data obtained has suggested possible correlation of the YE1/48 expression with lymphocyte transformation, and will be useful in prospective studies.

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CHAPTER TWO

MATERIALS AND METHODS

2.1 SOURCES OF MATERIALS

2.1.1 Animals

C57BL/6 (B6), B10.BR, BALB/c and C3H mice were obtained from Charles River Canada, Quebec, Canada. Time-mated pregnant C57BL/6 mice were obtained from the Jackson Laboratory, Bar Harbor, ME.

2.1.2 Cell Lines

The T lymphoma cell lines EL-4 and MBL-2 are of C57BL/6 mouse origin. EL-4 cells were chemically induced and MBL-2 cells were induced by Moloney MuLV. They differ in the expression of several cell surface markers including the retrovirus envelope glycoprotein gp70, the Ly-6 related antigens, and the PC-1-like antigens. BW5147 is an AKR thymic leukemia. NS-1 is a BALB/c myeloma. B10A/A2/2.2 and BALB/c/A1 are two uncharacterized Abelson MuLV-transformed leukemias of B10 and BALB/c origins, respectively. All of the above cell lines were obtained from Dr. E.S. Lennox (MRC Laboratory of Molecular Biology, Cambridge, UK). Variant clones of the MBL-2 cell line, MBL-2(4.1) and MBL-2(2.6), were derived in our laboratory by cloning twice the parental line in methyl cellulose media. AK-1 is an AKR spontaneous thymic leukemia and BM-3 is a BALB/c Moloney MuLV-transformed T leukemia. They were generated in our laboratory from explants of leukemic thymocytes from the appropriate mice. 2PK3 and A20 are BALB/c B cell lines obtained from Dr. R. McMaster (University of British Columbia, Vancouver, BC). P815, a DBA/2 mastocytoma and L1210, an uncharacterized DBA/2 leukemia, were obtained from Dr. J. Levy (Univeristy of British Columbia, Vancouver, BC). All of the above cell lines were grown in Dulbecco's modified minimum essential medium containing 5% fetal calf serum (FCS), 50 U/ml penicillin and 50 µg/ml streptomycin. B6SutA1 is a hemopoietic progenitor cell line derived from Friend MuLV-infected culture of C56B1.5 bone marrow cells and was kindly

provided by Dr. G. Krystal (Terry Fox Laboratory, Vancouver, BC). It was propagated in RPMI 1640 medium containing 20% FCS and 5% pokeweed mitogen-stimulated mouse spleen cell conditioned medium.

A panel of pre-B cell lines were obtained from Dr. F. Lemoine (Terry Fox Laboratory, Vancouver, BC). All of them were initially derived from long term lymphoid bone marrow cultures established by the method of Whitlock and Witte (1982). H9 is a spontaneously transformed BALB/c pre-B cell line (Lemoine et al., 1988a). AB_n, AB_nB_2, AB_pD_4 and AB_nH_5 are Abelson MuLV-transformed pre-B cell lines (Lemoine et al., 1988b), all of (C57BL/6 x C3H/HeJ) F_1 hybrid origin. B_nB_2 and B_pD_4 are the parental untransformed lines of AB_nB_2 and AB_pD_4 respectively. All of the transformed pre-B cell lines were maintained as feeder-independent cultures in RPMI 1640 medium containing 5% FCS and 50 μM β-mercaptoethanol (2ME).

2.1.3 Monoclonal Antibodies

The YE1 series of rat MAb's was generated in our laboratory from a fusion between 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 and YE1/32.8.5 recognize the same molecule which is the antigen studied in this thesis. YE1/9.9.3 reacts with the transferrin receptor in all proliferating mouse cells (Takei, 1983). YE1/21.2.1 reacts with the CD45 (T200 or LCA) antigen (Trowbridge, 1978). YE1/30.4.1 reacts with the Thy-1 molecule (Williams and Gagnon, 1982). YE6/26.1.1, generated against the MBL-2 cell line in a similar fashion (Takei, 1987), reacts with the Moloney MuLV envelope protein gp70 (Nowinski et al., 1972). TIB 105 (Ledbetter and Herzenberg, 1979) reacts with the murine CD8 (Lyt-2) antigen and was purchased from the Americal Type Culture Collection. GK1.5-177 (Dialynas et al., 1983) which reacts with the murine CD4 (L3T4) antigen was a gift from Dr. D. Kilburn

(University of British Columbia, Vancouver). KJ16-133 reacts with the variable region of the β chain of the murine TCR-α/β (Epstein et al., 1985; Roehm et al., 1985) on 20% of peripheral T cell and 10% of thymocytes (Haskins et al., 1984; Roehm et al., 1984). It was a gift from Drs. P. Marrack and J. Kappler (National Jewish Hospital and Research Center, Denver, CO). Undiluted culture supernatants of the hybridomas, which were grown in the same media as for the leukemic cell lines above, were used as MAb's.

2.1.4 Xenoantisera

Polyclonal antisera containing rabbit anti-mouse Ig (RaMIg), rabbit anti-rat Ig (RaRIg) and mouse anti-rat Ig (MaRIg) antibodies were developed and affinity purified in our laboratory. A rat immune antiserum was also generated by immunizing a Fisher 344 rat with affinity purified YE1/48 antigen (see 2.3.2 below). R3497 is a rabbit antiserum that reacts with the murine TCR-α/β on all T cells (Bekoff et al., 1986). It was a gift from Dr. R. Kubo (National Jewish Center for Immunology and Respiratory Medicine, Denver, CO).

2.1.5 Materials for Genetic Studies

The oligo(dT)-cellulose was obtained from Pharmacia, Uppsala, Sweden. All DNA reaction enzymes and restriction enzymes used in this study were also obtained from Pharmacia, except exonuclease III and S1 nuclease which were purchased from New England Biolabs, Beverly, MA. Nitrocellulose filters were obtained from Schleicher & Schuell, Keene, NH, and nylon membranes (Zeta-Probe) were obtained from BIO-RAD Laboratories, Richmond, CA. All ^{32}P-labeled nucleotides were purchased from New England Nuclear, Boston, MA.

2.2 GENERAL TECHNIQUES FOR BIOCHEMICAL STUDIES

2.2.1 Cell Preparations

Single cell suspensions from different tissues were prepared by physical agitation. Erythrocytes were removed by lysis in Tris-NH_4Cl (pH7.2) from contaminated tissues such as spleen and bone marrow. Separation of thymocytes by peanut lectin agglutination was carried out as described by Reisner et al. (1976). In brief, thymocytes (2×10^7 cells/ml) were incubated for 10 minutes at 20°C in phosphate buffered saline (PBS) (pH 7.5) containing 2.5% FCS and 0.5 mg/ml peanut agglutinin (PNA). The mixture was then layered onto a cushion of PBS containing 50% FCS and the agglutinated cells were sedimented for 20 minutes at 20°C. The top and bottom fractions were collected separately as PNA^- and PNA^+ cells respectively. Each fraction was washed twice in 0.25 M D-galactose to remove PNA from the cell surface. Mitogenic stimulation of spleen cells was performed by incubating 2×10^7 cells in 10 ml RPMI 1640 containing 10% FCS, 50 U/ml penicillin, 50 µg/ml streptomycin, 50 µM 2ME, 10 mM N-2-hydroxyethylpiperazine (HEPES) and 2 µg/ml Con A or 10 µg/ml LPS for 2 days. Con A was subsequently removed by washing the cells with 0.1 M α-methylmannoside.

Spleen T cells from 4-10 weeks old C57BL/6 mice were purified by a single passage of total spleen cells through scrubbed nylon wool column in RPMI 1640 containing 5% FCS (Dougherty et al., 1986). Spleen B cells were purified by panning on plastic surfaces coated with 1/100 dilution of unpurified RaMIg antiserum. In brief, total spleen cells were incubated in PBS (pH 7.5) containing 5% FCS (panning medium) on antiserum-coated plastic dishes for 30 minutes at 4°C. After thorough washing and incubation in the same buffer for one hour at 37°C, the adherent B cells were recovered by agitation.

T cell subpopulations defined by CD8 (Lyt-2) and CD4 (L3T4) phenotypes were also purified by a similar panning method except that the selection was

in a negative manner and non-adherent cells were recovered. In the preparation of $CD8^-CD4^-$ thymocytes, total thymocytes from 4-5 weeks old mice were pre-incubated in a 1:1 mixture of TIB 105 and GK1.5-177 for one hour at 4^oC, whereas in the preparation of $CD8^+CD4^-$ and $CD8^-CD4^+$ spleen T cells, spleen T cells were pre-incubated with either GK1.5-177 or TIB 105, respectively. The cells were washed three times in panning medium before incubating for 30 minutes at 4^oC on plastic dishes pre-coated with purified RaRIg antiserum. Non-adherent cells were then recovered by gentle swirling. The non-adherent populations were further enriched by subjection to two more cycles of panning procedures.

The purified cell subpopulations were analysed by flow cytometry as described below (2.2.2), for Thy-1, CD45 (T200), and surface immunoglobulin (sIg) expression using YE1/30.4.1, YE1/21.2.1 and RaMIg, respectively.

2.2.2 Flow Cytometry (FACS Analysis)

Approximately 0.5-1 x 10^6 cells were incubated with 50 µl MAb for 30 minutes at 4^oC. They were then washed twice with RPMI 1640 containing 10% FCS, 10 mM HEPES and 0.1% NaN_3 (or with PBS containing 5% FCS and 0.1% NaN_3 if they were freshly purified lymphocytes) before incubating for 30 minutes at 4^oC in 50 µl pre-titrated fluorescein isothiocyanate (FITC)-conjugated purified $(Fab')_2$ fragments of second antibodies. FITC-conjugated $(Fab')_2$ goat anti-rat IgG (FITC-GaRIg) was purchased from Cappel Laboratories (Cooper Biomedical, West Chester, PA). FITC-conjugated $(Fab')_2$ mouse anti-rat kappa light chain (FITC-MaRIg$_K$) was prepared and kindly provided by Dr. P. Lansdorp (Terry Fox Laboratory, Vancouver, BC). Conjugated $(Fab')_2$ fragments of purified RaRIg was prepared in our laboratory. In general, FITC-RaRIg or FITC-GaRIg were used in most analyses and FITC-MaRIg$_K$ was used in the analysis of spleen cells and pre-B cells. Dead cells were stained by 10 µg/ml propidium iodide (5 minutes, 4^oC) and were gated out on the basis of red

fluorescence. The cells were washed thrice before being analysed by flow cytometry (model FACS 440, Becton Dickenson Immunocytometry Systems, Mountain View, CA). In the analyses of purified lymphoid cells, the dead cells were not stained by propidium iodide. Appropriate gates were set by light scatter parameters to exclude the dead cells after the total cells were fixed by 1% formalin in PBS and stored overnight.

As negative controls, cells were stained by the second FITC-antibody alone or by an unrelated MAb in the first incubation. As positive controls, other MAb's to surface markers highly expressed on the test cells were used. When unpurified cell populations were analysed and discrete fluorescent peaks given by the test MAb were detected on a logarithm scale, the high fluorescent peak could be directly translated into the percentage of positive cells. The crossover channel number between the high and low fluorescent peaks in the mixed cell population was then used to determine the percentage of positive cells in the subsequent purified subpopulations. The percentage of cells in the purified subpopulation to the right of this channel was taken as positive. This method was reliable and the selected channel number has always coincided with the crossover point between the negative control and the test MAb curves of the purified subpopulation cell sample.

2.2.3 Surface Labeling Of Cells (Iodination)

Cell surface proteins were radiolabeled by the iodogen method (Markwell, 1978). In brief, 2-3 x 10^7 cultured cells or 5 x 10^7 fresh lymphocytes were agitated in an iodogen-coated (100 µg) vial in 0.5 ml PBS containing 0.5 mCi ^{125}I (Amersham Corporation, Arlington Heights, IL) for one hour at 20°C. In the case of thymocytes, 1.0 mCi was used and the cells were incubated for 30 minutes to reduce cell death. Radiolabeled cells were then washed four times in PBS to remove residual unreacted ^{125}I.

2.2.4 Immunoprecipitation

Radiolabeled cells were lysed in 2-3 ml 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. After removal of the nuclei and insoluble materials by microfuging for 10 minutes at 4°C, 30 µl MAb or 50 µl antiserum was added to the lysates and incubated on ice for one hour. Agarose beads (30-50 µl of 50% suspension) coupled with purified RaRIg antibodies (2-4 mg/ml) were then added to the mixture for another two hour incubation at 4°C with mild rotary mixing. For the immunoprecipitation from spleen cells, MaRIg-coupled agarose beads were used to reduce non-specific binding. For the immunoprecipitation with the rabbit antiserum R3497, protein A-coupled agarose beads (Sigma, St. Louis, MO) were used in place of RaRIg-coupled beads. After the incubation, the beads were washed with the same lysis buffer without BSA, and the bound immune complex was eluted by boiling the beads in 50 µl SDS-PAGE sample buffer for 5 minutes.

2.2.5 Sequential Immunoprecipitation

EL-4 cells (3×10^7) were surface labeled with ^{125}I and 3 ml of cell lysate was prepared as described above (2.2.3 and 2.2.4). The cell lysate was divided into three aliquots. The first aliquot was incubated with 50 µl of YE1/48.10.6 MAb-coupled agarose beads (4 mg antibody/ml beads, 50% suspension of beads) overnight at 4°C. The second aliquot was incubated with 20 µl R3497 (anti-T cell receptor antiserum) for 2 hours on ice. Protein A-coupled agarose beads were then added to the mixture and incubated overnight at 4°C. The third aliquot was incubated overnight at 4°C without the addition of antibodies. After the incubation, the agarose beads were removed by centrifugation. The resultant immunodepleted cell lysates were then subjected to immunoprecipitation using YE1/48.10.6, R3497, control rat MAb and normal rabbit serum.

2.2.6 SDS-PAGE Analysis

Sodium dodecylsulphate (SDS) polyacrylamide gel electrophoresis (PAGE) analysis was carried out by standard methods (Laemmli, 1970) either using a Protean apparatus (16 cm x 18 cm x 1.2 mm slab gel, BIO-RAD Laboratories, Richmond, CA) or a Mini-Slab apparatus (8 cm x 10 cm x 1 mm, Idea Scientific, Corvallis, OR). Non-reduced protein markers were prepared in our laboratory, consisting of human transferrin (90,000 molecular weight (MW)), BSA (67,000 MW), and ovalbumin (43,000 MW). Reduced protein markers were obtained from BIO-RAD Laboratories and include phosphorylase B (92,500 M_r), BSA (66,200 M_r), ovalbumin (45,000 M_r), carbonic anhydrase (31,000 M_r), soybean trypsin inhibitor (21,500 M_r), and lysozyme (14,400 M_r). Both sets of markers were visualized by Coomassie blue staining. In the analysis of immunoprecipitates from pre-B cell lines, prestained reduced protein markers were used. They were obtained from BIO-RAD Laboratories and include phosphorylase B (130,000 M_r), BSA (75,000 M_r), ovalbumin (50,000 M_r), carbonic anhydrase (39,000 M_r), soybean trypsin inhibitor (27,000 M_r), and lysozyme (17,000 M_r). Specific radiolabeled antigens were detected by autoradiography on KODAK XAR films with Dupont Cronex intensifying screens (Du Pont, Wilmington. DE).

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

Two-dimensional gel analysis (IEF vs SDS-PAGE) was performed according to the O'Farrell's method (1975) using ampholines of isoelectric points (pI) 3.5-10.0 (LKB, Bromma, sweden), a homemade IEF apparatus for tube gels (13 cm long), and the Protean Slab Gel apparatus (Bio-Rad Laboratories, Richmond, CA). A mixture of three proteins with known pI's were included in each sample and visualized by Coomassie blue staining after separation; they were human transferrin (pI 5.9), BSA (pI 4.9) and ovalbumin (pI 4.5).

2.2.8 Diagonal Gel Analysis

Samples were separated on 10% SDS-PAGE gels under non-reducing and reducing conditions in the first and second dimensions, respectively. The first-dimensional electrophoresis was performed in tube gels. The tube gels were then equilibrated in reducing sample buffer (containing 5% 2ME) before being turned 90° and laid across the top edge of the second dimension slab gels. Protein markers, BSA (67,000 MW), ovalbumin (43,000 MW) and carbonic anhydrase (31,000 MW), were included in each sample and their separation was visualized by Coomassie blue staining.

2.2.9 Tryptic Peptide Mapping

The tryptic peptide mapping procedure was adapted from the modified method of Kappler et al. (1983). The immunoprecipitate sample was reduced and the subunits were separated by two-dimensional gel analysis as described above (2.2.7). The gel was fixed with 25% isopropanol/10% acetic acid and then washed sequentially with 10% acetic acid and 25% isopropanol. SDS was removed by extensive washing with 10% methanol. The gel was then washed once with distilled water before being dried between dialysis membranes. The two subunits were located by autoradiography and excised. The gel slices were rehydrated with 1 ml 0.05 M NH_4HCO_3 containing 50 µg TPCK-treated trypsin (Sigma, St. Louis, MO). After rehydration, additional 3 ml of 0.05 M NH_4HCO_3 was added and the mixtures were shaken for 28 hours at 37°C. Supernatants containing the tryptic peptides were then collected and lyophilized thrice to remove NH_4HCO_3. Lyophilized tryptic peptides were dissolved in a minimal volume of the acidic electrophoresis buffer (acetic acid : formic acid : distilled water = 15 : 5 : 80). Approximately 5-10 µl of the sample containing 6000 to 8000 counts per minute (cpm) was spotted onto a 20 x 20 x 0.01 cm cellulose-coated thin-layer glass plate (an E. Merck product, from BDH Chemicals Canada Limited, Toronto, ON). The first-dimensional electrophoresis

was performed at 1000 volts for one hour (Horizontal Electrophoretic Unit, BIO-RAD Laboratories, Richmond, CA). The second-dimensional separation was carried out by thin-layer chromatography in n-butanol : pyridine : acetic acid : distilled water = 35.5 : 25 : 5 : 20. The tryptic peptide fingerprints were visualized by autoradiography.

2.2.10 Endoglycosidase F Analysis

Endoglycosidase F digestion was carried out according to McIntyre and Allison (1984). A YE1/48 immunoprecipitate was prepared from 2 x 10^7 surface iodinated EL-4 cells. The resultant immunocomplex, composed of the YE1/48.10.6 MAb and the antigen bound to RaRIg-coupled agarose beads, was resuspended in 40 µl of 0.1 M sodium phosphate buffer (pH 6.1) containing 50 mM EDTA, 1% (v/v) Nonidet P-40, 0.1% SDS and 0-5 units of endoglycosidase F enzyme (New England Nuclear, Boston, MA). It was incubated at 37°C for 3 hours (with 0.5 unit of enzyme) or 22 hours (with 0.0, 2.0, 5.0 units of enzyme). The reaction was terminated by washing the beads with lysis buffer, 10 mM Tris-HCl (pH 7.5) containing 1% Triton X-100, 0.15 M NaCl and 0.01% NaN_3. The digested antigen was eluted from the beads by boiling for 5 minutes in SDS-PAGE sample buffer containing 5% 2ME. It was then analysed by 10% SDS-PAGE under reducing conditions.

2.3 PURIFICATION OF YE1/48 ANTIGEN

2.3.1 Large Scale Preparation Of Cell Lysates

MBL-2(4.1) cells (0.7-1.0 x 10^{10}) were washed three times in PBS and lysed in 10 mM Tris-HCl buffer (pH 7.5) containing 1% Triton X-100, 0.15 M NaCl and 0.01% NaN_3 (1.5 litres per 10^{10} cells). The lysis mixture was stirred on ice for 30 minutes and then centrifuged for 90 minutes at 14,000 revolutions per minute (rpm) (JA-14 rotor in a Beckman J2-21 Centrifuge), 4°C,

to remove the nuclei and insoluble materials. The lysate was then immediately used in affinity purification or stored at -20°C for later use. For the purpose of tracing the course of antigen purification, a 2 ml lysate prepared from 2 x 10^7 surface iodinated cells was added to the large-scale lysate just prior to affinity chromatography.

2.3.2 Affinity Chromatography

The YE1/48 Mab was purified from ascites fluid by $(NH_4)_2SO_4$ precipitation (50% saturation) followed by DEAE Affi-gel blue chromatography (BIO-RAD Laboratories, Richmond, CA). The column fractions were analysed for IgG purity by SDS-PAGE as well as for specific antibody activities by indirect binding assay (Takei, 1983) using MBL-2(4.1) cells as the target. The purified antibody fractions were then pooled, dialysed against 0.1 M $NaHCO_3$ (pH 8.0) and coupled to Affi-gel 10 agarose beads (Bio-Rad Laboratories, Richmond, CA) 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 active sites. The beads were prewashed extensively with the eluting buffer (see below) followed by lysis buffer immediately before each subsequent use.

The large scale MBL-2(4.1) cell lysate was incubated with about 3 ml of YE1/48.10.6 MAb-coupled agarose beads on ice for 4 hours with constant agitation. The beads were then packed into a column and were thoroughly washed overnight with 10 mM Tris-HCl buffer (pH 7.5) containing 1% Triton X-100, 0.5 M NaCl and 0.01% NaN_3 until no radioactivity could be detected in the flow-through. The column was briefly washed with 10 mM Tris-HCl buffer (pH 7.5) containing 0.1% Triton X-100, 0.15 M NaCl and 0.01% NaN_3 before the adsorbed antigen was eluted with 100 mM glycine-HCl buffer (pH 2.9) containing 0.05% Triton X-100, 0.15 M NaCl and 0.01% NaN_3. The radioactive fractions were pooled and immediately neutralized with a few drops of 1 M Tris-HCl

buffer (pH 7.5).

2.3.3 Preparative SDS-PAGE

Preparative SDS-PAGE was carried out using the Protean apparatus from BIO-RAD. Precautions were taken to minimize the destruction of amino acid residues on the purified protein by free radicals and oxidants which were trapped in the gel matrix (Hunkapillar et al., 1983; Guellaen et al., 1984). The acrylamide gels were polymerized overnight and extensively preelectrophoresed with a protein of low MW (such as lysozyme) before use. Sodium thioglycolate was always added at 0.1 mM in the cathodic buffer before electrophoresis.

The affinity purified YE1/48 antigen was concentrated by ultrafiltration in a Centricon 30 microconcentrator (Amicon Corp., Danvers, MS) at 20°C. An equal volume of SDS-PAGE sample buffer was added to the concentrated antigen which was then denatured for 15 minutes at 55°C to minimize protein aggregation (Hunkapillar et al., 1983). It was subsequently separated on a 7.5% preparative SDS-PAGE gel. The gel was heat-dried between two sheets of dialysis membranes and the 90,000 M_r YE1/48 antigen was located by autoradiography. The radioactive band was excised, rehydrated in 1/2 x SDS-PAGE running buffer (25 mM Glycine, 12.5 mM Tris and 0.05% SDS), and subjected to electrophoretic elution in a sealed dialysis membrane tubing. The eluant was concentrated in a Centricon 30 microconcentrator. The antigen was then reconstituted to 0.1 M Tris-HCl (pH 8.0), 2% SDS and 50 mM dithiothreitol, and was incubated in a boiling water bath for 10 minutes. Upon cooling, iodoacetamide was added to a final concentration of 50 mM and the mixture was incubated in the dark at 37°C for 45 minutes. The reduced and alkylated antigen was then electrophoresed on a 10% preparative SDS-PAGE gel after the addition of an equal volume of sample buffer. The 45,000 M_r antigen subunits, in a single protein band, were located, eluted and concentrated as described

above. The purified antigen was stored at -20°C until use.

2.3.4 Assessment Of Purity and Yield

Small aliquots of the YE1/48 antigen purified from the initial affinity chromatography and the final reducing preparative SDS-PAGE were analysed on a 10% SDS-PAGE mini-gel. The gel was fixed and silver stained. The intensities of the purified antigen bands were then compared visually to those of BSA loaded on the same gel at 0.03-0.5 µg quantities.

2.4 PARTIAL AMINO ACID SEQUENCING

2.4.1 Tryptic Digestion Of The Purified Antigen

The procedure for protein precipitation and tryptic digestion was essentially based on the method described by Stearne et al. (1985) with some modifications. Approximately 55 µg (500-600 pmoles) of the purified antigen was mixed with 9 volumes of precooled (-20°C) high performance liquid chromatography (HPLC) grade methanol (BDH Chemicals, Toronto, ON) in a siliconized corex tube, immediately followed by the addition of TPCK-treated trypsin to 1% of the antigen (0.55 µg). The mixture was incubated overnight at -20°C before centrifugation for 45 minutes at 18,000 rpm (JA-20 rotor in a Beckman J2-21 centrifuge) and -5°C. This procedure removed the polyacrylamide polymers in the sample which might cause severe artefacts in the subsequent HPLC separation of tryptic peptides and phenylthiohydantoin (PTH) derivatives of amino acids. The protein precipitate was air-dried and resuspended in 100 µl of 0.1 M NH_4HCO_3 buffer (pH 8.0) containing 2 mM $CaCl_2$ and TPCK-treated trypsin (1% of the antigen), making the final weight ratio of protein to trypsin 50 : 1. The mixture was then incubated for 24 hours at 37°C and subsequently subjected to peptide separation by HPLC.

2.4.2 HPLC Separation Of Tryptic Peptides

Following tryptic digestion, the sample was immediately reconstituted to 3 M guanidine chloride and 0.1% (v/v) trifluoroacetic acid (TFA) in 1 ml volume. It was then loaded directly onto a μBONDAPACK C_{18} reverse phase HPLC column (Waters Associates, Milford, MS; 3.9 mm x 30 cm) equilibrated in 0.1% TFA. A gradient of 0% to 60% (v/v) acetonitrile in 0.1% TFA was run over a 105 minute period at a flow rate of 1 ml/min. A Waters HPLC system with two model 510 pumps, a U6K manual injector, a 660 automated gradient controller, a 490 programmable multi-wavelength detector and a SE 120 recorder was used. Absorbance at 215 nm was recorded at a chart rate of 0.5 cm/min and a scale of 0.0-0.2 absorbance. The peptide peaks were manually collected into glass tubes and stored at -20°C. Selected peptide peaks were sequenced by a gas phase sequencer at the Tripartite Microsequencing Centre (University of Victoria, Victoria, BC).

2.5 cDNA CLONING

2.5.1 Synthetic Oligonucleotide probes

Three 17 bases long (17-mer) antisense oligonucleotides FT1, FT2 and FT3 of 24-, 48- and 32-fold redundancies, respectively, were derived from three tryptic peptide sequences (see Chapter Five, Table X), and were synthesized by an Applied Biosystems DNA synthesizer (Dr. M. Smith's laboratory, University of British Colulmbia, Vancouver, BC). They were 5′ end-labeled using T4 polynucleotide kinase and [γ-^{32}P]ATP (3000 Ci/mmol).

2.5.2 Screening of λgt10 cDNA library

A non-size-selected complementary DNA (cDNA) library of MBL-2(4.1) was constructed in λgt10 vector in our laboratory. The method employing RNase H and polymerase I was used in the synthesis of the second strand of cDNA in

order to enhance full length construction (Gubler and Hoffman, 1983). The cDNA library was plated on E.coli C600Hfl at 3 x 10^4 plaque forming units (pfu) per 22 x 22 cm^2 plate, which was lifted onto nitrocellulose filters and lysed in situ (0.5 M NaOH). The filters were baked for 2 hours at 80°C under vacuum. They were prewashed for one hour at 42°C in 50 mM Tris-HCl (pH 8.0), 1 M NaCl, 1 mM EDTA and 0.1% SDS as described by Maniatis et al. (1982), followed by prehybridization in 6 x SSC (1 x SSC = 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0), 10 x Denhardt's (1 x Denhardt's = 0.2 mg/ml Ficoll, 0.2 mg/ml polyvinylpyrrolidone, and 0.2 mg/ml BSA), 0.1% SDS, 50 mM sodium phosphate (pH 6.5), and 0.1 mg/ml salmon sperm DNA for 2-3 hours at the hybridization temperatures (see below).

FT2 was used for the initial screening of the cDNA library. Hybridization was carried out overnight in 6 x SSC, 10 x Denhardt's, 50 mM sodium phosphate (pH 6.5), 1 mM EDTA and salmon sperm DNA, at 39°C, as derived from the formula ΣGC x 4°C + ΣAT x 2°C - 5°C (Suggs et al., 1981). The filters were then washed in 2 x SSC as described by Davis et al. (1986), for 2 x 15 minutes at 39°C and for 3 x 30 minutes at 29°C. The positive clones were isolated and purified. In order to distinguish from the cross-hybridization of redundant probes with λgt10 vector DNA, DNA isolated from these positive clones was digested with EcoRI and tested for hybridization with FT1 and FT3 probes by Southern blot analysis. The temperatures for hybridization with FT1 and FT3 were 43°C and 37°C, respectively, and the stringency washes were done at 10°C lower.

2.5.3 DNA Sequencing

The cDNA insert was excised by EcoRI digestion from the phage DNA of the positive cDNA clone M3-2, and was subcloned by standard methods (Maniatis et al., 1982) into the EcoRI site of pTZ19R plasmid (United States Biochemical Corporation, Cleveland, OH). The pTZ19R plasmid contains a priming site on

either side of the multiple cloning sites and thus allows double-strand DNA sequencing. Two series of deletion clones spanning across the cDNA insert were prepared by timed digestion with exonuclease III (Henikoff, 1984). DNA sequencing of the coding strand was performed by the dideoxy chain-termination method (Sanger et al., 1977) using [α-^{32}P]dATP (800 Ci/mmol). In addition, two internal sequences of the non-coding strand were determined using two synthetic 17-mer oligonucleotide primers derived from sequences of the coding strand.

2.6 GENETIC ANALYSES USING cDNA CLONE

2.6.1 cDNA Insert as Probes

M3-2 cDNA insert excised by EcoRI from either the original λgt10 clone or the subcloned PTZ19R plasmid clone was used for the genetic analyses to be described in the following. In addition, cDNA fragments of 514 base pairs (bp) (M3-2/514), 400 bp (M3-2/400) and 245 bp (M3-2/245) in length were prepared by HinfI digestion of the M3-2 cDNA insert, and a 151 bp fragment (M3-2/151) was prepared by FokI digestion. All the cDNA insert and fragments were isolated and purified by agarose gel electrophoresis and electroelution. They were radiolabeled by oligolabeling (Feinberg and Vogelstein, 1983) (Oligolabeling Kit, Pharmacia, Uppsala, Sweden) using [α-^{32}P]dCTP (3000 Ci/mmol), except for M3-2/151 which was labeled by primer-extension.

2.6.2 Northern Blot Analysis

Total cellular RNA was extracted from cultured cells and tissue cells using the acid guanidinium thiocyanate/phenol/chloroform method of Chomczynski and Sacchi (1987). Exceptions were EL-4, MBL-2(4.1), MBL-2(2.6), BW5147, AK-1 and NS-1 cells, for which the urea sarkosyl/CsCl method of Glisen et al. (1974) was used. Poly(A)$^+$RNA was purified from total cellular RNA of MBL-

2(4.1), MBL-2(2.6) and NS-1 cells by oligo(dT)-cellulose affinity chromatography. Total RNA (20 µg) or poly(A)$^+$RNA (5 µg) was electrophoresed in 0.66 M formaldehyde/1.0% agarose gels (Davis et al., 1986), transferred onto nylon membranes in 20 x SSC by centrifugation (Wilkins and Snell, 1987), and fixed under ultraviolet illumination (Khandjian, 1986). Prehybridization and hybridization were performed for 1-2 hours and overnight, respectively, at 65°C in 1.5 x SSPE (1 x SSPE = 0.18 M NaCl, 10 mM sodium phosphate (pH 7.0), and 10 mM EDTA), 1% SDS, 0.5% (w/v) no-fat powdered milk and 0.5 mg/ml salmon sperm DNA. The blots were then washed with a final stringency wash for 30 minutes at 60°C in 0.1 x SSC and 1.0% SDS. Bound cDNA probe was removed at 65°C by agitation for 2 x 30 minutes in 50% formamide and 10 mM sodium phosphate (pH 6.7), followed by 0.1 x SSC and 1% SDS (Parkhurst and Corces, 1987). The blots were then rehybridized as above with a chicken β-actin probe radiolabeled by nick translation (Nick Translation Reagent Kit, Bethesda Research Laboratories Life Technologies, Gaithersburg, MD) using [α-^{32}P]dCTP (800 Ci/mmol). Signals on all Northern blots were quantified by laser scanning densitometry (Model GS300, Hoefer Scientific Instruments, San Francisco, CA).

2.6.3 Genomic Southern Blot Analysis

Genomic DNA was prepared from cultured cells and tissue cells by the SDS/proteinase K method (Gross-Bellard et al., 1977) followed by phenol/chloroform extraction. Either 5 or 10 µg of DNA was electrophoresed in 0.8% agarose gels in Tris-acetate buffer and alkaline (0.4 M NaOH) blotted onto nylon membranes. Prehybridization and hybridization conditions were the same as those in Northern blot analyses, except that they were performed at 68°C and the final stringency wash was performed at 50°C.

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CHAPTER THREE

EXPRESSION AND BIOCHEMICAL ANALYSES OF A T CELL RECEPTOR α/β-LIKE MOLECULE, YE1/48,

Data Reported in J. Immunol. 136:1346-1353; February 1986
and J. Immunol. 140:161-169; January 1988

3.1 INTRODUCTION

The T cell antigen receptor (TCR) on human and murine T cells has recently been identified by using T cell clone-specific MAb's (for reviews, see Allison et al., 1984 and Reinherz et al., 1984). It is a dimeric glycoprotein consisting of 40-50,000 M_r disulphide-linked polypeptide chains, α and β (Allison et al., 1982; Haskins et al., 1983; Kappler et al., 1983b; Marrack et al., 1983; Meuer et al., 1983a, 1983b; Kaye and Janeway, 1984;). The two chains have different isoelectric points (pI's) and can be separated by two-dimensional gel electrophoresis, using SDS-PAGE versus IEF or nonequilibrated pH gradient electrophoresis (NEPHGE). Peptide analysis of the α and β chains has shown that they consist of variable and constant regions (Acuto et al., 1983b; Kappler et al., 1983a; McIntyre and Allison, 1983). Several cDNA clones that are derived from rearranged genes and are expressed specifically in T cells were isolated, and their sequences were found to be homologous to the immunoglobulin (Ig) genes, composed of variable, diversity, joining and constant regions (Chiens et al., 1984; Hedrick et al., 1984a, 1984b; Saito et al., 1984; Siu et al., 1984; Yanagi et al., 1984). Upon comparison of the predicted amino acid sequences deduced from the nucleotide sequences of the cloned cDNA's with the partial amino acid sequences obtained from purified human α and β chains (Acuto et al., 1984; Hannum et al., 1984), these cDNA's have been confirmed to code for the human TCR-α/β. Although the amino acid sequences of the murine α and β chains were not determined, the close similarities between the nucleotide sequences of the human TCR-α/β cDNA's and the putative murine receptor cDNA's have indicated that the cloned murine cDNA's encode the receptor molecules (Caccia et al., 1984; Clark et al., 1984; Jones et al., 1985). The constant region genes have been identified for the α and β chain genes. There appears to be no preferential expression of the constant genes in different T cell subsets (Royer et al.,

1984; Hedrick et al., 1985; Kronenberg et al., 1985). Initial studies on the rearrangement and transcription of the β chain gene in various T cell lines have shown that the joining and constant segments of the β chain gene are deleted in most of the suppressor T cell lines tested (Hedrick et al., 1985; Kronenberg et al., 1985), leaving a possibility for the existence of other types of TCR molecules. However, both α and β gene rearrangements have been detected in several suppressor cell lines (Royer et al., 1984; Toyonaga et al., 1984; Yoshikai et al., 1984a, 1984b; Ballinari et al., 1985; de Santis et al., 1985; Imai et al., 1986; Modlin et al., 1987), and the α/β heterodimer has been described in at least two reports (Bensussan et al., 1984; Modlin et al., 1987). Nevertheless, another TCR distinct from the α/β dimer has recently been identified. The TCR-γ/δ heterodimer can either be disulphide-linked or non-covalently associated. It was found on TCR-α/β$^-$CD3$^+$ T cells and Thy-1$^+$ dendritic epidermal cells (Borst et al., 1987; Brenner et al., 1987; Ioannides et al., 1987; Koning et al., 1987; Moingeon et al., 1987; Nakanishi et al., 1987).

We have isolated two rat MAb's that react with a TCR-α/β-like molecule, called YE1/48. The MAb's immunoprecipitate a disulphide-linked dimer of 45-50,000 M_r under reducing conditions and 90-95,000 M_r under non-reducing conditions. Their pI values are comparable to those of the TCR-α/β. In this chapter, the biochemical characterization of the YE1/48 antigen and its expression in lymphoid populations are described.

3.2 RESULTS

3.2.1 Two Monoclonal Antibodies Define The YE1/48 Antigen

The YE1/48.10.6 (IgG_{2b}) and YE1/32.8.5 (IgG_{2a}) MAb's were rat MAb's generated in our laboratory against a T cell hybrid of EL4-BU and Con A-activated AKR spleen cells (see Chapter Two, section 2.1.3). By flow

cytometric analysis using indirect fluorescent antibody staining, neither of the MAb's showed detectable binding to the surface of normal murine lymphoid populations, such as spleen cells and thymocytes, or other cell lines tested except two T lymphoma cell lines EL-4 and MBL-2(4.1) (Figure 1 and Table VI). The antigen molecules immunoprecipitated from the surface iodinated EL-4 and MBL-2(4.1) cells by both YE1/48.10.6 and YE1/32.8.5 MAb's exhibited similar M_r in SDS-PAGE analysis, at approxiamtely 90-95,000 M_r under non-reducing conditions and 45-50,000 M_r under reducing conditions (Figure 2) (as previously reported by Takei, 1983), indicating that the molecule has a disulphide-linked dimeric structure. By using sequential immunoprecipitation in which immunodepletion by either one MAb had led to the loss of the immunoprecipitation signal by the other MAb, it was confirmed that the two MAb's react with the same protein molecule. However, the two MAb's appear to bind to different epitopes because they showed limited competitive inhibition of binding (16-43%) with each other (data not shown). The antigen is designated YE1/48.

3.2.2 Biochemical Analysis Of The YE1/48 Antigen

Since the M_r and the dimeric structure of the YE1/48 antigen were similar to those of the murine TCR α and β chains, and the specific reactivities of the two MAb's with only two T lymphoma cell lines resemble the clonotypic binding of many anti-TCR-α/β MAb's, the antigen was considered as TCR-α/β-like and was dedicated to further characterization. By two-dimensional gel electrophoresis analysis (IEF vs SDS-PAGE), the two subunits of the YE1/48 antigen could be separated by their difference in pI's (Figure 3) into an acidic chain and a more basic chain, similar to the α and β chains of the murine TCR-α/β heterodimer. The heterogeneity in the pI values of each subunit indicated that they were both glycopolypeptides. The two YE1/48 subunits were isolated from the two-dimensional gel and were separately

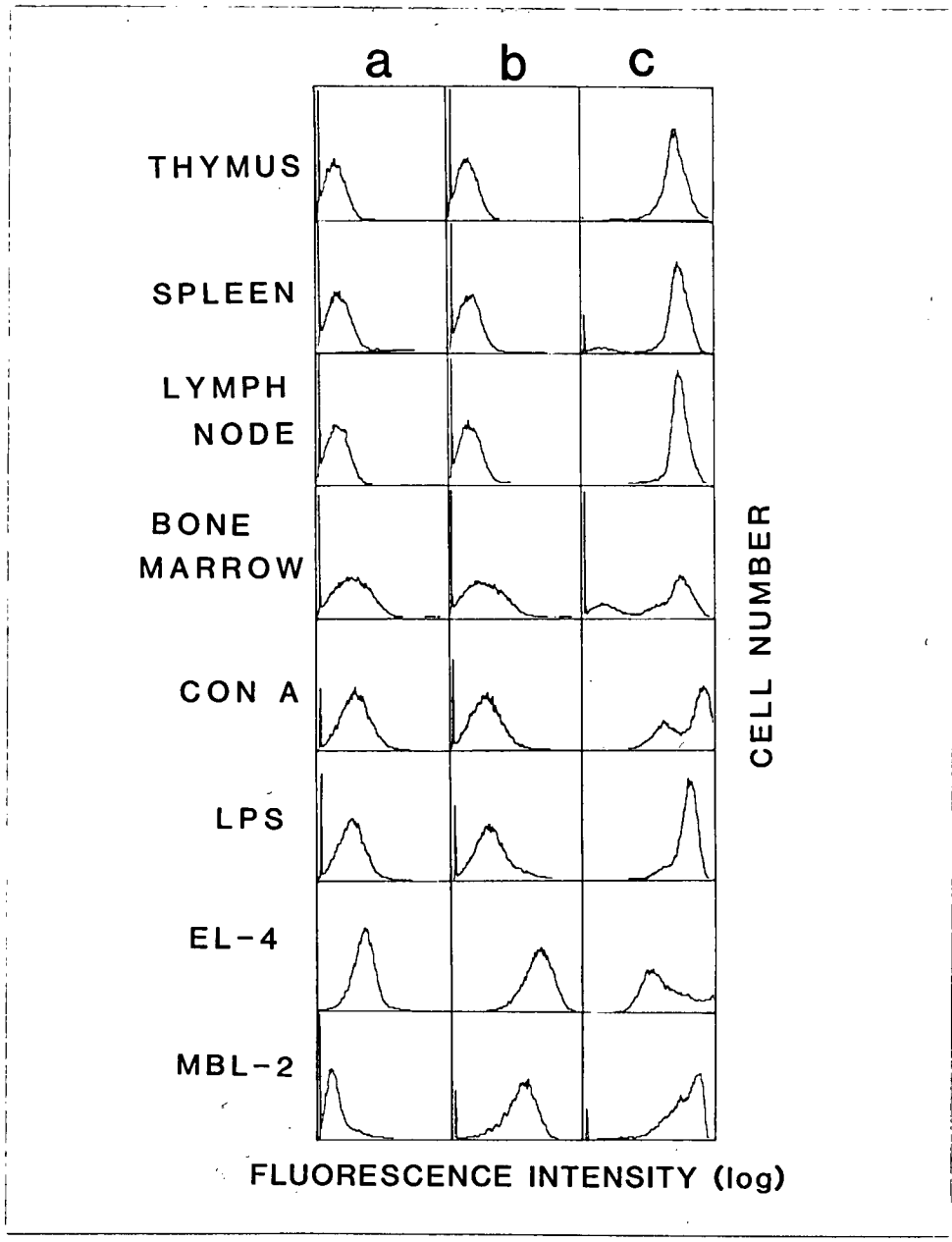

Figure 1. Flow cytometric analysis of YE1/48 expression on normal lymphoid tissue cells, and EL-4 and MBL-2(4.1) cells. C57BL/6 thymocytes, spleen cells, lymph node cells, bone marrow cells, Con A- and LPS-stimulated spleen cells, EL-4 and MBL-2(4.1) cells were indirectly stained by (a) an unrelated IgG_{2b} MAb as a negative control, (b) YE1/48.10.6 MAb, and (c) YE1/21.2.1 MAb (anti-CD45) as a positive control, followed by FITC-conjugated $(Fab')_2$ mouse anti-rat Ig as the second antibody. Dead cells were stained by propidium iodide and were gated out on the basis of red fluorescence. The same fluorescence gain was applied for all samples except EL-4 and MBL-2(4.1), for which a lower gain was used.

TABLE VI

SURFACE BINDING OF THE YE1/48.10.6 AND YE1/32.8.5 MAb'S ON NORMAL CELLS AND CELL LINES

Normal Tissues[a]	FACS
Thymus[b,c]	-
Spleen[c]	-
Bone marrow	-
Lymph nodes	-
Con A-stimulated spleen	-
LPS-stimulated spleen	-
Erythrocytes	-
Liver[b]	-

Cell Line	Origin	Cell Type	FACS
EL-4	C57BL/6	T cell line	+
MBL-2(4.1)	C57BL/6	T cell line	+
BW5147	AKR	T cell line	-
AK-1	AKR	T cell line	-
AK-2	AKR	T cell line	-
SAK 8	AKR	T cell line	-
SL-3	AKR	T cell line	-
BM-1	BALB/c	T cell line	-
BM-3	BALB/c	T cell line	-
BM-4	BALB/c	T cell line	-
A20	BALB/c	B cell line	-
2PK3	BALB/c	B cell line	-
B9	BALB/c	Pre-B cell line	-
B10	BALB/c	Pre-B cell line	-
B12	BALB/c	Pre-B cell line	-
17-1B	BALB/c	Pre-B cell line	-
L1210	DBA/2	Uncharacterized	-
B10A/A2/2.2	B10A	Uncharacterized	-
BALB/c/A1	BALB/c	Uncharacterized	-
NS-1	BALB/c	Myeloma	-
P815	DBA/2	Mastocytoma	-
B6SutA(JG-1)	C57BL/6	Hemopoietic progenitor line	-
$Ltk^-/8$	C3H	Fibroblast line	-

[a] C57BL/6 and B10Br mice tested.
[b] Fetal tissues tested also.
[c] New born mice tested also.

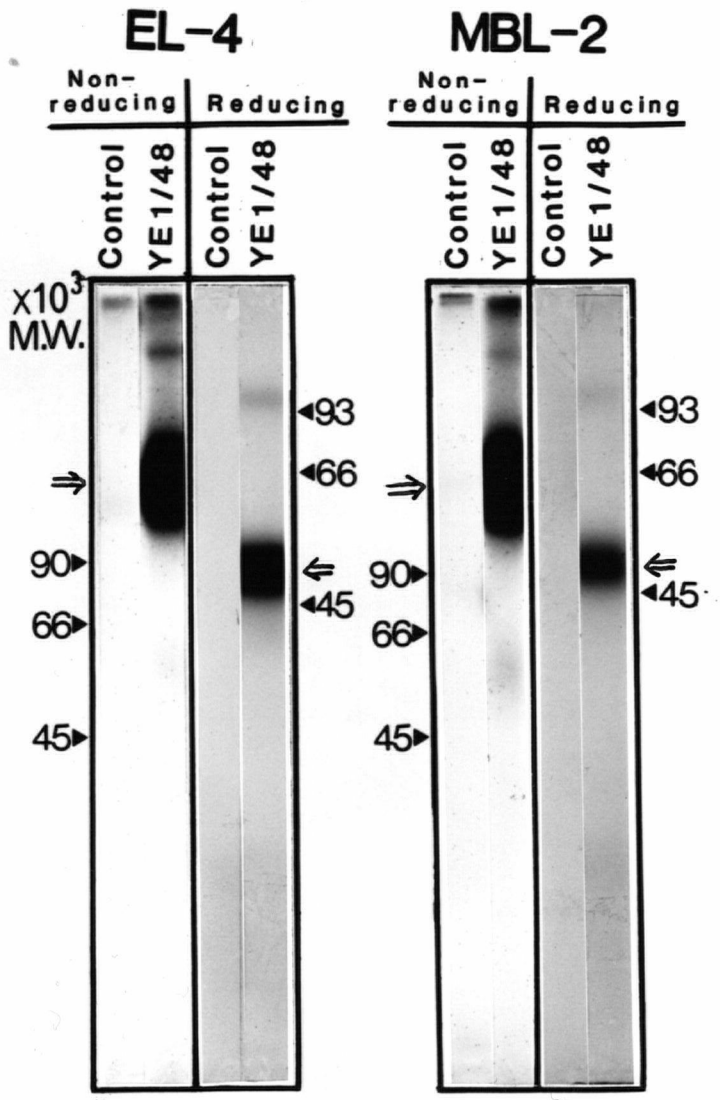

Figure 2. Immunoprecipitation of the YE1/48 antigen from EL-4 and MBL-2(4.1) cells. ^{125}I surface labeled cell lysates were first incubated with YE1/48.10.6 MAb or the parental myeloma Y3 culture supernatant (control), and then with sepharose beads conjugated with rabbit anti-rat Ig antibodies. The resultant immunoprecipitates were analysed by 10% SDS-PAGE under non-reducing and reducing conditions. Open arrows show the YE1/48 antigen precipitated.

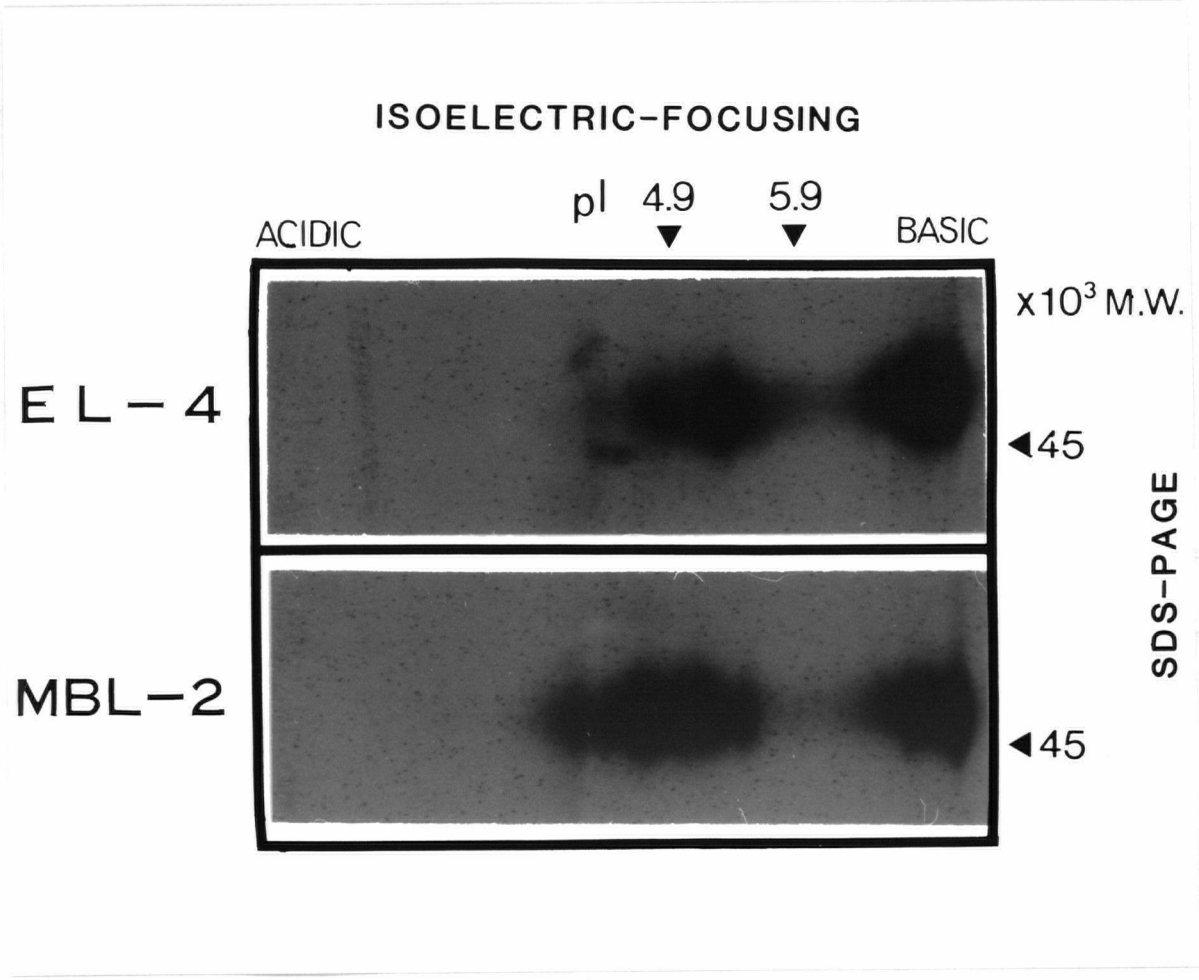

Figure 3. Two-dimensional gel analysis (IEF vs SDS-PAGE) of the YE1/48 antigen. YE1/48 immunoprecipitates from ^{125}I surface labeled EL-4 and MBL-2(4.1) cells were reduced and analysed by isoelectric focussing in the first dimension and 10% SDS-PAGE in the second dimension. Separation of human transferrin (pI 5.9) and bovine serum albumin (pI 4.9) on the same gels was shown by arrows for reference.

analysed by tryptic peptide mapping (Figure 4). The majority of the tryptic peptides were shared between the two antigen subunits (solid spots) with only a few peptides being different (striped or open spots). This observation strongly indicates that the two YE1/48 subunits, unlike the TCR α and β chains, are very similar to each other.

The YE1/48 antigen was analysed for glycosylation characteristics by endoglycosidase F digestion. The enzyme cleaves both the high-mannose and complex types of N-linked carbohydrate side chains which are attached to the polypeptide at specific asparagine residues. As shown by SDS-PAGE analysis under reducing conditions, the 45-50,000 M_r YE1/48 subunits were diminished in size to 42,000 M_r, 38,000 M_r and 32,000 M_r at a suboptimal dose of the enzyme (Figure 5, lanes b & c). This suggests that there are at least three N-linked glycosylation side chains attached to the YE1/48 polypeptides. This is comparable to the glycosylation characteristics of the murine TCR-α/β (McIntyre et al., 1984). Equal amounts of the 38,000 M_r and 32,000 M_r bands persisted when an increased level of enzyme was used for digestion (Figure 5, lane e). This has several implications. First, the two YE1/48 subunits may have different polypeptide lengths of 38,000 M_r and 32,000 M_r. Alternatively, the two subunits may have the same protein core size of 32,000 M_r, and the 38,000 M_r entity represents the partial resistance of a carbohydrate side chain to complete digestion. It is also possible that the two subunits may contain different amounts of O-linked carbohydrates insensitive to the endoglycosidase F digestion. Attempts to clarify these possibilities by two-dimensional gel analysis (IEF vs SDS-PAGE) of the deglycosylated antigen have not given conclusive results, because deglycosylation changes the pI values of the subunits making their separation inefficient. Hence, with the current data, whether the two persisting deglycosylated forms represent one common protein core or two distinct polypeptide subunits remains unresolved.

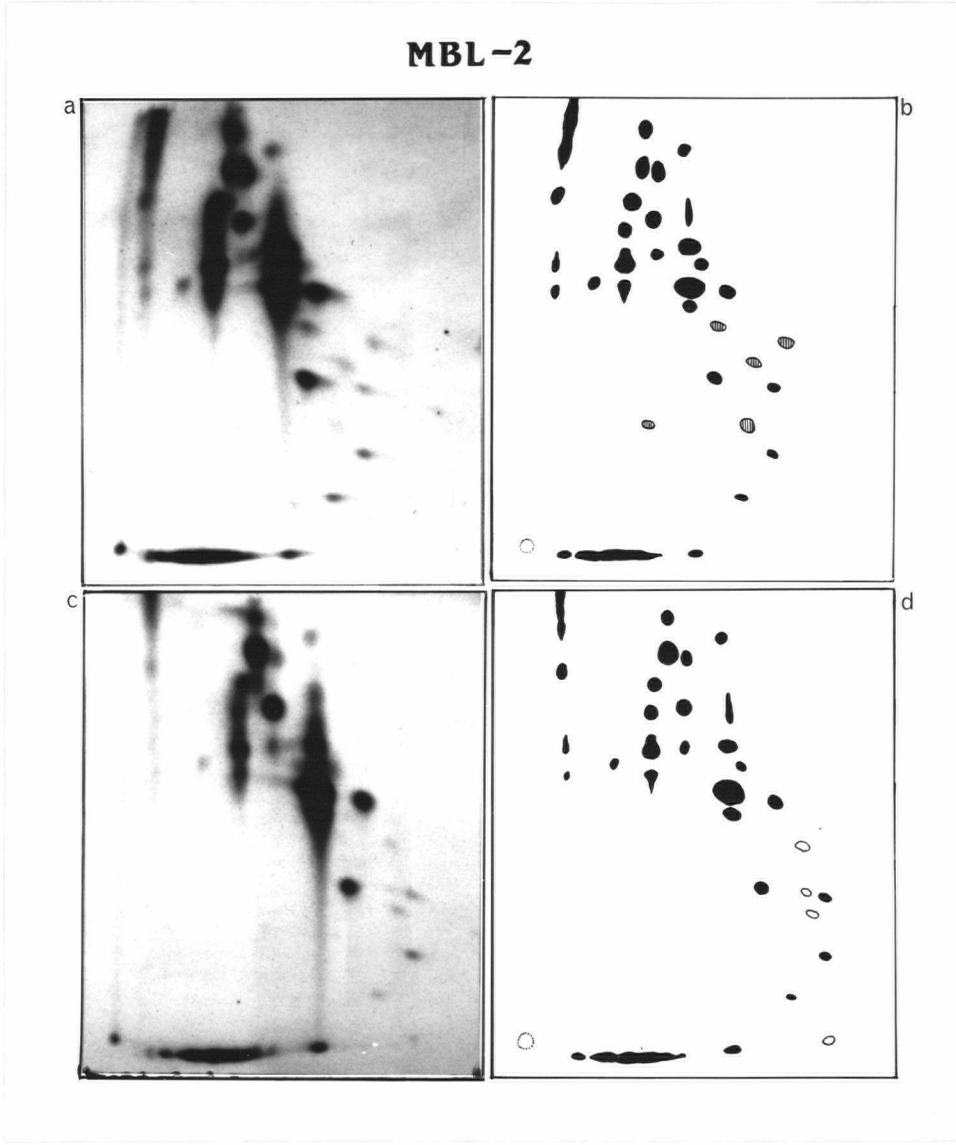

Figure 4. Tryptic peptide analysis of the YE1/48 subunits from MBL-2(4.1) cells. The subunits of the YE1/48 immunoprecipitate from MBL-2(4.1) cells were separated as described in Figure 3. After fixation, SDS was removed from the gel and the two polypeptide subunits were located by autoradiography. The subunits were then digested by TPCK-treated trypsin while being eluted by agitation at 37°C. The eluted tryptic peptides were concentrated and analysed by electrophoresis (from left to right from the origin marked by dotted circle) and thin-layer chromatography (from bottom to top). Schematic representations (b, d) of the autoradiographs (a, c) were shown to indicate peptides common to both YE1/48 subunits (solid spots) and those unique to the acidic (b) or basic (d) subunits (open spots).

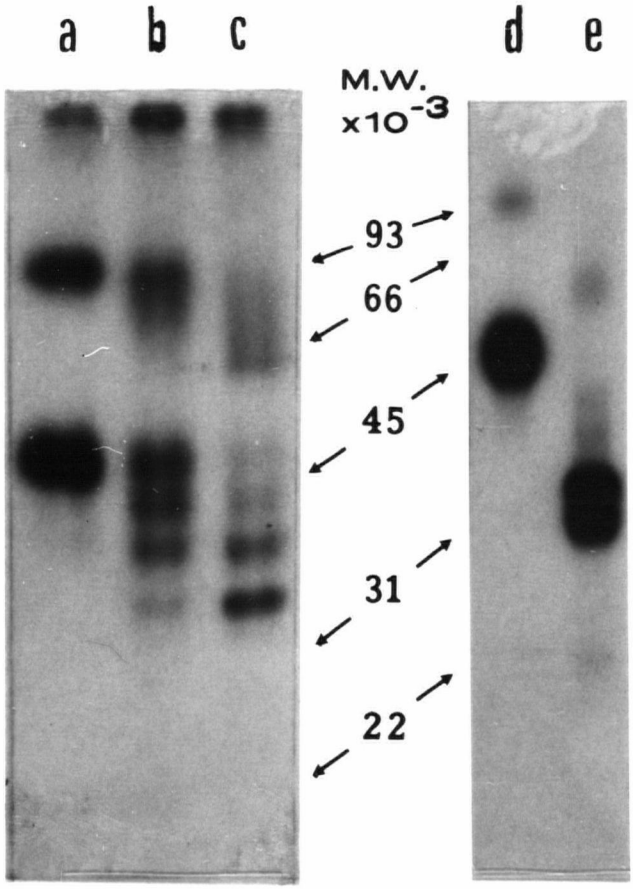

Figure 5. Endoglycosidase F digestion of the YE1/48 antigen from EL-4 cells. The YE1/48 immunprecipitate prepared from surface iodinated EL-4 cells was subjected to digestion by either (a & d) 0 unit/ml, (b) 12.5 units/ml, (c) 50 units/ml, or (e) 125 units/ml of endoglycosidase F at 37°C for 22 hours (except for 12.5 units/ml digestion in 3 hours). After removal of the released carbohydrates, the digests were analysed on 10% (a-c) or 12.5% (d, e) SDS-PAGE gels under reducing conditions.

3.2.3 Expression On Normal Lymphoid Cells

A) A Rat anti-YE1/48 Antiserum

Although clonotypic MAb's have been used in identifying TCR-α/β on T cell clones by blocking antigen-specific binding and antigen-mediated T cell activation, as well as in studying the biological effects of the activation, they cannot be used to study the expression of the receptor in normal T cell populations. To study the TCR-α/β in normal T cell populations, polyclonal antisera or MAb's capable of reacting with the constant regions of the receptor on the cell surface are required. Considering the possibility that the YE1/32.8.5 and YE1/48.10.6 MAb's may react with the clonotypic epitopes on YE1/48 on EL-4 and MBL-2(4.1) cells, and that YE1/48 may have constant regions similar to TCR-α/β, a rat immune antiserum was generated against the purified YE1/48 antigen in order to detect non-clonotypic determinants on the surface of YE1/48 antigen. The immunizing antigen was partially purified by affinity chromatography from EL-4 cells which express the antigen at high levels ($1.5\text{-}3.0 \times 10^5$ molecules per cell). The anti-YE1/48 antiserum thus generated, although specifically immunoprecipitating a molecule of similar M_r from EL-4 and MBL-2(4.1) cells, did not show detectable binding to the surface of normal thymocytes or spleen cells when tested by flow cytometry.

B) Immunoprecipitation As The Assay For Expression

The two MAb's and the anti-YE1/48 antiserum were further tested for their reactivities with normal lymphoid cell populations by immunoprecipitation in light of two technical differences between immunoprecipitation and flow cytometric analysis. In flow cytometry, the binding of antibodies to antigens on the surface of intact cells is detected by indirect fluorescence staining, whereas in immunoprecipitation, the cell membrane is partially solubilized by non-ionic detergents before the antibody binding is tested, and the signal is detected as radioactivity on the surface antigens bound by the antibodies. Second, the methods differ in their sensitivities of detection. In general,

antigens expressed at lower than 1000 molecules per cell on the cell surface are not readily detectable by flow cytometry. In immunoprecipitation, the sensitivity of detection depends on the efficiency of surface iodination of the antigen studied, which in turn relies on the number of tyrosine residues in the extracellular domain of the antigen and the spatial orientation of these residues favorable for the iodination reaction. For the YE1/48 antigen, it can readily be surface iodinated and its detection by immunoprecipitation is very efficient. MBL-2(2.6) cells, which show no YE1/48.10.6 MAb binding by flow cytometry, give detectable immunoprecipitation signals. The antigen can also be detected in a cell lysate prepared from a mixture of 95.5% YE1/48$^-$ L1210 cells and 0.5% EL-4 cells, both of which are transformed cell lines of similar cell size.

It was found that the YE1/48 antigen can be immunoprecipitated by both YE1/32.8.5, YE1/48.10.6 MAb's and the anti-YE1/48 antiserum from normal C57BL/6 thymocytes and spleen cells (Figure 6), albeit at much lower levels than detected from EL-4 and MBL-2(4.1) cells. It suggests that the epitopes defined by both MAb's are not clonotypic as previously speculated. It seems that either these epitopes are somehow not accessible to binding on the surface of intact cells unless the cell membrane is partially solubilized, or the expression of the YE1/48 antigen on normal cells is below the detection limit of flow cytometry. (This question will be further addressed in Chapter Five, section 5.3.4.)

C) T and B Lymphocytes

Immunoprecipitation was used as an assay for YE1/48 expression in various lymphoid cell populations and subpopulations. The results are summarized in Table VII. The antigen is expressed on both C57BL/6 spleen T cells (75% Thy-1$^+$, 12% sIg$^+$) and spleen B cells (95% sIg$^+$, 2% Thy-1$^+$) with the former detection signal considerably higher than the latter. Its expression on total spleen cells is however diminished after Con A or LPS stimulation. Among the

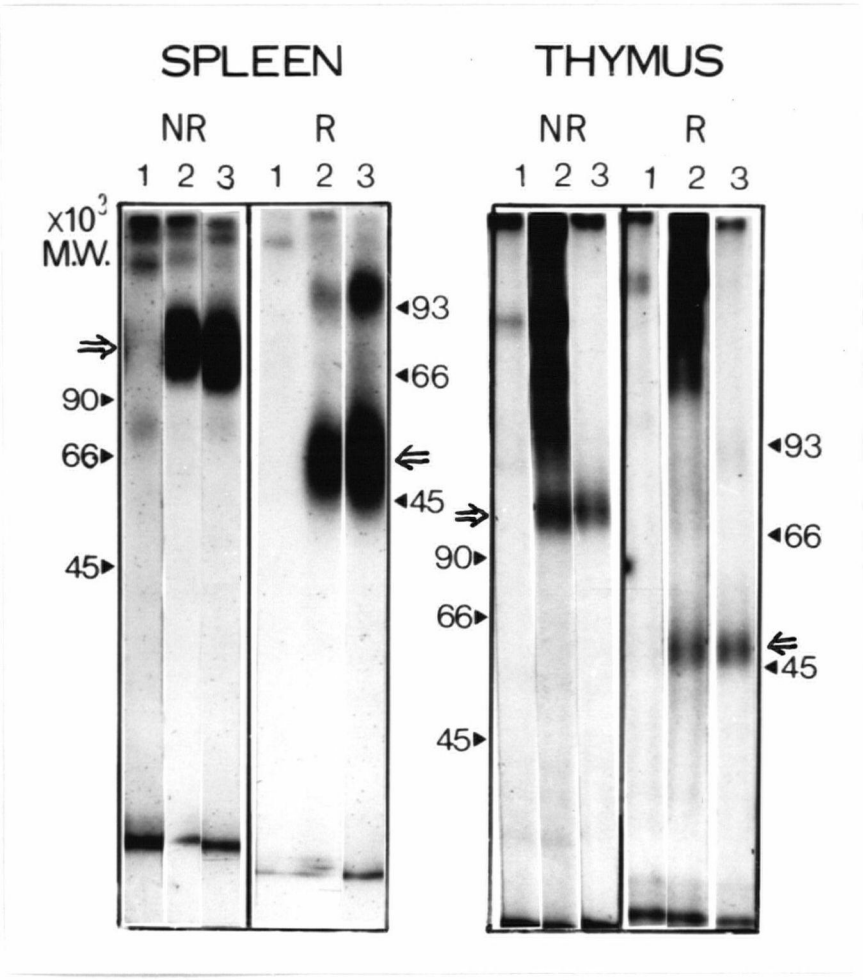

Figure 6. Immunoprecipitation of the YE1/48 antigen from thymocytes and spleen cells. Cell lysates of ^{125}I surface labeled C57BL/6 thymocytes and spleen cells were subjected to immunoprecipitation as described in Figure 2, using (1) the parental myeloma Y3 culture supernatant as negative control, (2) YE1/32.8.5, and (3) YE1/48.10.6 MAb's. The resultant immunoprecipitates were analysed by 10% SDS-PAGE under non-reducing (NR) and reducing (R) conditions. Open arrows show the YE1/48 antigen detected.

TABLE VII

IMMUNOPRECIPITATION OF THE YE1/48 ANTIGEN FROM NORMAL LYMPHOCYTES

Cell Population[a]	Immunoprecipitation
Thymocytes	+
$CD8^-CD4^-$	+
PNA^+	+
PNA^-	+
Spleen cells	+
Spleen T	+
Spleen B	w[b]
Con A-stimulated	+
LPS-stimulated	w
$CD8^+CD4^-$	+
$CD8^-CD4^+$	+
Bone marrow cells	w
Lymphoid-depleted	-
Mouse strains (spleen)	
C57BL/6 (B6)	+
B10.BR	+
BALB/c	w
C3H	-

[a] From C57BL/6 mice unless otherwise stated
[b] w = weak

spleen T cells, the antigen is detected at comparable levels in both the $CD8^+CD4^-$ (55% $CD8^+$, 0% $CD4^+$, 16% sIg^+) and $CD8^-CD4^+$ (0% $CD8^+$, 68% $CD4^+$, 13% sIg^+) subpopulations. Several T and B cell lines were also tested (Table VIII). None of them showed detectable expression of the YE1/48 antigen except EL-4, MBL-2(4.1), and MBL-2(2.6) cells. EL-4 and MBL-2(4.1), as reported above, express very high levels of the YE1/48 antigen, whereas MBL-2(2.6) expresses a very low level. It is interesting to note that C57BL/6 and B10.BR spleen cells gave a stronger YE1/48 signal than BALB/c and C3H spleen cells did (Table VII). It is possible that the YE1/48 expression is differentially regulated in different mouse strains or that the YE1/48 gene exhibits allelic polymorphism. This phenomenon of differential detection in different mouse strains may then help explain the absence of YE1/48 detection in the other T cell lines tested, all of which are of origins other than C57BL/6. The possibility of the YE1/48 antigen being polymorphic will be discussed in Chapter Five (section 5.3.3).

The expression of the YE1/48 antigen in thymocyte subpopulations was tested by immunoprecipitation (Table VII). It was found that the YE1/48 antigen can be detected in $CD8^-CD4^-$ (3% $CD8^+$ and/or $CD4^+$) C57BL/6 thymocytes which represent the most immature and the putative precursors of all thymocytes (Fowlkes et al., 1985; Kingston et al., 1985). Thymocytes from C57BL/6 mice were also separated by their PNA agglutination properties. The PNA^+ subpopulation is generally considered as immature and the PNA^- subpopulation as more mature (Reisner et al., 1976). As shown in Figure 7, the YE1/48 antigen was immunoprecipitated from both PNA^+ and PNA^- thymocytes. These observations are comparable to the observations of murine TCR-α/β at the protein (Roehm et al., 1984) as well as the mRNA levels (Raulet et al., 1985). It should be noted that the PNA^- cells gave a more intensive band than the PNA^+ cells did, suggesting that the YE1/48 antigen might be more abundant on the mature thymocytes than on the immature thymocytes. However, it remains

TABLE VIII

IMMUNOPRECIPITATION OF THE YE1/48 ANTIGEN FROM LYMPHOID CELL LINES

Cell Line	Origin	Cell Type	Detection
EL-4	C57BL/6	T	+
MBL-2(4.1)	C57BL/6	T	+
BW5147	AKR	T	-
AK-1	AKR	T	-
BM-3	BALB/c	T	-
2PK3	BALB/c	B	-
A20	BALB/c	B	-
L1210	DBA	Uncharacterized	-

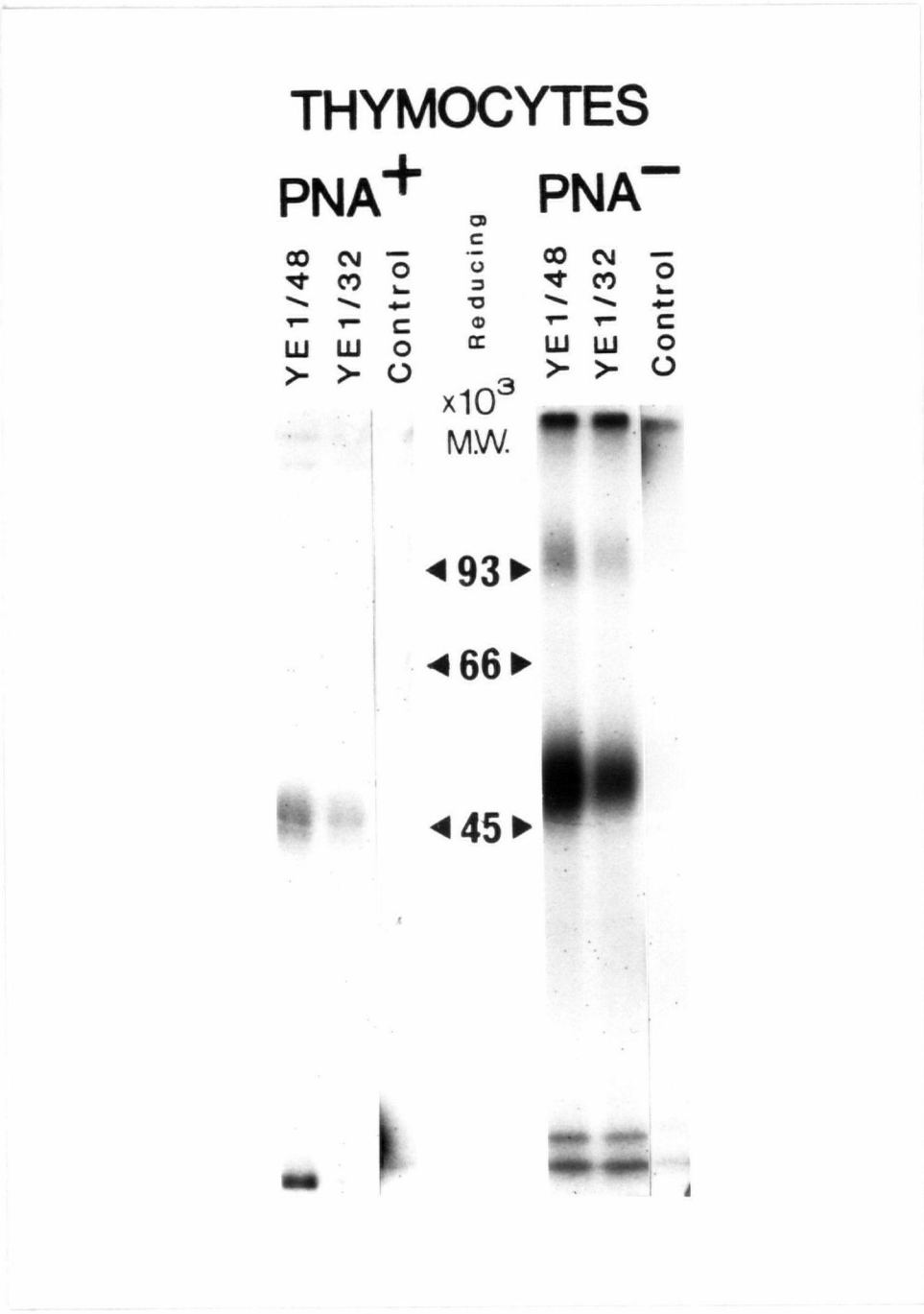

Figure 7. Immunoprecipitation of the YE1/48 antigen from PNA$^+$ and PNA$^-$ thymocytes. Cell lysates of ^{125}I surface labeled C57BL/6 PNA$^+$ and PNA$^-$ thymocytes were subjected to immunoprecipitation as described in Figure 2, using the parental myeloma Y3 culture supernatant, YE1/32.8.5, and YE1/48.10.6 MAb's. SDS-PAGE (10%) analysis of the resultant immunoprecipitates under reducing conditions was shown.

unknown whether it is due to a higher surface expression on individual cells or a larger subpopulation of cells expressing the antigen.

D) Bone Marrow Cells

Cells from other lineages in the hemopoietic system were tested for their expression of the YE1/48 antigen (Table VII). C57BL/6 bone marrow cells gave a weak immunoprecipitation detection signal relative to the lymphoid cells described above. After they were cultured for three days in long term culture conditions described by Dexter et al. (1977), which should lead to the depletion of most cells of the lymphoid lineage while the myeloid and erythroid cells survive, no YE1/48 antigen was detected. It suggests that some lymphoid cells in the bone marrow, either originating from the blood circulation or as prothymocytes or immature precursor B cells, are expressing the YE1/48 antigen. The expression in bone marrow will also be reexamined by Northern blot analysis and will be described in Chapter Five (see section 5.3.2). No antigen was detected on the B6SutA1 (C57BL/6 hemopoietic progenitor line) and NS-1 (BALB/c myeloma) cell lines.

3.3 DISCUSSION

The YE1/48 antigen described in this study resembled the murine TCR-α/β in two major properties. The antigen is composed of two 45-50,000 M_r disulphide-linked glycopolypeptide subunits, similar to TCR-α/β. The subunits exhibit pI values comparable to the TCR α and β chains. In addition, the YE1/48 antigen was initially identified by specific reactivities of two MAb's with EL-4 and MBL-2(4.1) cells, in similar manner to the identification of the TCR-α/β by clonotypic MAb's. No other T cell surface antigens with similar features had been identified at the time of this work (the TCR-γ/δ had not been described and human CD28 (T44) had not been well characterized). In

fact, diagonal gel analysis (non-reducing vs reducing SDS-PAGE) of surface iodinated T cell surface molecules had shown that dimeric proteins in general are quite rare on T cells (Allison et al., 1982; Acuto et al., 1983a), and TCR-α/β might be the only membrane protein that has a dimeric structure in this M_r range. Hence, it was of interest to characterize the YE1/48 antigen and elucidate its possible relationship with TCR-α/β. Other than the biochemical analyses and the antigen expression described in this chapter, the YE1/48 antigen has also been directly compared to the TCR-α/β which will be described in the next chapter.

3.3.1 Homodimer Or Heterodimer

Unlike the heterodimeric α and β chains of TCR which differ considerably from each other in tryptic peptide fingerprints (Acuto et al., 1983b; Reinherz et al., 1984), the two YE1/48 subunits had very similar tryptic peptide maps. In addition, the tryptic peptide maps of both YE1/48 subunits were also very different from those of the TCR-α/β chains even though the same mapping procedure was used. It could not be decided if the minority of the tryptic peptides unique to each YE1/48 subunit represented some differences in post-translational modifications of two identical protein backbones, indicative of a homodimer, or if the YE1/48 subunits indeed had some differences in their protein sequences, indicative of a heterodimer. The results of the endoglycosidase F analysis were also inconclusive. Because of the persistence of the 32,000 M_r and 38,000 M_r bands at a high dose of the enzyme, one could suggest that these bands represent the protein cores of the two subunits. The two protein cores however must have very similar primary structures in order to account for the two tryptic peptide maps. On the other hand, it was possible that the 38,000 M_r band represented an incompletely deglycosylated product of the 32,000 M_r protein core due to secondary structures of the polypeptide or the presence of O-linked carbohydrates insensitive to

endoglycosidase F. These analyses thus suggested that the YE1/48 antigen was unlikely to be identical to the TCR-α/β dimer. Whether the YE1/48 antigen dimer was a homodimer or a heterodimer could not be determined by the current data. In Chapter Five (section 5.3.5), this issue will be discussed again based on the YE1/48-encoding cDNA clone.

3.3.2 Expression of YE1/48 On Lymphocytes

The two MAb's YE1/32.8.5 and YE1/48.10.6, which were initially speculated to react with clonotypic determinants specifically expressed on EL-4 and MBL-2(4.1) cells, were shown to react with a wide range of T cells in normal lymphoid populations in the present study. This observation clearly indicated that the MAb-defined epitopes were not clonotypic. By immunoprecipitation, the YE1/48 antigen was detected in the thymus and spleen, including $CD8^-CD4^-$ and PNA^+ immature thymocytes, PNA^- mature thymocytes, $CD8^+CD4^-$ and $CD8^-CD4^+$ spleen T cells. However, the percentage of positive cells and the expression levels in individual cell subpopulations could not be determined because neither of the MAb's gave detectable binding to normal T cell surface. The radioactivity of the YE1/48 immunoprecipitates from EL-4 and MBL-2(4.1) cells is at least 30 fold higher than that from normal thymocytes or spleen cells. Nonetheless, immunoprecipitation signals do not give reliable representation of the expression level, because the efficiency of surface iodination and cell lysis, and the quality of the cell lysates, may vary among different cell sources, and thus may affect the amount of specific proteins immunoprecipitated. The relative expression levels will be addressed in a more quantitative manner by Northern blot analysis of the YE1/48 transcript levels (see Chapter Five, section 5.2.4). At this point, there were two possibilities, either the frequency of $YE1/48^+$ normal T cells might be fairly low, or the antigen was expressed on the majority of thymocytes and spleen cells at a much lower density. Among 23 T cell hybrids generated in our

laboratory by fusing BW5147 AKR thymoma with C57BL/6 thymocytes and 10 hybrids by fusing BW5147 with C57BL/6 spleen T cells, none was YE1/48$^+$ by immunoprecipitation, indicating that the YE1/48$^+$ cells might constitute less than 5% of thymocytes and 10% of spleen T cells. Alternatively, the BW5147 thymoma which is YE1/48$^-$ may possess trans-acting suppressor activity that inhibits the expression of YE1/48 in the hybrid clones.

Much weaker detection signals have also been noted on spleen B cells, indicating that either the YE1/48 antigen is expressed at very low levels on B cells or very few B cells express the antigen. On the other hand, these data do not exclude the possibility that detection of the antigen was attributable to the 2% contaminating Thy-1$^+$ T cells in the B cell preparation because of the high sensitivity of the YE1/48 immunoprecipitation assay. This question was addressed by Northern blot analysis using the YE1/48 cDNA, the results of which suggest that the YE1/48 antigen is indeed expressed on B cells (see Chapter Five, section 5.2.4). Neither Con A nor LPS stimulation of spleen cells had led to an increase in the expression level of YE1/48 antigen as detected by immunoprecipitation. The detection of YE1/48 antigen in bone marrow cells appeared to be contributed by circulating lymphocytes since lymphoid cell depletion by selective culture conditions removed the immunoprecipitation signal. The possible expression of YE1/48 in the bone marrow will be described and discussed again in Chapter Five (see section 5.2.4).

3.3.3 Differential Expression And Accessibility of MAb-defined Epitopes

The detection of the YE1/48 antigen in a wide range of normal T cells has initially suggested that the YE1/32.8.5 and YE1/48.10.6 MAb's may be reacting with the constant portions of the molecule analogous to TCR-α/β. However, the YE1/48 antigen can be detected on normal lymphoid cells only by immunoprecipitation and not by flow cytometry. There are at least two

possible explanations. Either the epitopes recognized by the MAb's (as well as the anti-YE1/48 antiserum) are somehow not accessible to binding on the surface of intact cells unless the cell membrane is partially solubilized, or the expression of YE1/48 on normal cells is below the detection limit of flow cytometry. Even the rat anti-YE1/48 antiserum which should be reactive to multiple determinants on the molecule did not show apparent binding to normal T cell surface. It is possible that the exposed surface of the YE1/48 antigen on normal cells is very conserved between mouse and rat. In analogy, some conventional antisera produced against TCR-α/β invariably fail to detect non-clonotypic determinants exposed on the T cell surface (McIntyre and Allison, 1983; Brenner et al., 1984). This is probably not surprising because TCR-α/β seems to be highly conserved between different species, as indicated by the high homology of the nucleotide sequences of the human and murine α and β genes (Caccia et al., 1984; Clark et al., 1984; Jones et al., 1985). Perhaps, in both the YE1/48 antigen and TCR-α/β, only those determinants which are normally masked on the surface of normal T cells are antigenic in rat. This could explain why the anti-YE1/48 antiserum cannot give detectable binding on normal T cell surface. Alternatively, if YE1/48 expression level is indeed below the detection limit of flow cytometry, the binding of the antiserum to multiple determinants on the surface of the antigen may not be detectable.

Previously, Haskins et al. reported a MAb which reacts with a non-clonotypic determinant on the murine TCR-α/β molecule (Haskin et al., 1984). The MAb KJ16-133 not only immunoprecipitates TCR-α/β from detergent-solubilized T cells but also binds to the surface of some normal T cells. However, the surface binding is temperature dependent. The MAb binds well at 37^{o}C in the presence of azide but poorly at 4^{o}C, which may be attributed to the increased membrane fluidity at higher temperatures. Unlike the KJ16-133 MAb, no detectable binding was observed by flow cytometry after the incubation of normal spleen cells with YE1/48.10.6 MAb at 37^{o}C in the presence of azide.

It will be interesting if the epitopes defined by YE1/32.8.5 and YE1/48.10.6 MAb's are indeed accessible to surface binding by the antibodies only on EL-4 and MBL-2(4.1) cells but not on normal thymocytes and spleen cells. There are three possible explanations for this unusual phenomenon: 1) the biochemical properties of the YE1/48 molecule on T lymphoma cell may be different from those on normal T cells; 2) there may be some associated molecules whose properties differ between the lymphomas and normal cells; 3) normal and transformed T cells may differ in general membrane compositions resulting in differences in the way the YE1/48 molecule is embedded in the lipid bilayer. CD3 is a glycoprotein complex known to non-covalently associate with the TCR-α/β and TCR-γ/δ heterodimers (Reinherz et al., 1982; Meuer et al., 1983a; Reinherz et al., 1983; Kaye and Janeway, 1984; Weiss and Stobo, 1984; Brenner et al., 1985) In mice, it is composed of five subunits: γ(21,000 M_r), δ(26,000 M_r), ε(25,000 M_r), p21(21,000 M_r) and ζ(a homodimer of 16,000 M_r chains) (Borst et al., 1983a, 1983b; Allison and Lanier, 1985; Samelson et al., 1985; Oettgen et al., 1986). They can be coimmunoprecipitated with the TCR's in cell lysates prepared in digitonin detergent. To test if the YE1/48 antigen is likewise non-covalently associated with other proteins and if some changes in such associated molecules can affect the accessibility of the MAb-defined epitopes on the YE1/48 antigen, a SDS-PAGE gel of YE1/48 immunoprecipitate from a Triton X-100 detergent lysate of MBl-2(4.1) cells was allowed for prolonged autoradiograpic exposure. Coimmunoprecipitates of 32,000 M_r, 24,000 M_r and 16,000 M_r were observed. However, they are probably not physically associated antigens because antisera raised separately against them can cross-react with the YE1/48 antigen, indicating that either they are degraded fragments of the antigen or they carry epitopes cross-reactive with the YE1/48.10.6 MAb. Furthermore, no coimmunoprecipitate was detected in the digitonin detergent lysates of MBL-2(4.1) cells. Hence, it appears unlikely that associated

molecules have contributed to the changes in epitope exposure on the YE1/48 antigen. It remains intriguing to speculate that the difference in the YE1/48 antigen on lymphomas and normal cells, whether it is epitope changes or differential expression, may correlate with the cellular transformation of lymphocytes. This subject will be discussed again in Chapter Five (see section 5.3.2) based on the data derived from Northern blot analysis using the YE1/48-encoding cDNA clone.

3.3.4 YE1/48 Allelic Polymorphism

It has been puzzling that, among all T cell lines tested, the YE1/48 antigen was only detected on EL-4, MBL-2(4.1) and MBL-2(2.6) by immunoprecipitation. A possible explanation emerges from the finding that YE1/48 is readily detected on C57BL/6 and B10.BR spleen cells but less so on BALB/c and C3H spleen cells. The three YE1/48 expressing cell lines are the only C57BL/6 cell lines tested. No cell lines of B10.BR origin were tested. From these data, it remains undetermined if the YE1/48 expression is differentially regulated in different mouse strains or the YE1/48 gene exhibits allelic polymorphism. Subsequent genomic Southern blot analysis using the YE1/48 cDNA clone has indicated that the differential immunoprecipitation signals may be due to allelic polymorphism of the YE1/48 gene (see Chapter Five, section 5.2.3). Since C57BL/6 carries a MHC b-haplotype whereas B10.BR carries a k-haplotype, the allelic difference does not appear to correlate with the MHC.

3.4 SUMMARY

A murine TCR-α/β-like antigen, YE1/48, with dimeric structure of 90-95,000 M_r under non-reducing and 45-50,000 M_r under reducing conditions, was detected by two rat MAb's YE1/32.8.5 and YE1/48.10.6 on the surface of two

murine T lymphoma cell lines EL-4 and MBL-2(4.1). By two-dimensional gel electrophoresis (IEF vs SDS-PAGE), the two YE1/48 subunits could be separated into an acidic and a more basic chains with pI values similar to those of the murine TCR-α/β chains. Endoglycosidase F analysis suggested that at least three N-linked carbohydrate side chains are attached to the polypeptides. Tryptic peptide fingerprints of the YE1/48 subunits however showed that, unlike the TCR-α/β, the subunits are very similar to each other. These results suggest that the YE1/48 antigen is unlikely identical to the TCR-α/β molecule. However, the current biochemical analyses alone have not definitively shown any possible relationship between the two molecules, and whether the YE1/48 antigen is a homodimer or a heterodimer.

The two MAb's which react with different epitopes on the YE1/48 molecule do not show detectable binding to the surface of normal lymphoid cells or cell lines except EL-4 and MBL-2(4.1) as tested by flow cytometry. A rat anti-YE1/48 antiserum does the same. In further analysis by immunoprecipitation from surface iodinated cells, the YE1/48 antigen was detected on normal C57BL/6 thymocytes and spleen cells. This indicates that, either prior solubilization of the cell membrane is required for MAb binding to the epitopes on normal cells and, for some unknown reasons, these epitopes are accessible for MAb binding on intact EL-4 and MBL-2(4.1) cells, or the YE1/48 antigen is expressed on normal cells at very low levels undetectable by flow cytometry. An attempt to differentiate these two possibilities will be presented in the discussion in Chapter Five (see section 5.3.4). By immunoprecipitation, the YE1/48 antigen can be detected in a wide range of thymocytes and spleen T cells. The present data cannot show conclusively whether or not the antigen is expressed on B cells. The YE1/48 expression on B cells will be further demonstrated by Northern blot analysis in Chapter Five (see section 5.2.4). Analysis of spleen cells from different mouse strains suggests that the YE1/48 expression may be differentially regulated or the

YE1/48 gene may be allelic.

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CHAPTER FOUR

COMPARISON OF THE YE1/48 ANTIGEN WITH THE T CELL RECEPTOR α/β

(Data reported in J. Immunol. 140:161-169, January 1988)

4.1 INTRODUCTION

Disulphide-linked dimeric proteins are relatively rare on the T cell surface (Goding and Harris, 1981; Ballinari et al., 1985). Only four major T cell dimeric antigens have been characterized in detail. They are TCR-α/β, TCR-γ/δ, CD8 (also known as Lyt-2,3 in mouse and T8 in human) and CD28 (T44) antigens. TCR-α/β is a heterodimeric molecule of 85,000 M_r with an acidic α and a basic β subunit each of approximately 40-45,000 M_r in mouse (Allison et al., 1982; Kappler et al., 1983; Samelson, 1985). In human, the α and β subunits are 49,000 and 43,000 M_r, respectively (Meuer et al., 1983a, 1983b). TCR-γ/δ is a heterodimer of 35,000(γ) and 45,000(δ) M_r subunits in mouse and 40,000(γ) and 43,000(δ) in human (Lew et al., 1986; Moingeon et al., 1986; Borst et al., 1987; Brenner et al., 1987; Moigeon et al., 1987; Nakanishi et al., 1987; Pardoll et al., 1987). Both the α/β and γ/δ receptors are glycoproteins encoded by T cell-specific rearranged genes, and are non-covalently associated with the CD3 complex. The α/β receptor is expressed on mature immunocompetent cells and recognizes foreign antigens in a MHC-restricted manner, leading to antigen-specific T cell activation. The γ/δ receptor is instead expressed on $CD3^+\alpha/\beta^-$ cells, including the $CD3^+CD8^-CD4^-$ immature thymocytes, some peripheral T cells and some Thy-1^+ dendritic epidermal cells. Most γ/δ^+ cell lines exhibit MHC non-restrcited cytolytic activities via the γ/δ CD3 complex (Bank et al., 1986; Moingeon et al., 1986; Alarcon et al., 1987; Borst et al., 1987; Brenner et al., 1987; Ferrini et al., 1987) but there is a report of one cell line that manifests MHC-linked proliferation and cytotoxicity (Matis et al., 1987). It is likely that the MHC non-restricted cytotoxicity is induced by IL-2 in the culture as the cytoxicity is lost upon factor depletion (Phillips et al., 1987; Borst et al., 1988). CD8 is a homodimer of 34,000 M_r subunits in human (Ledbetter et al., 1981a; Snow and Terhorst, 1983), and a heterodimer in mouse, composed of a

30,000 M_r (β, Lyt-3) subunit linked to either a 38,000 M_r(α of Lyt-2) or a 34,000 M_r(α′ of Lyt-2) subunit (Ledbetter et al., 1981b; Jay et al., 1982). Both the human and the mouse molecules can further exist in homomultimers or heteromultimers. It is thought to be a receptor for non-polymorphic regions of the MHC class I molecule and to be involved in enhancing T cell activation (Emmrich et al., 1986; Dembic et al., 1987; Gabert et al., 1987; Ratnofsky et al., 1987; Takada and Engelman, 1987). CD28, so far identified only in human, exists primarily as a homodimer of 44,000 M_r subunits although monomers are also detected (Martin et al., 1986). It is expressed on the majority of $CD4^+$ helper/inducer and $CD8^+$ CTL's but not on $CD8^+$ suppressor cells (Hara et al., 1985). Since its perturbation by MAb can enhance TCR-α/β/CD3-mediated T cell activation, CD28 has been speculated to be a receptor for accessory signals (Martin et al., 1986; Lesslauer et al., 1986; Poggi et al., 1987).

Other disulphide-linked dimeric antigens have also been identified on T cells. A1 is a murine 90,000 M_r homodimer of 45,000 M_r subunits which shows no homology with the human CD28 (T44) molecule in peptide mapping analysis. It is detected on two T lymphomas EL-4 and C6VLB and no function has yet been identified (Nagasawa et al., 1987; Spiazzi et al., 1987). CD27 (Tp55) is a 120,000 M_r homodimeric differentiation antigen of 55,000 M_r subunits expressed on a large subset of peripheral T cells and most medullary thymocytes (van Lier et al., 1987). The transferrin receptor is a 180,000 M_r homodimer of 95,000 M_r subunits expressed in all proliferating cells (Omary and Trowbridge, 1981a, 1981b; Trowbridge and Lopez, 1982). It transports iron-bound transferrin molecules into dividing cells in which Fe^{2+} ions are released for use in DNA synthesis and electron transfer chain-reactions. An unidentified 96,000 M_r molecule which is reduced to 32,000 M_r under reducing conditions has also been detected on a T lymphoma cell line (Ballinari et al., 1985). Finally, on many retrovirus-transformed T cell lines, the viral envelope proteins gp70 and p15 are often expressed as a disulphide-linked heterodimer

although p15 is not radiolabeled by cell surface iodination (Ledbetter and Nowinski, 1977).

We have identified by two rat MAb's, YE1/32.8.5 and YE1/48.10.6, a 90-95,000 M_r disulphide-linked dimer of two 45-50,000 M_r subunits called YE1/48. It is expressed on a wide range of thymocytes and spleen T cells, and two T lymphomas EL-4 and MBL-2(4.1). The antigen resembles the murine TCR-α/β in its molecular size and pI values, and thus has been described as a TCR-α/β-like antigen. No function of the YE1/48 antigen has been obtained comparable to that of TCR-α/β because the two MAb's as well as the anti-YE1/48 antiserum do not give detectable surface binding on normal T cell populations or established T cell lines except the two T lymphomas. In Chapter Three tryptic peptide mapping of the YE1/48 subunits has suggested that YE1/48 is unlikely to be identical to TCR-α/β. Yet it has not been definitively concluded whether or not the YE1/48 antigen is related to either of the α/β chains, and whether YE1/48 is a homodimer or a heterodimer. In this chapter, we have clearly demonstrated that the YE1/48 antigen is distinct from TCR-α/β by their sequential immunoprecipitation from EL-4 cells, the differential expression of the two molecules on two variant MBL-2 clones, and the direct comparison of partial amino acid sequences of the YE1/48 antigen with the published TCR sequences.

4.2 RESULTS

4.2.1 YE1/48 Antigen Is Likely Not The T Cell Receptor α/β

It was initially thought that the murine TCR-α/β is the only major dimeric antigen on T cell surface with 40-50,000 M_r under reducing and 80-90,000 M_r under non-reducing conditions. Based on this assumption, diagonal gel analysis, in which SDS-PAGE is performed under non-reducing conditions in the first dimension and reducing conditions in the second, was used for the detection of TCR-α/β when no MAb was available to detect it in

polyclonal cell populations. As demonstrated in Figure 8a, from a surface-iodinated thymocyte lysate, a single spot was detected under the diagonal line at the region to which a protein of 80-90,000 M_r under non-reducing and 40-50,000 M_r under reducing conditions would separate. Such a protein had generally been thought to be the TCR-α/β heterodimer. Indeed, the murine TCR-α/β immunoprecipitated by the KJ16-133 MAb which reacts with 10% of thymocytes (Roehm et al., 1984) was detected in the diagonal gel as a similar spot (Figure 8, b3). No difference in separation could be noted in the YE1/48 antigen immunoprecipitated from the same lysate (Figure 8, b2).

Given the similarity between YE1/48 and TCR-α/β in their mobility on SDS-PAGE and diagonal analyses, experiments were carried out to test if they may be the same molecule. Since no cell line was available that reacts with both KJ16-133 and YE1/48.10.6 MAb's, a rabbit antiserum R3497 (Bekoff et al., 1986) which recognizes TCR-α/β on all T cells, was used in conjunction with YE1/48 MAb to sequentially immunoprecipitate from EL-4 cell lysates. Upon depletion of all detectable YE1/48 antigen from the EL-4 cell lysate (Figure 9a, lane B2), the murine TCR-α/β could still be immunoprecipitated by R3497 (Figure 9b, B). Conversely, when the amount of TCR-α/β was drastically reduced by immunoprecipitation with R3497 (Figure 9b, C), the amount of the YE1/48 antigen remained unchanged (Figure 9a, lane C2). The treatment of the lysate with either antibody did not affect the amount of an unrelated T cell surface antigen CD45 (T200) (Figure 9a, lanes 3), indicating that the antigen depletion was specific. Hence, the YE1/48 antigen and the murine TCR-α/β appear to be distinct molecules.

The difference between the YE1/48 antigen and the murine TCR-α/β was further shown by their differential expression on the MBL-2(4.1) and MBL-2(2.6) variant clones. The two clones have identical levels of cell surface antigen expression with YE1/48 being the only exception known. The high

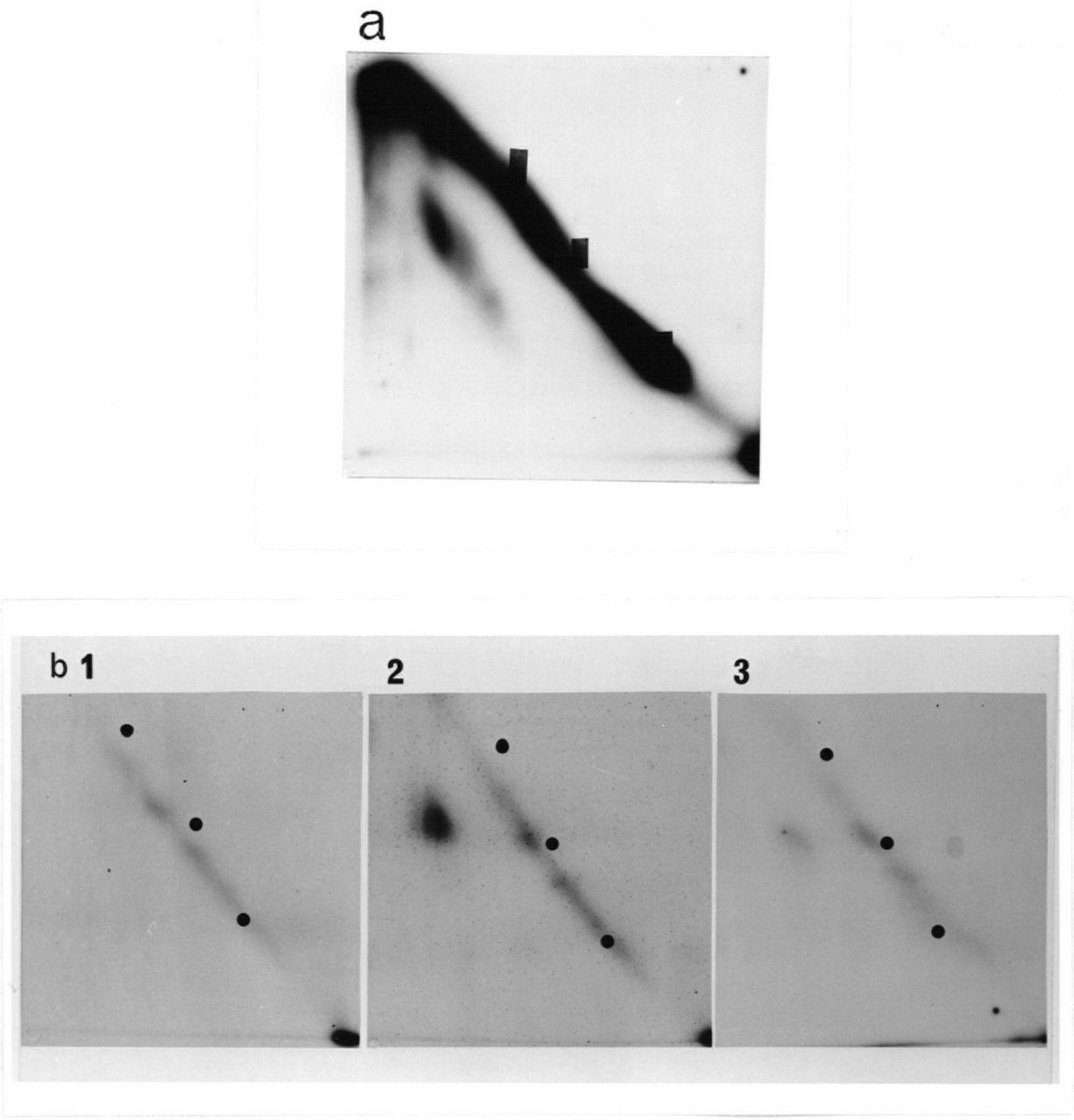
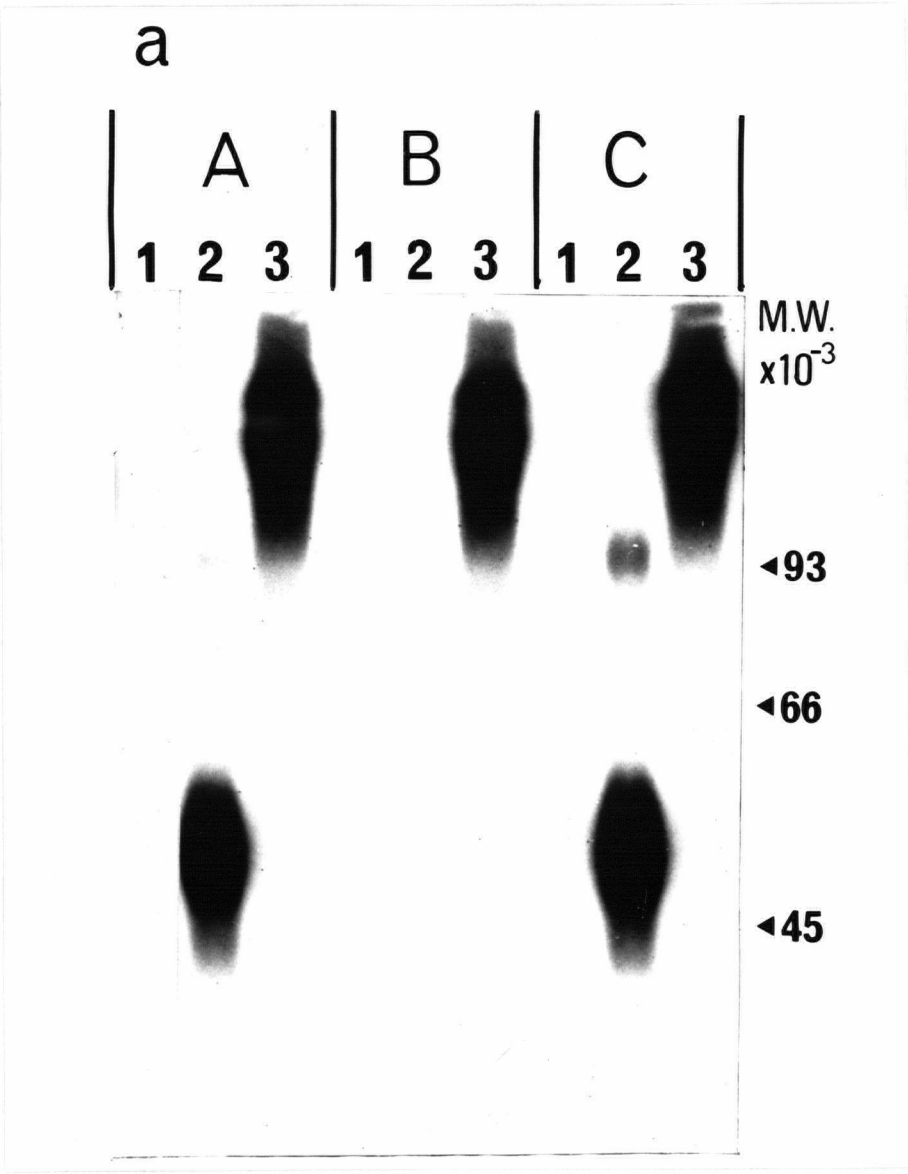


Figure 8. Diagonal gel analysis of the YE1/48 antigen and the T cell receptor α/β from thymocytes. The cell lysates of surface iodinated C57BL/6 thymocytes were directly analysed (a) by diagonal SDS-PAGE gels (10% under non-reducing versus 10% under reducing conditions), or (b) were subjected to immunoprecipitation, using (1) a negative control, YE6/26.1.1 (anti-gp70), (2) YE1/48.10.6, and (3) KJ16-133 MAb's before analysis. Three protein markers, bovine serum albumin (67,000 MW), ovalbumin (43,00 MW), and carbonic anhydrase (31,000 MW) were included in the samples and were visualized by Coomaise blue staining (solid squares and dots).

Figure 9. (next page) Sequential immunoprecipitation of the YE1/48 antigen and the T cell receptor α/β from EL-4 cells. A cell lysate prepared from surface iodinated EL-4 cells was pretreated with either (A) no antibody, (B) YE1/48 MAb, or (C) the rabbit anti-T cell receptor antiserum R3497 before subjected to immunoprecipitation using (1) a negative control, YE6/26.1.1 (anti-gp70), (2) YE1/48.10.6, (3) a positive control, YE1/21.2.1 (anti-CD45), a normal rabbit serum (not shown), and (b) the rabbit antiserum R3497. The resultant immunoprecipitates were analysed on a 10% SDS-PAGE gel(a) under reducing conditions except for the R3497 immunoprecipitates which were analysed on 10% diagonal gels(b). The normal rabbit serum immunoprecipitates were not shown. The protein markers on the diagonal gels were the same as in Figure 8.


a
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B
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1 2 3 1 2 3 1 2 3
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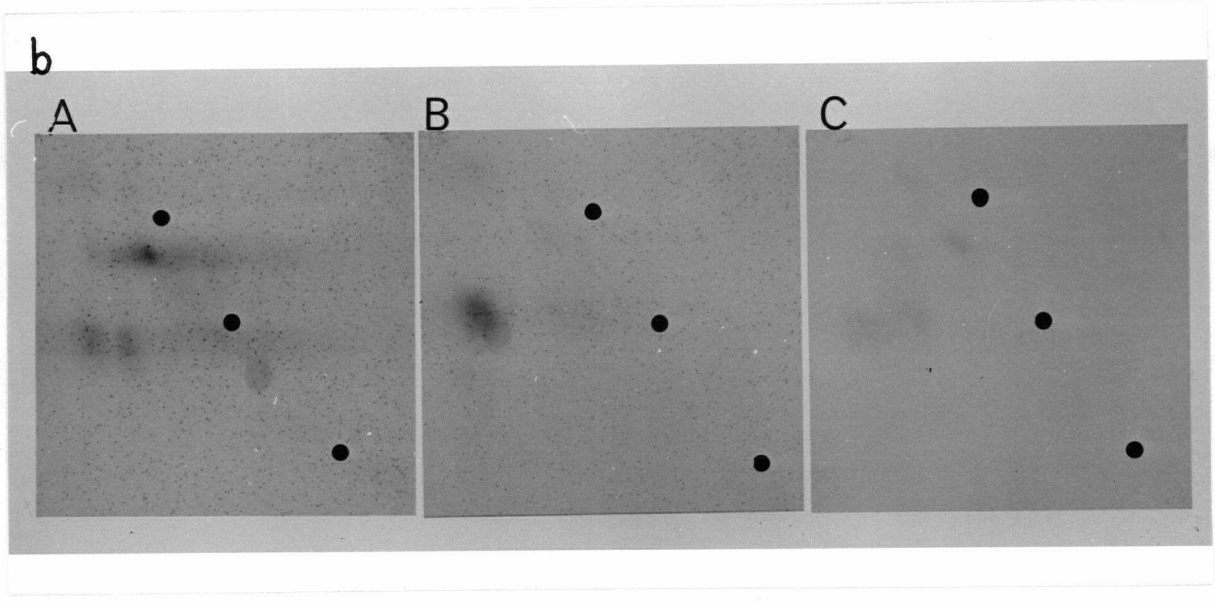
b
A
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C

variant clone MBL-2(4.1) expresses 2.3-4.5 x 10^5 molecules of YE1/48 antigen per cell, as determined by saturation binding of ^{125}I-labeled YE1/48.10.6 MAb. The low variant clone MBL-2(2.6), based on the observation that no YE1/48 binding is detected by flow cytometry, expresses less than 1000 molecules per cell, at least 200 fold lower than MBL-2(4.1) does. The difference in the level of the YE1/48 antigen was readily shown by the difference in the intensities of the bands on SDS-PAGE (Figure 10, lanes c). In contrast, the amount of TCR-α/β immunoprecipitated by R3497 from the low variant clone MBL-2(2.6) was no less than that from the high variant MBL-2(4.1). (Figure 10d). The two clones also express approximately the same amounts of other unrelated surface antigens, such as transferrin receptor and CD45 (T200) (Figure 10, lanes a and b). These results again indicate that the YE1/48 antigen and the murine TCR-α/β are likely two distinct molecules.

4.2.2 Purification Of The YE1/48 Antigen

The YE1/48 antigen was purified from MBL-2(4.1) cells using a three step purification scheme: affinity chromatography and two cycles of preparative SDS-PAGE under non-reducing and reducing conditions. Batches of lysates derived from 0.7-1.0 x 10^{10} cells were subjected to purification with YE1/48.10.6 MAb affinity column. When an aliquot of the eluted fraction was analysed on a SDS-PAGE gel followed by silver staining (Figure 11, lane f), a 90-95,000 M_r band was detected as a major component with a few minor contaminating protein bands. The faint 50,000 M_r contaminant band was previously found to have pI identical to that of the more basic YE1/48 subunit and hence might represent a monomer of the antigen. The typical yield of the 90,000 M_r affinity purified YE1/48 antigen was 100 µg (1.1 nmoles)/10^{10} cells. This corresponds to an extraction of 6.6 x 10^4 molecules per cell, which is in reasonable agreement with the estimated antigen density of 2.3-4.5 x 10^5 molecules/cell on the MBL-2(4.1) cell line considering inevitable protein loss

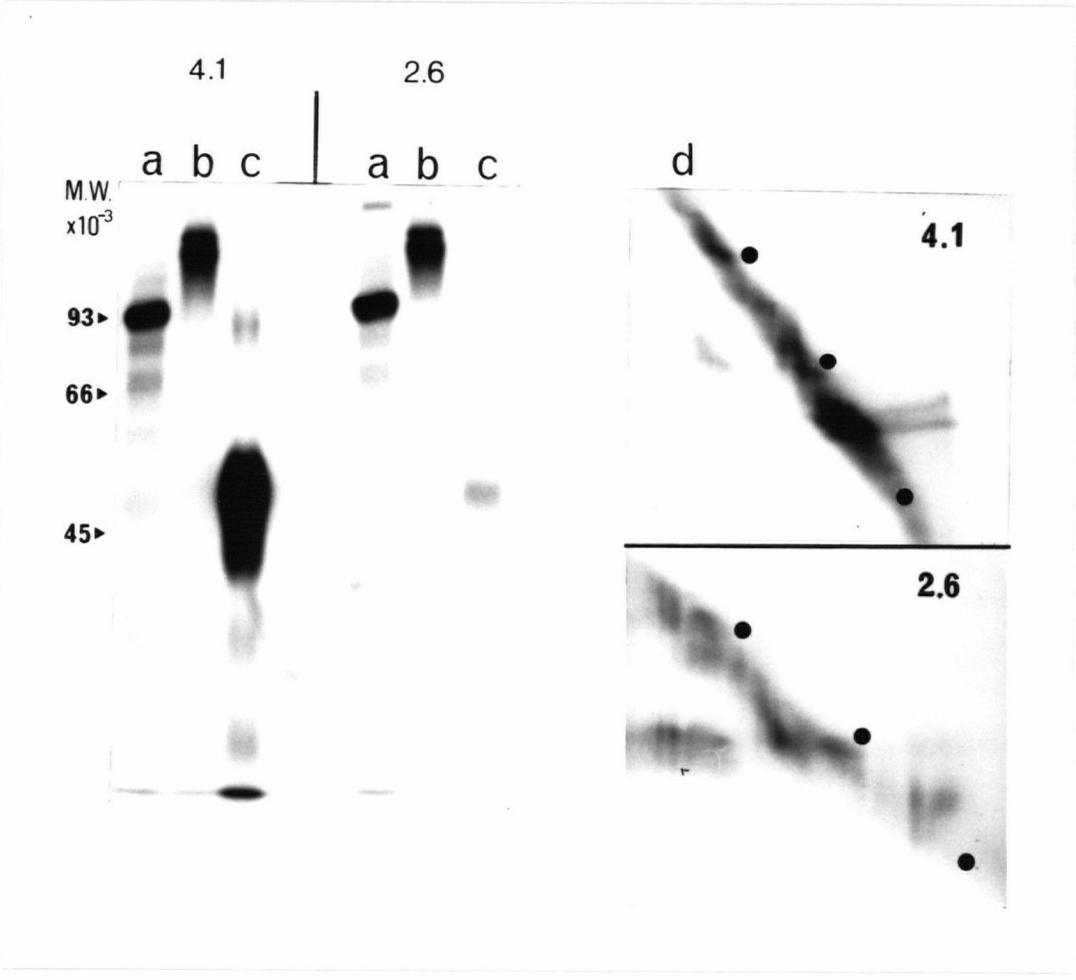

Figure 10. Comparison of the YE1/48 antigen level and the T cell receptor α/β expression on two MBL-2 variant clones. Surface iodinated cells of the MBL-2(4.1) and MBL-2(2.6) clones were subjected to immunoprecipitation using two positive controls (a) YE1/9.9.3 (anti-transferrin receptor) and (b) YE1/21.2.1 (anti-CD45), (c) YE1/48.10.6, and (d) the rabbit anti-T cell receptor antiserum R3497. The immunoprecipitates were analysed on a 10% SDS-PAGE gel under reducing conditions except for the R3497 immunoprecipitates which were analysed by 10% diagonal gel analysis. The protein markers on the diagonal gels were the same as in Figure 8.

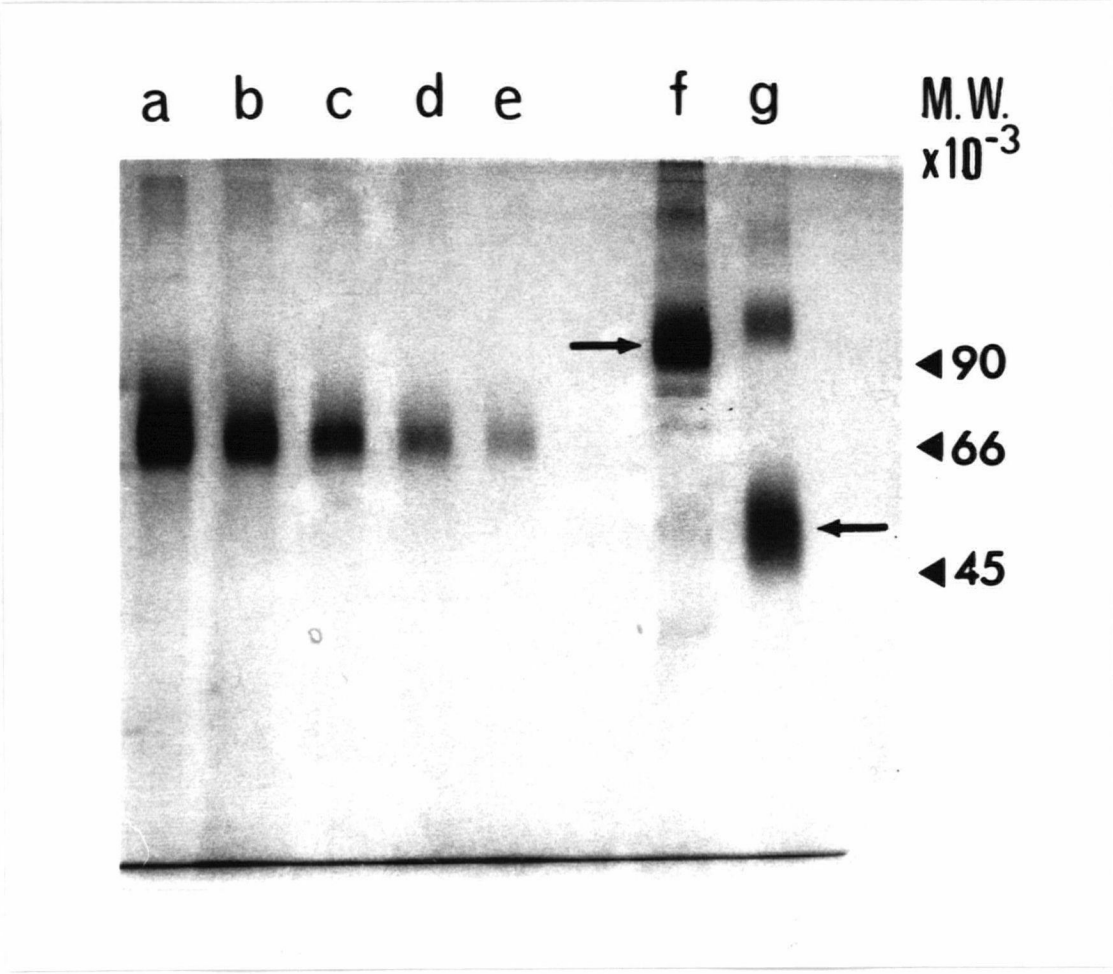

Figure 11. Assessment of the purity and the yield of the purified YE1/48 antigen. Small aliquots of the YE1/48 antigen purified from the initial affinity chromatography(f) and the final reducing preparative SDS-PAGE(g) were analysed on a 10% SDS-PAGE gel under non-reducing conditions. Solid arrows indicate the YE1/48 proteins in the initial disulphide-linked form and the final unlinked form. Bovine serum albumin was included in quantities of (a) 0.5 µg, (b) 0.25 µg, (c) 0.13 µg, (d) 0.06 µg and (e) 0.03 µg as references for quantitative assessment.

in the large scale cell lysate preparation and the affinity purification procedure.

The affinity enriched YE1/48 antigen was subjected to preparative SDS-PAGE to eliminate the contaminating proteins of higher and lower M_r. The 90,000 M_r protein band excised was reduced, alkylated and separated by preparative SDS-PAGE again. This reduction/alkylation step was essential because most contaminating 90,000 M_r proteins are likely to be monomeric proteins and, therefore, would be removed when the 45,000 M_r reduced YE1/48 antigen was isolated. Furthermore, reduction and alkylation was necessary to cleave the intrachain as well as interchain disulphide bonds, in order to prevent the formation of disulphide-linked tryptic fragments. If not removed, these disulphide-linked tryptic fragments, which possess more than one amino-terminus, might give rise to mixed amino acid signals during subsequent sequencing. The analysis of the SDS-PAGE purified reduced/alkylated antigen by silver staining on an independent SDS-PAGE gel showed that the purified sample was essentially pure by M_r, containing a major portion as the 45-50,000 M_r monomer and a minor fraction as the 90-95,000 M_r dimer (Figure 11, lane g). The persistence of the dimer band was probably due to an incomplete alkylation of the antigen such that redimerization of the purified 45-50,000 M_r monomer had occurred. A faint band at higher M_r may also represent a multimeric product of the 45-50,000 M_r monomer, suggesting that the YE1/48 antigen may have a strong tendency to form multimeric complexes. The highest yield obtained with several batches of antigen purification was 50-60 µg (550-660 pmoles) per 10^{10} cells, which corresponds to a 50-60% efficiency of the SDS-PAGE procedure as calculated from the affinity enriched 90,000 M_r antigen. Due to a poor recovery of the two subunits from two-dimensional gels (IEF vs SDS-PAGE), no attempts were made to separate and purify each subunit.

4.2.3 Separation Of The Tryptic Peptides By HPLC

Despite the various precautions taken to minimize the destruction or modification of amino acid residues during the preparative SDS-PAGE procedure (see Chapter Two, section 2.3.3), no amino acid sequences of the purified YE1/48 antigen (16 µg; 180 pmoles) could be obtained, suggesting that the amino-terminus might be blocked. The purified antigen (55 µg; 600 pmoles) was therefore digested with trypsin which cleaves specifically after each lysine and arginine residue except when arginine is followed by a proline. The tryptic fragments were separated on a C_{18} reverse phase HPLC column, and the separation profile is shown in Figure 12. All of the tryptic peptides were eluted within the range of 5-40% acetonitrile in 0.1% trifluoroacetic acid (TFA). The majority of the peptides were relatively hydrophilic and were eluted in 10-20% acetonitrile. Very few hydrophobic peptides were detected. A similar profile of peptide separation was obtained with another independent purification and digestion experiment. It is likely that some of the hydrophobic sequences did not dissolve in 3 M guanidine chloride and were lost in the prefiltration of sample prior to the HPLC separation. The observation that a substantial amount of radioactivity was lost in the prefiltration step supports this possibility.

4.2.4 Amino Acid Sequences

Twelve tryptic peptides separated from the HPLC column were selected for amino acid sequencing (Tripartite Microsequencing Centre, University of Victoria, Victoria, BC). While two of them were probably blocked at the amino-termini and another two contained mixed signals of amino acid residues, nine sequences were determined from eight peptide fractions (Table IX). Eight out of the nine peptides were sequenced to completion with either arginine or lysine as the last residues. These nine sequences were reasonably unambiguous although the phenylthiohydantoin (PTH) derivatives of most amino acid residues

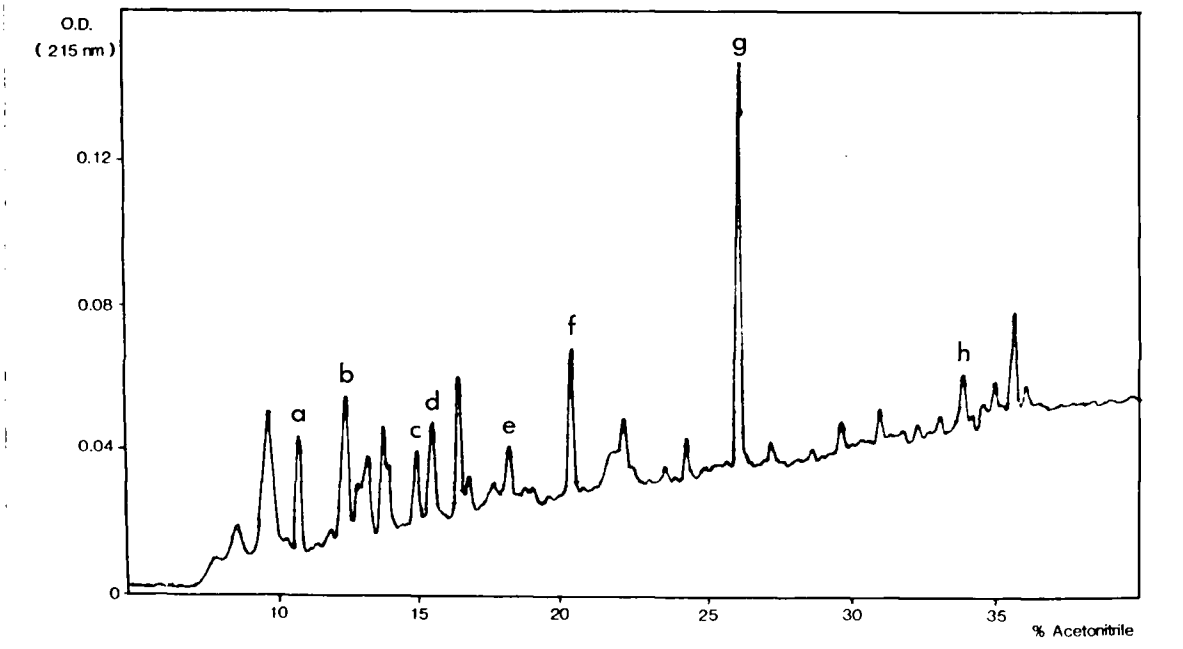

Figure 12. Separation of the YE1/48 tryptic peptides by C_{18} reverse phase HPLC chromatography. Approximately 55 µg of the purified reduced and alkylated YE1/48 antigen was digested with trypsin and subjected to peptide separation on a C_{18} column using a 0-60% acetonitrile gradient in 0.1% TFA. Only the 5-40% acetonitrile elution profile which contained the major eluted peptides was shown here. The peaks marked a-h contained the peptides which were subsequently sequenced and presented in Table IX.

TABLE IX

AMINO ACID SEQUENCES OF THE YE1/48 TRYPTIC PEPTIDES

Peptide		Sequence
a		Gln-Val-Arg-Pro-Glu-Glu-Thr-Lys
b		Thr-Glu-Ser-Gly-Glu-Lys
c	*	Ile-Asp-Asp-Glu-Asp-Glu-Leu-Lys
d1		Leu-Ala-Leu-Asn-Thr-Arg
d2		Leu-Ala-Leu-Asn-Thr-Pro-Ser-Lys
e		Ile-Phe-Gln-Tyr-Asp-Gln-Gln-Lys
f	*	Thr-Val-Leu-Asp-Ser-Leu-Gln-His-Thr-Gly-Arg
g	*	Asp-Trp-Ala-Trp-Ile-Asp-Asn-Arg-Pro-Ser-Lys
h		Ser-Ile-Glu-Cys-Asp-Leu-Glu-Ser-Leu-Asn-#

* Confirmed by two independent purification and tryptic digestion experiments.

The tryptic peptide was not sequenced to completion.

were detected at a level of 10-150 pmoles. Indeed three peptide sequences c, f and g were confirmed by two independent purification, digestion and HPLC separation experiments. In peptide fraction d, two amino acid signals, arginine and proline, were detected at the sixth position. Since the seventh position was not proline and the quantity of signals at the seventh and eighth positions decreased drastically from that at the sixth position, it is possible that the arginine at the sixth position defines the cleaved end of one tryptic peptide whereas the proline at the same position belongs to another peptide ending in lysine at the eighth position. The two sequences d1 and d2, sharing the first five amino acid residues, probably possess similar hydrophobicity and thus were eluted as a single peak on the acetonitrile gradient.

4.3 DISCUSSION

4.3.1 YE1/48 Is Distinct From The T Cell Receptor α/β

Based on the sequential immunoprecipitation from EL-4 cells and the differential expression on MBL-2(4.1) and MBL-2(2.6) variant clones, it appears that the YE1/48 antigen is unlikely to be identical to TCR-α/β. However, these data cannot conclusively rule out the possibility that, in the sequential immunoprecipitation experiment, the YE1/48.10.6 MAb and the R3497 rabbit antiserum were recognizing two differentially glycosylated forms of the same protein. Compatible with this hypothesis is the glycosylation characteristics of the YE1/48 antigen. Endoglycosidase F analysis of the YE1/48 antigen has revealed a minimum of three N-linked carbohydrate side chains. Similarly, three N-linked carbohydrate side chains have been demonstrated on each of the 27-32,000 M_r murine TCR-α/β polypeptide cores as well as predicted from their cDNA clones (Chien et al., 1984; Hedrick et al., 1984; Kaye and Janeway, 1984; McIntyre and Allison, 1984). Nevertheless, two

lines of evidence substantiate the distinction between the YE1/48 antigen and TCR-α/β. First, unlike the heterodimeric α and β chains of TCR which differ considerably from each other in tryptic peptide fingerprints (Acuto et al., 1983; Reinherz et al., 1984), the two YE1/48 subunits have very similar tryptic peptide maps. In addition, the tryptic peptide maps of both YE1/48 subunits are also very different from those of the TCR-α/β even though the same mapping procedure was used. Second, the amino acid sequences of the purified YE1/48 antigen have shown no homology with the published murine TCR-α/β sequences. It is further confirmed by similar comparison of the YE1/48-encoding cDNA sequence with the published α and β sequences which will be described in Chapter Five (see section 5.2.2). Therefore, it can be concluded that the YE1/48 antigen is not identical to the TCR-α/β molecule.

4.3.2 Comparison Of YE1/48 With Other Known Proteins

The YE1/48 antigen was purified and several amino acid sequences of the tryptic peptides were determined. The two subunits of the YE1/48 antigen were not separated from each other in the purification because of difficulties in isolating the two subunits separately with a high recovery. Therefore, the sequences of the tryptic peptides generated from the purified antigen cannot be assigned to each subunit at this time. Since the tryptic peptide mapping has previously shown that the two subunits share the majority of their tryptic peptides, it is likely that many of the sequences of the tryptic peptides reported here are shared by both protein subunits.

Amino acid sequences of nine tryptic peptides generated from the purified YE1/48 antigen were analysed for possible homology with over 4000 other protein sequences in the National Biomedical Research Foundation (NBRF) data base (Release 11.0, January 1987). The WORDSEARCH program (University of Wisconsin, Genetics Computer Group, Madison, WI) based on the algorithm of Wilbur and Lipman (1983) was used for sequence alignment. Using a word size

of two for locating the sequence matches, the highest alignment scores obtained with the nine tryptic sequences ranged from 6 to 11. When a word size of one was used, which generally provides a higher sensitivity but less specificity of the sequence alignment, the highest scores were 8 to 14 and additional protein sequences emerged at the top of the score list. On using the GAP program (University of Wisconsin Genetics Computer Group) for pair-wise sequence alignment allowing no gap insertions, the similarities of the tryptic peptide sequences with the protein sequences of the highest scores ranged from 37.5% to 83.3%. The proteins showing relatively high scores include bacterial and viral proteins, cellular and nuclear enzymes, neuropeptides, interferons, acetylcholine receptor, Ig superfamily members ($T3_{\delta}$, CD8 and CD4) and oncogene products. The major proteins which share similarities with more than one tryptic peptide sequence are oncogene products (versus peptides a, c, f and e), Ig superfamily members (versus peptides a and b), viral coat proteins (versus peptides a and h) and, at lower similarities, leukemia viral gag proteins (versus peptides a, d, f, g and h). The significance of these similarities has yet to be justified because in many cases, combinations of several amino acid residues can be found in apparently unrelated proteins. Furthermore, since the tryptic peptides are only 6 to 11 residues in length, matches in a few residues of a random cause may have given some deceivingly favourable scores in similarities. In addition, as mentioned above, the sequences of the tryptic peptides cannot be assigned to each of the two subunits of the YE1/48 antigen, making it difficult to determine whether the proteins sharing homology with more than one peptide are significant. Hence, the similarities observed are not considered to be convincing and no homology with any known proteins can yet be determined. Longer primary sequences are needed for the analysis of possible homology of the YE1/48 antigen with other proteins (see Chapter Five, section 5.2.2).

The purified YE1/48 antigen has a strong tendency to form dimers and

multimers, suggesting the presence of multiple cysteine residues within the molecule. Although only one cysteine residue has been identified in nine tryptic peptides, the complete YE1/48 protein sequence deduced from the cDNA clone isolated later indicates a relatively high cysteine content in the molecule (see Chapter Five, section 5.2.2).

Finally, our data show that the YE1/48 antigen appears to be a novel murine T cell surface antigen. Its internal amino acid sequences are clearly different from the TCR α, β and γ polypeptides. The human CD28 (T44) is another disulphide-linked molecule with similar molecular size as YE1/48. The MAb 9.3 which reacts with CD28 does not cross-react with mouse T cells (our unpublished observation), hence no direct comparison with the YE1/48 antigen by sequential immunoprecipitation is possible. The relationship of YE1/48 with CD28 (T44) was at the time unknown since the sequence of CD28 was not available during the amino acid sequencing of the YE1/48 antigen. When the human CD28 cDNA sequence was subsequently available, the YE1/48-encoding cDNA had also been isolated. The comparison of their sequences has revealed no significant homology (see Chapter Five, section 5.2.2).

With the recent report on the A1 molecule (Nagasawa et al., 1987; Spiazzi et al., 1987), we speculate that the YE1/48 antigen may be identical to A1. The YE1/48 antigen is similar to A1 in molecular size, N-linked glycosylation patterns, their substantially easier detection by immunoprecipitation from two C57BL/6 T lymphomas compared to that from normal T lymphocytes, and the high tendency for their subunits to redimerize. Similar to the YE1/48 antigen, no functional data of the A1 molecule has been derived because, in both cases, the MAb's do not show detectable binding to normal T cells in the thymus and spleen. Possible identity of YE1/48 with the A1 molecule is until now unclarified since the A1 MAb is not available and the primary sequence of A1 has not been described.

4.4 SUMMARY

The YE1/48 antigen is a disulphide-linked dimer of two 45-50,000 M_r subunits expressed on murine T lymphocytes. It resembles the TCR-α/β in molecular size and pI values, but it appears different from the α/β receptor by tryptic peptide fingerprints (see Chapter One). To further compare YE1/48 with TCR-α/β, their expression on EL-4 cells and MBL-2 variant clones were studied and the partial amino acid sequences of YE1/48 were determined. The two molecules can be distinguished on EL-4 cells by sequential immunoprecipitation using YE1/48.10.6 MAb and a rabbit antiserum reactive with all TCR-α/β molecules. The MBl-2(4.1) and MBL-2(2.6) variant clones, which differ in the level of YE1/48 expression by more than 200 fold, express comparable amounts of the α/β receptor. Hence, the YE1/48 antigen and TCR-α/β appear to be different molecules. The YE1/48 antigen was purified from MBL-2(4.1) cells by affinity chromatography and preparative SDS-PAGE, digested with trypsin and the resultant peptides were separated by reverse phase HPLC. The amino acid sequences of several YE1/48 tryptic peptides were determined. Upon comparison with the protein sequences in the data base, no identical sequences were detected including the published TCR sequences. It therefore confirms that YE1/48 is distinct from the TCR α, β and γ gene products, and that YE1/48 is likely a novel T cell antigen not previously described. However, the possibility of homology with other proteins remains undetermined because the tryptic peptides are too short to yield meaningful statistical comparison with the data base. Further comparisons have been made possible after the isolation of a YE1/48 cDNA clone (see Chapter Five).

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CHAPTER FIVE

ISOLATION OF A cDNA CLONE ENCODING THE YE1/48 ANTIGEN

5.1 INTRODUCTION

In the preceeding two chapters, the biochemical analyses of the YE1/48 antigen and its detection in normal lymphoid populations by immunoprecipitation have been described. It has also been demonstrated that YE1/48 is distinct from the murine T cell receptor α/β. Nevertheless, because the YE1/48.10.6 and YE1/32.8.5 MAb's do not show detectable binding on intact normal cell surface, no biological effects upon the perturbation of the YE1/48 antigen could be derived. In order to elucidate the possible function of the YE1/48 antigen and its correlation with other known antigens, we undertook the cDNA cloning of YE1/48. In this chapter, we describe the isolation of a cDNA clone encoding the YE1/48 antigen and its deduced primary structure. Using the isolated cDNA as a probe, the expression of the YE1/48 gene in lymphoid cell populations was analysed and its possible correlation with cellular transformation was implicated. Genomic Southern analysis has also demonstrated the allelic polymorphism of the YE1/48 gene and the presence of other genes with homologous sequences.

5.2 RESULTS

5.2.1 Isolation Of A YE1/48 cDNA Clone

Three mixtures of anti-sense synthetic oligonucleotides of 17 bases in length were made based on three tryptic peptide sequences previously reported (see Chapter Four). Two of the three tryptic sequences were determined in duplicate antigen purification and digestion experiments. The oligonucleotide mixtures contain all possible sequences encoding the hexapeptides; they correspond to codon degeneracies of 24, 32 and 48 (Table X). They were used to screen 100,000 recombinant plaques from a MBL-2(4.1) λgt10 cDNA library under low stringencies (see Chapter Two, section 2.5.2). A cDNA clone named

TABLE X

SEQUENCES OF THE REDUNDANT SYNTHETIC OLIGONUCLEOTIDE PROBES USED IN THE ISOLATION OF THE YE1/48-ENCODING CLONE M3-2

Probe	Redundancy	Amino acid versus oligonucleotide sequences
FT1	24	W A W I D N ACCCGAACCTAACTATT G G G T T C
FT2	48	I D D E D E TAACTACTACTTCTACT G G G C G T
FT3	32	Q Y D Q Q K GTTATACTAGTTGTTTT C G G C C

M3-2 that hybridized to all three probes was isolated. It has an insert size of approximately 1.3 kb. It appears to be close to full length because Northern blot analysis using the cDNA insert as a probe showed a single 1.4 kb band in the poly(A)$^+$RNA extracted from MBL-2(4.1) cells.

5.2.2 Nucleotide And Deduced Protein Sequences Of The cDNA Clone

The YE1/48 cDNA was sequenced by the dideoxy chain-termination method, in conjunction with the preparation of deletion clones and the use of internal synthetic oligonucleotide primers (Figure 13a). The cDNA is 1272 bp in length. The longest open reading frame, beginning with an ATG methionine codon, extends from nucleotides 181 to 966, encoding a polypeptide of 262 amino acids (Figure 13b). Within this protein sequence, all of the tryptic peptide sequences previously reported can be located (Table XI). However, a few discrepant residues were noted: Trp at position 160 versus Glu in tryptic peptide b; Cys at position 163 versus Glu in the same tryptic peptide; Gly at position 223 versus Arg in peptide d1 and Pro-Ser in peptide d2. Neither of these discrepancies can be explained by single base pair changes in the cDNA sequence. In light of the low molar quantities of these amino acid residue signals (40 pmoles and below), we consider the cDNA-deduced protein sequence more reliable than those determined by peptide sequencing.

Within the same reading frame, another ATG Met codon is located at nucleotide 208 (amino acid position 10), 25 nucleotides downstream to the first one. By the criteria that a purine (A at position 178) resides at the third nucleotide upstream to the first ATG codon but a pyrimidine (T at position 205) upstream to the second ATG, and that the flanking sequence of the first ATG codon better conforms to the consenses sequence CCACCATGG (Kozak, 1986, 1987) critical to translation by eukaryotic ribosomes, it is likely that the first ATG initiation codon is used. Thus, the cDNA contains a 5′ untranslated sequence of at least 180 bp and a 3′ untranslated sequence of

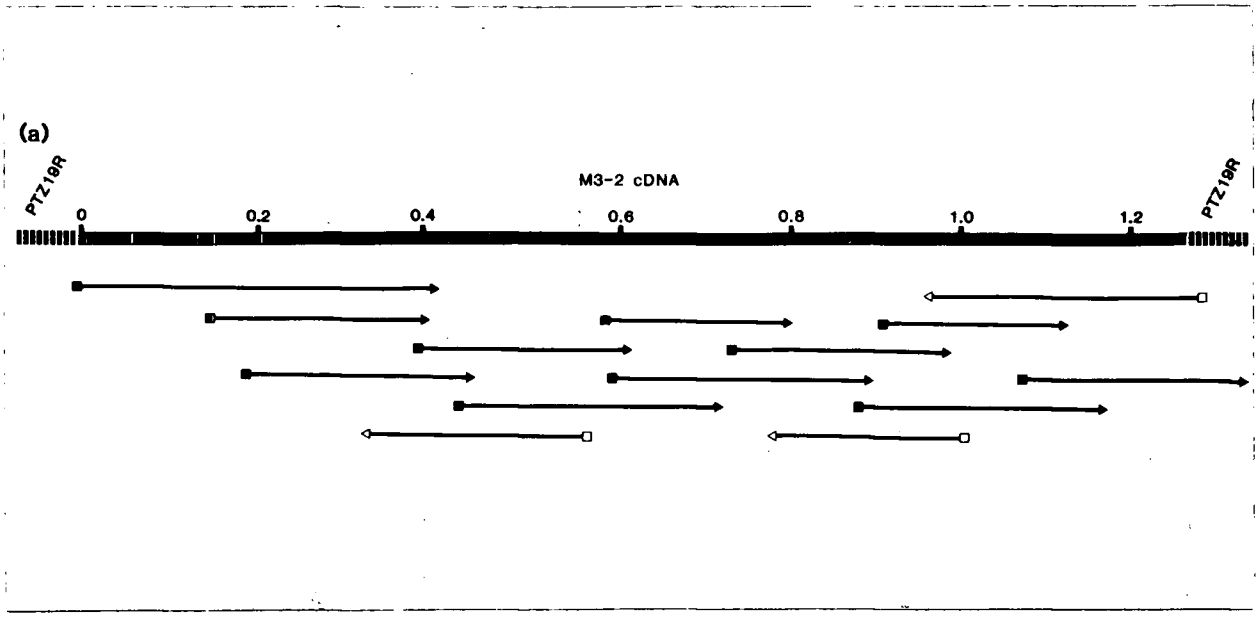


Figure 13. Strategy of DNA sequencing and the nucleotide and deduced amino acid sequences of the cDNA clone M3-2. (a) The coding strand (solid squares) and the non-coding strand (open squares) of the cDNA were sequenced by the dideoxy chain-termination method. The forward and reverse priming sites in the pTZ19R pasmid vector were used to determine the end sequences. Two series of deletion clones prepared by exonuclease III digestion were used to determine twice the coding strand sequence of the entire cDNA. Two synthetic oligonucleotide primers were used to determine two sequences in the non-coding strand. Distance on the cDNA was marked in kilobases. (b) (next page) Nucleotides are numbered on both sides of the cDNA sequence and amino acid residues are numbered in parentheses on both sides of the deduced protein sequence. The potential polyadenylation signal is indicated by broken underline. Protein sequences corresponding to the tryptic peptides in Table XI are underlined. The transmembrane domain is marked by a thick broken line above the amino acid sequence, and on either side is marked by an arrow the cytoplasmic or extracellular portion. Positions of potential N-linked glycosylation sites are indicated by "CHO". Cysteine residues are marked by asterisks. The tripeptide Arg-Gly-Asp is highlighted as "RGD" above the amino acid sequence.

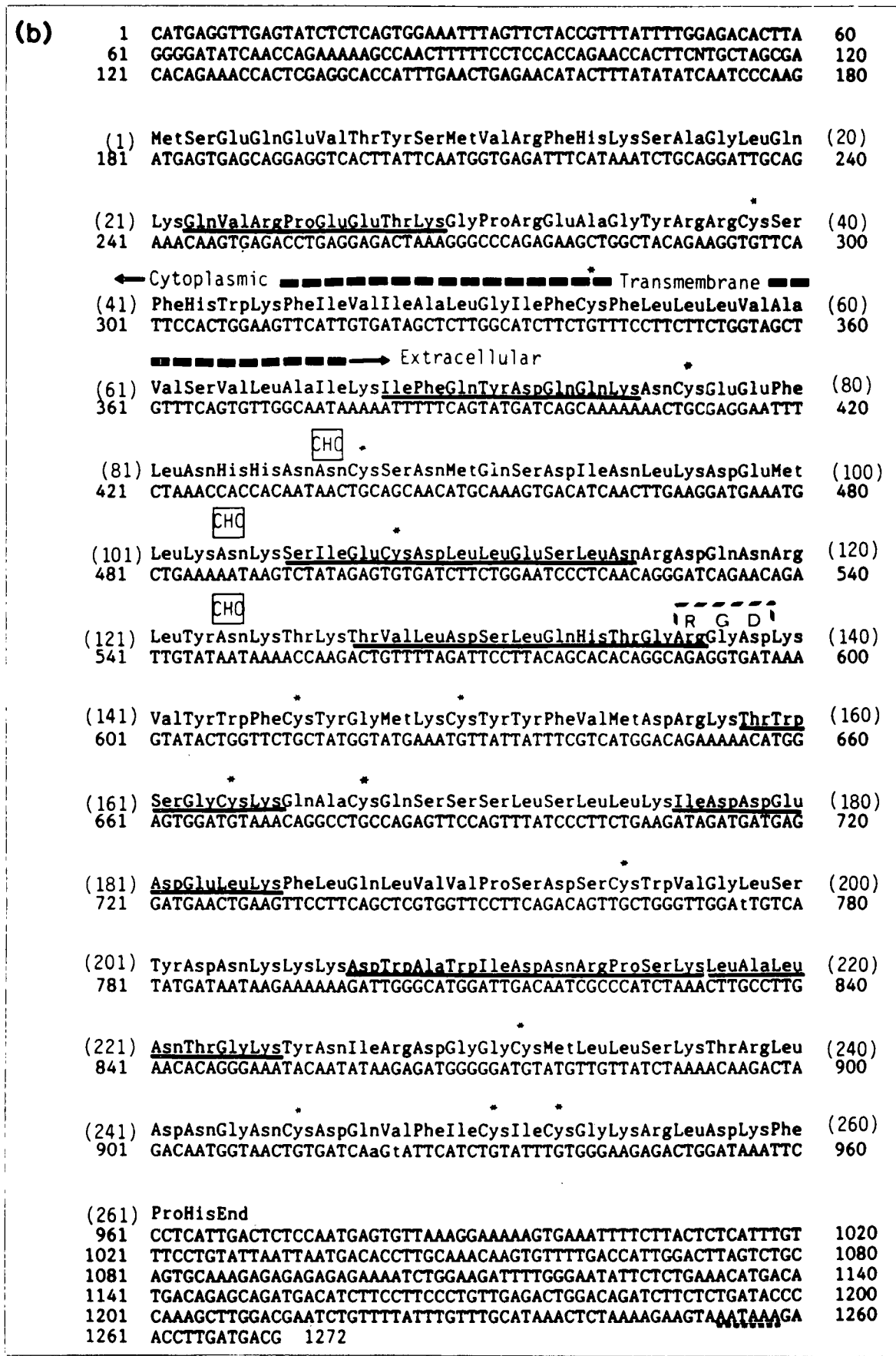

(b)
1 CATGAGGTTGAGTATCTCTCAGTGGAAATTTAGTTCTACCGTTTATTTTGGAGACACTTA 60
61 GGGGATATCAACCAGAAAAAGCCAACTTTTTCCTCCACCAGAACCACTTCNTGCTAGCGA 120
121 CACAGAAACCACTCGAGGCACCATTTGAACTGAGAACATACTTTATATATCAATCCCAAG 180
(1) MetSerGluGlnGluValThrTyrSerMetValArgPheHisLysSerAlaGlyLeuGln (20)
181 ATGAGTGAGCAGGAGGTCACTTATTCAATGGTGAGATTTCATAAATCTGCAGGATTGCAG 240
(21) LysGlnValArgProGluGluThrLysGlyProArgGluAlaGlyTyrArgArgCysSer (40)
241 AAACAAGTGAGACCTGAGGAGACTAAAGGGCCCAGAGAAGCTGGCTACAGAAGGTGTTCA 300
←Cytoplasmic Transmembrane
(41) PheHisTrpLysPheIleValIleAlaLeuGlyIlePheCysPheLeuLeuLeuValAla (60)
301 TTCCACTGGAAGTTCATTGTGATAGCTCTTGGCATCTTCTGTTTCCTTCTTCTGGTAGCT 360
Extracellular
(61) ValSerValLeuAlaIleLysIlePheGlnTyrAspGlnGlnLysAsnCysGluGluPhe (80)
361 GTTTCAGTGTTGGCAATAAAAATTTTTCAGTATGATCAGCAAAAAAACTGCGAGGAATTT 420
CHO
(81) LeuAsnHisHisAsnAsnCysSerAsnMetGlnSerAspIleAsnLeuLysAspGluMet (100)
421 CTAAACCACCACAATAACTGCAGCAACATGCAAAGTGACATCAACTTGAAGGATGAAATG 480
CHO
(101) LeuLysAsnLysSerIleGluCysAspLeuLeuGluSerLeuAsnArgAspGlnAsnArg (120)
481 CTGAAAAATAAGTCTATAGAGTGTGATCTTCTGGAATCCCTCAACAGGGATCAGAACAGA 540
CHO
R G D
(121) LeuTyrAsnLysThrLysThrValLeuAspSerLeuGlnHisThrGlyArgGlyAspLys (140)
541 TTGTATAATAAAACCAAGACTGTTTTAGATTCCTTACAGCACACAGGCAGAGGTGATAAA 600
(141) ValTyrTrpPheCysTyrGlyMetLysCysTyrTyrPheValMetAspArgLysThrTrp (160)
601 GTATACTGGTTCTGCTATGGTATGAAATGTTATTATTTCGTCATGGACAGAAAAACATGG 660
(161) SerGlyCysLysGlnAlaCysGlnSerSerSerLeuSerLeuLeuLysIleAspAspGlu (180)
661 AGTGGATGTAAACAGGCCTGCCAGAGTTCCAGTTTATCCCTTCTGAAGATAGATGATGAG 720
(181) AspGluLeuLysPheLeuGlnLeuValValProSerAspSerCysTrpValGlyLeuSer (200)
721 GATGAACTGAAGTTCCTTCAGCTCGTGGTTCCTTCAGACAGTTGCTGGGTTGGAtTGTCA 780
(201) TyrAspAsnLysLysLysAspTrpAlaTrpIleAspAsnArgProSerLysLeuAlaLeu (220)
781 TATGATAATAAGAAAAAAGATTGGGCATGGATTGACAATCGCCCATCTAAACTTGCCTTG 840
(221) AsnThrGlyLysTyrAsnIleArgAspGlyGlyCysMetLeuLeuSerLysThrArgLeu (240)
841 AACACAGGGAAATACAATATAAGAGATGGGGGATGTATGTTGTTATCTAAAACAAGACTA 900
(241) AspAsnGlyAsnCysAspGlnValPheIleCysIleCysGlyLysArgLeuAspLysPhe (260)
901 GACAATGGTAACTGTGATCAaGtATTCATCTGTATTTGTGGGAAGAGACTGGATAAATTC 960
(261) ProHisEnd
961 CCTCATTGACTCTCCAATGAGTGTTAAAGGAAAAAGTGAAATTTTCTTACTCTCATTTGT 1020
1021 TTCCTGTATTAATTAATGACACCTTGCAAACAAGTGTTTTGACCATTGGACTTAGTCTGC 1080
1081 AGTGCAAAGAGAGAGAGAGAAAATCTGGAAGATTTTGGGAATATTCTCTGAAACATGACA 1140
1141 TGACAGAGCAGATGACATCTTCCTTCCCTGTTGAGACTGGACAGATCTTCTCTGATACCC 1200
1201 CAAAGCTTGGACGAATCTGTTTTATTTGTTTGCATAAACTCTAAAAGAAGTAAATAAAGA 1260
1261 ACCTTGATGACG 1272

TABLE XI

TRYPTIC PEPTIDE SEQUENCES USED TO CORROBORATE THE YE1/48 cDNA CLONE

Peptide	Probe[a] (Redundancy)	Sequence[b]	Position
a		Gln-Val-Arg-Pro-Glu-Glu-Thr-Lys	22-29
b		Thr(Glu)Ser-Gly(Glu)Lys	159-164
c	FT2 (48)	Ile-Asp-Asp-Glu-Asp-Glu-Leu-Lys	177-184
d1		Leu-Ala-Leu-Asn-Thr(Arg)	218-222
d2		Leu-Ala-Leu-Asn-Thr(Pro-Ser)Lys	218-224
e	FT3 (32)	Ile-Phe-Gln-Tyr-Asp-Gln-Gln-Lys	68-75
f		Thr-Val-Leu-Asp-Ser-Leu-Gln-His-Thr-Gly-Arg	127-137
g	FT1 (24)	Asp-Trp-Ala-Trp-Ile-Asp-Asn-Arg-Pro-Ser-Lys	207-217
h		Ser-Ile-Glu-Cys-Asp-Leu-Leu-Glu-Ser-Leu-Asn-[c]	105-115

[a] Redundant antisense 17-mer probes are based on the amino acid residues underlined.
[b] Amino acid residues different from predicted by cDNA nucleotide sequence are indicated in parentheses.
[c] This tryptic peptide was not sequenced to completion.

over 300 bp. Both of these untranslated regions contain termination codons in all reading frames. A polyadenylation signal sequence (AATAAA) is located 14 bp from the 3′ end of the cDNA although a poly(A)-tail is missing.

By hydrophobicity plotting (Kyte and Doolittle, 1982), no hydrophobic sequence characteristic of a leader sequence is found at the amino(N)-terminus of the deduced protein sequence. Beginning from residue 45 is a hydrophobic sequence of 22 amino acids, 19 of which are non-polar and none is charged. It is preceded by a cluster of basic amino acids (Arg-Arg-X-X-X-His-X-Lys from position 17 to 24) which is a common feature of the cytoplasmic domain just proximal to the transmembrane segment in all integral membrane proteins (Blobel, 1980; Sabatini et al., 1982). It indicates that the 22 residues-long segment is a transmembrane sequence and the N-terminal domain is located in the cytoplasm. Thus, YE1/48 is a type II membrane protein in which the transmembrane sequence serves as a leader segment in the translocation of proteins across the cell membrane as well as an anchorage of the protein in the membrane (Singer et al., 1987). It has 44 amino acids in the N-terminal cytoplasmic domain and 196 amino acids in the C-terminal extracellular domain. The predicted protein product is approximately 30,500 MW and contains 3 potential N-linked glycosylation sites (Asn-X-Ser/Thr) at amino acid positions 86, 103 and 123 in the C-terminal extracellular domain. The predicted molecular size is in close agreement with the 32,000 M_r core size suggested by previous endoglycosidase F analysis (see Chapter Three, section 3.2.2). The digestion pattern by endoglycosidase F also indicates that all of the three potential N-linked glycosylation sites are likely used. The predicted protein sequence contains 5.3% cysteine residues which is higher than the reported average of 2.8% in eukaryotic proteins (Klapper, 1977). This may explain the strong tendency of the YE1/48 protein to redimerize after reduction (see Chapter Four, section 4.2.1). A cysteine residue, which may be a site of fatty acylation via a thioester bond (Rose et al., 1984), is present within

the transmembrane sequence at position 77. A serine residue at position 40 in the N-terminal cytoplasmic domain is located in a Arg-Arg-X-Ser sequence which is a common site of phosphorylation by many cAMP- and cGMP-dependent protein kinases (Krebs and Beavo, 1979). However, no phosphorylation of the YE1/48 antigen has been detected so far.

No significant homology was found when the YE1/48 cDNA and the deduced protein sequences were compared with the GENBANK nucleic acid (release 52.0, August 1987) and NBRF protein (release 13.0, June 1987) data base using the WORDSEARCH program (University of Wisconsin Genetics Computer Group, Madison, WI) based on the algorithm of Wilbur and Lipman (1983). Further comparison with sequences of other membrane antigens which are not included in the above database, such as human CD28 (T44) (Aruffo and Seed, 1987), human CD2 (Sewell et al., 1986), and the shared β subunit of human LFA-1, Mac-1 and p150,95 (Kishimoto et al., 1987), also revealed no homology. Two interesting features were however noted. An Arg-Gly-Asp (RGD) tripeptide sequence, which accounts for the cell adhesive properties of many extracellular matrix proteins (Ruoslahti and Pierschbacher, 1987), is located at position 137-139 in the C-terminal extracellular domain. In this domain is also found a Cys-X_4-Cys-X_{12}-Cys-X_3-Cys sequence from position 145 to 167. This sequence resembles a consensus pattern often found in zinc-binding proteins such as metallothionein and in finger proteins that can bind DNA (Berg, 1986; Evans and Hollenberg, 1988). In the consensus zinc-binding domain of the DNA-binding proteins, a zinc ion engages in hexahedral coordinate bonding to four cysteine residues and the long peptide sequence between the inner two cysteines forms the DNA-binding finger. In the DNA-binding finger, the C-terminal half often takes the configuration of an α-helix that spans all the way through the two Cysteine residues downstream. A Chou and Fasman propensity measure for α-helices and β-sheets (1978) of the deduced YE1/48 protein sequence, however, does not indicate a tendency for α-helix formation in the potential zinc-

binding domain. Whether these four cysteine residues on the YE1/48 antigen can bind zinc is yet unknown.

5.2.3 Genomic Southern Analyses

Southern blot analysis of EL-4 and C57BL/6 mouse kidney genomic DNA digested with six different restriction enzymes and hybridized with the M3-2 cDNA insert probe did not show any rearrangement of the YE1/48 gene in EL-4 cells as compared to the germline (Figure 14a). Similar analysis of genomic DNA digested with XbaI from different sources including C57BL/6 liver, spleen, thymocytes and bone marrow cells, from C57BL/6 T cell lines expressing YE1/48 including EL-4, MBL-2(4.1) and MBL-2(2.6), and from two other cell lines not expressing YE1/48 such as BW5147 (AKR T cells) and A20 (BALB/c B cells), did not reveal any gene amplication or translocation rearrangement of the YE1/48 gene (Figure 14b).

Interestingly, Southern blot analysis of genomic DNA from C57BL/6, BALB/c and C3H mice digested with EcoRI and HindIII showed two different patterns (Figure 15a), indicating that the YE1/48 gene is at least dimorphic in that C57BL/6 mice carry an allele different from that in BALB/c and C3H mice. Moreover, since there is no EcoRI site within the M3-2 cDNA sequence and there is only one for HindIII, the multiple banding patterns observed with both enzymes in either allele present two possibilities: either there are many introns within the gene, spanning a large region of DNA (about 40 kb), or there are other genes with highly conserved sequences. In an attempt to distinguish the above possibilities, three M3-2 fragments were prepared by HinfI digestion, representing 514 bp of the 5′ end (M3-2/514 probe), 400 bp of the middle portion (M3-2/400 probe) and 245 bp of the 3′ end (M3-2/245 probe) of the cDNA insert. Hybridization using these three probes to a genomic Southern blot showed that the multiple banding patterns are primarily localized in the middle portion of the YE1/48 gene (Figure 15b, ii). If

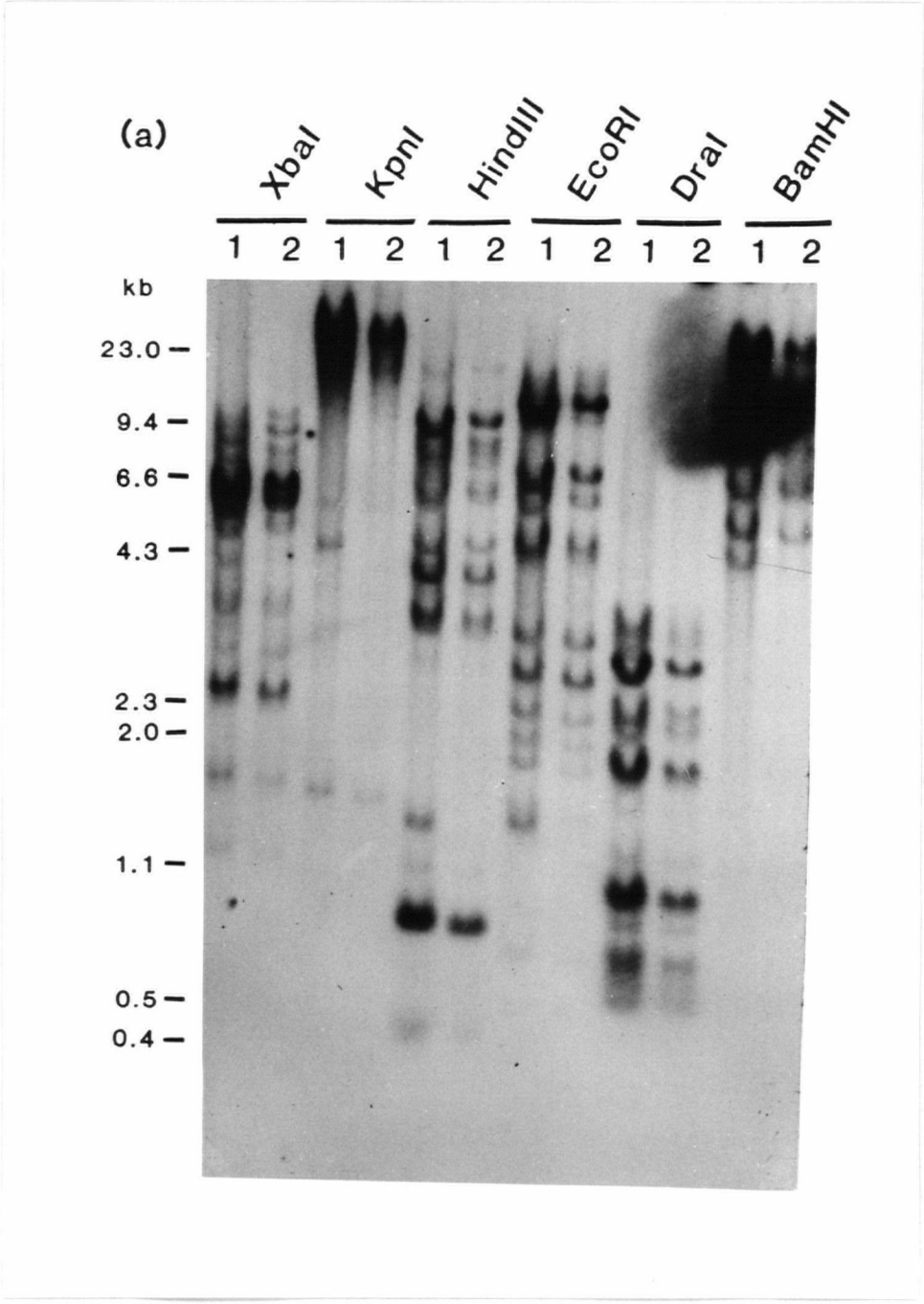


Figure 14(a). Genomic Southern analysis showing no rearrangement of the YE1/48 gene. Genomic DNA (5 µg) from (1) C57BL/6 kidney or (2) EL-4, were digested with various restriction enzymes, separated on a 0.8% agarose gel, and alkaline blotted onto a zeta-probe nylon filter. The blot was then hybridized with the radiolabeled M3-2 cDNA insert probe.

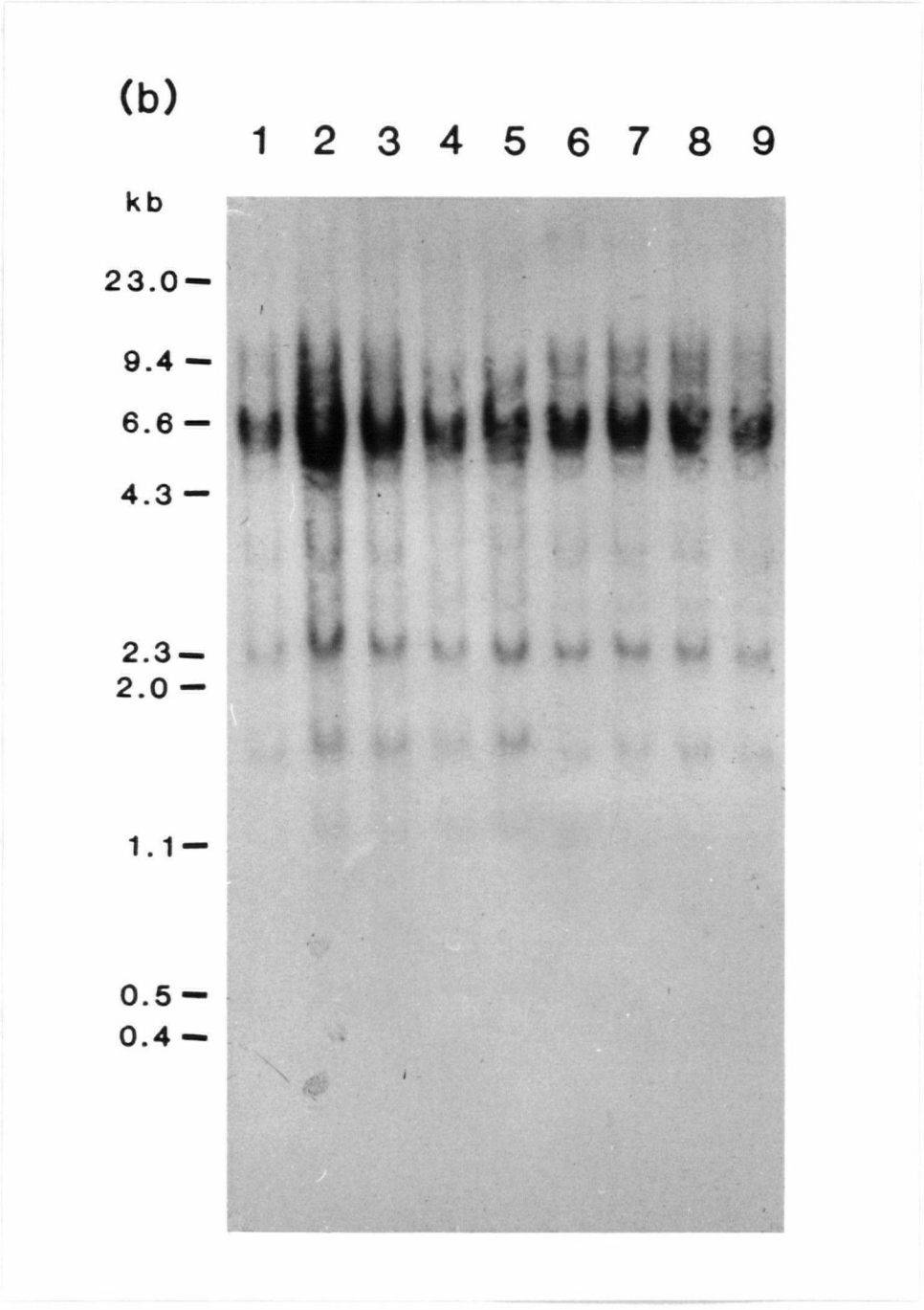


Figure 14(b). Genomic Southern analysis showing no amplification of the YE1/48 gene. Genomic DNA (10 µg) from C57BL/6 (1) liver, (2) spleen, (3) thymus, (4) bone marrow, (5) a BALB/c B cell line, A20, (6) an AKR T cell line, BW5147, (7) MBL-2(2.6), (8) MBL-2(4.1), and (9) EL-4 cells, were digested with XbaI, electrophoresed, blotted and hybridized as described in Figure 14(a).

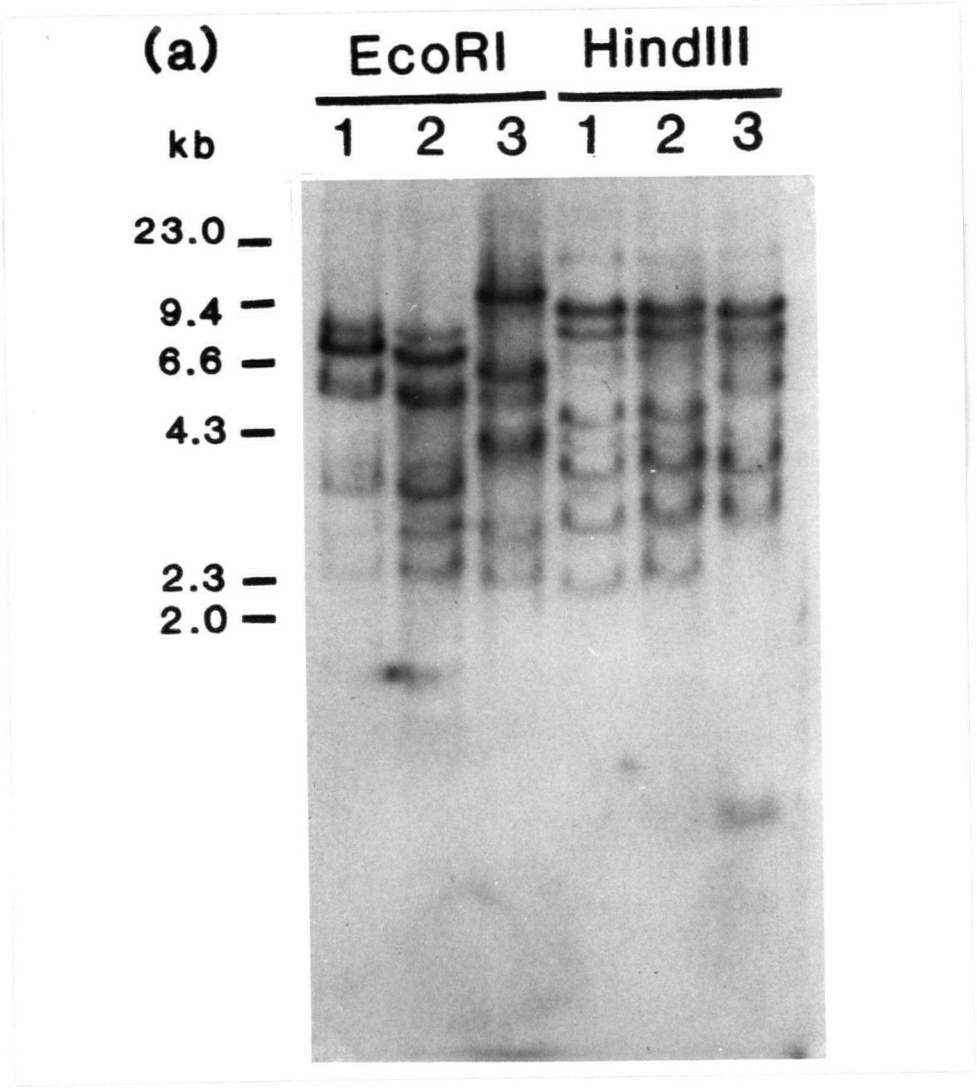
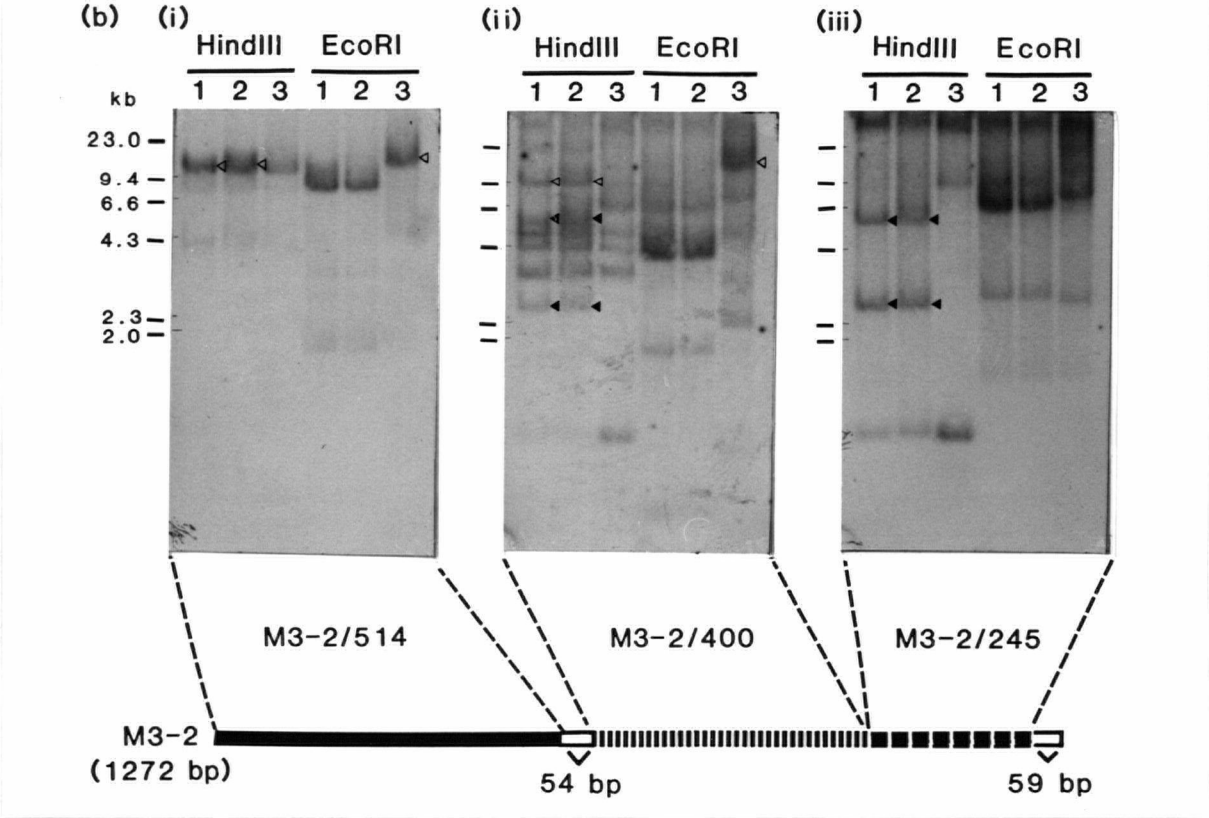
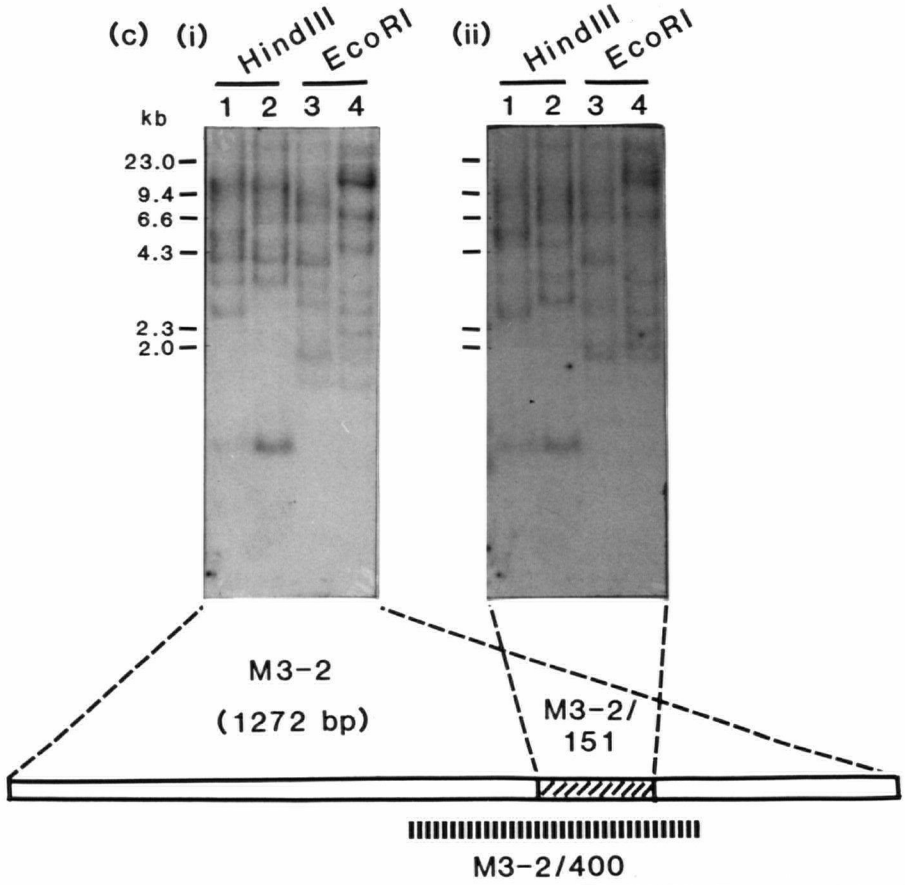


Figure 15. Genomic Southern analysis showing allelic polymorphism of the YE1/48 gene and possible existence of other related genes. (a) 3 µg of genomic DNA from (1) C3H, (2) BALB/c, and (3) C57BL/6 spleen cells were digested with HindIII and EcoRI, separated on a 0.8% agarose gel, blotted and hybridized with the M3-2 cDNA insert probe as described in Figure 14. (b) (next page) A similar DNA blot was hybridized consecutively with three radiolabeled M3-2 fragment probes of (i) 514 bp (M3-2/514), (ii) 400 bp (M3-2/400) and (iii) 245 bp (M3-2/245) in length. The closed and open arrows mark two sets of DNA fragment bands detected by more than one probe. (c) (next page) 10 µg of genomic DNA from (1) BALB/c and (2) C57BL/6 spleen cells were digested with HindIII and EcoRI and prepared on a nylon filter as described above. It was hybridized consecutively with (i) the M3-2 insert probe and (ii) a M3-2 fragment of 151 bp (M3-2/151). The M3-2/151 sequence is located within that of M3-2/400 (b, ii).


(b) (i)
HindIII EcoRI
1 2 3 1 2 3
kb
23.0
9.4
6.6
4.3
2.3
2.0
(ii)
HindIII EcoRI
1 2 3 1 2 3
(iii)
HindIII EcoRI
1 2 3 1 2 3
M3-2/514
M3-2/400
M3-2/245
M3-2
(1272 bp)
54 bp
59 bp


(c) (i)
HindIII EcoRI
1 2 3 4
kb
23.0
9.4
6.6
4.3
2.3
2.0
(ii)
HindIII EcoRI
1 2 3 4
M3-2
(1272 bp)
M3-2/
151
M3-2/400

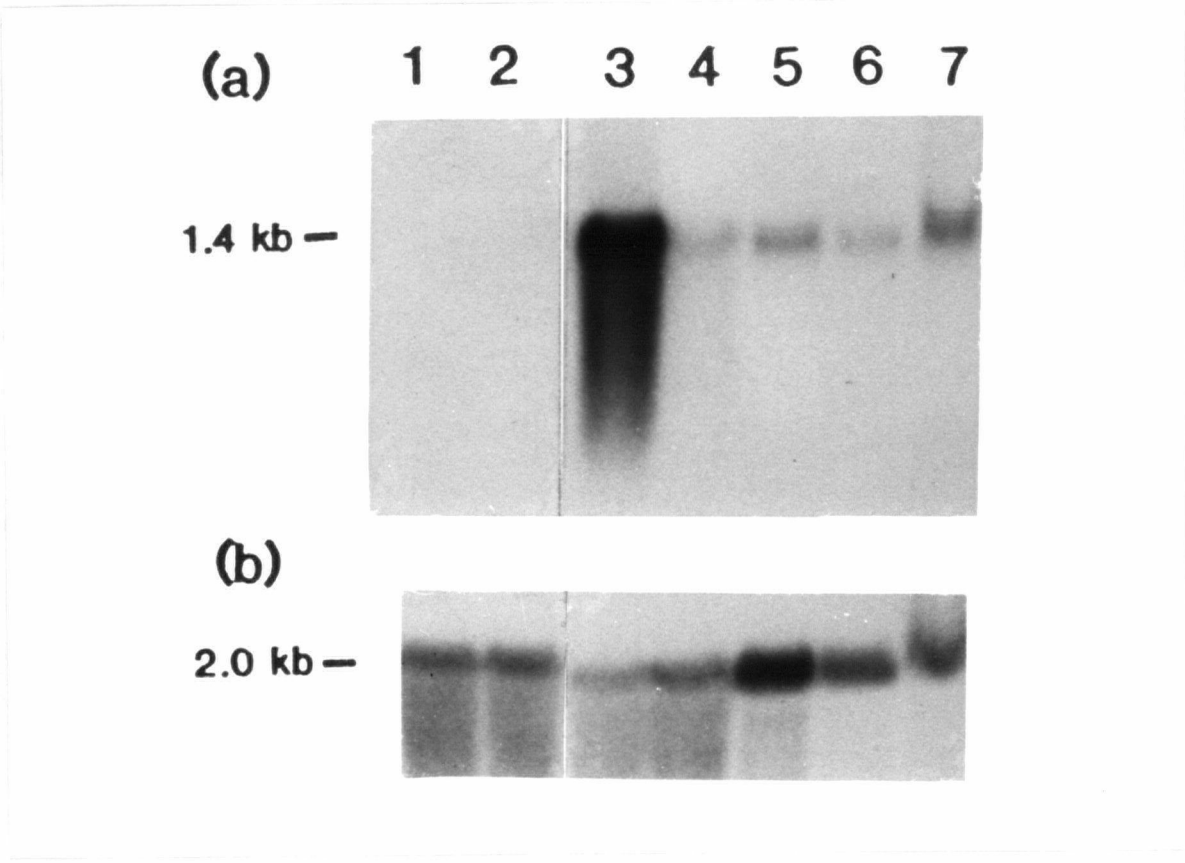
YE1/48 is a single gene containing many introns, any two fragment probes that are closely proximal to each other should detect a single overlapping genomic restriction fragment. As shown in Figure 15b, i and ii, single overlapping bands (marked by open arrows) were shared by the M3-2/514 and M3-2/400 probes. In contrast, two overlapping bands (marked by solid arrows in Figure 15b, ii and iii) were shared by the M3-2/400 and M3-2/245 probes. It therefore implicates that other related genes may exist, and that the mere existence of numerous introns in the YE1/48 gene cannot fully explain the multiple banding patterns observed. To further test this possibility, a 151 bp FokI fragment (M3-2/151) located within the M3-2/400 sequence was prepared. Comparable multiple banding patterns were detected when the FokI fragment was used for Southern blot hybridization (Figure 15c, ii), strongly implicating the existence of other genes with homologous sequences.

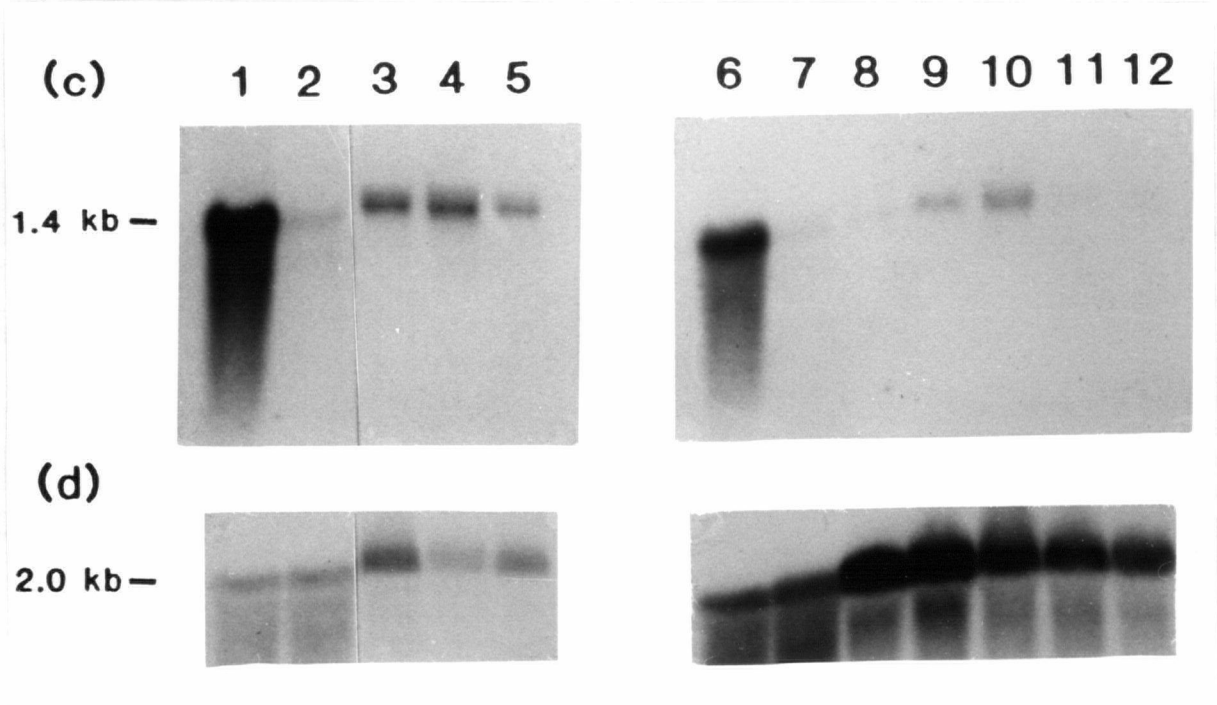
5.2.4 Northern Blot Analyses

The expression of YE1/48 in lymphoid cell populations and established cell lines was analysed by Northern blot hybridization of total RNA using the M3-2 cDNA insert (Figure 16). To quantitatively compare the expression levels of YE1/48 in different cell populations, all Northern blots were rehybridized for actin message which presumably maintains a relatively constant level in all cell types. The signal intensity was monitored by laser scanning densitometry. In agreement with previous immunoprecipitation data (see Chapter Three, section 3.2.3 C), YE1/48 transcripts were detected in MBL-2(4.1) (a, lane 3) and EL-4 (data not shown) cells but not in two other T cell lines BW5147 and AK-1 cells (a, lanes 1 and 2). The signal from MBL-2(2.6) cells (a, lane 4) is about 40 fold lower than from MBL-2(4.1), approximately in parallel with the 200-fold difference in YE1/48 antigen expression on their cell surface.

The levels of YE1/48 transcripts in C57BL/6 bone marrow cells (a, lane 5)

Figure 16. (next page) Northern blot analysis of YE1/48 mRNA in lymphoid cell populations. Total RNA (20 µg) from different cell sources were separated on 0.66 M formaldehyde/1.0% agarose gels, centrifugally blotted onto zeta-probe nylon filters, and hybridized with the radiolabeled M3-2 cDNA insert probe (a and c). The blots were then stripped and rehybridized with a radiolabeled chicken β-actin plasmid probe (b and d) to show the relative amount of RNA loaded in each lane. RNA samples are as follows:
In part (a) and (b), lane (1) BW5147, an AKR T lymphoma, (2) AK-1, an AKR T lymphoma, (3) MBL-2(4.1), (4) MBL-2(2.6), (5) C57BL/6 bone marrow, (6) C57BL/6 thymocytes and (7) C57BL/6 spleen cells.
In part (c) and (d), lane (1) MBL-2(4.1), (2) MBL-2(2.6), (3) C57BL/6 total spleen, (4) C57BL/6 spleen T cells, (5) C57BL/6 spleen B cells, (6) MBL-2(4.1), (7) MBL-2(2.6), (8) LPS-stimulated C57BL/6 spleen, (9) Con A-stimulated C57BL/6 spleen, (10) C57BL/6 spleen, (11) BALB/c spleen, and (12) C3H spleen.


(a)
1 2 3 4 5 6 7
1.4 kb —
(b)
2.0 kb —


(c)
1 2 3 4 5
6 7 8 9 10 11 12
1.4 kb —
(d)
2.0 kb —

and thymocytes (a, lane 6) are comparable to that in MBL-2(2.6), allowing for the relative intensity of the YE1/48 and actin bands (b, lanes 4-6). No transcripts were detected in day 16.5 fetal thymocytes (63% $CD4^-CD8^-$) and day 14 fetal liver (data not shown). However, by previous immunoprecipitation, the YE1/48 antigen could be detected from $CD4^-CD8^-$ adult thymocytes (97% $CD4^-CD8^-$) (data not shown). The failure to detect YE1/48 mRNA in the Northern blot analysis of fetal thymocytes may be due to a lower sensitivity of the Northern blot analysis compared to the immunoprecipitation assay. Alternatively, it may be due to a lower percentage of specific message because of mRNA instability or slow protein turnover, or an inherent difference in properties between the fetal and adult $CD4^-CD8^-$ thymocytes. The signal in spleen cells (a, lane 7) is approxiamately 5 fold higher than that in adult total thymocytes and is primarily contributed by T cells because a comparable signal was detected in spleen T cells (88% Thy-1^+, 96% $T200^+$, and 4% sIg^+) (c, lane 4), whereas the signal in spleen B cells (96% sIg^+, 2% Thy-1^+, and 99% $T200^+$) (c, lane 5) is half the level of total spleen cells (c, lane 3). Activation of spleen cells by Con A or LPS did not increase the YE1/48 mRNA levels (c, lanes 9 and 8); in fact it lowered them.

Interestingly, the YE1/48 transcript level in spleen cells from BALB/c mice is about three fold lower than that from C57BL/6 mice and was undetected in C3H spleen cells (c, lanes 10 to 12), comparable to their relative YE1/48 immunoprecipitation signals (see Chapter Three, section 3.2.3 C). It suggests that either the YE1/48 expression is differentially regulated in different mouse strains or the YE1/48 coding regions in different mouse strains exhibit substantial sequence heterogeneity.

5.2.5 Expression Of YE1/48 On Transformed Pre-B Cell Lines

The YE1/48 mRNA was undetected on two normal pre-B cell lines of (C57BL/6J x C3H/HeJ) F_1 hybrid origin, B_nB_2 and B_pD_4 (Figure 17a, lanes 8 and

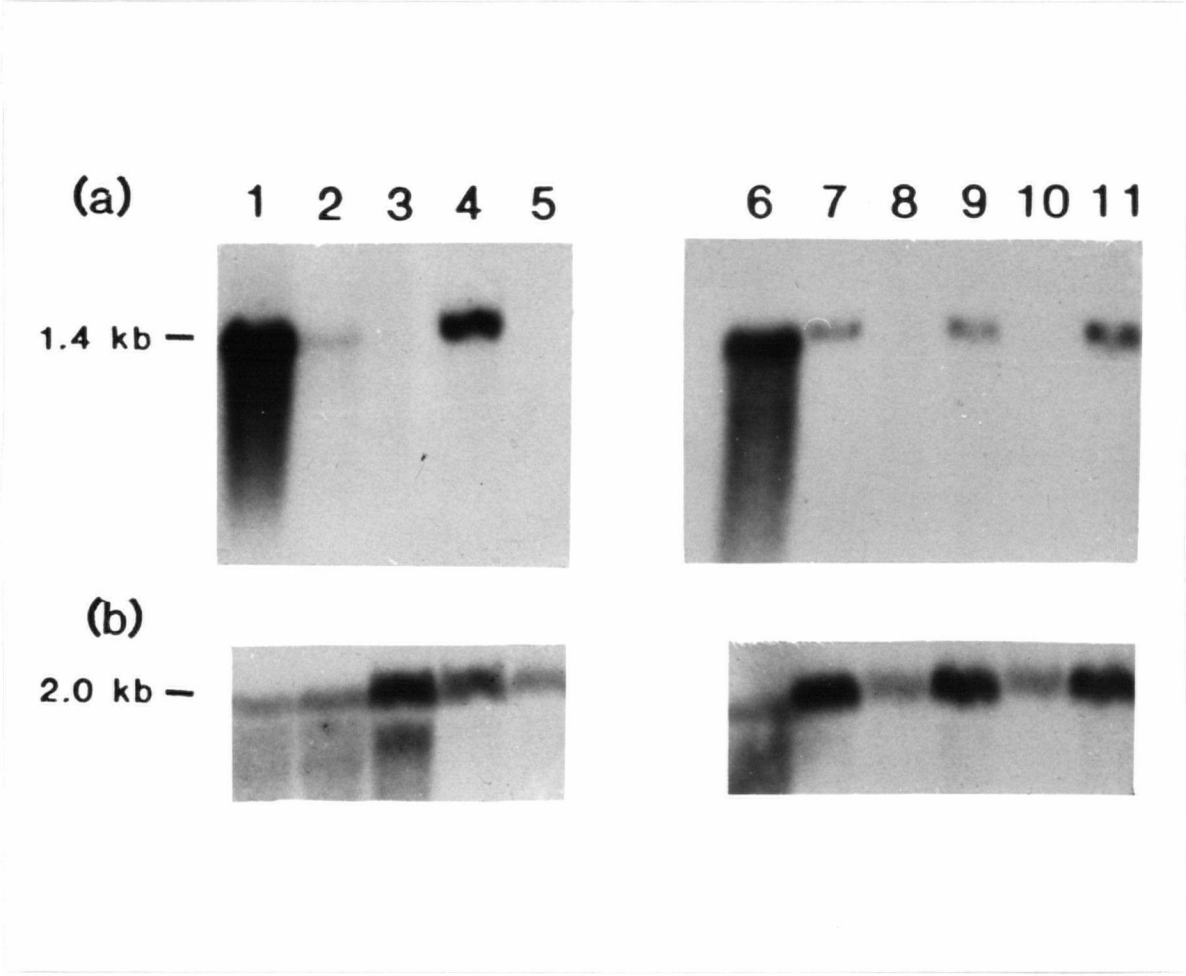


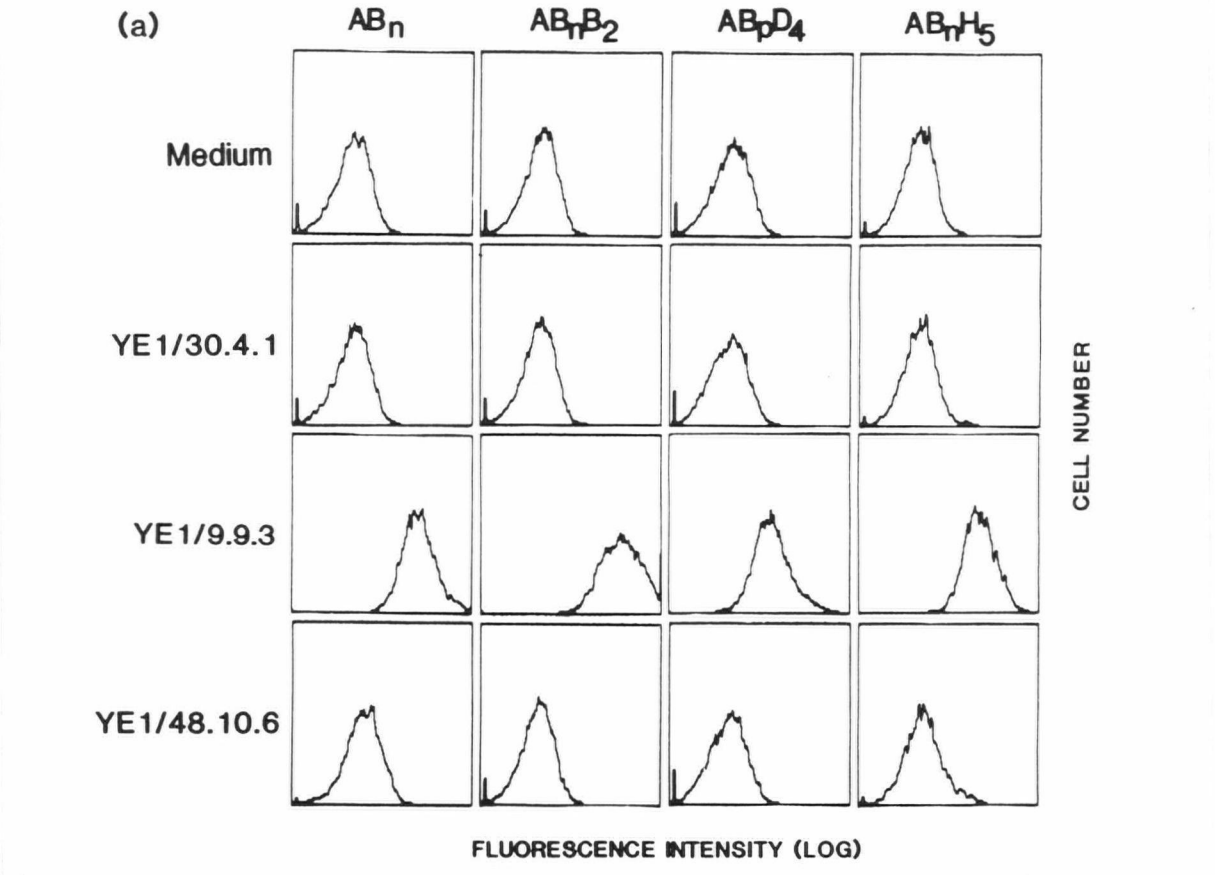
Figure 17. Northern blot analysis of YE1/48 mRNA in Abelson MuLV-transformed pre-B cell lines indicating possible correlation of YE1/48 expression with transformation. Total RNA (20 µg) from different sources were separated, prepared on nylon filters, and hybridized with the (a) M3-2 insert and (b) β-actin probes as described in Figure 16. Lane (1) MBL-2(4.1), (2) MBL-2(2.6), (3) B6SutA1, a C57BL/6 hemopoietic progenitor line, (4) AB_n, an Abelson MuLV-transformed pre-B line, (5) H9, a spontaneously transformed pre-B line, (6) MBL-2(4.1), (7) AB_nB_2, an Abelson MuLV-transformed pre-B line, (8) B_nB_2, the untransformed parent of AB_nB_2, (9) AB_pD_4, an Abelson MuLV-transformed pre-B line, (10) B_pD_4, the untransformed parent of AB_pD_4, and (11) AB_nH_5, an Abelson MuLV-transformed pre-B line. All pre-B cell lines are of (C57BL/6J x C3H/HeJ) F_1 hybrid origin except H9 which is of BALB/c origin.

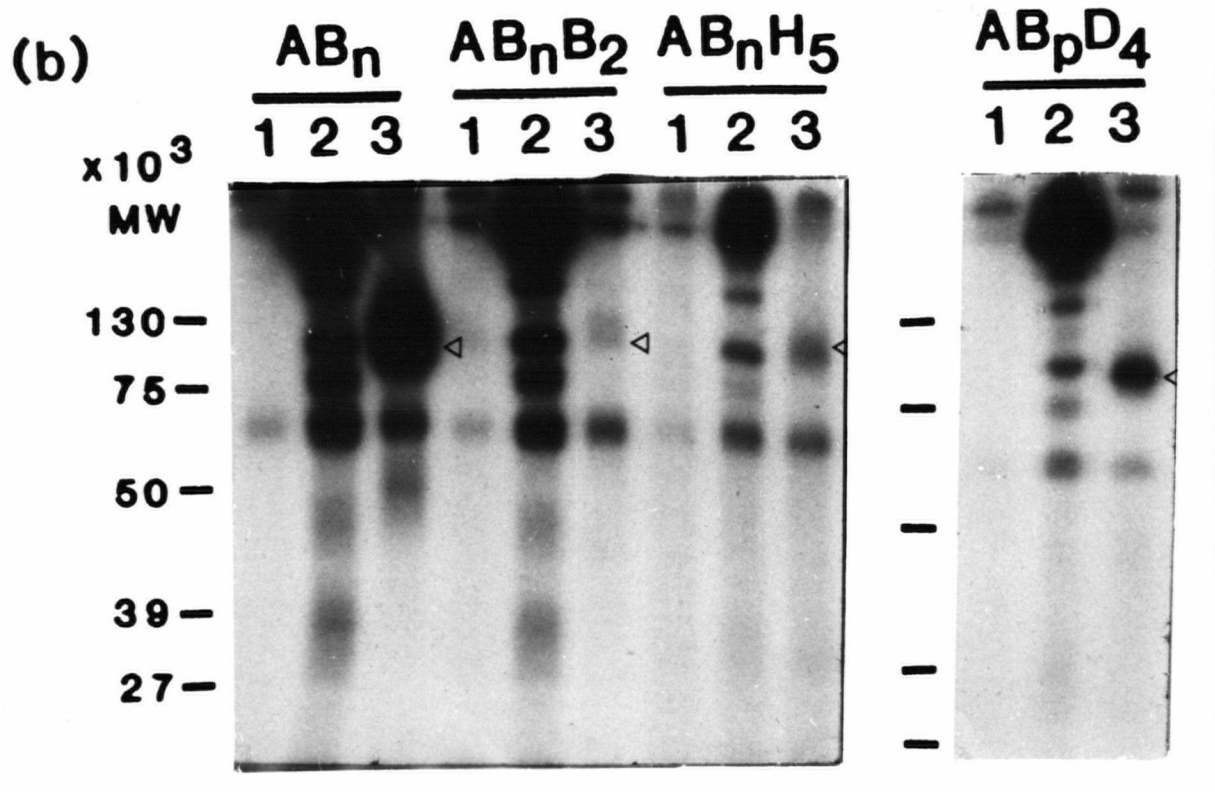
10), which were maintained in long term cultures (Whitlock and Witte, 1982). However, two transformed pre-B cell lines, AB_nB_2 and AB_pD_4 (lanes 7 and 9), generated by Abelson MuLV infection of the above normal pre-B cell cultures were positive. Two additional Abelson MuLV-transformed pre-B cell lines, AB_n and AB_nH_5 (lanes 4 and 11), were also positive. The amounts of the YE1/48 mRNA varied from comparable levels (AB_nB2 and AB_pD4) to seven fold higher (AB_n) than those observed in MBL-2(2.6) (lane 2) and C57BL/6 adult total thymocytes. The protein product of the YE1/48 gene was also detected on their cell surface by immunoprecipitation (Figure 18b, lanes 3). Despite the relatively high transcript level and immunoprecipitation signal, none of the four pre-B cell lines could be surface stained by YE1/48.10.6 MAb as analysed by flow cytometry (Figure 18a). No transcript signal was detected in a BALB/c spontaneously transformed pre-B cell line H9 and a C57BL/6 hemopoietic progenitor cell line B6SutA1 (Figure 17a, lanes 5 and 3).

5.3 DISCUSSION

A YE1/48-encoding cDNA clone, M3-2, has been isolated by screening a MBL-2(4.1) λgt10 library with three redundant synthetic oligonucleotide probes based on the tryptic peptide sequences previously determined. The identity of the YE1/48 cDNA clone is primarily confirmed by the presence of eight tryptic peptide sequences in the cDNA-deduced protein sequence. It is also supported by Northern blot analysis showing a single 1.4 kb mRNA signal of M3-2 in poly(A)$^+$RNA isolated from MBL-2(4.1) cells and a much lower signal from MBL-2(2.6) cells, which agrees with the differential YE1/48 expression detected on these two variant clones (see Chapter Three, section 3.2.3 B). No similar mRNA signal was detected in a YE1/48$^-$ myeloma line NS-1.

Figure 18. (next page) Detection of the YE1/48 antigen on Abelson MuLV-transformed pre-B cell lines by flow cytometric analysis and immunoprecipitation. (a) Cells were stained by the YE1/48.10.6 MAb using FITC-conjugated $(Fab')_2$ mouse anti-rat kappa light chain as the second antibody. As negative controls, medium alone or YE1/30.4.1 MAb (anti-Thy-1) was used for staining. As a positive control, YE1/9.9.3 MAb (anti-transferrin receptor) was used. Dead cells were stained by propidium iodide and were gated out on the basis of red fluorescence. (b) Cell lysates in 1% Triton-X 100 detergent were subjected to immunoprecipitation by (1) YE1/30.4.1 (anti-Thy-1), (2) YE1/9.9.3 (anti-transferrin receptor), and (3) YE1/48.10.6 MAb's, using sepharose-bound rabbit anti-rat Ig antiserum as the second antibody. The immunoprecipitates were analysed by SDS-PAGE (10%) under non-reducing conditions. The YE1/48 dimer was marked by open arrows.


(a)
ABn
ABnB2
ABpD4
ABnH5
Medium
YE1/30.4.1
YE1/9.9.3
YE1/48.10.6
CELL NUMBER
FLUORESCENCE INTENSITY (LOG)


(b)
ABn
ABnB2
ABnH5
ABpD4
1 2 3 1 2 3 1 2 3 1 2 3
x10³
MW
130
75
50
39
27

5.3.1 Possible Functional Sites And Domains

The M3-2 cDNA-deduced protein sequence predicts that the YE1/48 antigen is a type II integral membrane protein with a 196 amino acid C-terminal extracellular domain and a 44 amino acid N-terminal cytoplasmic domain. Type II membrane proteins are not as common as type I proteins which have N-terminal extracellular domains and C-terminal cytoplasmic domains. Examples of type II proteins are the transferrin receptor (Schneider et al., 1984), the asialoglycoprotein receptor (Drickamer et al., 1984; Spiess et al., 1985), the HLA-DR invariant chain (Strubin et al., 1984), and 4F2 on activated human lymphocytes (Lumadue et al., 1987). No correlation between the type II protein orientation in the cell membrane and their functional properties has been described.

No apparent homology of the YE1/48 cDNA and the deduced protein sequences with other known sequences has been identified, including the TCR α, β and γ products, and the T cell activation antigen CD28 (T44). The predicted protein carries an Arg-Gly-Asp (RGD) tripeptide which is the active binding site on many extracellular matrix proteins and platelet adhesion proteins such as fibronectin, vitronectin, type I collagen, fibrinogen, von Willebrand factor and osteopontin. Receptors for these ligands are collectively called the integrins (Hynes, 1987; Ruoslahti and Pierschbacher, 1987). All integrins are non-covalently linked heterodimers that carry homologous α and homologous β subunits. The RGD-binding integrins connect the extracellular matrix outside the cells indirectly to the cytoskeleton inside the cells. This recognition system provides cells with anchorage, traction for migration, and signals for polarity, position, differentiation, and possibly growth. RGD sequences are however uncommon on membrane proteins. Examples other than YE1/48 are the HLA class I and class II antigens (Larhammar et al., 1983; Strachan et al., 1984), the human EGF-R (Lin et al., 1984; Ullrich et al., 1984; Xu et al., 1984), the human glucocorticoid receptor (Hollenberg et al., 1985), and the human

transferrin receptor (McClelland et al., 1984; Schneider et al., 1984). No adhesive properties dependent on the RGD sequence have been demonstrated in these membrane proteins. It is likely that some uncharacterized protein sequences flanking the RGD tripeptide can significantly influence the adhesive property of the RGD-carrying domain. It is not known if the RGD sequence on the YE1/48 protein functions in adhesive interactions.

The predicted YE1/48 protein sequence contains 5.3% cysteine residues, above the reported average of 2.8% in eukaryotic proteins (Klapper, 1977); 12 out of 14 cysteine residues are in the extracellular domain. Many cysteine-rich proteins are known to bind metals such as zinc, cadmium, iron or copper (Berg et al., 1986). They include metallothionein (Furey et al., 1986) and some cellular enzymes and proteins (Berg, 1986). More recently, cysteine-rich proteins carrying the consensus configuration of Cys-X_{2-4}-Cys-X_{4-15}-Cys/His-X_{2-4}-Cys/His have been correlated to zinz-binding and DNA-binding properties (Berg, 1986; Evans and Hollenberg, 1988). Examples include intracellular steroid hormone receptors (Arriza et al., 1987; Petkovich et al., 1987; Sabbah et al., 1987) and nuclear factors that influence transcription by RNA polymerases II and III (Hanas et al., 1983; Smith et al., 1984; Kadonaga et al., 1987). An extracellular sequence within YE1/48 (residues 145-167) conforms to this consensus configuration. However, no eukaryotic cell surface integral proteins have yet been described to have metal-binding properties, DNA-binding properties, or be capable of translocating from the cell membrane to the nucleoplasm. The high content of cysteines in the extracellular domain of YE1/48 may just be a property of the highly disulphide-bonded protein secondary structure. It may account for the strong tendency of the YE1/48 subunits to form dimers and multimers (See Chapter Four, section 4.2.2). Indeed, cysteine-rich domains have frequently been found in other integral membrane proteins and no unique implications have been specified.

5.3.2 Expression On Lymphoid Cells And Its Possible Correlation With Cellular Transformation

Northern blot analysis using the M3-2 cDNA insert as a probe has provided a quantitative and reliable estimation of YE1/48 expression in lymphoid cell populations. The results are in close parallel with the previous immunoprecipitation data. YE1/48 is primarily expressed on spleen T cells but it is also expressed on other lymphocytes at lower levels, including adult thymocytes and spleen B cells. Its expression on total spleen cells decreases upon mitogenic stimulation by Con A. It appears to be expressed on cells of the lymphoid lineage in general and is not preferentially restricted to a particular T cell differentiation stage. The YE1/48 transcript was also detected in bone marrow cells. Considering that its expression level in bone marrow is comparable to that in total thymocytes, the mRNA detected is unlikely to be completely contributed by mature circulating lymphocytes. It is not known which bone marrow cells express the YE1/48 gene. As suggested by previous immunoprecipitation of the YE1/48 antigen from long term cultured bone marrow cells, the YE1/48$^+$ cells in the bone marrow are probably of lymphoid origin (see Chapter Three, section 3.2.3 D).

Although the YE1/48 gene is expressed on a wide range of lymphocytes, its level is quite low when compared with that on the T lymphoma lines EL-4 and MBL-2(4.1). It is undetectable on two normal pre-B cell lines maintained in tissue culture but they become positive after the transformation by Abelson MuLV. One of the transformed pre-B lines, AB_n, expresses the gene at a level comparable to those in EL-4 and MBL-2(4.1) cells. Since the M3-2 derived protein sequence does not show any homology with viral sequences in the data base, the YE1/48 gene is likely a cellular gene induced during the transformation of pre-B lymphocyte. Other than EL-4 and MBL-2(4.1), two murine T lymphomas TIMI.4 (C57BL/6 origin) and R1.1 (C58/J origin) have recently been tested in our laboratory. TIMI.4 expresses the YE1/48 antigen

at a high level whereas R1.1 does not (data not shown). The expression on TIMI.4, like that on EL-4 and MBL-2(4.1), can be detected by flow cytometry. Thus, YE1/48 is highly expressed in all three T lymphoma cell lines of C57BL/6 origin so far tested.

5.3.3 Allelic Polymorphism And Possible Existence Of Related Multigenes

In the Southern blot analysis of genomic DNA from three mouse strains, C57BL/6, BALB/c and C3H, multiple banding patterns were detected with the entire M3-2 cDNA probe on the genomic blots. Similar multiple bands were detected even by the shortest fragment probe M3-2/151. Since it is unlikely that the majority of introns are localized within a coding sequence of 151 bp, other related genes with homologous sequences must be present. These related genes are not expressed in MBL-2(4.1) cells as no additional mRNA bands were detected in the Northern blot analysis allowing 20% sequence mismatch with the M3-2 probe (data not shown). Whether the cross-hybridizing genes detected in the genomic Southern analysis are members of a multigene family expressed in other cell types or if they are pseudogenes is not known.

The genomic Southern blot analysis has also implicated possible restriction fragment length polymorphism of the YE1/48 gene. This observation parallels with our earlier immunoprecipitation data in which the YE1/48 antigen was more readily detected by both YE1/32.8.5 and YE1/48.10.6 MAb's from C57BL/6 spleen cells than from BALB/c and C3H spleen cells (see Chapter Three, section 3.2.3 C). We have previously speculated that the differential immunoprecipitation signal is either due to differential regulation of the YE1/48 gene expression, or due to allelic polymorphism among the mouse strains. The finding of possible restriction fragment length polymorphism of the YE1/48 gene supports the latter possibility. On the other hand, the current Northern blot analysis results also suggest a differential YE1/48 mRNA detection, indicative of a differential regulation of the YE1/48 gene. To

intepret these observations, it should be kept in mind that other related genes detected by the YE1/48 cDNA probe may contribute to the restriction fragment length polymorphism observed. It is possible that the mouse strains exhibit both allelic polymorphism and differential gene expression. However, until further analysis of the YE1/48 genomic sequences of different mouse strains is done, no definitive conclusion can be derived.

5.3.4 Differential Accessibility Of Epitopes On T Lymphomas And Normal T Lymphocytes

As discussed in Chapter Three (see section 3.2.3 B and 3.3.3), the YE1/32.8.5 and YE1/48.10.6 MAb's, which readily detect the YE1/48 antigen on intact EL-4 and MBL-2(4.1) cells, do not seem to bind to the surface of normal C57BL/6 thymocytes and spleen cells. However, the MAb's can immunoprecipitate the antigen from detergent-solubilized normal cells. Since the normal thymocytes and spleen cells express YE1/48 at a substantially lower level than EL4 and MBL-2(4.1) cells do based on their relative immunoprecipitation signals, the failure to surface stain YE1/48 on normal cells may be due to the difference in the sensitivities between flow cytometric analysis and immunoprecipitation. Assuming a close parallel of protein expression with the corresponding mRNA level, the current Northern blot data has indicated that total thymocytes and spleen cells may express the YE1/48 protein at a level detectable by flow cytometric analysis. Moreover, the Abelson MuLV-transformed pre-B cell line AB_n, which exhibits a relatively high level of YE1/48 mRNA and an intense YE1/48 immunoprecipitation signal, do not show detectable binding of the anti-YE1/48 MAb's on the cell surface by flow cytometry. Therefore, it is likely that the epitopes defined by the two MAb's are masked and are not accessible for MAb binding on transformed pre-B cell lines as well as intact normal lymphocytes. It is intriguing that these epitopes seem to become exposed on three T lymphomas, EL4, MBL-2(4.1) and

TIMI.4.

5.3.5 Homodimer Or Heterodimer

Previous tryptic peptide mapping data has shown that the YE1/48 antigen subunits exhibit very similar fingerprints (see Chaper Three, Figure 4). It has however not been determined if the two subunits differ in their protein core sequences or they differ only in post-translational modifications. All of the tryptic peptide sequences derived from the mixture of reduced YE1/48 subunits (see Chapter Four, Table VI) are now located in the M3-2 cDNA-deduced protein sequence. Assuming that all eight tryptic peptides non-selectively represent both YE1/48 subunits, it appears that the M3-2 sequence encodes both polypeptides. It in turn implies that YE1/48 is likely a homodimer with subunits differing in post-translational modifications, giving rise to their distinct pI characteristics. Alternatively, if the YE1/48 subunits are derived from two dissimilar mRNA's, the two mRNA's should share most of the sequence to account for the closely related tryptic peptide fingerprints. No additional mRNA bands other than the 1.4 kb band was detected in Northern blot analysis of RNA from MBL-2(4.1) cells, using the M3-2 insert probe under conditions allowing a 20% sequence mismatch. Hence, unless the hypothetical mRNA has the same size of 1.4 kb, there is unlikely a distinct mRNA which encodes a distinct YE1/48 subunit to form a heterodimer.

Although it is unlikely that there is another mRNA encoding a YE1/48 subunit distinct from that predicted by the M3-2 cDNA clone, it remains possible that the single M3-2 mRNA may give rise to two translated proteins because two in-phase Met codons (25 nucleotides apart) are found at the start of the M3-2 open reading frame. The alternate use of Met codons on a single mRNA is common in viruses, but it has also been demonstrated in eukaryotic cells (Strubin et al., 1986). By comparing to the initiator consensus sequence common to most eukaryotic mRNA's, the first Met codon in the M3-2

cDNA sequence likely has a higher efficiency in initiating translation than the second one. Although initiation from the second Met codon remains possible, two observations seem to argue against the possiblity that another polypeptide produced by initiation from the second Met codon is used to form a heterodimer with the polypeptide produced by initiation from the first Met codon. First, only two charged amino acids (positions 3 and 5) are found between the two methionine residues, which probably cannot account for the difference in pI's of the YE1/48 subunits observed in two-dimensional gel analysis (see Chapter Three, Figure 3). Second, if the YE1/48 subunits use both initiation codons, only one different tryptic peptide will be predicted because there is no lysine or arginine residue between the two Met codons. Instead, four to five unique tryptic peptides are allocated to each subunit in the fingerprints. Therefore, it seems unlikely that both initiation codons are used to generate sequence-related polypeptides to form a YE1/48 disulphide-linked heterodimer. Current findings suggest that YE1/48 may be a homodimer with subunits that differ in post-translational modifications. However, it is also possible that the pI characteristics and the tryptic peptide fingerprints of the YE1/48 subunits reflect some technical artefacts. For example, the basic YE1/48 subunit separated in two-dimensional gel analysis may represent protein aggregation near the top of the IEF tube gel, and the minority of tryptic peptides unique to each YE1/48 subunit may be due to protein contamination in sample preparation. The YE1/48 antigen may in fact be a homodimer with identical subunits.

5.4 SUMMARY

YE1/48 is a murine cell surface disulphide-linked dimeric antigen of two 45-50,000 M_r subunits expressed on thymocytes and spleen cells. The two MAb's reactive with the antigen do not give detectable binding on the surface of

normal lymphocytes and no functional data has yet been obtained. A YE1/48-encoding cDNA clone, M3-2, has now been isolated based on the amino acid sequences determined from the purified antigen. The 1.3 kb cDNA clone represents a major portion of the full length 1.4 kb mRNA and includes the entire open reading frame encoding a polypeptide of 262 amino acids and 30,500 MW. The predicted protein has a type II membrane protein orientation with 44 amino acids in the N-terminal cytoplasmic domain, 22 amino acids in the transmembrane domain, and 196 amino acids in the C-terminal extracellular domain. There are three potential N-linked glycosylation sites in the extracellular domain all of which are probably used. No signigicant homology can be identified with other known protein sequences in the data base or with human CD28. Hence YE1/48 is likely a novel murine cellular antigen undescribed. In the cDNA-deduced protein sequence, there are found a potential cell-adhesive binding site (RGD tripeptide), and a domain with potential metal-binding property. However, no activities of these potential functional sites have yet been indicated.

The isolation of the cDNA clone M3-2 has allowed further analyses of the YE1/48 protein structure, its expression on lymphocytes, and its genetic structure. The failure to detect another mRNA in MBL-2(4.1) cells closely homologous to the M3-2-defined mRNA has suggested that YE1/48 is a homodimer with subunits exhibiting differential translational processing, rather than a heterodimer with two sequence-related subunits. Genomic Southern analysis has suggested that the YE1/48 gene may have two alleles, one in C57BL/6 and the other in BALB/c and C3H mice. The genomic analysis has also strongly suggested the existence of other genes with sequences highly homologous to the YE1/48 gene.

The YE1/48 gene appears to be expressed at low levels on a wide range of T lymphocytes with no restriction to their differentiation stages, and on spleen B cells as well as bone marrow cells. The expression is not increased

by mitogenic stimulation of either spleen T or B lymphocytes. However, the high YE1/48 expression on some of the transformed T and pre-B cell so far tested suggests a possible correlation of the YE1/48 expression with lymphoid transformation. Furthermore, the two epitopes on YE1/48 antigen defined by the YE1/48.10.6 and YE1/32.8.5 MAb's appear to be masked on intact normal cells but are specifically exposed on the C57BL/6 T lymphomas EL-4, MBL-2(4.1), and TIMI.4.

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CHAPTER SIX

SUMMARY AND PERSPECTIVES

In the immune system, T lymphocytes are known to play central roles in the execution and the regulation of an immune response. Many of the T cell functions rely on cell surface antigens as receptors for extrinsic signals, either in the form of soluble regulatory factors or in the form of surface components on other cells. Characterization of these T cell surface antigens is important in the understanding of the molecular mechanisms by which an immune function takes place. In the past, MAb's have proven to be a valuable tool in the identification of novel T cell antigens. Moreover, they may provide important clues to how T cell surface molecules function since some MAb's influence T cell functions by mimicking natural ligands that act on the T cell surface or blocking the receptor-ligand interactions. However, the precise functions of many T cell surface antigens are not yet known.

In our preliminary studies, rat MAb's were generated against mitogen-activated murine T lymphocytes to identify surface antigens which might be involved in immune functions. Two MAb's, YE1/48.10.6. and YE1/32.8.5, were found by flow cytometric analysis to react with only two T lymphoma cell lines EL-4 and MBL-2(4.1), but not with normal resting or proliferating lymphocytes. Both MAb's define an identical disulphide-linked dimeric molecule of 90-95,000 M_r, composed of 45-50,000 M_r subunits of distinct pI characteristics. It was designated the YE1/48 antigen. The molecular size and the apparent heterodimeric structure of the YE1/48 antigen resemble properties of the murine TCR-α/β dimer. Moreover, the specific reactivity of the MAb's with only two T cell lines suggested that they might recognize clonotypic epitopes on the YE1/48 antigen similar to those present on the murine TCR-α/β. The

TCR-α/β antigen, which recognizes foreign antigens in the context of MHC molecules, is an essential element in the antigen-specific T cell immune response. MAb's to TCR-α/β have been valuable tools in studying the molecular events involved in antigen-dependent T cell activation as well as T cell differentiation. Since no other dimeric molecules of similar size had been identified at that time, it appeared that YE1/48 might be the authentic murine TCR-α/β. Therefore, the YE1/48 antigen was further characterized and compared with the murine TCR-α/β.

In my initial studies, the YE1/48 antigen was subjected to biochemical analyses. Endoglycosidase F digestion experiments showed that YE1/48 has glycosylation characteristics similar to those of TCR-α/β. However, the tryptic peptide maps of the YE1/48 subunits demonstrated substantial differences from the murine TCR-α/β, and suggested that YE1/48 might not be a heterodimer. Furthermore it was found that, although the YE1/48.10.6 and YE1/32.8.5 MAb's did not show detectable binding to normal lymphocytes by flow cytometry, they could be used to immunoprecipitate the YE1/48 antigen from detergent-solubilized normal thymocytes and spleen cells, albeit at a much lower level than that detected from EL-4 and MBL-2(4.1) cells. Its detection on normal mixed populations of lymphocytes by immunoprecipitation, had once led to the speculation that the MAb's might recognize epitopes on the constant domain of a TCR-like antigen. Thus, I attempted to determine if the YE1/48 antigen was the authentic murine TCR-α/β, or if it was a related molecule. Sequential immunoprecipitation of the YE1/48 antigen and the TCR-α/β dimer from EL-4 cells suggested that YE1/48 and TCR-α/β are likely distinct molecules. The differential expression of YE1/48 on two variant MBL-2 clones which express similar levels of TCR-α/β also implied that YE1/48 is different from TCR-α/β. Finally, direct comparisons of the partial amino acid sequence of YE1/48 antigen with the murine TCR sequences definitively distinguished the YE1/48 antigen from products of the TCR α, β and γ genes.

During the progress of the above studies, it had become apparent that the human CD28 (T44) dimeric antigen which may play significant roles in immune functions also has similar molecular size as YE1/48 does. Little was known about the molecular structure of CD28 at that time. Only lately a human CD28 cDNA clone was isolated. Since the YE1/48.10.6 and YE1/32.8.5 MAb's do not show detectable binding on intact normal cell surface, no biological effects upon the perturbation of the YE1/48 antigen could be derived. Thus, in order to elucidate the possible function of the YE1/48 antigen and its correlation with other known antigens, I undertook the molecular cloning of the YE1/48 molecule based on the partial amino acid sequences derived from the purified antigen. The isolated cDNA clone M3-2 contains the entire coding sequence of the YE1/48 antigen and represents a single 1.4 kb YE1/48 mRNA in cells expressing the antigen. It encodes a polypeptide of 262 amino acids and 30,5000 MW. The predicted protein has a type II membrane protein orientation with 44 amino acids in the N-terminal cytoplasmic domain and 196 amino acids in the C-terminal extracellular domain. Three potential N-linked glycosylation sites in the extracellular domain are likely used as suggested by the previous endoglycosidase F digestion experiments. An Arg-Gly-Asp (RGD) tripeptide and a domain with potential zinc-binding property are also found in the extracellular domain. However, it remains unknown if they are functional in cell adhesion and metal-binding. So far, no significant sequence homology has been identified with other known proteins in the data base or with human CD28.

Despite our failure to elucidate the function of the YE1/48 antigen from its primary sequence, the isolation of the YE1/48 cDNA clone has allowed a better understanding of its molecular structure. The comparison of the cDNA sequence to the partial amino acid sequences previously determined implicates that YE1/48 is likely a homodimer of related subunits resulted from differential post-translational processing. Genomic Southern analyses suggest

that YE1/48 may have two alleles among different mouse strains, and that there may exist other genes with highly homologous sequences, which are probably not expressed on the T cell lines and lymphocytes so far tested.

The expression of YE1/48 on lymphocytes presents the most intriguing characteristics of the YE1/48 antigen. By Northern blot analyses, the YE1/48 gene was found to be expressed at low levels in a wide range of T cells with no restriction to their differentiation stages, and on spleen B cells as well as bone marrow cells. The expression on spleen lymphocytes is not related to cell proliferation as the mRNA levels were not increased by mitogenic stimulatin of either the T or B cell population. However, the YE1/48 expression appears to be induced by the Abelson MuLV-transformation of pre-B cells, at a level comparable to that observed on EL-4 and MBL-2(4.1) cell lines. In contrast to EL-4 and MBL-2(4.1) cells, the high level of YE1/48 antigen on the transformed pre-B cells could not be detected by YE1/48.10.6 and YE1.32.8.5 MAb's by flow cytometry although it could be immunoprecipitated. This difference in flow cytometric analysis and immunoprecipitation experiments is similar to the previous findings of YE1/48 expression on normal thymocytes and spleen cells. It thus appears that the two MAb-defined epitopes on YE1/48 are masked on intact normal cells but are specifically exposed on the T lymphomas EL-4 and MBL-2(4.1). Another T lymphoma TIMI.4 was recently found to exhibit similar exposure of the two epitopes. It is tantalizing to speculate a correlation of the high expression of YE1/48 on transformed T and pre-B cells, as well as the differential exposure of YE1/48 epitopes on transformed T cells versus normal lymphocytes, with the cellular transformation process.

A number of questions concerning the YE1/48 antigen remain to be addressed. With respect to the function of YE1/48, the present studies were limited by the lack of reactivity of the YE1/48.10.6 and YE1.32.8.5 MAb's with the antigen on intact normal cell surface. To test for the possible

functions, one approach is to generate MAb's which recognize the YE1/48 antigen on intact normal cell surface. The presently available cDNA clone can be utilized in the large scale preparation of materials for immunization purposes. In view of the previous failure in generating appropriate antiserum in rats, other animals like hamsters may be used. The appropriate MAb's generated may then be tested for their effects on T cell functions such as activation, proliferation and cell adhesion. The possible correlation of high level YE1/48 expression with the Abelson MuLV-transformation of pre-B cells is one of the intriguing findings in the present studies. It may be of interest to isolate a cDNA clone of the human homologue of YE1/48 and test whether its expression is correlated to leukemias/lymphomas. Moreover, the kinetics of YE1/48 expression during the course of Abelson MuLV-transformation of murine pre-B cells in culture can be determined. Finally, the existence of other related genes has suggested that YE1/48 may be a member of a multigene family. It therefore implies that the YE1/48 antigen may have some important functions which are conserved among a family of similar gene products. The identificaton of the other multigenes and the analysis of their expression on other cell types may add insight to the functions and roles of the YE1/48 antigen on the T cell surface.