

Lorita Maria Rebellato. **GENERATION AND CHARACTERIZATION OF
A MoAb AGAINST A UNIQUE T200 MOLECULE IN RHESUS MONKEYS.**

(Under the direction of Judith M. Thomas, PhD) Department
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The leukocyte common antigen (T200), now termed CD45, is present on all leukocytes. T200 molecule was first found in the rat using a heterologous antiserum, has also been identified in humans and mice, and the human form has been designated CD45. Biochemical analysis indicates that at least four polypeptides carry epitopes identified by many monoclonal antibodies (moAbs). Some antibodies, now termed CD45R, were found to precipitate only the two higher molecular weight polypeptides. These antibodies were found to react predominantly with B lymphocytes. Recently, it has been determined that these B restricted or CD45R moAbs recognize a set of T cells.

This study describes the generation, characterization, tissue distribution, and some functional properties of anti-7H5, a moAb which appears to recognize a unique T200 molecule on rhesus monkey mononuclear leukocytes. The role of this molecule on the alternative pathway of activation of T lymphocytes, and the enhancing effect of anti-7H5 on ConA cultures remains to be elucidated. The results of these studies suggest 7H5 molecule may function in association with other cytoplasmic and/or cell surface molecules to influence the activation of T lymphocyte

function.

This common leukocyte antigen has a restricted distribution, similar to that exhibited by CD45R molecules in rats and humans. However, the 7H5 determinant is antigenically distinct from the CD45R determinant defined by anti-2H4 moAb. Furthermore, these molecules show differences in two bands in SDS-PAGE gels. The 7H5 molecule exhibits a unique very high mw band not seen in other descriptions of CD45R molecules. 7H5 also is unique from other CD45R molecules in that binding of anti-7H5 does not block NK lysis, and it selectively potentiates ConA, but not PHA responses. Collectively, these results suggest 7H5 is a unique variant of a CD45R molecule in rhesus monkeys and may play a central role in the function of lymphoid cells, perhaps in association with both cytoplasmic molecules and others at the cell surface.

GENERATION AND CHARACTERIZATION
OF A UNIQUE
T200 MOLECULE IN RHESUS MONKEYS

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LIST OF ABBREVIATIONS

AMLR	Autologous Mixed Lymphocyte Reaction
cDNA	Complementary DNA
ConA	Concanavalin A
CML	Cell Mediated Lympholysis
CTL	Cytotoxic T Lymphocyte
CTLp	Precursor Cytotoxic T LYmphocyte
ELISA	Enzyme-Linked Immunosorbent Assay
E/T	Effector Target Ratio
FACS	Fluorescence-Activated Cell Sorter
FCS	Fetal Calf Serum
HSA	Human Serum Albumin
HPLC	High Pressure Liquid Chromatography
IFN	Interferon
IL-2	Interleukin 2
IL-2R	Interleukin 2 Receptor
LC-A	Leukocyte Common Antigen
LDA	Limiting Dilution Analysis
MHC	Major Histocompatibility Complex
MLC	Mixed Lymphocyte Culture
MoAb	Monoclonal Antibody
NK	Natural Killer
NMS	Normal Monkey Serum
PBMC	Peripheral Blood Mononuclear Cell
PEG	Polyethylene Glycol
PHA	Phytohemagglutinin

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INTRODUCTION

The leukocyte common antigen (T200), now termed CD45, is present on all leukocytes. Biochemical analysis indicate that at least four polypeptides carry epitopes identified by different antibodies. Some antibodies, termed CD45R, were found to precipitate only the two higher molecular weight polypeptides (Dalchau and Fabre, 1981). These antibodies were first thought to react predominantly with B lymphocytes, but recently Morimoto et al. 1985a have shown that CD45R moAbs recognize a subset of T cells. Expression of CD45 polypeptides is closely regulated within the hemopoietic system. The genes coding for mouse, rat, and human CD45 have been cloned recently (Saga et al. 1986 and Ralph et al. 1987). These results showed homology in sequence and organization among species. cDNA clones isolated from different cell types or cell lines revealed that alternative splicing of a single gene is a major mechanism for heterogeneity on various leukocyte types. CD45 molecule is rich in attachment sites for O-linked sugars. The extensive glycosylation of the molecule could provide another mechanism for generating heterogeneity in CD45 molecules (Lefrancois et al. 1985). The function of CD45 is not clear. Direct evidence for the precise role of the CD45 molecule is lacking. A role in cell-cell interaction and signal transmission have been postulated. Antibodies to CD45 may potentiate or inhibit proliferative

responses and inhibit NK or specific CTL (Tighe et al. 1987). The unusual molecular structure and ubiquity of CD45 in leukocytes, suggest that it plays a central role in the function of hematopoietic cells, maybe in association with other molecules.

REVIEW OF THE LITERATURE

MONOCLONAL ANTIBODIES

The monoclonal antibody technology has revolutionized the study and practice of immunology as well providing tools of immense value in many areas of biology and medicine. In 1975, Kohler and Milstein demonstrated that myeloma cells could be fused with antibody producing cells called B lymphocytes, and the resultant hybrids produced antibodies. When the fusion process and selection of single cell clones is complete, the daughter cells are known as "hybridomas". The hybrid cells inherit some characteristics from both parents, grow as malignant cells, and produce monospecific antibody.

Hybridoma production usually involves fusion between immunized spleen cells which contain the antibody-producing B lymphocytes and mutant myeloma cell partners of the same inbred strain. Selection of hybridomas involves the use of enzyme deficient parental myeloma mutant cells and appropriate selective media. Thymidine kinase (TK) and hypoxanthine-guanine phosphoribosyl transferase (HGPRT) are necessary for the salvage pathway of DNA synthesis. The mutant cells (HGPRT⁻) normally synthesize purines and pyrimidines de novo. A common agent used in selection of hybridomas is Hypoxanthine/Aminopterin/Thymidine (HAT) medium. In the selection medium HAT, aminopterin is the main component. Thymidine and hypoxanthine serve as

substrate for TK and HGPRT enzymes respectively.

Aminopterin is a dihydropholic acid analog which blocks the de novo synthesis of DNA. Since the myeloma mutant cells do not have the HGPRT enzymes, they are incapable of using the salvage pathway. However, hybrid cells will grow in the presence of aminopterin, since they obtained their HGPRT enzymes from the normal lymphocytes fusion partners.

Myeloma cells and myeloma-myeloma hybrids will not survive in aminopterin-containing medium. Unfused spleen cells can also use the salvage pathway, but these cells do not tend to grow for long periods of time in culture medium devoid of the required growth factors.

Monoclonal antibodies are immunoglobulins of immense value and interest. This methodology allows the production of endless quantities of a single antibody against a particular antigen. Monoclonal antibodies are widely used in biological research today, and in newly developed clinical applications such as diagnosis and treatment of cancer, hematopoietic tumors, and in bone marrow and kidney transplantation.

A BRIEF OVERVIEW OF THE DIFFERENTIATION AND FUNCTION OF T LYMPHOCYTES

Advances in T Cell Immunobiology

Understanding of complex cell-cell interactions involved in the induction and regulation of T cell responses has been facilitated in recent years by advances

in several areas. These include: 1) the development of in vitro methods for characterization and identification of T lymphocyte subsets by cell surface markers; 2) the development of new techniques for the isolation of highly purified subclasses of T lymphocytes on the basis of expression of cell surface markers; 3) the development of in vitro techniques to discriminate functional properties and interactions of the isolated subsets of T lymphocytes with other cells; 4) the correlation of normal and abnormal in vitro functional properties of T lymphocyte subpopulations with in vivo disorders of the immune response; 5) and lastly recent advances in the field of molecular biology which have led to a better understanding of the function and regulation of gene expression in the cells of the immune system. These areas will be briefly discussed below.

Functionally Distinct Subsets of T cells

An extraordinary degree of heterogeneity exists within the T cell population despite the morphologic similarity of T lymphocytes. The genetic program of the human T lymphocyte is complex and includes immunoregulatory functions, the capacity to recognize specific antigens, and the ability to execute unique effector functions. T lymphocytes proliferate in response to soluble and cell surface antigens as well as polyclonal activators,

including the mitogens phytohemagglutinin (PHA) and concanavalin A (Con A) (Chess et al. 1974). They mediate cytotoxic killer activity in cell-mediated lympholysis (CML) (Sondel et al. 1975). T cells also produce mediators and are involved in virtually all regulatory interactions, including helper, inducer, and suppressor cell function (Reinherz et al. 1979a-f).

Correlation of Functional Properties of Lymphoid Cell Subsets and Surface Marker Phenotypes

Understanding of the heterogeneity of lymphoid populations was made possible through the discovery of selective expression of specific cell surface antigens on lymphoid cells and the description of functional properties of the cells possessing these antigens. Extensive classification of lymphocyte subsets has been accomplished through the development of specific monoclonal antibodies coupled with the analytical and separation capabilities of fluorescence-activated cell sorting. Important in this regard is the fact that the expression of a particular cell surface antigen may correlate with functional properties or the differentiation state of that cell. However, in many cases the role of the cell surface antigen is not known. Monoclonal antibodies to membrane molecules can be used to positively or negatively select identified subpopulations of cells for further functional, biochemical or genetic studies and also to study the function of the molecule

directly by monoclonal blocking studies.

Cluster Differentiation (CD) Antigens of T cell Subsets

T cells are subdivided into two major subclasses, CD4 and CD8 cells (Reinherz and Schlossman, 1980a). The CD4 and CD8 glycoproteins are nonpolymorphic members of the immunoglobulin gene superfamily which are expressed on the surface of functionally distinct populations of T lymphocytes. These surface molecules are identified by monoclonal antibodies. In humans the CD4 molecule is identified by antibodies OKT4 and Leu3. The human CD8 molecule is identified by OKT8 and Leu2. The mouse homologues are L3T4 for CD4 and LyT2/LyT3 for CD8. Both CD4 and CD8 are thought to be markers of T-cell differentiation. The expression of these molecules is correlated with discrete stages in maturation of thymocytes and T cells. There is a small fraction of thymocytes that express neither CD4 nor CD8; these immature "double negative" cells are possible precursors to thymocytes that express these antigens. The majority of thymocytes are double-positive cells expressing both CD4 and CD8; and the more mature thymocytes as well as peripheral T cells, exclusively express either CD4 or CD8.

CD4 Cell Subsets

The CD4 population is heterogeneous for expression of several phenotypic determinants. Thomas et al. 1980 showed

expression of OKT17 antigen defined a T4+ subset comprising radiosensitive suppressor inducer cells and radioresistant helper cells, whereas the T4+OKT17- subset contains only radiosensitive helper cells and is devoid of suppressor/inducer activity. More recently Morimoto and co-workers, (1985a); described a moAb which was obtained by immunization of mice with primate Aotus trivirgatus PBL. Binding by their moAb, anti-2H4 defined on functional separation of CD4 cells as suppressor inducer cells or as helper cells. T4+2H4+ cells induce T8+ suppressor cells, and T4+2H4- cells induce B cell immunoglobulin synthesis in humans and rhesus monkeys. Another moAb obtained by immunization of mice with Sanguinus oedipus lymphocytes (Morimoto et al. 1985b) called anti-4B4, positively defines the human helper inducer subset of lymphocytes, i.e., the reciprocal population to that defined by anti-2H4. The heterogeneity of the CD4 subset of lymphocytes has been defined functionally, with the new moAbs described above, as well as with a series of antibodies which react with distinct non-competing epitopes of the T4 molecule.

T Cell Subsets based on MHC Restriction

The majority of T lymphocytes, and those best-characterized at the present time, recognize foreign antigens as peptide fragments associated with self major histocompatibility complex (MHC)-encoded proteins. The

reason for the association of T cell receptors with self MHC-encoded cell surface molecules has been difficult to understand, and controversy surrounds many aspects of MHC restriction in T cell specificity. There are two major classes of MHC molecules. MHC class I molecules are found on the surface of most somatic cells, while MHC class II molecules are expressed selectively on the surface of cells involved in immune responses such as B cells, activated T cells and macrophages. T cells are subdivided by the cell surface expression of the CD4 and CD8 markers. CD4T cells recognize foreign protein antigen fragments associated with self class II MHC molecules, while CD8T cells recognize foreign protein antigen fragments associated with self class I MHC molecules. T cells also respond to polymorphic differences in MHC molecules. Recognition of allogeneic or non-self allogeneic MHC molecules largely follows the pattern of foreign antigen recognition, with CD4 T cells predominantly recognizing class II MHC polymorphisms, and CD8 T cells largely recognizing class I MHC polymorphisms.

Diverse Functions of CD4 Cells

CD4 cells comprises a subset of mature T lymphocytes which possesses regulatory functions and includes helper cells and suppressor inducer cells (Janeway et al. 1988).

The roles of CD4 cells are very diverse and include:

a) Proliferation: CD4+ cells proliferate in response to both soluble antigens and cell associated antigen;

- b) Helper cell function: CD4 cells are able to induce B cells to secrete immunoglobulin and precytotoxic T cells to become cytotoxic effector cells (Reinherz et al. 1980b);
- c) Lymphokine release: functional activity of CD4T cells can be assessed by release of effector molecules upon antigenic stimulation. CD4 cells produce a variety of lymphokines. Production of interleukin-4 (IL-4) correlates with the ability to help normal B cells to make specific antibody (Mosmann et al. 1986 and Killar et al. 1987). Production of interleukin-5 (IL-5) is involved in both specific and polyclonal B cell activation (West et al. 1987); while the production of interferon s (IFNs) and lymphotoxin (LT) correlate with delayed type hypersensitivity (DTH) and cytolytic capacity of CD4T cells clones (Tite et al. 1985 and Mosmann et al. 1986). Interferon s activates macrophages and upregulates membrane expression of MHC gene products;
- d) Induction of suppression: interactions between CD4+ and CD8+ cells are required to induce suppression of immunoglobulin production in systems driven by antigens, pokeweed mitogen (PWM) and in autologous mixed leukocyte cultures (MLC) (Thomas et al. 1980; Morimoto et al. 1981; Morimoto et al. 1982; Gatenby et al. 1982); CD4 suppressor cells also have a role in allograft tolerance (Bruce Hall et al. 1988);
- e) CD4 T cell have a functional dichotomy. One functional

set is the major source of help in antibody responses. The other set's major function is to activate macrophages by the secretion of IFNs and to mediate cellular cytotoxicity (Janeway et al. 1988).

CD4 Molecule/Immunoglobulin Supergene Family

Sequence analysis of CD4 (Maddon et al. 1985) and CD8 (Littman et al. 1985) cDNA clones has demonstrated that they are both homologous to immunoglobulin genes, making them members of the immunoglobulin supergene family. The CD4 and CD8 receptors are believed to enhance T-cell responsiveness by increasing intercellular adhesion and or by transmitting an intracellular signal. That CD4 and CD8 molecules are capable of enhancing T-cell responsiveness has been demonstrated by the ability of anti-CD4 and anti-CD8 moAbs to inhibit the responsiveness of CD4+ and CD8+ T cells, respectively. This concept has been substantiated in transfection studies with recombinant cDNA for CD8 and CD4. Sleckman et al. (1987) and Gay et al. (1987) showed that when CD4 cDNA was transfected into cells that normally do not express CD4 these cells demonstrated a CD4 enhancement of responsiveness as compared with non-CD4 expressing cells. A similar pattern was found in the CD8 system (Ratnofsky et al. 1987; and Gay et al. 1987). CD4-mediated enhancement was observed when MHC class II molecules were expressed on the surface of the antigen-

presenting-cell (APC). Recently, Doyle et al. (1987) showed that MHC class II molecules bind the CD4 receptor. This suggest the ligand for the CD4 receptor is the MHC class II molecule. Similarly, Ratnofsky et al. (1987) suggested that MHC class I molecules are the ligand for CD8.

LEUKOCYTE COMMON ANTIGEN:

FAMILY OF GLYCOPROTEINS

The leukocyte common antigen (LC-A) family of glycoproteins was originally described in the rat by Trowbridge et al. (1975) and Fabre et al. (1977) by using conventional absorbed rabbit anti-rat lymphocyte sera. This antigen was detected on thymocytes, bone marrow cells, peripheral lymphocytes and macrophages, but not on other tissues. Sunderland et al. (1979) described a method for purification of a LC-A from rat thymocytes. The membrane fraction obtained after purification was used to immunize Balb/c mice. A moAb produced by this immunization procedure precipitated a 150 Kd glycoprotein which was found to be present on 95% of thymocytes, bone marrow cells and thoracic duct lymphocytes.

The LC-A were described in mouse by immunoprecipitation of solubilized thymocytes lysates with rabbit anti-mouse thymocyte sera, (Trowbridge et al. 1975). Using ^{125}I surface labelled thymocytes, two major bands were seen in autoradiographs. One of these bands with a

molecular weight (mw) of 25 Kd is the Thy-1 antigen. This is a polymorphic T cell antigen which is expressed differentially at different stages of T cell maturation. The other band contained a membrane glycoprotein of 200 Kd. The latter molecule, termed T200, was found to be a major differentiation antigen of mouse lymphoid tissue. Later on, Trowbridge, (1978) developed a monoclonal antibody against the mouse lymphocyte surface glycoprotein, T200. Using this antibody to examine spleen and thymocyte cell lysates labelled with ^{125}I , these investigators showed that there were some structural differences between the T200 antigens expressed on B and T lymphocytes. The B-cell glycoprotein consisted of at least one component of mw 220 Kd, while the T-cell glycoprotein had a mw of 190 kd. Coffman et al. (1981) described what they called B220: a B cell-specific member of the T200 glycoprotein family. This was accomplished by the characterization of a moAb generated by immunization with a pre-B cell tumor derived by infection of a mouse with the Abelson murine leukemia virus. This antibody was specific for the 220 Kd form of T200. Human LC-A were described by Dalchau et al. (1980). A murine moAb was prepared after immunization with a human lymph node preparation which was depleted of surface immunoglobulin positive cells and by doing so this population was enriched for T lymphocytes. The moAb produced recognized the human homologue of the LC-A. It was

found that the human homologue of the LC-A is a major component of human leukocyte membranes, with identical mw and tissue distribution to the rat LC-A. Dalchau et al. (1980) also showed that B lymphocytes have less LC-A than T lymphocytes, and that the LC-A content of human thymocytes increases as they mature.

In 1981 this same group reported findings that indicated that the LC-A or "molecule" is not a single, discrete entity, but a family of structurally related molecules with possibly different functions; they described a moAb that reacted strongly with B lymphocytes and did not react with granulocytes, and reacted only weakly with a small subpopulation of thymocytes and peripheral T lymphocytes; biochemical studies showed that this moAb recognized a 215 Kd glycoprotein. These findings were different from that seen with moAbs to the LC-A, where 100% of the leukocyte population were positive.

Houssaint et al. (1987) described the LC-A in chickens; they described a moAb that detected early stages of embryonic development in various hematopoietic compartments. The chicken moAb precipitates two antigens present on the surface of PBL with mw of 200 and 215 Kd which are probably the homologues of the T-200 and B-220 antigens previously described.

Sheep LC-A was described by Mackay et al. (1987). They showed the cellular expression of different LC-A molecules

in sheep using two moAbs which react with specific epitopes of LC-A present either on all leukocytes, or uniquely expressed on thymocytes or as the p220 component of LC-A, expressed only by B cells and a unique lymphocyte subset.

Differential Expression of T200 antigen on T cell subpopulations:

Rat T cell subsets defined by T200

Spickett et al. (1983) proposed that rats contain at least three functionally distinct subsets of T cells on the basis of expression of the MRC OX-22 antigen. MoAb to MRC OX-22 detects rat T cells that mediate graft-versus-host reactions and those responsible for the suppression of antibody synthesis in hosts undergoing these reactions. MRC OX-22 is also expressed on all B cells, 50% of bone marrow cells and 2% of thymocytes. Biochemical studies revealed that this antibody reacts with the high molecular weight form of the LC-A family. Comparative studies on human LC-A suggest that the molecular heterogeneity of this group of glycoproteins has been conserved between rat and man.

Human CD4 cells defined by T200

CD4/2H4 subset of T lymphocytes has been recently subdivided on the basis of the presence of the 200 and 220 Kd molecules which have been associated with the T200 family of glycoproteins (CD45). The moAb which subdivides the CD4 subset is called anti-2H4 and was described by

Schlossman group in 1985 (as discussed above); this antibody defines a determinant on human and on rhesus monkey T lymphocytes expressed on a subpopulation of cells which activates the CD8-bearing suppressor cell. This study demonstrated that the antigenic determinant recognized by anti-2H4 is closely conserved in phylogenetically disparate primate species and suggested that the T-cell subpopulation which anti-2H4 defines, an inducer of suppression, must be functionally important.

Specific Functions of 2H4 cells

Since the original description of 2H4, there have been several reports on the functional and structural significance of the 2H4 molecule.

2H4 antigen expression and concanavalin induced immunoregulatory function

The 2H4 molecule may play a crucial role in the generation of suppressive signals of Concanavalin-A (Con-A)-activated cells (Morimoto et al. 1986). Con A-induced suppressor T cells have been shown to down-regulate the proliferative responses to antibody response to specific antigens (Morimoto et al. 1978) and the proliferative response to soluble antigens (Hubert et al. 1976); to mitogens (Shou et al. 1976 and Sakane et al. 1977); and to alloantigens in mixed lymphocyte reactions (MLR) (Shou et al. 1976 and Sakane et al. 1977). Morimoto et al.

(1986) showed that the 2H4+ subset of T cells can be activated by Con A to suppress both antibody production by B cells and T cell proliferation in the MLR. They also showed a correlation between the level of suppressive activity within the 2H4 subset and the membrane density of 2H4 antigen expression. They found increasing the concentration of Con A resulted in a increase of 2H4 expression, as well as a concomitant increase in suppression.

2H4 expression and the AMLR

Takeuchi et al. (1987a) and Takeuchi et al. (1987b) reported similar results using the autologous mixed lymphocyte reaction (AMLR) system. The AMLR is though to play an important role in generating suppressor inducer signals and in down-regulating the immune response following self MHC recognition. In this study 2H4 antigen expression was shown to be increased on CD4+ cells during activation in AMLR. Furthermore, treatment of AMLR activated CD4 cells with anti-2H4 but not anti-T3 or T4 antibody abolished the suppressor inducer function of these cells, suggesting that the 2H4 molecule but not the T3-receptor complex plays a important role in the generation of suppressor inducer signals in the AMLR system.

2H4 expression in autoimmune disease

Abnormalities of suppressor T cell function have been reported in patients with systemic lupus erythematosus (SLE) by Abdou et al. (1976) and others. Morimoto et al. (1987a) reported the findings that SLE patients has a significant smaller percentage of circulating T4+2H4+ suppressor-inducer cells than normal individuals, they also reported that active SLE and renal disease patients had the greatest reduction in the % of this subset. Patients with multiple sclerosis which is though to be a T cell-mediated autoimmune disease associated with abnormalities in immunoregulation; a selective loss of the suppressor-inducer T-cell subset (T4+2H4+) was found among this patients (Morimoto et al. 1987b).

Molecular Analysis of T200 antigens

Biochemical studies done by Rudd et al. (1987) showed that the 2H4 antigen is comprised of at least three distinct polypeptides of 125, 200 and 220 Kd. The 200 and 220 Kd polypeptides are associated with the highest isoform of a pool of five distinct LC-A /T200 antigens. Pulse-chase analysis and endoglycosidase treatment revealed that the 125 Kd form of the 2H4 antigen is nonglycosylated, while the 200 and 220 polypeptides are structurally related and derived by N-and O-linked glycosylation of two nascent subunits of 150 and 160 Kd mw. The function of the T4+2H4+ subset could be blocked by an antibody reactive with the

LC-A/T200 isoforms enriched with O-linked oligosaccharides at 200 and 220 Kd.

The genetic basis for the generation of the diversity of LC-A molecules was demonstrated in the studies of Streuli et al. (1987). These investigators showed that a single human LC-A gene can generate multiple forms of mRNAs that encode proteins of different structures. The isolation of the LC-A clones and Northern blot analysis demonstrated the existence of at least five distinct LC-A mRNAs. These different mRNAs result from differential usage of three exons by alternative splicing.

Comparisons in the amino acid sequence between human LC-A and mouse revealed that three regions of LC-A, the signal peptide, transmembrane peptide, and cytoplasmic domain are highly conserved (91, 91, and 84% respectively). The extracellular domain is poorly conserved (39%). However a high conservation of cysteine residues has been found in the extracellular domain which suggested maintenance of a basic structural order in the LC-A family of proteins. LC-A proteins are among the largest of defined membrane protein molecules.

Other large membrane proteins are the receptor of epidermal growth factor (EGF), the platelet-derived growth factor, the product of the protooncogene c-fms and the oncogene product neu. Although no sequence homology has been found among LC-A and these growth factors receptors and

epidermal growth factor receptor-related proteins, the similarities in overall architecture suggest that LC-A may function as cell-surface receptors. Alternatively these molecules could facilitate the interactions of lymphocytes among themselves and with other cells of the immune system by acting as signal transducers.

Streuli et al. (1987) reported the molecular basis of the 2H4 epitope expression by using cDNA clones encoding four of the LC-A. LC-A diversity is generated from a single gene by differential usage of three exons near the NH₂-terminus. They synthesized in vitro the extracellular domains of the LC-A by using cDNA clones which corresponded to four of the different forms of the LCA molecules. These molecules when immunoprecipitated with anti-2H4 antibody demonstrated that exon A is required for the expression of the 2H4 epitope.

In the last 3rd International Workshop on Human Leukocyte Differentiation Antigens (Oxford, 1986), the LC-A antigens were divided into two groups. One group is termed conventional antigens. Another group is termed restricted antigens. Conventional antigens bind to all leukocytes (CD45) and restricted antigens recognize a more limited set of cells (CD45R). This distinction correlates with different molecular specificities revealed by immunoprecipitation.

Five epitopes of the human CD45 antigen have been

defined and there is suggestive evidence for at least three others. In addition, there are at least two restricted (CD45R) epitopes.

T200 antigens and T cell Activation responses

Modulation of a function by antibody to membrane antigen implies a relationship between the function and the antigen. It has been reported by Harp et al. (1983) that modulation of proliferation in the the mixed lymphocyte culture (MLC), and the generation of allospecific cytotoxic cells, by moAb to a nonpolymorphic determinant on the T200 molecule. Thus, moAb to T200 molecule can alter the course of T cell alloimmune response to alloantigens by murine splenocytes. These modulations are manifested as late suppression of both responses. Thus, crosslinking the T200 molecule seems to be able to transduce either net positive or negative signals to the murine system. This very likely depends on the nature of the subset being recognized. In some cases enhancement of the response is seen. The enhancing effect is not seen in all cases and is only seen when the anti-T200 antibody is added to the assay at day 0 of MLC.

Ledbetter et al. (1985) identified a functional role of the p220 molecule by adding purified anti-p220 antibody to cultures of T cells activated by mitogens to measure antibody-specific modulation of proliferation rates. These antibodies at suboptimal doses of PHA caused strong

augmentation of proliferation without affecting PHA binding. Several lines of evidence suggest that the triggering of p220 molecules on T cell activation is via IL-2 receptor. More recently, Martorell et al. (1987) provided evidence that the T200 molecule could be involved in T cell proliferation by giving a second signal to the cell necessary for the induction of IL-2 secretion.

Structural and Functional Relationships of T200 antigens in Natural Killer Cell Activity

The "real" function(s) of the T200 antigens are yet not completely understood, although a role in cell-cell interaction has been postulated by Morishima et al. (1982). One possible role of the T200 molecules is in natural cytotoxic mechanisms. Natural killer cell activity is present in the nonadherent lymphoid cells of normal individuals. Natural killer (NK) effector activity to tumor target cells is associated with the large granular lymphocyte population and among other markers, T200 is expressed on NK cells.

Sparrow et al. (1983) described a moAb that recognizes the human homologue of T200, and had ability to specifically inhibit NK cytolytic activity against K562 target cells. They postulated that human T200 might have a receptor function in Nk cell recognition and binding to target cells because moAb-coated NK effector cells have a

reduced capacity to bind to K562 target cells. Similar findings have been reported for the murine system, in which antisera to the Ly-5 allodeterminants that reside on the T200 molecules Siadak et al. (1980) can specifically inhibit murine NK activity Kasai et al. (1979) and Cantor et al. (1979).

Not all antibodies to the human T200 molecule have an inhibitory effect on NK cell function. Comparisons made by Newman et al. (1983), showed two inhibitory anti-T200 antibodies and two noninhibitory anti-T200 moAbs, all were the same isotype. Studies by radiobinding assays showed that blocking antibodies bound to a T200 region called "A" and nonblocking to a region called "B" which was shown to reside between the membrane and region A. These results indicated that inhibition of NK lysis by T200 antibodies is a function of the site on the molecule to which the antibody binds.

Starling et al. (1987) showed mixtures of antibodies to different epitopes of the restricted or B-220 and non-restricted or CD45 molecule enhanced the NK inhibitory activity of weakly inhibitory moAbs. The inhibition was at post-conjugation formation stage of lysis at the effector cell level, and was seen with different target cells. This suggested that T200 molecules are involved in cytolytic signal transduction rather than only cell-cell recognition between NK effectors and targets.

T200 antigens and cytotoxic T cell effector activity

Many T cell surface molecules have been implicated in cytotoxic function by the ability of specific moAbs to inhibit T cell activity in vitro. It has been shown by Tighe et al. (1987) that moAbs to the LC-A prevent cytotoxic T cell effector function. Two different epitopes on LC-A molecule are able to inhibit antigen-specific lysis by cytotoxic T cells. Lefrancois et al. (1984) described neoantigenic determinants called CT; these CTL-specific activation antigens have been detected by moAbs that inhibit CTL function. These determinants are associated with the T200 glycoproteins and two other proteins of 140 and 85 Kd; also CT determinants are present on a secreted protein, gp155. Pulse-chase experiments suggested that CT determinants are added late in T200 biosynthesis and that these glycans are O-linked. Interleukin-2 (IL-2) was shown to influence the expression of the CT oligosaccharides present at the CTL cell surface (Lefrancois et al., 1984).

SUMMARY OF T200 MOLECULE

The human T200 molecule (CD45) is a polypeptide of 1120 aminoacids. Within the first 170 aminoacids, three alternative splicing arrangements have been detected. The rest of the molecule, consisting of 222 extracellular aminoacids, a transmembrane sequence and a large intracytoplasmic domain of 707 aminoacids, is the same in

all cDNA isolated (Ralph et al. 1987). T200 molecule is rich in attachment sites for O-linked sugars; thus suggesting a mechanism for polymorphisms within CD45 molecules (Lefrancois et al. 1985).

The function of CD45 remains unclear. The large intracytoplasmic domain has suggested an analogy with neural cell adhesion molecule (N-CAM) (Ralph et al. 1987) and this domain may also interact with the cytoskeletal protein fodrin (Bourguignon et al. 1985), suggesting a role in cell-cell interaction. The phosphorylation of the cytoplasmic domain described by Omary et al. (1980), suggests that T200 molecule may also act in signal transmission.

Antibodies to CD45 may potentiate or inhibit proliferation responses and inhibit NK or specific CTL. These data plus unusual molecular structure and ubiquity of CD45 on leukocytes, suggest that this molecule may play a central role in function of hematopoietic cells, perhaps in association with both cytoplasmic molecules and others at the cell surface.

MATERIALS AND METHODS

ISOLATION OF LYMPHOID CELLS

Normal rhesus monkey peripheral blood mononuclear cells (PBMC) obtained from heparinized blood samples were isolated on a ficoll hypaque density gradient (Pharmacia,

Piscataway, NJ, s.g. 1.077) as previously described (Thomas et al., 1982). The cells at the interface were washed 3X with RPMI 1640 containing 2mM L-glutamine (Gibco, Grand Island, NY) and resuspended to the desired concentration according to the experiment being performed.

PRODUCTION AND SCREENING OF MONOCLONAL ANTIBODIES

Balb/c mice were immunized with 1×10^7 rhesus monkey HLA-DR depleted PBMC and boosted after 4 weeks followed by fusion at 4th day after final boost. Spleen cells from immunized mice were fused with the myeloma cell line Sp2-0 using purified PEG solution to increase the frequency of hybrid cells and a standard procedure for fusion (Kohler et al. 1975).

Hybrids were screened initially for immunoglobulin production by an ELISA assay. The clones that were producing immunoglobulin were further screened by binding to rhesus monkey PBMC. Percentage Binding was detected by immunofluorescence after staining with FITC tagged goat anti-mouse Ig (Jackson Lab., West Grove, PA) and subsequent analysis in a fluorescence activated cell sorter (FACS). The selected hybrids were cloned and recloned by limiting dilution analysis in the presence of Balb/c thymus cells as feeders. Cloned cells were retested for binding to monkey PBMC. Hybridoma cells were maintained in vitro in RPMI 1640 containing 10% fetal calf serum (Hyclone, Logan, UT), 10% NCTC-109 medium for hybridomas (Whittaker, M.A.Bioproducts,

Walkersville, MD), 1X MEM non-essential amino acid solution (Sigma, St. Louis, MO), 1X insulin-transferrin-sodium selenite media supplement (Sigma, St. Louis, MO).

ELISA ASSAY FOR DETECTION OF IMMUNOGLOBULIN PRODUCING HYBRIDS

Limbro 96 well EIA plates were coated with 200 μ l of goat anti-mouse IgG (1.3 mg/ml) in carbonate buffer pH 9.6 and incubated overnight at 4°C. Plates were washed 5x with 0.05% Tween-PBS buffer, followed by blocking step with 5% HSA-PBS at 37°C for 30 minutes. Plates were washed with 0.05% Tween-PBS and incubated with 50 μ l of controls and hybridoma supernatants at room temperature for 2 hours. After incubation wells were washed 5x with 0.05% Tween-PBS and 50 μ l of 1:1000 dilution of goat anti-mouse alkaline phosphatase added to each well followed by incubation at room temperature for 1 hour. Plates were washed 5x with 0.05% Tween-PBS and incubation with 200 μ l of p-nitrophenyl phosphate disodium (1mg/ml) (Sigma, St. Louis, MO) in carbonate buffer pH 9.6 for 30 minutes at room temperature; plates were kept in dark. Reaction was stopped with 25 μ l of 3M NaOH and plates were read on a microplate reader.

ASCITES PRODUCTION

Balb/c mice were pristaned with 0.5 ml of pristane (2,6,10,14-tetramethylpentadecane; Aldrich Chemical Company, Milwaukee, WI) intraperitoneally prior to the

injection of hybridoma cells. Hybridoma cells were washed 2x prior injection into mice and approximately 5×10^6 to 1×10^7 cells were injected.

ISOTYPE OF IMMUNOGLOBULINS

Hybrid cell isotype was determined by using a single radial immunodiffusion (SRID) system Diffugen Tago (Tago, Inc; Burlingame, CA).

ANTIBODY PURIFICATION

Monoclonal antibody anti-7H5 was purified initially by 18% sodium sulfate precipitation of immunoglobulins present in the ascites, followed by gel filtration high pressure liquid chromatography (Waters 990) using a Protein-Pak DEAE SPW, 7.5mm x 7.5 cm column. Purified anti-7H5 monoclonal antibody was used for the studies listed below.

BIOTINYLATION OF PURIFIED ANTI-7H5 ANTIBODY

Biotin was linked to anti-7H5 monoclonal antibody by using a solution of DEAE-purified immunoglobulin and N-hydroxysuccinimidobiotin (NHS-biotin) (Sigma, St. Louis, MO) as described (Updyke et al. 1986). Briefly: 1 mg of purified immunoglobulin in 0.1 M Hepes, (pH 8.0) was added to 0.932 ml. 68 ul of NHS-biotin (2mg/ml in dimethyl sulfoxide) was added and after 4 hours of incubation at room temperature the tube was transferred to a Centriprep 30 microconcentrator tube (Amicon, Danvers, MA) and washed

five times with 2 ml of 0.1 M Hepes (pH8.0), 0.05% sodium azide (NaN₃). Biotinylated anti-7H5 antibody was reconstituted at 2 mg/ml with 0.1M Hepes (pH8.0), 0.05% NaN₃, and stored at -70°C.

FACS ANALYSIS ON RHESUS MONKEY CELLS

Rhesus monkey PBMC, bone marrow, spleen, lymph node cells and human cells and cell lines were washed with phosphate-buffered saline (PBS)/2.5% newborn calf serum/0.025% NaN₃. Samples of 2×10^5 cells were incubated with unconjugated and/or conjugated (Becton-Dickinson, Mountain View, CA) monoclonal antibodies. A previously established optimal titer of moAb was used in the binding studies. The final concentration of moAb was approximately 1.2 ug per 2×10^5 cells in a 0.1 ml volume. Cells which were sensitized with unconjugated moAb were subsequently washed twice and incubated with secondary goat anti-mouse immunoglobulin conjugated to fluorescein (Jackson Laboratories, West Grove, PA). Cells sensitized with biotinylated moAbs were washed and incubated with avidin (Becton-Dickinson, Mountain View, CA) conjugated to phycoerythrin or fluorescein isothiocyanate. Coated cells were washed twice with PBS-0.05% azide fixed in 1% paraformaldehyde and analyzed on a FACS system (Becton-Dickinson, Mountain View, CA). A panel of anti-human monoclonal antibodies that cross reacted with rhesus monkey PBMC were utilized in double labelling experiments with

anti-7H5 moAb. They are listed in table 8.

DEPLETION OF 7H5 POSITIVE CELLS WITH moAb AND MAGNETIC BEADS

Depletion of cells populations which express 7H5 was done by incubating the cells with anti-7H5 for 45 minutes at 4°C, followed by washing 3x with RPMI 1640 and adding Dynabeads M-450 coated with goat anti-mouse IgG (Dynal Inc., Fort Lee, NJ). Prior to adding the anti-mouse IgG coated Dynabeads, a sample of labelled cells was taken to determine the percentage of 7H5 positive by FACS analysis. Dynabeads were added at a ratio of 15 beads per positive cell. Incubation of cells and Dynabeads was done at 4°C for 45 min. with constant orbital shaking. Magnetic beads were removed by placing cell/beads mixture against a magnetic field, and cells were removed with a pasteur pipette. Cells were washed twice, counted and resuspended with the appropriate media according to the experiment being performed. A control for non 7H5 depletion was done by incubating a sample of "depleted" cells and goat anti-mouse FITC and FACS analysis. This cell population should be negative for 7H5 antigen, as compared with pre-sort sample.

HUMAN CELL LINES

B cell lines (Raji and Daudi), T cell lines (HSB and Jurkat), monocytic leukemia line U937, eritroleukemia line K562 and myeloid leukemia line HL60 were available in the

laboratory. These cell lines were used to test the reactivity of anti-7H5 moAb in human tissues.

IN VITRO PROLIFERATIVE RESPONSES OF T LYMPHOCYTES

In these studies, rhesus monkey PBMC were cultured at 1×10^6 cells/ml (100 μ l) in triplicate samples in 96-well microtiter plates in RPMI 1640 containing penicillin, streptomycin, 2mM glutamine, and 12.5% pooled normal rhesus monkey serum (NMS). The mitogens used to stimulate the PBMC were: phytohemagglutinin (PHA) (Wellcome Diagnostics, Greenville, NC), Concanavalin-A (ConA) (Calbiochem, La Jolla, CA), and Staphylococcus aureus (SPA) (Pharmacia, Upsala, Sweden). Dose response curves were always done. After 3 days in culture, cells were pulsed for 18 hours with 1 μ Ci of 3 HThymidine/well (NEM; 6.7 Ci/mmol specific activity). Cells were harvested onto glass fiber filters by using a cell harvester, and radioactivity was measured in a liquid scintillation counter.

CELL SURFACE 125 I-LABELLING; IMMUNOPRECIPITATION AND GEL ELECTROPHORESIS

Rhesus monkey PBMC were surface labelled with 125 I as described elsewhere (Hubbard et al. 1976). After iodination cells were washed twice with PBS containing 5 mM potassium iodide and then extracted into cell lysis buffer containing 10mM Iodoacetamide (Sigma, St. Louis, MO), 1% Nonidex P-40 (NP40) (Particle Data, Inc., Elmhurst, IL), 50mM

Tris, 150 mM NaCl, 0.02% NaN₃, 1mM Phenylmethyl sulfonyl fluoride, and 0.2 TIU (trypsin inhibitor unit)/ml, 20 KI U/ml of Aprotinin pH 8.0 (Sigma, St. Louis, MO). The mixture was incubated for 45 min. at 4°C. After incubation the cell lysate mixture was centrifuged at 12000 rpm in a Eppendorf microcentrifuge. The resulting supernatant lysates containing 10⁷-10⁸cpm of ¹²⁵I were mixed with 100 ul of anti-7H5 IgG1 mouse monoclonal antibody or an equivalent amount of irrelevant murine IgG1 myeloma diluted in PBS or Tris NaCl buffer. This mixture was incubated at 4°C on a rocker for a minimum of 4 hrs. After incubation, 50-75 ul of recombinant Protein-G sepharose (Zymed laboratories Inc., San Francisco, CA) was added and incubated at 4°C on rocker (1-3 hrs). Following six washes with PBS-0.5% NP40, 100 ul of reducing or non-reducing SDS-buffer was added to the pellet, which was boiled for 5 minutes, and then centrifuged for 3 minutes in Eppendorf microcentrifuge. Immunoprecipitates were analyzed on one dimensional 7.5% sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE) by the method of Laemmli., (1980). Autoradiographs were prepared by using intensifying screens (Cronex Lightning-Plus, Dupont de Nemours & Co., Wilmington, DE) with Kodak X-OMAT film at -70°C.

NK CYTOTOXICITY ASSAY

The assay was performed according to the method developed in our laboratory for rhesus monkey (Carver et

al. 1988). NK cells, freshly obtained from peripheral blood mononuclear cells were used as effector cells. Target cells were the human myeloid, NK sensitive cell line K562. Target cells were labelled with ^{51}Cr (150 $\mu\text{Ci}/10^6$ cells) for an hour at 37°C , washed 3x with RPMI containing 10% FCS and adjusted to 1×10^4 cells/ml. Effector and target cells were mixed in round bottom microtiter plates. Effectors were titrated in doubling dilutions to obtain E/T ratios of 20:1 down to 2.5:1. The assay was performed in triplicate in 0.2 ml RPMI 1640 containing 2mM L-glutamine and 10% FCS. The cultures were centrifuged for 5 minutes at $50 \times g$, and incubated at 37°C for four hours in 5% CO_2 incubator. After dispersal of the cell pellets and subsequent centrifugation at 1000 rpm for 7 minutes, the supernatants were collected with a supernatant collection system (Skatron, McLean, VA). Radioactivity in the supernatant was measured in a Beckman gamma counter. Spontaneous release of radioactivity was determined in culture medium without effector cells and was usually 10-15% of the maximum release. Maximum release of ^{51}Cr was determined by adding 5% liquinox detergent solution in place of the effector cells. The percentage of cell mediated lysis was calculated by the formula:

$$\frac{\text{CPM experimental release} - \text{CPM spontaneous release}}{\text{CPM maximum release} - \text{CPM spontaneous release}} \times 100$$

$$\text{CPM maximum release} - \text{CPM spontaneous release}$$

CELL MEDIATED LYMPHOLYSIS (CML)

Preparation of effectors

The rhesus monkey CML technique was developed in Dr. J. Thomas' lab. (Thomas et al. 1988). Briefly, responder and stimulator PBMC were passed over a Sephadex G10 column to deplete adherent cells, using a modification of the method of Ly and Michael., (1974) as described previously. Stimulator cells received 2000 rads by using a $^{137}\text{Cesium}$ source. Responder and stimulator cells were resuspended in Clicks media (Altick and Associates, River Falls, WI), supplemented with 2.5% fresh autologous serum plus 25 U/ml of recombinant interleukin-2 (rIL-2) (Amgen, Thousand Oaks, CA). Cells were plated into a flat bottom 96 well plate at 2×10^5 cells per well in 100 ul and 7×10^3 stimulator cells were added in a 100 ul. Plates were incubated at 37°C in 5% CO_2 , cultures were fed on day 2 with Clicks containing 2.5% autologous serum by removing 100 ul of supernatant and adding 100 ul of fresh medium. On day 6, effector cells were transferred to V bottomed plates and centrifuged at 700 rpm for 7 minutes. After centrifugation, 100 ul of supernatant were removed and 100 ul of fresh Clicks medium added back to wells. The plates were centrifuged again. After centrifugation, 100 ul of supernatant were removed and 50 ul of 50% pooled NMS in Clicks was added to wells.

Preparation of targets

The PBMC target cells were prepared from defibrinated blood samples. Cells were isolated on Ficoll and passed over Sephadex as described above. Nonadherent cells were cultured for 48 hours at a concentration of $3 \times 10^6/\text{ml}$ in RPMI 1640 containing 10% pooled NMS and 100ul (900ug) of stock PHA (Burroughs Wellcome, Greenville, NC) at 37°C in 5% CO_2 incubator. PHA blast target cells were centrifuged and resuspended in 200 ul of RPMI 1640 and 75 uCi of ^{51}Cr added, followed by one hour incubation at 37°C . The ^{51}Cr target cells were then washed 3x with 10% NMS-RPMI, counted and resuspended to $5 \times 10^4/\text{ml}$ in Clicks medium, and 50 ul were added to effector cells.

Calculation of results

Spontaneous release was calculated in the wells that did not contain effector cells. Maximal release values were obtained by incubation of the target cells with 5% liquenox. Supernatants were harvested after 6 hours of incubation at 37°C using the Skatron supernatant collection system (Skatron, McLean, VA) and counted in a Beckman gamma 7000 counter. The percent lysis were calculated as in the NK assay described above.

REMOVAL OF ADHERENT CELLS

Adherent cells were depleted on Sephadex G-10 (Pharmacia, Piscataway, NJ) according to the method of Ly

and Michael, (1974) with minor modifications (Thomas et al. 1982). PBMC were resuspended at 2×10^7 /ml in RPMI containing 2.5% human serum albumin (HSA) and applied to a 5 ml column of Sephadex G-10 which was equilibrated with HSA-RPMI. After 45 minutes at 37°C, cells were eluted with 20 ml of warm HSA-RPMI medium and washed 2X with RPMI.

BLOCKING STUDIES WITH PHA AND ConA

Blocking studies with PHA and ConA were performed by incubating 1×10^6 monkey PBMC/ml in RPMI 1640 containing 2 mM L-glutamine (Gibco, Grand Island, NY) with different concentrations of PHA (3, 30, and 300 ug/ml) or ConA (1.6, 16, and 160 ug/ml). Tubes containing cells were placed at 37°C for 1 and 1/2 hrs. Cells were washed 2x with RPMI 1640 and one set of the cells that were incubated with ConA were further incubated with 25 mM α -methyl mannoside in RPMI 1640 for 1/2 hr at room temperature. Cells were washed 3x in RPMI 1640 followed by incubation with anti-7H5 moAb for 45 min at 4°C. Cells were washed 2x and incubated with secondary goat anti-mouse immunoglobulin conjugated to fluorescein (Jackson laboratories, West Grove, PA). Cells were washed 2x with PBS-0.05% azide, fixed in 1% paraformaldehyde and analyzed on a FACS system (Becton-Dickinson, Mountain View, CA).

CAPPING STUDIES AND VERIFICATION IF 2H4 IS COCAPPED BY 7H5

These studies consisted of incubating 2×10^5 monkey

PBMC/tube with 2.5 ug of purified anti-7H5 moAb in 100 ul of RPMI 1640/2.5% fetal calf serum at 37°C for 30 min. After incubation, cells were washed 2x with RPMI 1640 and placed back at 37°C for time course incubations in RPMI 1640/2.5% fetal-calf serum. At different incubation times cells were removed from the incubator, washed 2x with RPMI 1640 and then:

- a) labelled with goat anti-mouse immunoglobulin conjugated with fluorescein, washed 2x with PBS-0.05% azide, fixed in 1% paraformaldehyde, and analyzed on a FACS system.
- b) labelled with anti-7H5 moAb biotinylated and anti-2H4 moAb conjugated to fluorescein and incubated for 45 min. at 4°C. Cells were washed 2x with PBS/2.5% newborn calf-serum/0.025% azide followed by incubation with streptavidin conjugated to phycoerythrin. Cells were washed 2x with PBS-0.05% azide, fixed in 1% paraformaldehyde and analyzed by two color analysis FACS analyzer.

RESULTS

PRODUCTION OF MURINE moAbs AGAINST MEMBRANE DETERMINANTS PRESENT ON RHESUS MONKEY PBMC

The attempts to generate murine moAbs against epitopes present in monkey PBMC are summarized in Table 1. Fusion 1 contamination problems were solved by improving sterile techniques and also by placing fusion plates on sterile trays inside incubator. Fusion 2 problems with PEG toxicity were solved by using purified PEG (generously given by Dr. Peter Cresswel from Duke University). Following successful fusion several steps were required in order to have a desirable antibody producing clone. These steps involved initial screening by ELISA assay to detect antibody secreting hybrids and by FACS analysis to determine the binding titer to monkey lymphocytes. The hybrids presenting the highest binding titer were selected and cloned by limiting dilution (Table 2). These selected hybrid clones (IIC2, IID4, IIB4 and IB4) were divided into four series (A,B,C ,and D) according to the percentage of monkey PBMC that bind to hybrid cells and were analyzed by FACS.

Figure 1 shows FACS analysis of these selected clones. The selection of antibody producing clones for expansion and further study were done in two steps. Table 3 summarizes the steps involved: Clones were studied by the antibody binding pattern in FACS analysis and further divided into series according to the

binding pattern. The binding pattern represents the number of cells that bind to monkey PBMC and the relative brightness of fluorescence in the cell population identified. Representatives of each pattern serial were titrated for antibody production by an ELISA assay. Series A,B,C,and D clones that had titers above 1:800 were further studied.

Series B (6/11) and Series C (4/8) clones presented antibody titer above 1:800. Figure 2 shows FACS analysis of some series B clones patterns that react with rhesus monkey PBMC. These 10 selected (Series B and C) clones were expanded as ascites preparations.

Table 4 summarizes the results obtained with series B and C clones. Ascites production in series B: 3/6 clones showed rapid growth and titers above 1:1600. In series C: 1/4 clones had rapid growth and antibody titers above 1:1600. Three moAbs from series B and one from series C were further studied by immunoprecipitation of monkey PBMC ^{125}I labelled membrane antigens with ascites moAbs.

Figure 3 shows the results obtained in immunoprecipitation studies of series B and C moAbs. SDS-PAGE studies of these moAbs revealed the presence of a high molecular weight protein of approximately 200 Kd by anti-7H5 moAB. Antibodies anti-2A12, and anti-2G1 immunoprecipitated a surface protein of approximately 120 Kd. Series C moAb, anti-2D10 immunoprecipitated a protein

of approximately 130 Kd.

High molecular weight membrane protein (T200) have been extensively studied in rats, mice, and humans. Having a T200 moAb to rhesus monkey lymphocyte determinant could provide a unique tool to study the biochemical and functional role of T200 molecules in the immune system of these primates. The main questions we faced at this point were whether anti-7H5 defined a T200 antigen in the monkeys and what functions might it have. Table 5 outlines the additional studies which were done with anti-7H5 moAb. These are discussed in detail below.

PURIFICATION OF moAb ANTI-7H5 AND ISOTYPE DETERMINATION

In order to obtain large amounts of anti-7H5 moAb, Balb/c mice were used for ascites production. Approximately 30 mice were used. Each mouse generated about 4 ml of ascitic fluid. Anti-7H5 moAb was purified from the ascitic fluid by HPLC using a DEAE-column.

Figure 4 shows the plot (time x wavelength) of purification process. Approximately 18.5 minutes after sample injection a sharp peak was observed. A sample collector was attached to the HPLC and samples of 0.5 ml were collected during the 35 minutes run. Each individual sample isotype was determined. The IgG1 fraction corresponded to the large peak that was observed at 18.5 min. Every sample collected was tested for binding to

rhesus monkey PBMC and binding pattern compared to the ascites preparation. Samples that were IgG1+ and had similar binding to monkey PBMC were pooled together.

BIOTINYLATION AND FACS ANALYSIS OF BIOTINYLATED ANTI-7H5

The biotinylation procedure as described in materials and methods, takes the advantage of the fact that when biotin is bound to a macromolecule, it is still available for high affinity interaction with avidin. Affinity purified anti-7H5 moAb (1 mg/ml) was used in the biotinylation procedure. To determine the best working dilution of biotinylated anti-7H5, several antibody dilutions and streptavidin PE or FITC conjugated were tested. These studies demonstrated that anti-7H5 biotinylated at 1:64 dilution and 4 ul of streptavidin give the best binding to rhesus monkey PBMC. Figure 5 shows FACS analysis of rhesus monkey PBMC with anti-7H5 biotinylated.

ONE COLOR FACS ANALYSIS TO DETERMINE DISTRIBUTION ON VARIOUS MONKEY AND HUMAN LYMPHOID CELLS

Affinity purified anti-7H5 moAb was used for one color FACS analysis of various monkey lymphoid tissues. The data in Tables 6 and 7 summarizes the reactivity of 7H5 antigen in different monkey and human tissues.

Approximately 10% of monkey bone marrow cells; 68% of spleen cells, 60% of lymph node cells, and 70-80% of PBL

express 7H5 antigen. These results show that 7H5 antigen is expressed in high percentage in monkey lymphoid cells.

Figure 6 shows the results of FACS analysis (FL1 versus SSC) of the expression of 7H5 antigen on monkey lymphoid tissues.

Figure 6A shows that 10% of monkey bone marrow cells are positive; Figure 6B shows that 68% of monkey spleen cells are positive; Figure 6C shows that 60% of monkey lymph node are positive; Figure 6D shows that 70-80% of peripheral blood lymphocytes express 7H5 antigen.

The FACS plot suggests these are heterogeneous populations in that the positive cells have varying large angle light scatter patterns. In contrast to the positive binding to monkey lymphoid cells, Table 7 shows the absence of 7H5 antigen in human tissues. A large panel of human cell lines (T and B lymphocytes, monocytes, erythroid and myeloid), PBL and thymocytes were tested, and all were negative for 7H5 staining. These cells and cell lines represent different lineages and stages of maturation. The lack of reactivity of anti-7H5 moAb with human tissues, indicate that the T200 epitope recognized by anti-7H5 is unique for nonhuman primate leukocytes.

TWO COLOR FACS ANALYSIS FOR DEFINITION OF 7H5 ANTIGEN COEXPRESSION ON DEFINED MONKEY PBMC SUBCLASSES

These studies were done by using a panel of anti-human moAbs that crossreact with monkey PBMC as shown in Table 8.

These anti-human moAbs react with different populations of lymphoid cells and detect different functional molecules within the immune system. The majority of the normal values determined by FACS analysis and presented in Table 8 were determined in our laboratory. To determine the percent of coexpression of 7H5 antigen with these defined subpopulations, two color FACS analysis studies were performed. Double color studies involved the use of biotinylated anti-7H5 moAb and the panel of anti-human moAbs (Table 8) conjugated to phycoerythrin or fluorescein isothiocyanate. Results of double color fluorescence were determined by FACS analysis. Table 9 summarizes the results of two color fluorescence with anti-7H5 and panel of moAbs that crossreact with rhesus monkey PBMC.

High coexpression of 7H5 was observed in B cells (99%) and FcIgG (FcR3) bearing cells (99%) represented by anti-Leu-16, anti-Leu-11a, and anti-Leu-11b moAbs respectively (Figure 7). The majority of T cells (CD2) were found to coexpress 7H5, but less homogeneously than B cells and FcR+ bearing cells. There was also a high coexpression of 7H5 within both major T cells subsets i.e., CD8+ cells (cytotoxic/suppressor) (75%) and CD4+ cells (helper/suppressor) (68%). Since the CD4 subset can be further subdivided into 2H4+ and 4B4+ populations, we examined 7H5 expression on these populations and found that both significantly coexpress 7H5 antigen: 2H4 (66%) and 4B4

(53%).

Approximately 37% of monocytes/macrophages recognized by anti-Leu-M3 coexpress 7H5, but neutrophils did not appear to express this determinant. Anti-Leu-M4 moAb showed negligible (< 0.5%) coexpression with anti-7H5 moAb.

Figures 7,8, and 9 shows two color FACS analysis for definition of 7H5 antigen coexpression on monkey PBMC subclasses. In the DR+, CD20, CD16, CD2, and CD4 subsets respectively, the FACS results suggested the 7H5+ cells represented a major population. In the other moAb defined subsets there may be several populations expressing the 7H5 antigen.

MoAb DEPLETION STUDIES TO DETERMINE EXPRESSION OF 7H5 ANTIGEN ON FUNCTIONAL MONKEY LYMPHOCYTE SUBPOPULATION

Attempts to define functional properties of cells which express 7H5 were approached by depleting the 7H5 positive cells with magnetic beads and assaying their function before and after depletion. The number of 7H5+ cells in each experiment was determined before depletion experiment. The number of positive cells was used to determine the number of magnetic beads to be added to cell population to be depleted. After depletion, a sample of cells was taken and by FACS analysis determined the number of remaining 7H5 cells (usually < 2%). Figure 10 shows a typical example of monkey PBMC 7H5 depletion by magnetic beads. Figure 10A shows

FACS analysis of the expression of 7H5 antigen on monkey PBMC (pre-depletion). Figure 10B shows the absence of 7H5 cell population after depletion.

NK assay- To determine the expression of 7H5 population in NK cells, 7H5 depleted PBMC were used in monkey NK assay. Figure 11 shows the expression of 7H5 antigen in NK cells. Control (NK) cells consisted of unfractionated effector PBMC which showed significant kill to target cells (human myeloid, NK sensitive cell line K562). Mock controls were obtained by incubating unfractionated effector cells with normal mouse IgG, followed by magnetic beads treatment. Mock treatment of effector cells did not affect the NK function of these cells. The removal of 7H5+ cells totally abolished NK activity to K562. Similar results were found by depletion with anti-2H4 moAb, which detects a T200 glycoprotein. These results indicate that the 7H5 antigen and 2H4 antigen are expressed on all circulating NK cells in the rhesus monkey.

CML assay - The effect of 7H5 antigen in the generation of monkey CML response is shown in Figure 12. RHLA incompatible responder and stimulators cells were tested for the generation of CML responses. Intact or 7H5-responders cells responses to the same stimulators cells were compared. As observed in both experiments, the removal of 7H5+ cells significantly reduced the % CML lysis. In

experiments 1 and 2 the control responses (intact responders) were 85% and 48% lysis; and the responses of 7H5- cells were 22% and 12%, respectively. These experiments showed a mean 75% reduction in lysis after depletion of CTLp with anti-7H5. Thus the majority of monkey CTLp express this determinant.

RESPONSE TO POLYCLONAL MITOGENS- The effect of depleting 7H5 antigen in proliferative responses to polyclonal mitogens is summarized in Figure 13. Polyclonal responses to PHA, ConA, and SPA were totally abolished when 7H5 depleted responder cells were used. Mitogens concentrations added to each individual well were: PHA:45 ug; ConA:25 ug; and SPA:13.5 ug. All values were done in triplicate and results values in figure 13 were subtracted from media control value (effector cells with no mitogen added).

Figure 13 comprises the results of two representative experiments done with different monkey PBMC. Baseline responses to polyclonal mitogens varies from monkey to monkey; nevertheless the effect of depleting the 7H5+ population in both experiments is consistent and significant. The data show that the monkey PBMC populations which mount proliferative responses to polyclonal mitogens in vitro all express the 7H5 determinant. The presence of 7H5 on various populations of T cells, B cells, and NK cells suggests this determinant is a common lymphocyte antigen.

MoAb BLOCKING STUDIES TO DEFINE ROLE OF 7H5 ANTIGEN IN VARIOUS FUNCTIONS OF MONKEY LYMPHOCYTE SUBSETS

These studies consisted of adding affinity purified anti-7H5 moAb to responding cells in culture or in the CML experiments pre-coating effector and stimulator cells separately and washing off antibody before adding coated cells to culture assay. These studies were performed on several lymphocyte function assays, and results are summarized bellow:

NK activity- The effect of pre-incubating responder cells with anti-7H5 moAb before adding NK target cells is summarized in Table 10. In these studies effector cells were pre-coated with 20 ug of anti-7H5 moAb for 30 minutes at 37°C. The effector cells were plated directly without washing out the moAb. Table 10 summarizes the results of 2 representative NK blocking assay. The data show that pre-coating responder cells with anti-7H5 does not significantly affect the percent of lysis to K562 target cells at any E/T ratio studied. These studies suggests that 7H5 antigen is not involved in the NK lytic mechanism.

CML activity- These studies were designed to investigate the effect of anti-7H5 moAb treatment on the function of responder and stimulator cells in allogeneic monkey CML. The responder and stimulator cells were pre-coated with antibody and washed 2X before being added to the culture

wells. Figure 14 shows results of two representative experiments. The data indicate that the pretreatment of responder but not stimulator cells with anti-7H5 moAb before plating cells in 6 day CML assay enhanced CTLp activation. The magnitude of CML responses of untreated monkey effector cells varied from monkey to monkey. Nevertheless, pretreatment of responder cells with anti-7H5 significantly enhanced CTLp activation in both experiments.

To determine whether the enhancing effect of 7H5 moAb was directly related to 7H5 moAb binding, a series of experiments were conducted comparing CML responses of cells pretreated with IgG1 anti-7H5 or normal mouse IgG1.

Figure 15 describes the results of these experiments. The data indicate that anti-7H5 moAb does not block allospecific CTL effector activity. Rather, the pretreatment of responder cells produced an increase in the percent specific lysis of allogeneic target cells. Enhancement of specific lysis was not observed when responder cells were preincubated with normal mouse IgG1. As pointed out above, there were variabilities in CML responses in each experiment. However, the enhancing effect of anti-7H5 was consistent showing a 50-150% increase in lysis.

In three experiments, anti-7H5 moAb was added to effector cultures 15 minutes before adding ^{51}Cr tagged target cells (day 6). Results of these experiments are

shown in Figure 16. In experiment 1 there was minimal autoreactivity and a minimal suppressive effect of anti-7H5 moAb. In experiments 2 and 3 there was lower alloreactive and relatively high autokill. In these latter experiments addition of anti-7H5 decreased lytic activity, and most significantly the autokilling. These data would suggest allospecific lysis by mature effector cells generated in 6 day CML cultures may be minimally effected by anti-7H5 moAb, but nonspecific autoreactive effector activity is inhibited.

Polyclonal mitogens- The effect of adding purified anti-7H5 moAb to monkey effector cells treated with polyclonal mitogens PHA and ConA was studied.

Figure 17 shows that anti-7H5 moAb does not significantly affect the proliferative response induced by PHA. Experiment 1 responses induced by PHA were non significantly increased in the presence of anti-7H5 moAb. In Experiment 2 the addition of anti-7H5 had almost no affect.

Figure 18 shows the effect of adding anti-7H5 moAb to ConA cultures. Two representative experiments were analyzed and shows that anti-7H5 moAb in ConA cultures significantly increased these responses. ConA (1:50) response was increased from about 80×10^3 cpm to 140×10^3 cpm ($p < 0.001$) with anti-7H5 present on culture. There was a

statistically significant increase in ConA responses in every dilution of ConA tested. The media controls indicate anti-7H5 moAb per se is not mitogenic. These results suggest an amplification role of anti-7H5 moAb in the polyclonal ConA response to monkey PBMC.

The data in Figure 19 compares the effect of anti-7H5 moAb and normal mouse IgG1 on rhesus monkey PBMC response to ConA. The graph shows the percent of unmodified ConA response when anti-7H5 moAb or normal mouse IgG1 were added to ConA cultures. In all three experiments the unmodified ConA response was significantly increased when anti-7H5 moAb was added to ConA cultures, compared slight increases when normal mouse IgG1 was added at the same concentration.

EFFECTS OF PREINCUBATION WITH PHA AND CONA ON BINDING OF ANTI-7H5 TO MONKEY PBMC

To determine if PHA or ConA and anti-7H5 might bind to a similar receptor, experiments were set up to pretreat the cells with ConA or PHA and assay for binding of anti-7H5 moAb.

Table 11 shows that pretreatment of monkey PBMC with different PHA concentrations (3, 30, and 300 ug/ml) does not interfere with the binding of anti-7H5 moAb. These studies were analyzed by FACS indicated that the percent of cells that express 7H5 antigen in monkey PBMC and had no pretreatment with PHA was not significantly different from those that were treated with PHA. Results on Table 11,

background fluorescence staining with goat anti-mouse IgG-FITC ranged between 1.2 and 4.8% and was subtracted from the percent positive cells stained with moAb and goat anti-mouse IgG-FITC. These results indicated that PHA and anti-7H5 do not compete for the same site.

Table 12 shows the results of preincubating monkey PBMC with ConA and subsequent labelling with anti-7H5 moAb plus goat anti-mouse FITC and analysis at FACS. These experiments indicated that pretreatment with different concentrations of ConA (1.6, 16, and 160 ug/ml) does not significantly interfere with the binding of anti-7H5 moAb to monkey PBMC. Thus, it is unlikely that ConA and anti-7H5 bind to the same receptor site.

CAPPING STUDIES TO DETERMINE IF ANTI-7H5 moAb MODULATES THE 7H5 ANTIGEN AND WHETHER THE KNOWN T200 ANTIGEN 2H4 IS COCAPPED BY 7H5

Figure 20 shows the results of capping experiments which attempted to determine whether 7H5 and 2H4 bind to the same molecule. These studies demonstrated that 7H5 molecule does not cap off the cell membrane. The goat anti-mouse values remained almost unchanged throughout the 20 hrs of experiment; meaning that 7H5 molecule was not modulated from the cell surface. Thus it was not possible to test this hypothesis by this approach.

In the double label tube, 2H4 positive cell values did

not change significantly during prolonged incubation in excess anti-7H5 moAb. The 7H5 positive cell values after 30 min. dropped. This was due to the 7H5 sites already been occupied by the unlabelled anti-7H5 (added at time 0) as indicated by the presence of mouse IgG on the cell surface. These indicated that anti-7H5 does not compete the binding of anti-2H4. Thus, anti-2H4 and anti-7H5 do not recognize the same epitope. Whether they detect the same T200 molecule remains to be proven.

TABLE 1

ESTABLISHING A SUCCESSFUL HYBRIDOMA
3 SETS OF FUSION EXPERIMENTS:

FREQUENCY OF ANTIBODY PRODUCING HYBRIDS TO RHESUS PBL		PROBLEMS
FUSION 1	12/24	CONTAMINATION
FUSION 2	15/24	PEG TOXICITY
FUSION 3	21/24	—

TABLE 2

FUSION 3: SCREENING HYBRIDS AND DEVELOPMENT OF CLONES

	ANTIBODY BINDING PATTERNS TO RHESUS PBMC			
	A	B	C	D
% PBMC BOUND BY FUSION 3	>95%	60-94%	24-59%	5-24%
ANTIBODY PRODUCING HYBRIDS	2	3	2	14
REPRESENTATIVE HYBRIDS SELECTED*	IIC2	IID4	IIB4	ID4
AFTER CLONING BY LDA (N=21)	75/300	60/300	90/300	27/300

* Hybrids selected for cloning by the highest binding titer

TABLE 3

SELECTION OF ANTIBODY PRODUCING CLONES
FOR EXPANSION AND FURTHER STUDY

A) BINDING PATTERN REVEALED BY FACS ANALYSIS:

Series A: 3 patterns

Series B: 11 patterns

Series C: 8 patterns

Series D: 5 patterns

B) TITRATION OF ANTIBODY PRODUCTION BY ELISA ASSAY:

Series A: Clones with titer > 1:800: 1/3

Series B: Clones with titer > 1:800: 6/11 *

Series C: Clones with titer > 1:800: 4/8 *

Series D: Clones with titer > 1:800: none

*Selected for ascites production

TABLE 4

ASCITES PRODUCTION AND PRELIMINARY
CHARACTERIZATION OF 4 moAbs

1) ASCITES PRODUCTION:

SERIES B: Clones with rapid growth and titer > 1:1600 : 3/6

SERIES C: Clones with rapid growth and titer > 1:1600 : 1/4

2) IMMUNOPRECIPITATION OF MEMBRANE ANTIGENS WITH ASCITES moAbs:

moAb	mW of Protein
SERIES B: Anti-7H5	~ 200,000
Anti-2A12	~ 120,000
Anti-2G1	~ 120,000
SERIES C: Anti-2D10	~ 130,000

TABLE 5

OUTLINE OF STUDIES DONE WITH ANTI-7H5

-
- 1) Ascites production: 30 mice
 - 2) Purification by HPLC and Isotype determination (IgG1)
 - 3) Biotinylation and FACS analysis of biotinylated anti-7H5
 - 4) One color FACS analysis to determine distribution on various monkey and human lymphoid cells
 - 5) Two color FACS analysis for definition of 7H5 antigen co-expression on defined monkey PBMC subclasses
 - 6) moAb depletion studies to determine expression of 7H5 antigen on functional monkey lymphocyte subpopulations
 - 7) moAb blocking studies to define role of 7H5 antigen in various functions of monkey lymphocyte subsets
 - 8) Effects of preincubation with PHA and ConA on binding of anti-7H5 to monkey PBMC
 - 9) Capping studies to determine if anti-7H5 moAb modulates the 7H5 antigen and whether the known T200 antigen 2H4 is cocapped by 7H5
-

TABLE 6

REACTIVITY OF ANTI-7H5 moAb TO RHESUS MONKEY
CELLS BY FACS ANALYSIS

TISSUE	% BINDING
Bone Marrow	10
Spleen	68
Lymph Node	60
PBL	70-80

TABLE 7

LACK OF REACTIVITY OF ANTI-7H5 moAb
TO HUMAN CELLS BY FACS ANALYSIS

HUMAN CELLS	% BINDING
NORMAL CELLS:	
THYMOCYTES	0
PBL	0
CELL LINES:	
T CELLS:	
HSB	0
JURKAT	0
B CELLS:	
RAJI	0
DAUDI	0
2751	0
MONOCYTES:	
U937	0
ERYTHROID:	
K562	0
MYELOID:	
HL60	0

TABLE 8

ANTI-HUMAN moAbs* DEFINING PBMC** SUBPOPULATIONS

moAb	CD	% BINDING		FUNCTIONAL SUBPOPULATION
		HUMAN PBMC	RHESUS PBMC	
Anti-Leu-5B	CD2	77.1±10.1 n=20	62.3±17.8 n=18	T cells, NK cells and some pre-T cells
Anti-OKT4	CD4	34.8±8.1 n=19	20.1±6.2 n=16	T helper/suppressor inducer cells
Anti-Leu-8	-	56.8±6.1 n=19	19.4±3.8 n=6	T helper cells
Anti-4B4	CDW29	55.0±15*** n=25	37.7±3.4 n=2	T helper cells
Anti-2H4	CD45R	22.1±11.3 n=11	22.8±4.9 n=3	T suppressor/inducer cells
Anti-Leu-2a	CD8	23.2±5.7 n=19	42.7±11.9 n=17	T cytotoxic/suppressor cells, NK cells
Anti-Leu-15	CD11b	28.2±6.8 n=18	18.7±6.7 n=13	T suppressor cells, NK, monocytes and granulocytes
Anti-Leu-16	CD20	6.9±2.8 n=20	14.8±3.5 n=10	B cells
Anti-Leu-11a	CD16	19.1±5.8 n=18	14.8±4.6 n=12	FcR IgG on NK cells and neutrophils
Anti-Leu-11b	CD16	15.0±7**** n=4	12.9±2.9 n=4	FcR IgG on NK cells and neutrophils
Anti-Leu-19	-	14.6±7.3	8.6	NK cells
Anti-HLA-DR	-	23.0±6.7 n=19	18.1±5.9 n=23	MHC class II framework antigen and activated T cells
Anti-Leu-M3	-	20.7±5.5	6.5±2.0	Monocytes/macrophages

* All crossreact with monkey PBMC

** Ficoll hypaque isolated PBMC

*** Coulter Immunology value

**** Becton Dickinson value

TABLE 9

TWO COLOR FLUORESCENCE WITH ANTI-7H5 AND
 PANEL OF moAbs IN RHESUS MONKEY PBMC

moAb	% OF moAb+ CELLS THAT COEXPRESS 7H5
Anti-Leu-16	99
Anti-Leu-11b	99
Anti-Leu-11a	94
Anti-Leu-5b	77
Anti-HLA-DR	75
Anti-Leu-2a	75
Anti-OKT4	68
Anti-2H4	66
Anti-4B4	53
Anti-Leu-8	52
Anti-Leu-19	40
Anti-Leu-M3	37
Anti-Leu-15	26
Anti-Leu-M4 (PMNs)	0.5

TABLE 10

NK ACTIVITY IS NOT BLOCKED BY ANTI-7H5

Pre-Treatment of Effector Cells*	Percent Lysis of K562 Target Cells			
	Effector:Target Ratio			
EXP.1	20:1	10:1	5:1	2.5:1
None	79.4	69.4	54.9	29.6
Anti-7H5	74.2	63.1	45.0	23.4
EXP.2				
None	64.9	60.1	56.8	37.4
Anti-7H5	66.4	69.4	51.7	30.2

* 6×10^5 Effectors were preincubated with 20 ug of anti-7H5 in a total volume of 0.6 ml for 30 minutes at 37°C, and plated without washing out the moAb.

TABLE 11

**PREINCUBATION WITH PHA DOES NOT BLOCK THE BINDING OF
ANTI-7H5 TO RHESUS MONKEY PBMC**

PRETREATMENT	ANTIBODY	% POSITIVE CELLS*
NONE	7H5	74.3
PHA 3 ug/ml	7H5	74.6
PHA 30 ug/ml	7H5	70.0
PHA 300 ug/ml	7H5	78.8

* Background fluorescence staining with goat anti-mouse IgG-FITC ranged between 1.2 - 4.8% and was subtracted from the % positive cells stained with moAb plus goat anti-mouse-IgG-FITC

TABLE 12

PREINCUBATION WITH ConA DOES NOT BLOCK THE BINDING OF
ANTI-7H5 moAb TO RHESUS MONKEY PBMC

PRETREATMENT	ANTIBODY	% POSITIVE CELLS*
None	7H5	74.3
ConA 1.6 ug/ml	7H5	69.8
ConA 1.6 ug/ml	a-MM** + 7H5	73.87
ConA 16 ug/ml	7H5	79.1
ConA 16 ug/ml	a-MM + 7H5	75.6
ConA 160 ug/ml	7H5	77.5
ConA 160 ug/ml	a-MM + 7H5	72.1

* Background fluorescence staining with goat anti-mouse IgG-FITC ranged between 1.2 - 4.8% and was subtracted from the % positive cells stained with moAb + goat anti-mouse IgG-FITC.

** 25 mM of a-methyl mannoside added per 10^6 cells after incubation with ConA.

Figure 1- Antibody binding pattern to rhesus PBMC.

Representative hybrids selected.

1A: Clone IIC2

1B: Clone IID4

1C: Clone IIB4

1D: Clone ID4

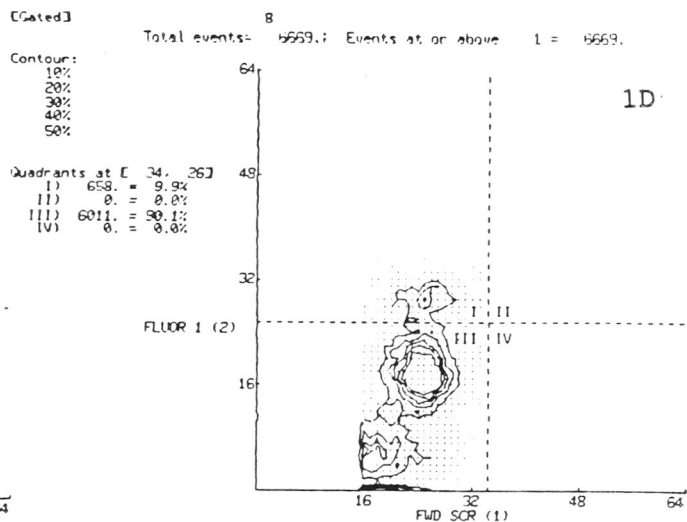
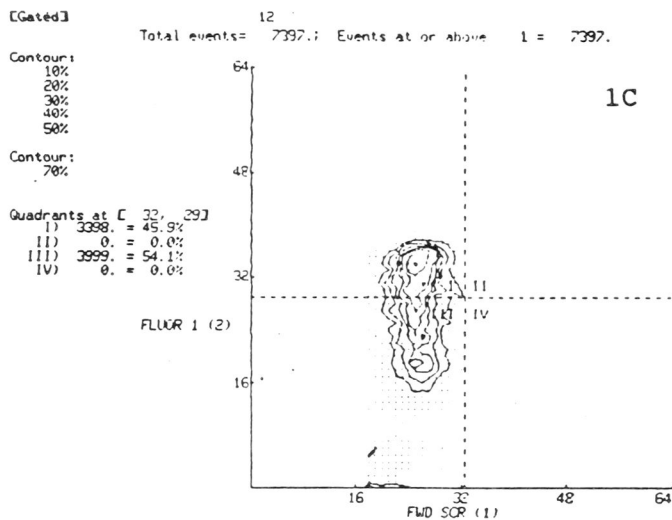
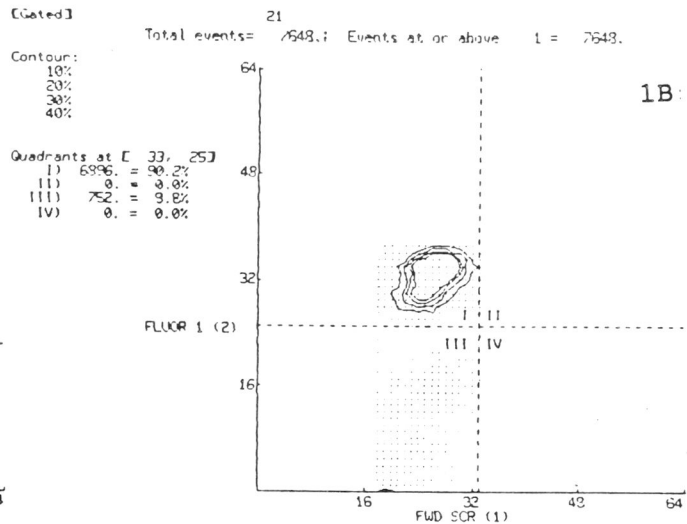
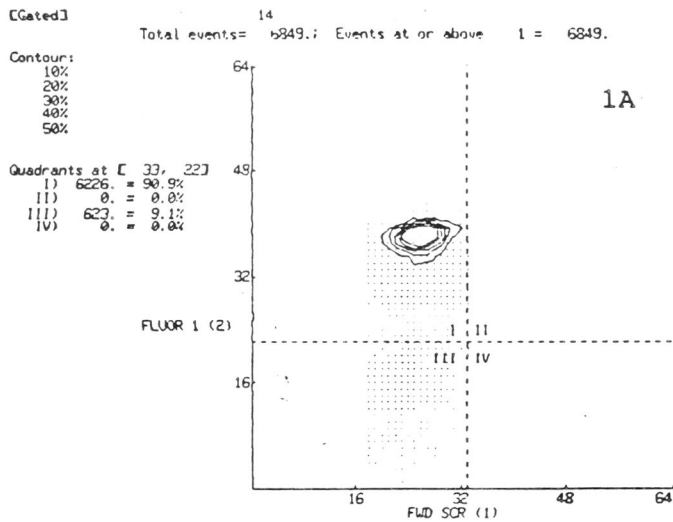


Figure 2- Examples of Series B clones patterns that reacted with rhesus monkey PBMC by FACS analysis.

DL2-110925.FCS

198

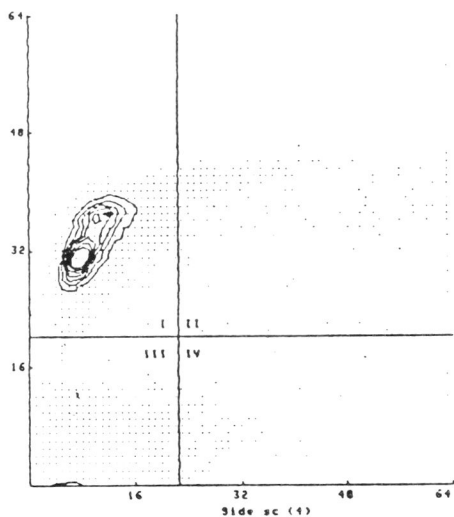
Total events= 10240 : Events at or above I = 10240

Contour:
 10%
 20%
 30%
 40%
 50%
 60%
 70%

Quadrants at E 22, 203

I) 8270. = 80.9%
 II) 250. = 2.4%
 III) 1622. = 15.8%
 IV) 90. = 1.0%

Green F (2)



DL2-110926.FCS

201

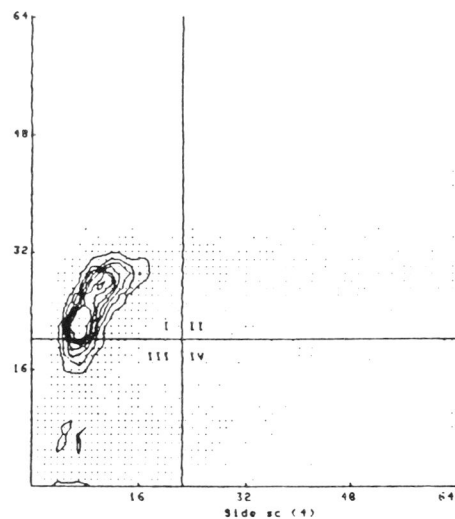
Total events= 10240 : Events at or above I = 10240

Contour:
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 20%
 30%
 40%
 50%
 60%
 70%

Quadrants at E 22, 203

I) 6918. = 67.6%
 II) 205. = 2.0%
 III) 1044. = 10.2%
 IV) 72. = 0.7%

Green F (2)



DL2-110923.FCS

176

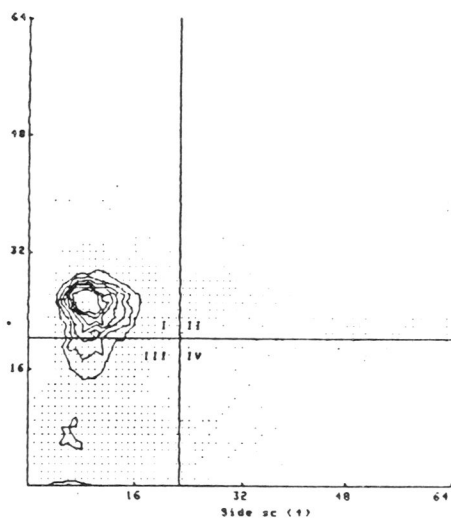
Total events= 10240 : Events at or above I = 10240

Contour:
 10%
 20%
 30%
 40%
 50%
 60%
 70%

Quadrants at E 22, 203

I) 6376. = 61.8%
 II) 200. = 2.0%
 III) 3353. = 32.7%
 IV) 103. = 1.0%

Green F (2)



DL2-110920.FCS

144

Total events= 10240 : Events at or above I = 10240

Contour:
 10%
 20%
 30%
 40%
 50%
 60%
 70%

Quadrants at E 22, 203

I) 6171. = 60.2%
 II) 401. = 4.7%
 III) 3150. = 30.8%
 IV) 110. = 1.3%

Green F (2)

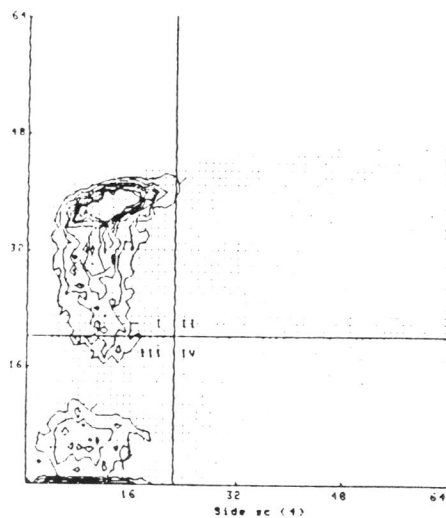


Figure 3- 7.5-15% SDS-PAGE of ^{125}I labelled monkey PBMC
and immunoprecipitation with Series B and C moAbs.
Lane 1: Anti-9.6 moAb
Lane 2: Anti-2D10 moAb
Lane 3: Anti-2A12 moAb
Lane 4: Anti-2G1 moAb
Lane 5: Anti-7H5 moAb

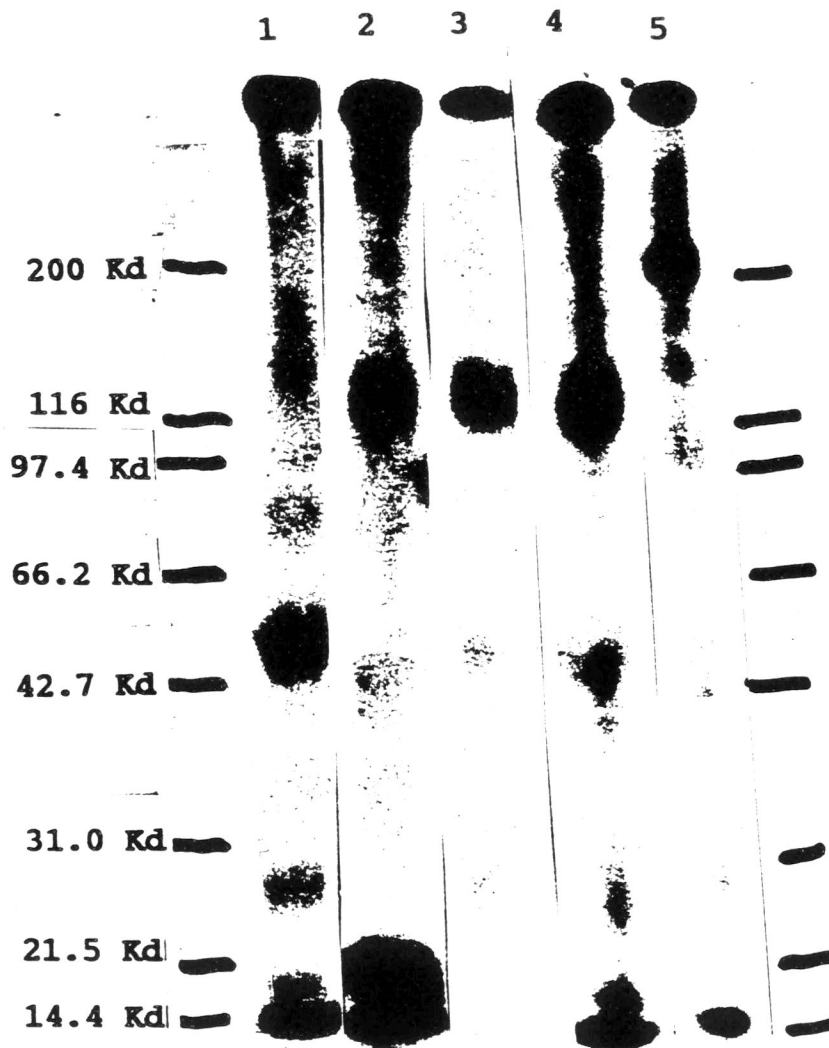


Figure 4- Purification of anti-7H5 moAb
by HPLC using DEAE column.

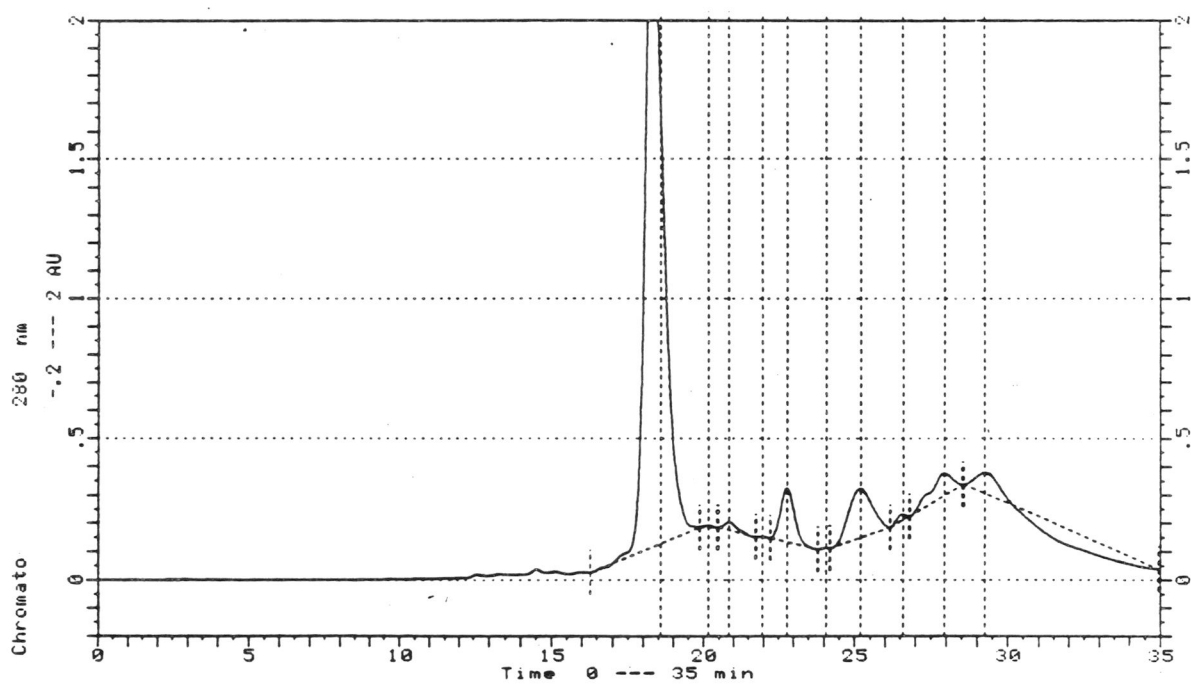


Figure 5- FACS analysis of rhesus monkey PBMC
with anti-7H5 biotinylated.

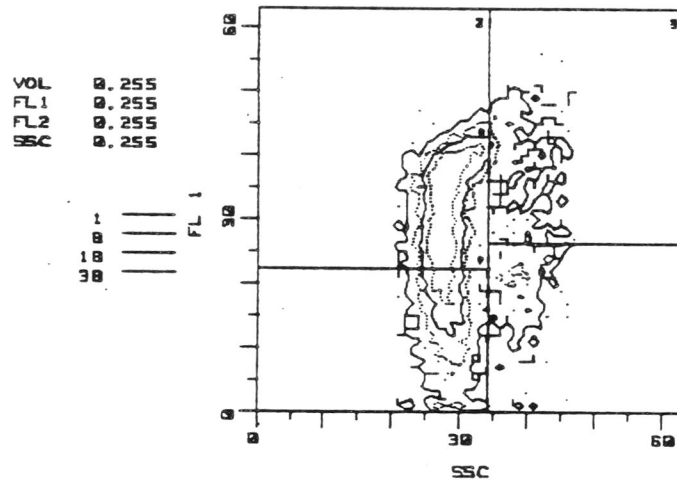


Figure 6- One color analysis of 7H5 reactivity on different monkey lymphoid tissues.

A: Bone marrow

B: Spleen

C: Lymphonode

D: PBL

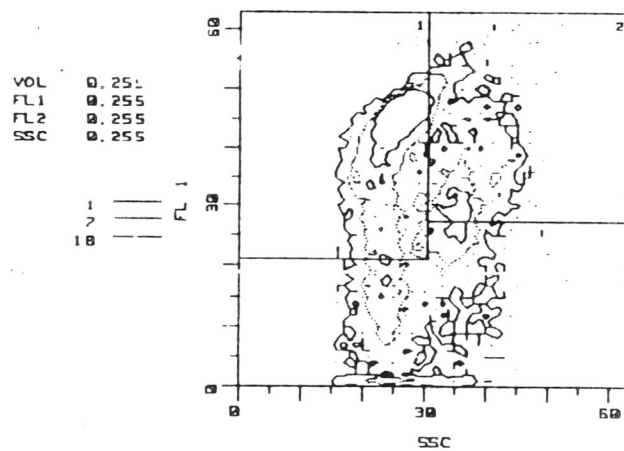
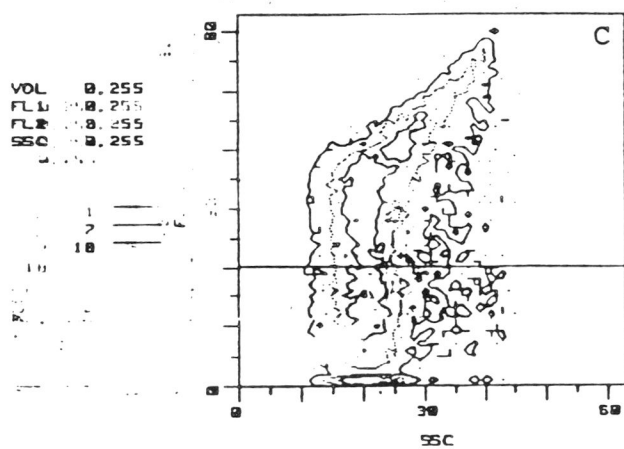
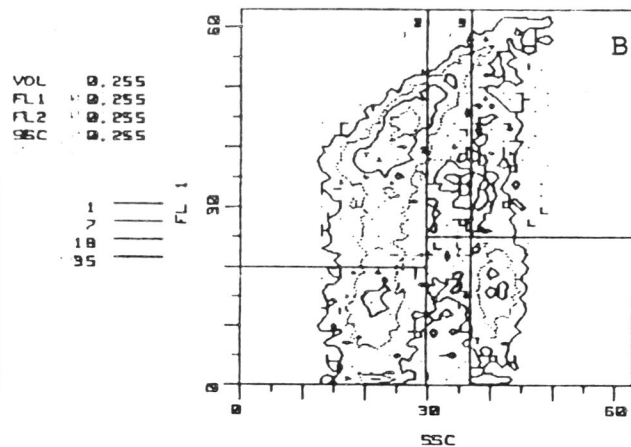
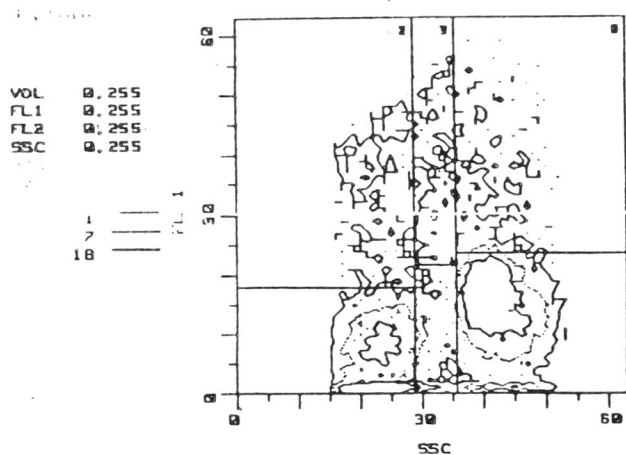


Figure 7- Two color FACS analysis for definition of 7H5 antigen coexpression on monkey PBMC subclasses.

A: Expression of 7H5 antigen on CD20+ monkey PBL.
Anti-7H5 is FL1, and anti-CD20 (Leu-16) is FL2.

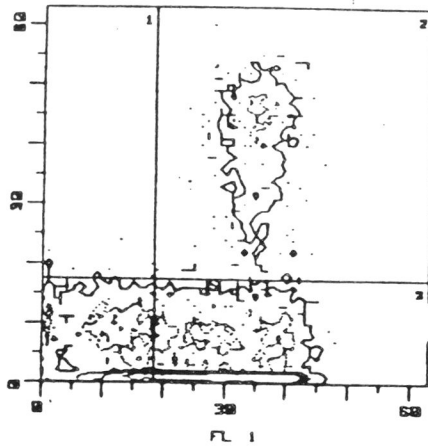
B: Expression of 7H5 antigen on CD16+ monkey PBL.
Anti-7H5 is FL1, and anti-CD16 (Leu-11b) is FL2.

C: Expression of 7H5 antigen on CD16+ monkey PBL.
Anti-7H5 is FL2, and anti-CD16 (Leu-11a) is FL1.

D: Expression of 7H5 antigen on CD2+ monkey PBL.
Anti-7H5 is FL2, and anti-CD2 (Leu-5b) is FL1.

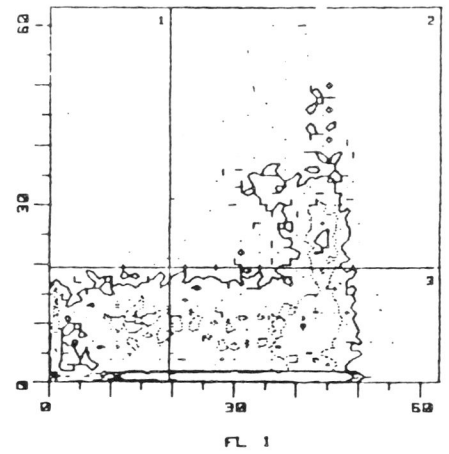
VOL 0.255
FL1 0.255
FL2 0.255
SSC 0.136

1
8
18
38



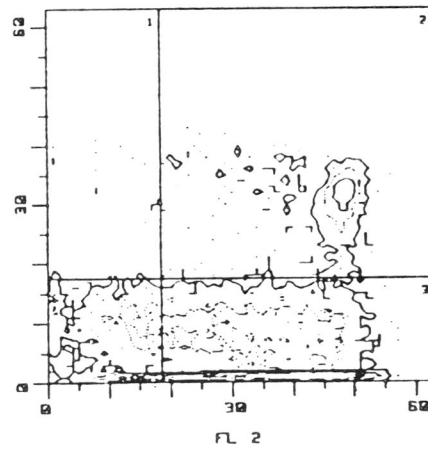
VOL 0.255
FL1 0.255
FL2 0.255
SSC 0.122

1
8
18



VOL 0.255
FL1 0.255
FL2 0.255
SSC 0.136

1
8
18
38



VOL 0.255
FL1 0.255
FL2 0.255
SSC 0.133

1
8
18

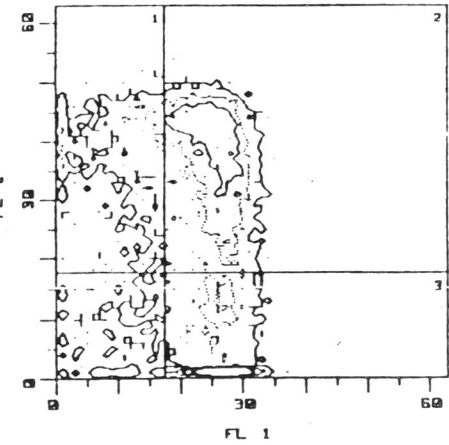


Figure 8- Two color FACS analysis for definition of 7H5 antigen coexpression on monkey PBMC subclasses.

A: Expression of 7H5 antigen on HLA-DR+ monkey PBL.
Anti-7H5 is FL1, and anti-HLA-DR+ is FL2.

B: Expression of 7H5 antigen on CD8+ monkey PBL.
Anti-7H5 is FL1, and anti-CD8 (Leu-2a) is FL2.

C: Expression of 7H5 antigen on CD4+ monkey PBL.
Anti-7H5 is FL2, and anti-CD4 (OKT4) is FL1.

D: Expression of 7H5 antigen on CD45R+ monkey PBL.
Anti-7H5 is FL2, and anti-CD45R (2H4) is FL1.

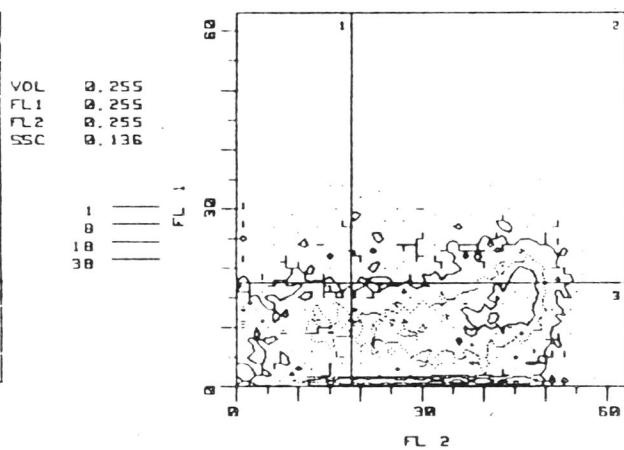
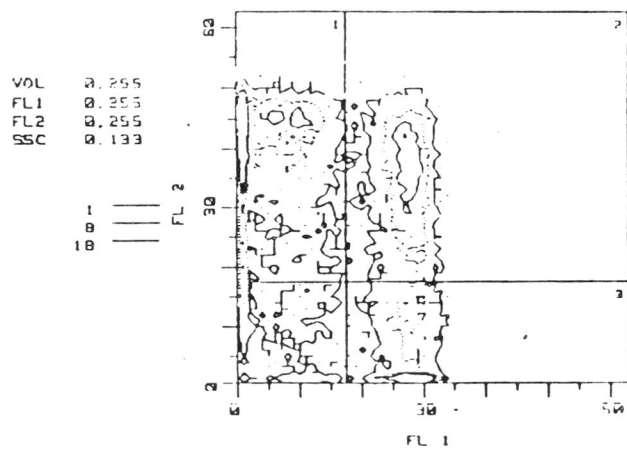
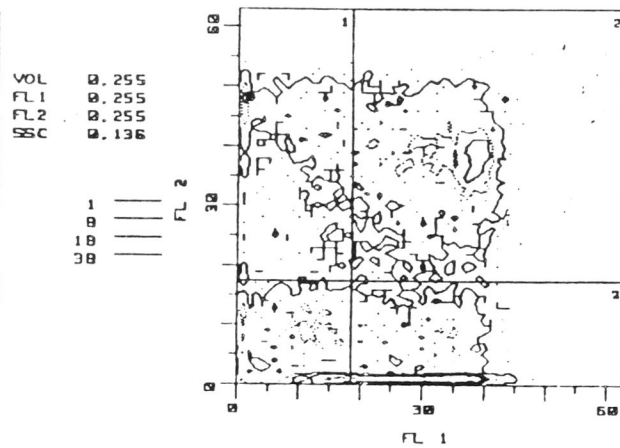
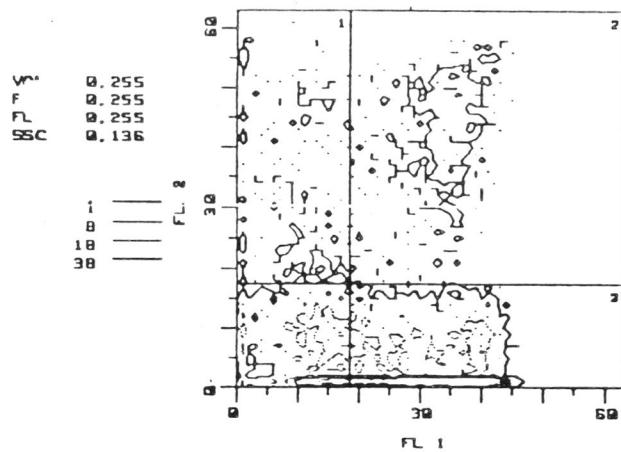


Figure 9- Two color FACS analysis for definition of 7H5 antigen coexpression on monkey PBMC subclasses.

A: Expression of 7H5 antigen on CDW29+ monkey PBL.
Anti-7H5 is FL2, and anti-CDW29 (4B4) is FL1.

B: Expression of 7H5 antigen on Leu-8+ monkey PBL.
Anti-7H5 is FL1, and anti-Leu-8 is FL2.

C: Expression of 7H5 antigen on Leu-19+ monkey PBL.
Anti-7H5 is FL1, and anti-Leu-19 is FL2.

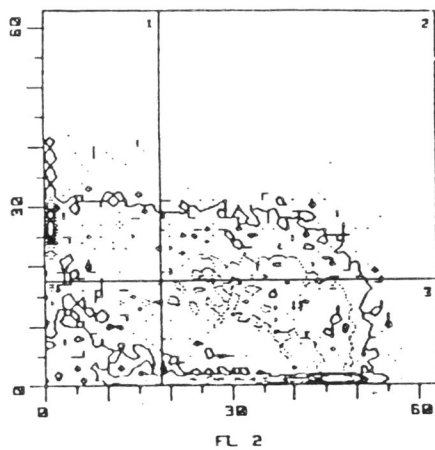
D: Expression of 7H5 antigen on Leu-M3+ monkey PBL.
Anti-7H5 is FL1, and anti-M3 is FL2.

E: Expression of 7H5 antigen on CD11b+ monkey PBL.
Anti-7H5 is FL1, and anti-CD11b (Leu-15) is FL2.

VOL 0.255
FL1 0.255
FL2 0.255
SSC 0.136

1
8
18
38

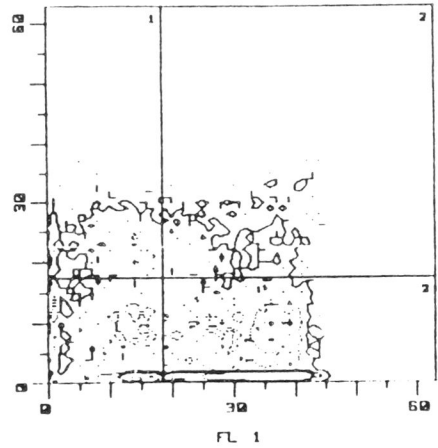
FL 1



VOL 0.255
FL1 0.255
FL2 0.255
SSC 0.136

1
8
18
38

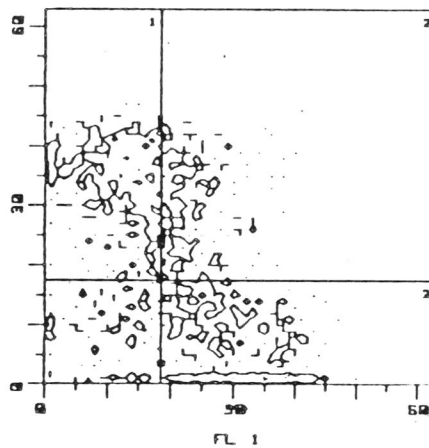
FL 2



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FL1 0.255
FL2 0.255
SSC 137.159

1
8
18
38

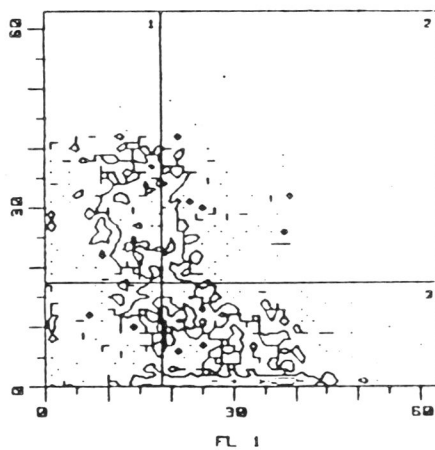
FL 2



VOL 0.255
FL1 0.255
FL2 0.255
SSC 137.159

1
8
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38

FL 2



VOL 0.255
FL1 0.255
FL2 0.255
SSC 0.255

1
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FL 2

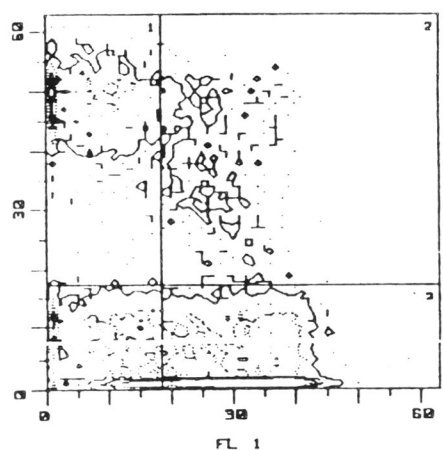


Figure 10- Depletion of 7H5+ cells from monkey PBMC
with moAb anti-7H5 and magnetic beads.

A: Pre-depletion

B: Post-depletion

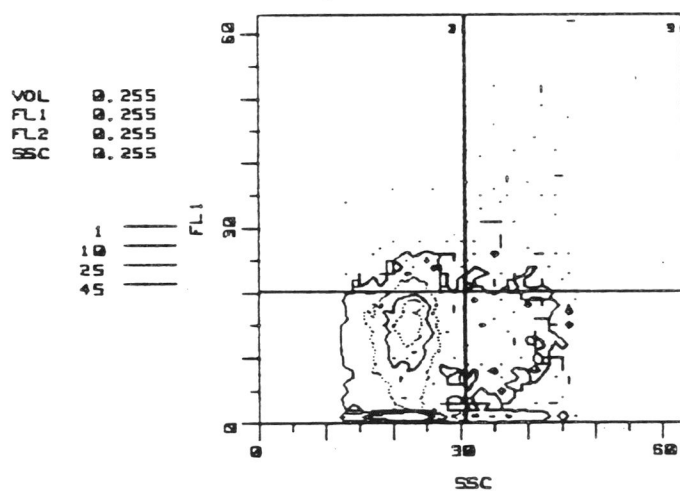
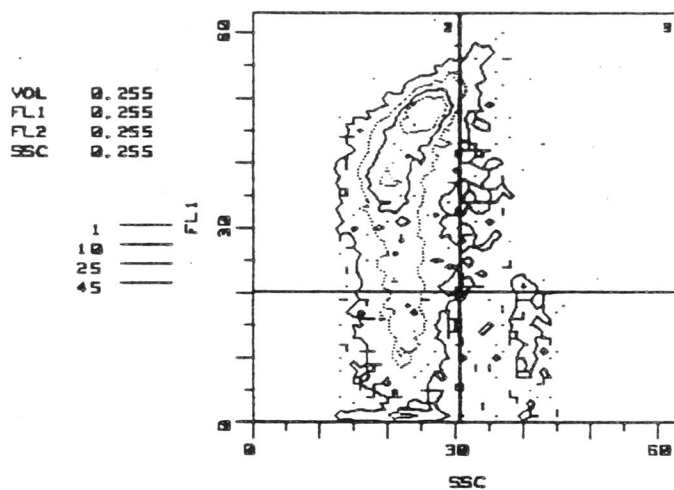


Figure 11- NK assay with 7H5 depleted effector cells shows expression of 7H5 on NK cells. Results from NK assay performed in 3 normal rhesus monkeys and are expressed as the mean % cytotoxicity $\pm$ SD obtained at 4 E/T ratios.

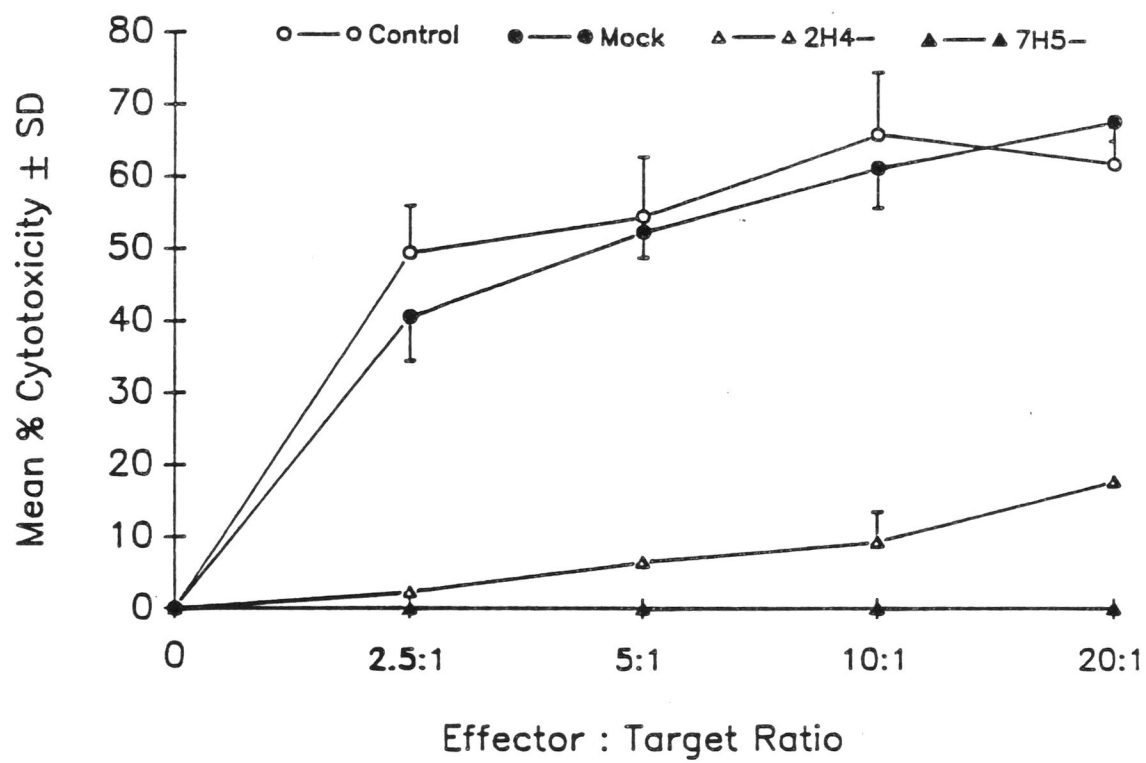


Figure 12- CML assay with 7H5 depleted responder cells shows expression of 7H5 on CTLp. Results are expressed on % CML lysis in two separate experiments. Responder cells are intact or 7H5 depleted.

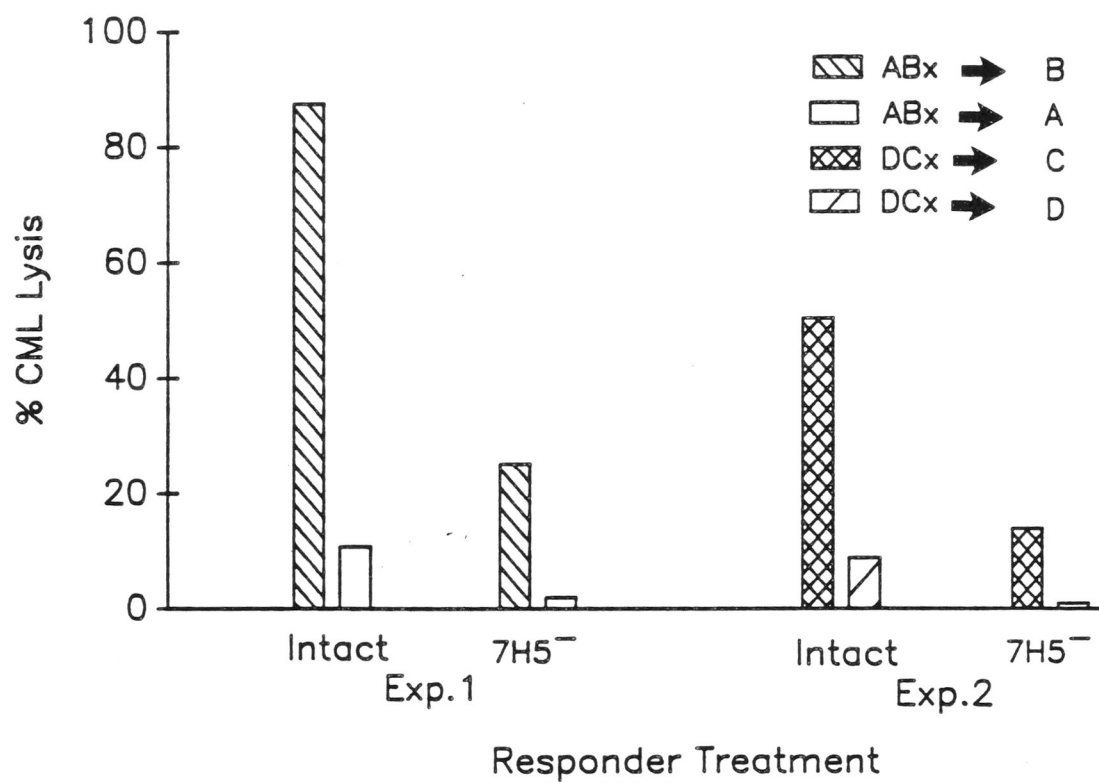


Figure 13- Depletion of PBMC expressing 7H5 abolishes proliferation responses to polyclonal mitogens. Data are expressed as [³H]Thymidine uptake(cpm) $\times 10^3$ of 2 experiments.

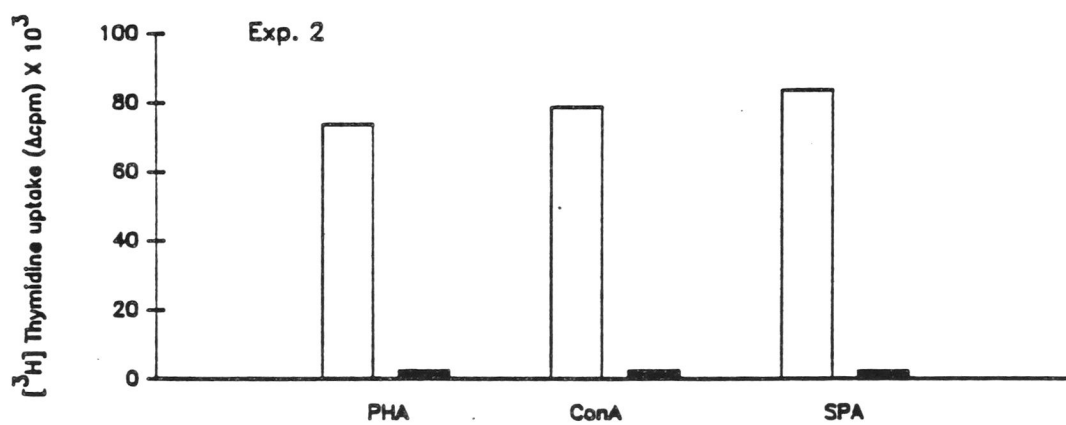
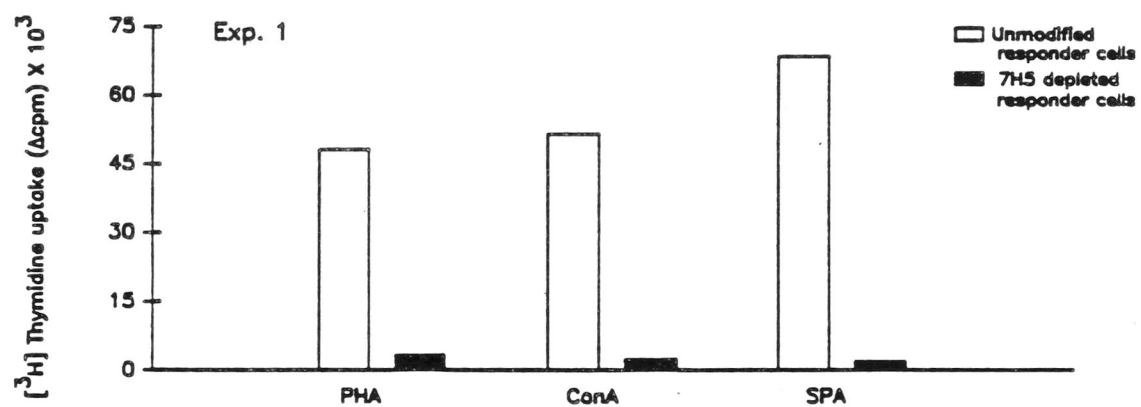


Figure 14- Pretreatment of responder but not stimulators with moAb anti-7H5 enhances CTLp activation. Results are expressed as % specific lysis. Either 2×10^5 responder or stimulator cells were pretreated with 1.2 ug of anti-7H5 for 30 minutes washed 3 times and plated in 6 day CML cultures. Stimulator cells were irradiated (2000R) after the moAb pretreatment.

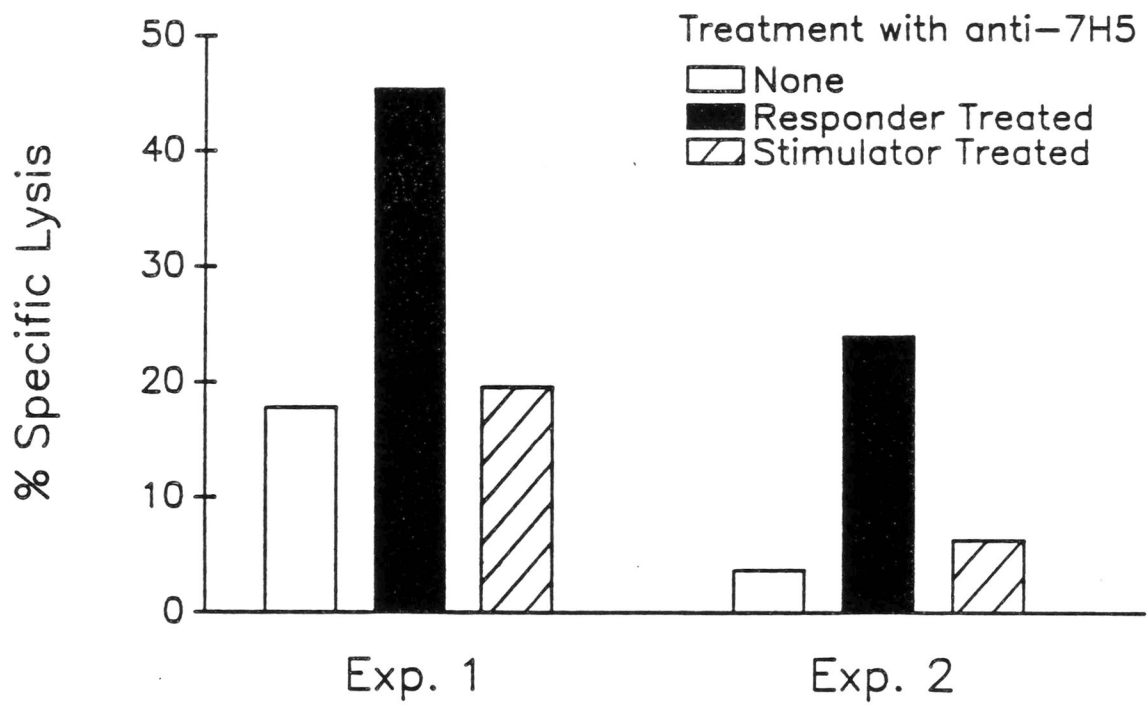


Figure 15- Enhancement of CTLp activation to allogeneic cells by treatment of responders with anti-7H5. Results of 4 experiments are expressed as percent specific lysis of allogeneic target cells. 2×10^5 responder cells were pretreated with 1.2 ug of anti-7H5 for 30 minutes, washed x3, and plated in 6 day cultures.

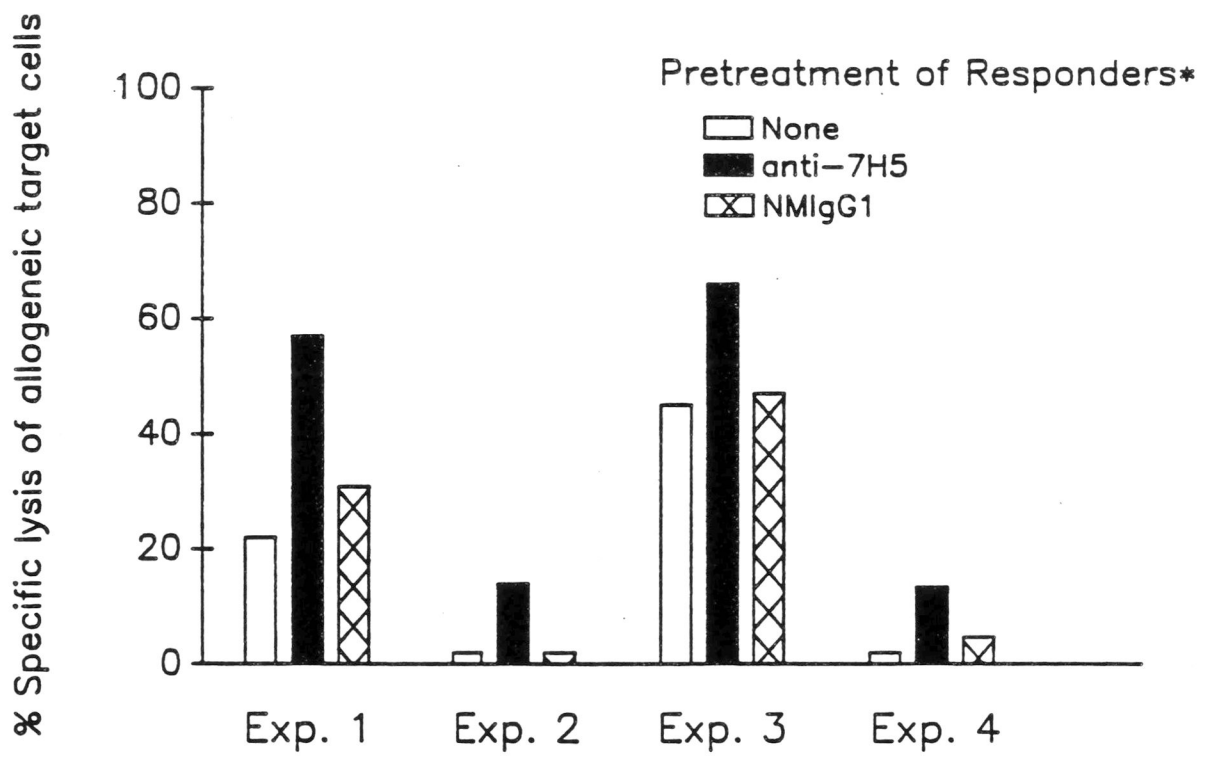


Figure 16- Data suggesting anti-7H5 does not block allospecific CTL effector activity. Results are expressed as percent specific lysis of 3 experiments. 1.2 ug of anti-7H5 moAb added to effector cultures 15 minutes before adding ^{51}Cr tagged targets.

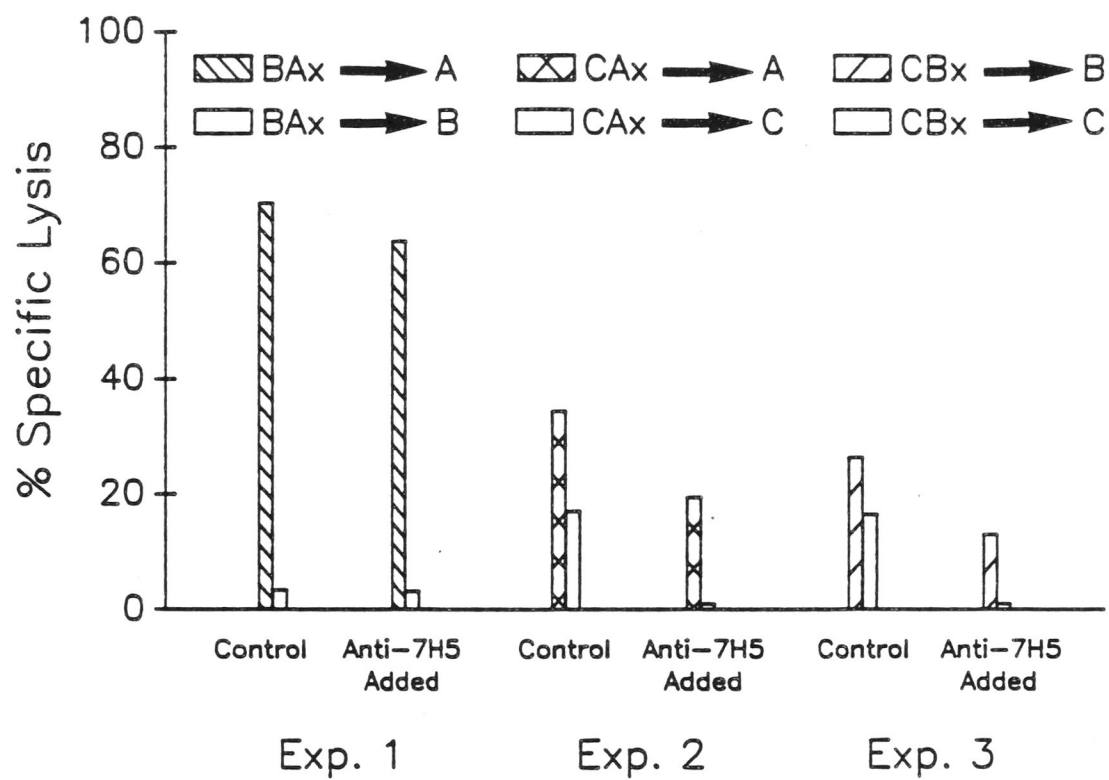


Figure 17- Addition of anti-7H5 to PHA cultures. Results are expressed as [^3H]Thymidine uptake (cpm) $\times 10^3$ of 2 experiments. 1.2 ug of anti-7H5 added per culture well.

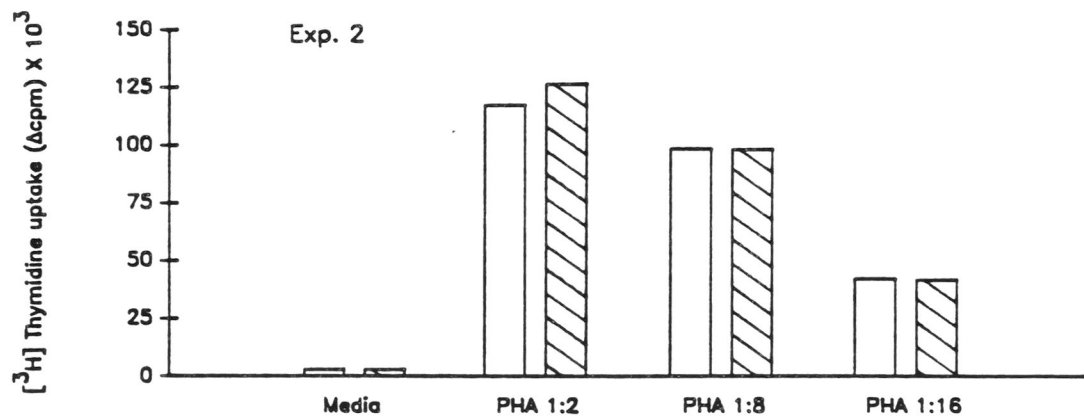
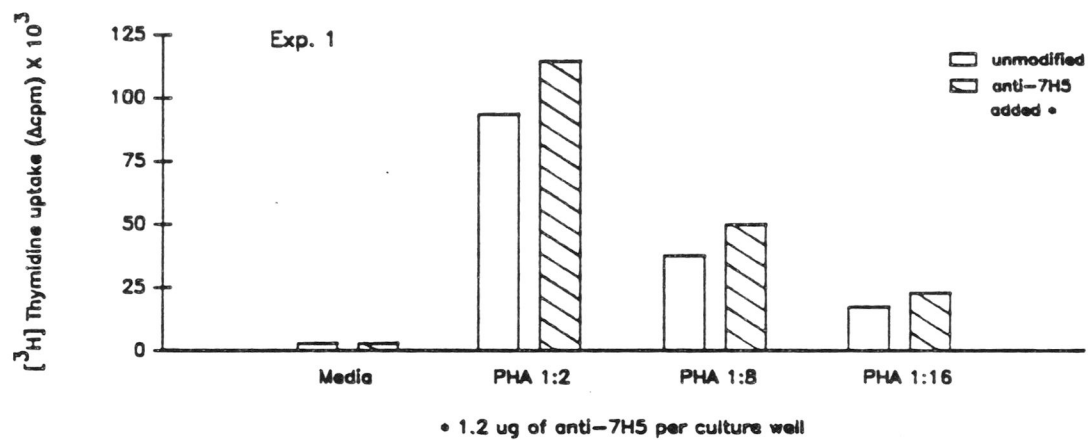


Figure 18- Addition of anti-7H5 to ConA cultures. Data are expressed as [^3H]Thymidine (cpm) $\times 10^3$ of 2 experiments. 1.2 ug of anti-7H5 was added per culture well.

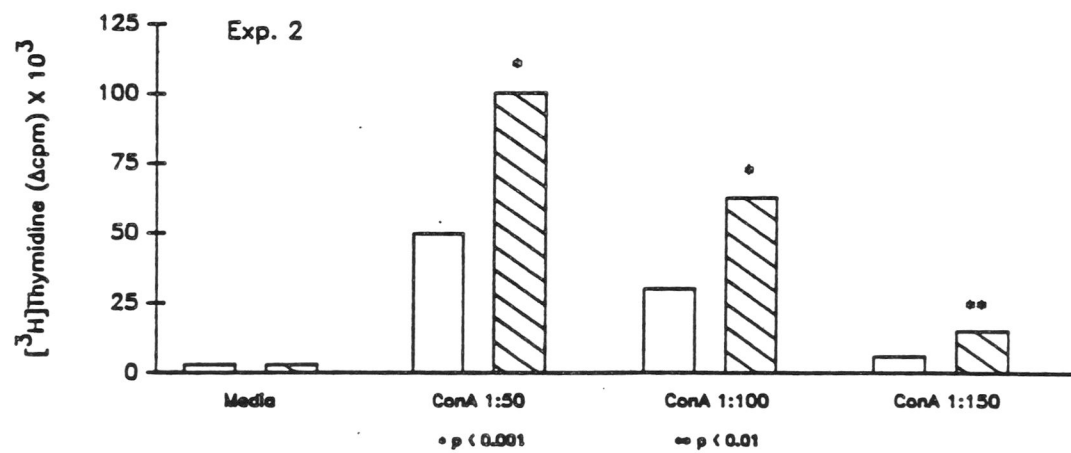
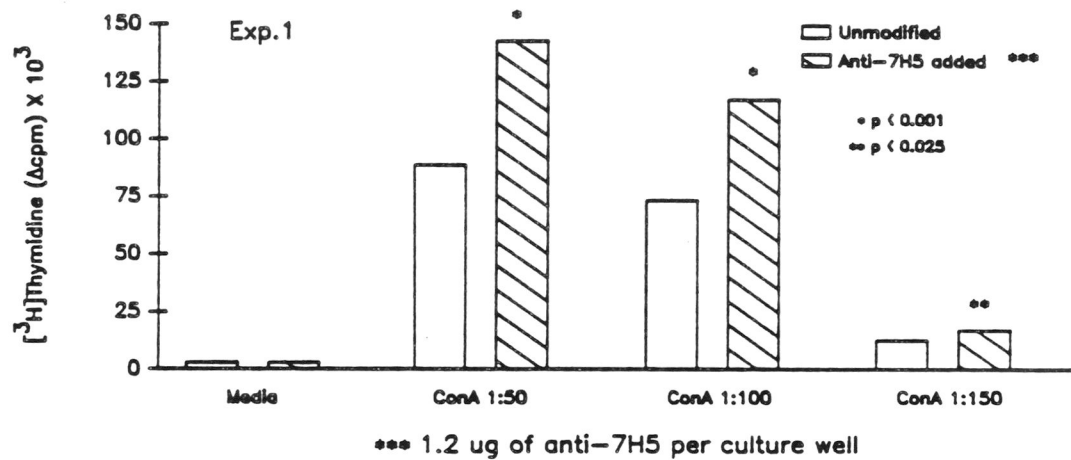


Figure 19- Enhancing effect of moAb 7H5 on rhesus PBMC response to ConA. Results are expressed as percent of unmodified ConA response of 3 experiments. 0.5 ug of ConA was added to cultures, and 1.2 ug of moAb 7H5 or NM1gG1 was added per culture.

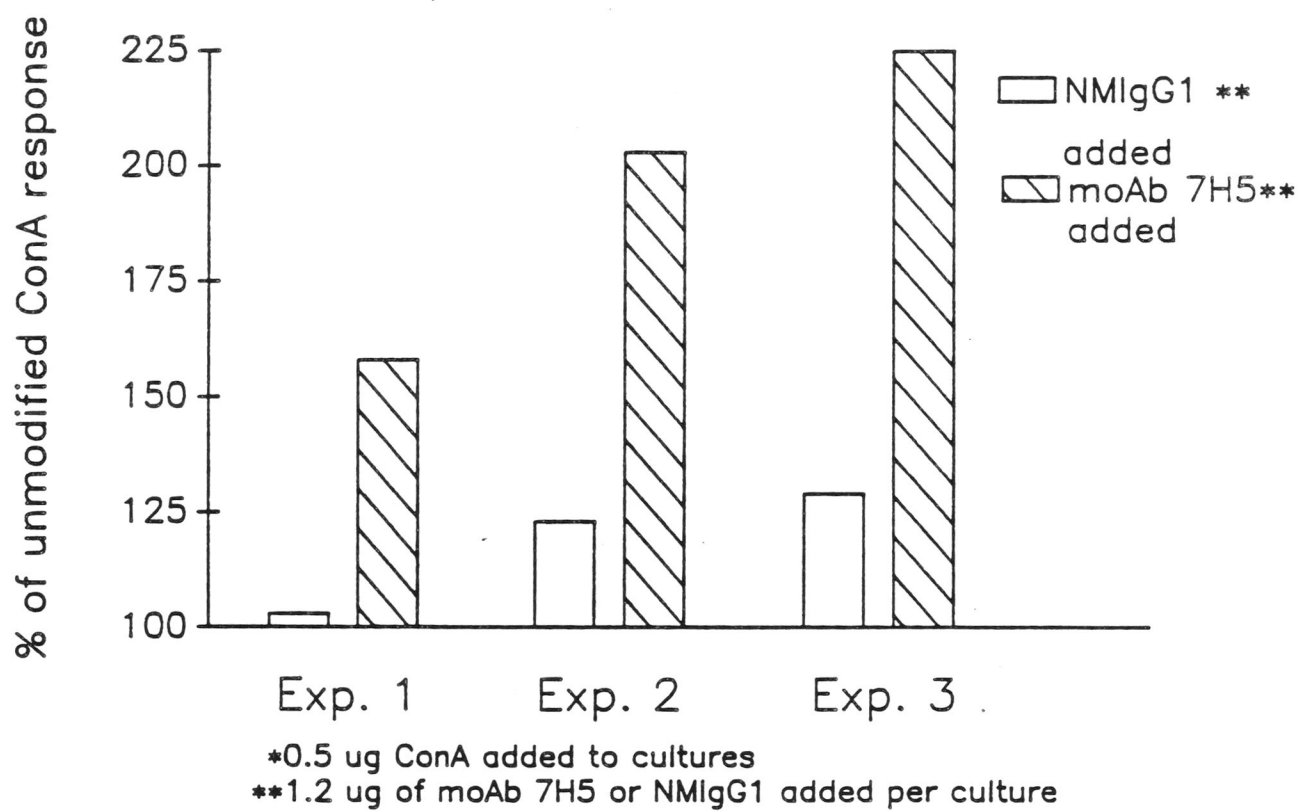
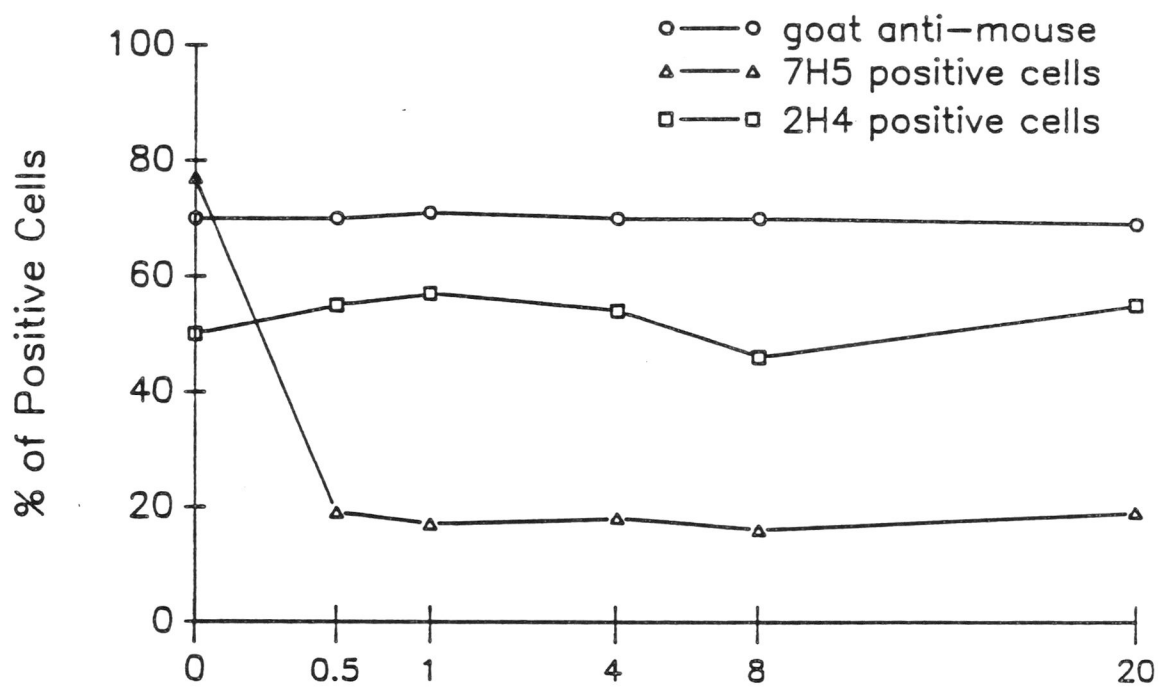


Figure 20- Capping studies with anti-7H5 on monkey PBMC. Results are expressed as percent of positive cells. Cells were incubated up to 20 hours at 37°C with anti-7H5 moAb.



Time of Incubation at 37°C with anti-7H5 moAb (hours)

DISCUSSION

These studies describe the results of original efforts to develop and characterize a murine monoclonal antibody to a common leukocyte antigen expressed on monkey PBMC.

The leukocyte common antigen (LC-A) CD45, also known as T200 (Trowbridge, 1978) or Ly-5 in mice (Omary et al. 1980) is a cell surface glycoprotein complex of approximately 200 Kd. T200 molecules have been identified in several species including rat (Fabre et al. 1977), mouse (Trowbridge et al. 1975), chicken (Houssaint et al. 1987), sheep (Mackay et al. 1987), and human (Dalchau et al. 1980). There is no previous description of a moAb prepared against rhesus monkey lymphocytes. However, there is evidence that rhesus monkey lymphocytes express the CD45R T200 determinant 2H4 which is also expressed in humans (Morimoto et al. 1985a). T200 molecules have shown to exhibit marked heterogeneity with regard to molecular mass, carbohydrate composition, and antigenicity depending on the cell lineage. These molecules are selectively expressed on all hemopoietic cells, excluding mature erythroid cells (Trowbridge, 1978; Dalchau et al. 1980; Omary et al. 1980; Standring et al. 1978). The present studies were prompted by the recognition that the availability of T200 moAbs to rhesus monkey could provide novel tools to study the biochemical and functional significance of this molecule in the immune system of

nonhuman primates.

The first step toward this goal was to establish a successful hybridoma. Stable hybrids were screened by their antibody binding pattern to rhesus monkey PBMC, and cloned by LDA. The selection of antibody producing clones was initially based on binding pattern revealed by one color FACS analysis and titration by ELISA assay. Several clones were selected for ascites production and four moAbs exhibited rapid growth as ascites and relatively high titers. Immunoprecipitation studies of one of these moAbs, an IgG1 antibody, called 7H5, identified a protein antigen of approximately 200 Kd. We anticipated that this determinant might be a T200 molecule and designed additional experiments to examine this hypothesis.

T200 molecules have been shown to have characteristic distribution patterns on lymphoid cells and tissues (Pulido et al. 1988). FACS analysis on the reactivity of anti-7H5 moAb to rhesus monkey cells indicated that this antigen is present in 10% of bone marrow cells, 68% of spleen cell, 60% of lymph node cells, and 70-80% of PBL cells. The distribution of the 7H5 antigen on monkey mononuclear leukocytes is similar to the distribution of a human anti-T200 moAb which labelled 22.0 ± 9.8 % of bone marrow cells; 48.0 ± 11.0 % of lymph node cells, and 52.5 ± 10.6 % of PBL cells (Dalchau et al. 1981). Monoclonal antibody, anti-2H4 is reactive with lymphocytes of a wide array of primate

species. In rhesus monkey, 2H4 represents 49.0 ± 8.0 % of PBL cells, in man 21.0 ± 5.0 % of PBL express 2H4 antigen (Letvin et al. 1985).

FACS studies revealed the anti-7H5 moAb did not bind to normal lymphoid cells or to transformed cell lines. These results indicate that the epitope recognized by anti-7H5 on the monkey 200 Kd molecule is not expressed on human cells. Thus, anti-7H5 recognizes a unique rhesus monkey determinant.

Two color fluorescence studies with anti-7H5 and a panel of anti-human moAbs that crossreact with rhesus monkey cells indicated that 7H5 is variably expressed on B cells, NK cells, monocytes, and subpopulations of T cells. The results indicated that virtually all B cells and CD16 (FcR3 bearing) express 7H5. The CD4 and CD8 subsets of T lymphocytes express a similar percentage of 7H5+ cells, 68% and 75% respectively. Monocytes appear to coexpress the 7H5 marker on a subset (37%). Apparently 7H5 is not expressed on neutrophils, since the anti-Leu-M4 moAb, which reacts with monkey neutrophils showed almost no crossreactivity with anti-7H5 moAb. These results indicate a restricted distribution of 7H5 antigen on hematopoietic tissues, suggesting this membrane determinant may be related to the molecule CD45R. Furthermore, the extensive coexpression of 7H5 antigen with other functionally important lymphoid cell markers, suggests a role of 7H5 cell surface molecule in

the immune system.

Immunoprecipitation studies with anti-7H5 moAb and ^{125}I labelled rhesus monkey PBL lysates revealed the presence of three high mw subunits of 205, 220 and a 235 Kd. Anti-2H4 moAb in rhesus monkey and man PBMC detects 205 and 220 Kd isoforms of the restricted form of T200 molecule. These studies showed that the 7H5 molecule exhibits a high mw band which is not seen in the 2H4 molecule.

Monkey T200 epitope recognized by anti-7H5 moAb showed not to be the same as the one recognized by anti-2H4 moAb which defines the human suppressor/inducer population. Results from FACS studies indicated 2H4 and 7H5 do not compete for binding in the monkey PBMC. It is possible that 7H5 and 2H4 are different splice products of the same gene. It is known that T200 glycoproteins are generated from a single gene by differential usage of three exons near the NH₂-terminus. Since 2H4 and 7H5 identify two similar mw subunits in the monkey T200 molecule and they differ in one subunit. Competition and cross absorption studies with SDS-PAGE are in progress and should help to initially elucidate whether 2H4 and 7H5 recognize the same molecule.

Depletion studies with anti-7H5 and magnetic beads showed that removal of 7H5+ cells totally impaired monkey NK activity. These results suggest that 7H5 molecule is expressed on all monkey NK cells. Blocking studies suggested that 7H5 molecule is not involved in monkey NK

lytic mechanism. Purified anti-7H5 moAb did not block the NK lysis to K562 target cells. Studies in mice and man showed that not all antibodies to the T200 molecule have an inhibitory effect on NK cell function (Newman et al. 1983; Seamn et al. 1981). Starling et al. (1987) demonstrated the ability of three moAbs against restricted and non-restricted leukocyte common epitopes to inhibit NK lysis. These findings suggest either the 7H5 molecule is not directly involved in NK lysis or that the epitope recognized by anti-7H5 moAb is a "non-blocking" region on the monkey T200 molecule. Recent observations could favor the latter interpretation. There is evidence that several epitopes on the human LC-A are functionally important in NK activity (Starling et al. 1987). Furthermore, Newman et al. (1983) defined a blocking region A (pronase-sensitive) and a non-blocking region B (pronase-insensitive) on the T200 molecule.

CML studies using 7H5 depleted effector cells showed a significant reduction of percent CML lysis indicating that monkey CTLp cells express the 7H5 antigen. Attempts to block CTLp activation with anti-7H5 were unsuccessful. Rather than blocking, we found that pretreatment of responders with anti-7H5 moAb at day 0 of CML cultures enhanced CTLp activation. The enhancement occurred when the responder cells were pretreated with 7H5 moAb but not when the stimulator cells were pretreated. Thus, moAb 7H5 seemed

to potentiate the activation of CTLp. The enhancement of CTLp activation was not observed when responder cells were treated with normal mouse IgG1, indicating that specific binding of anti-7H5 moAb was necessary to augment the CML response. It is possible that 7H5 moAb is binding to a T200 associated complex other than T-cell receptor/CD3 complex and somehow facilitating a transduction signal for activation. Alternative pathways of activation for CTLp have been described. One pathway involves activation via moAb 9.3, which is directed against the CD28 (Tp 44) T cell surface determinant (Hara et al. 1985 and Moretta et al. 1985). MoAbs directed to epitopes of the CD2 antigen (T11.1 and T11.2) are also associated with the alternative pathway of activation of cytotoxic T lymphocytes. Antibodies to CD2 activate T lymphocytes to up-regulate interleukin-2 receptors (IL-2R) and to proliferate (Meuer et al. 1984; Brottier et al. 1985, and Holter et al. 1986). Further studies will be necessary to determine if the stimuli caused by anti-7H5 moAb and activation of the alternative pathway caused maturation and cytotoxic function. An important question that remains to be resolved in the monkey CML is whether anti-7H5 induced enhancement is nonspecific or specific and whether this moAb can induce cytotoxicity in the absence of stimulator cells. Likewise, the question of whether anti-7H5 may affect expression of surface molecules involved in alternate pathways of CTL

activation (e.g. CD2, CD28, CD25 and others) needs to be determined. Depletion of monkey PBMC expressing 7H5 abolished proliferative responses to polyclonal mitogens. Mitogens used were PHA, ConA, and SPA. These studies indicated that the monkey lymphocytes that proliferate in response to these mitogens all express these common 7H5 antigen.

When anti-7H5 was added to cultures responding to PHA or ConA mitogens, divergent results were obtained.

Addition of anti-7H5 moAb to PHA cultures did not significantly change the proliferative responses of monkey PBMC. Ledbetter et al. (1985) reported a role of p220 (T200) in T cell proliferation. Anti-p220 antibody augments human T cell proliferation driven by PHA. This enhancement was via IL-2 receptor expression. Sanchez-Madrid et al. (1987) found that antibodies to CD45 molecule inhibited proliferative response to PHA. This divergent results may be explained by the different specificities of the antibodies used.

It is likely that the ligand formed between anti-7H5 and the membrane epitope recognized does not augment IL-2/IL-2R expression, since augmentation of PHA responses did not occur.

FACS analysis of monkey PBMC showed that preincubation with PHA does not block the binding of anti-7H5 to these cells.

In contrast to the results in PHA response, addition of anti-7H5 moAb to ConA cultures significantly increased ConA responses ($p < 0.001$). Early studies by Hoessli et al. (1979) and Favero et al. (1982), indicated that high-molecular weight glycoproteins receptors are targets for the mitogenic action by ConA. They showed that ConA receptors were a complex of glycoproteins of approximately 200 Kd. The enhancing effect of moAb anti-7H5 on rhesus PBMC response to ConA suggests that one or more of the high-molecular-weight glycoproteins recognized by anti-7H5 may be the site of ConA mitogenic action in these primates. Since pretreatment of lymphocytes with ConA did not affect 7H5 binding on FACS analysis, ConA does not appear to bind the epitope defined by anti-7H5. It is possible that ConA binds to other sites on the T200 molecule, and studies are ongoing to test this hypothesis.

Capping studies with anti-7H5 moAb indicated that 7H5 molecule does not cap. Figure 20 summarizes these studies, and shows 7H5 molecule did not cap during the 20 hours of the experiment. These observations are consistent with studies showing a lack of modulation of T200 molecules by antibodies in other species (Takeuchi et al. 1987b). This observation may be due to the large size of 7H5 molecule, or maybe due to the structural conformation of the protein in the membrane and possible interactions with other membrane components (Tackeuchi et al. 1987b).

CONCLUSION

These studies described the generation, characterization, tissue distribution, and some functional properties of anti-7H5, a moAb which appears to recognize a unique T200 molecule on rhesus monkey mononuclear leukocytes. The role of this molecule on the alternative pathway of activation of T lymphocytes, and the enhancing effect of anti-7H5 on ConA cultures remains to be elucidated. The results of these studies suggest 7H5 molecule may function in association with other cytoplasmic and/or cell surface molecules to influence the activation of T lymphocyte function. This common leukocyte antigen has a restricted distribution, similar to that exhibited by CD45R molecules in rats and humans. However, the 7H5 determinant is antigenically distinct from the CD45R determinant defined by anti-2H4 moAb. Furthermore, these molecules show differences in two bands in SDS-PAGE gels. The 7H5 molecule exhibits a unique very high mw band not seen in other descriptions of CD45R molecules. 7H5 also is unique from other CD45R molecules in that binding of anti-7H5 does not block NK lysis, and it selectively potentiates ConA, but not PHA responses. Collectively, these results suggest 7H5 is a unique variant of a CD45R molecule in rhesus monkeys and may play a central role in the function of lymphoid cells, perhaps in association with both cytoplasmic molecules and others at the cell surface.

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