# FUNCTIONAL AND BIOCHEMICAL CHARACTERIZATION OF A T CELL DETERMINANT DEFINED BY A MONOCLONAL ANTIBODY (TH5.2) WHICH IS INVOLVED IN THE IL 2 PRODUCING AND PROLIFERATIVE CAPABILITIES OF T CELLS.

by

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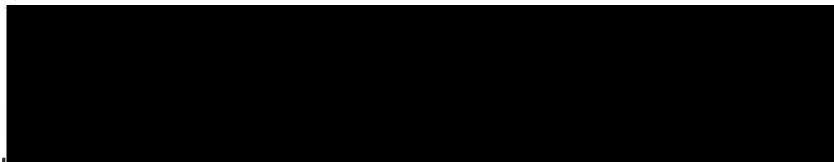
A DISSERTATION

Presented to the Department of
Microbiology and Immunology and the
Oregon Health Sciences University
School of Medicine
in partial fulfillment of
the requirements for the degree of

Doctor of Philosophy

May 1986

APPROVED:



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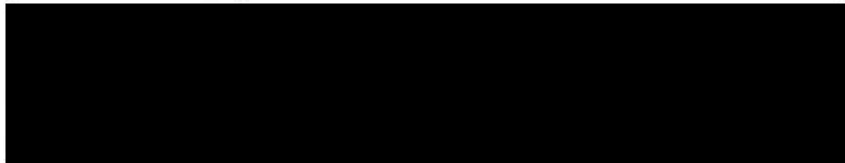

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# ABSTRACT

Utilizing an OKT-4 -positive human T cell lymphoma cell line as immunogen, we have produced a monoclonal antibody (MAb), designated TH5.2, which is capable of augmenting the IL 2 production and proliferation of antigen-activated T cells. TH5.2 MAb by itself is not mitogenic for T cells; the enhanced IL 2 production and proliferation requires co-stimulation with either antigen or mitogen. TH5.2 MAb recognizes all peripheral blood T cells at varying intensity, but does not react with monocytes. Using dual-color fluorescence analysis, it was determined that TH5.2 MAb reacts at higher intensity on Leu-3a positive T cells compared to Leu-2a positive cells. Sorting T cells on the basis of fluorescent intensity with TH5.2 MAb demonstrated that the T cells reacting at high TH5.2 intensity were able to respond to mitogen at a higher rate (5-10X), as well as produce higher concentrations of IL 2 in response to mitogen as compared to the low TH5.2 intensity cells. Cytotoxically treating peripheral blood mononuclear cells (PBMC) with TH5.2 MAb plus complement, which resulted in an approximate eight percent decrease in the total population, significantly reduced the ability of the cells to proliferate to both mitogen and antigen. Addition of recombinant IL 2 (RIL 2) to the cultures was able to restore the proliferative capability of the cells. In further analyzing the ability of TH5.2 MAb to augment the proliferative capability of PBMC to antigen and mitogen stimulation <u>in</u>

*vitro*, it was determined that TH5.2 MAb was capable of acting synergistically with antigen or mitogen in increasing the IL 2 producing capability of T cells. Biochemical analysis demonstrated that the T cell surface antigen recognized by TH5.2 MAb is a glycoprotein of 55,000 to 60,000 molecular weight. The molecule is not part of a disulfide-linked dimeric structure, and contains only a few percent of tunicamycin-sensitive carbohydrate structures. As shown in sequential immunoprecipitation studies, the TH5.2 antigen is on a molecule distinct from the IL 2 (Tac) receptor and the T4 molecule. Cell surface antigen modulation experiments indicate that the TH5.2 antigen does not co-modulate with, and therefore is distinct from, the T3, T4, T8 and Leu 5(T11) T cell antigens.

Our studies have determined that the TH5.2 determinant is involved in the proliferative capability of T cells. The TH5.2 determinant is a distinct molecule from a number of other characterized membrane antigens and may play a role in the ability of T cells to synthesize and/or secrete IL 2.

# INTRODUCTION

The ability of T lymphocytes to become activated and subsequently proliferate in response to antigen stimulation is a complex process involving the participation of a number of cell surface receptors and determinants. Identification and characterization of these membrane structures is an initial step in elucidating the mechanism of T cell activation. Considerable research has been conducted in the recent past concerning the nature and function of these receptors and determinants, as well as the identification and role of soluble growth mediators that are required for T cell proliferation. One concept which is emerging from these studies is the complexity of the activation and proliferation pathways that are utilized by T cells. Although much is understood about the process, it is evident that additional effort and research is required for a more complete understanding of the events to evolve. The purpose of the research presented in this dissertation is to add to the body of knowledge of T cell activation by defining and characterizing novel membrane determinants involved in the process. Presented in this introduction will be a review of previous investigations which have contributed to the current knowledge of the activation process. This review will hopefully assist in providing a context for understanding the basis of the studies described in this dissertation.

It has been well established that T lymphocytes are capable of responding to antigen only in the context of haplocompatible determinants encoded by the major histocompatibility (MHC) gene complex (1-5). T lymphocytes can be divided into two subpopulations: one committed to recognizing antigen in the context of MHC class II encoded determinants (predominantly cells of the helper/inducer phenotype), and the other restricted to (cytotoxic/suppressor) class I antigen recognition (6-9). The helper/inducer T cells require the expression of haplocompatible class II determinants along with antigen on the surface of antigen presenting cells for the activation event to occur. A number of cell types have been shown to be capable of presenting antigen to class II restricted T cells including monocytes (10,11), endothelial cells (12,13), dendritic cells (14,15), Langerhans cells (16,17), epithelial cells (18), and B lymphocytes (19). All of these cell types are capable of expressing MHC class II determinants. The nature of the interaction between receptors on the T cell, and antigen and class II determinants expressed on the presenting cell is not well understood. It has been hypothesized that a determinant expressed on class II-restricted T lymphocytes, the T4 molecule is involved in the recognition of class II determinants expressed on the antigen presenting cell (9,20,21). The T4 molecule is expressed predominantly on the helper/inducer subpopulation of T cells, although cytotoxic T4-positive cells are known to occur (22). Anti-T4 MAb are capable of inhibiting activation of T cells by MHC class II compatible antigen

presenting cells plus antigen (20). In addition, antibodies against T4 are capable of inhibiting the cytotoxic activity of T4-positive T cells against target cells bearing class II determinants (9,21). These observations are consistent with the hypothesis that T4 molecules expressed on T cells function as receptors for class II determinants. Bank and Chess (23) have hypothesized that anti-T4 antibodies inhibit T4-positive T cell function not by blocking class II determinant recognition, but rather by inducing a negative signal to the cells. They determined that activation of T4-positive T cells under certain conditions could be accomplished using class II determinant-negative accessory cells. Anti-T4 MAb was nevertheless still capable of inhibiting the activation, indicating that an inhibitory signal was being delivered to the T cell independent of class II recognition.

Recently, it was determined that antigen and MHC class II structures are closely associated with each other on the surface of monocytes. Puri and Cantor (24) isolated a moiety from supernatants of antigen-pulsed adherent speen cells which consisted of antigen tightly bound to MHC class II (I-A) molecules. The I-A-antigen complexes were capable of binding to solid support affinity columns of the correct antigen specificity, as well as to haplocompatible MHC class II supports but not to haplo-incompatible columns. Radiolabelled antigen-I-A complexes were capable of binding only to T cell clones of the correct antigen and I-A specificities. In addition, sequential immunoprecipitation experiments with anti-I-A and anti-antigen

antibodies confirmed that the complexes consisted of tightly bound antigen and I-A moieties. These results confirmed previous observations that antigen shed by an adherent cell population was capable of reacting with anti-Ia affinity columns (25). In these experiments it was additionally determined that the antigen-I-A complex was 100-1000-fold more immunogenic in vitro than antigen alone and that this increased immunogenicity could be removed by anti-Ia columns. These results indicate that binding structures for antigen and MHC class II determinants may be closely associated with each other on the T cell membrane. The identification and characterization of the T cell antigen receptor is critical in understanding the antigen presentation process and until recently the successful elucidation of the structure has eluded immunologists.

In 1983, Haskins and Marrack (26), Meuer et al (27) and others (28,29) described the production of MAb which were specific for individual antigen-specific T cell clones or T hybridoma lines, and which also were capable of inhibiting T cell functions. The MAb (termed anti-Ti by Reinherz) recognized a di-sulfide-linked heterodimer (Ti) of about 40,000 and 43,000 molecular weight (mouse) and 49,000 and 43,000 molecular weight (human) on the surface of T cells. Functional studies demonstrated that the MAb were capable of 1) inhibiting antigen-driven proliferation (26), 2) inhibiting cytotoxicity of T cell clones to their specific targets (30,31), and 3) under the correct conditions totally replace antigen in inducing the appropriate clone to proliferate (32). In addition to these functional properties of the anti-Ti MAb, it was shown that the polypeptide chains (termed $\alpha$ and $\beta$)

comprising the heterodimer recognized by the MAb, displayed considerable structural heterogeneity when different antigen-specific T cell clones were compared (33). This heterogeneity would be expected of an antigen recognition molecule. Taken together, the studies suggested that the anti-Ti MAb recognize a structure on T cells that is involved in antigen recognition by T cells.

Following an entirely different approach in the identity and characterization of the T cell antigen receptor, Hedrick and Davis (34) and Yanugi et. al. (35) cloned cDNA copied from mRNA transcripts that were specific for T lymphocytes, and screened these clones for any that might potentially encode the antigen receptor. The studies revealed the existence of gene segments which occurred in a rearranged sequence only in T cells, compared to a "germ-line" non-rearranged sequence in other cell types including B cells. These gene segments showed homology to that of immunoglobulin genes not only in sequence but also in general organization exhibiting rearrangeable V, D, J, and C segments. It was hypothesized by the authors that the gene segment encoded a polypeptide that might be involved in antigen recognition by T cells in an analogous fashion to immunoglobulin for B cells. N-terminal amino acid sequencing established that these DNA segments encodedd one of the polypeptide ($\beta$) chains of the putative T cell receptor heterodimer described earlier (36). The sequencing data linked the functional results provided by the use of anti-Ti MAb, with the molecular/biochemical data, thus providing compelling evidence that the heterodimer is the T cell antigen receptor. Chien and Davis (37) and Saito and Tonegawa (38) subsequently cloned gene segments

corresponded to the other polypeptide chain ($\alpha$) of the heterodimer completing the initial characterization of the molecule.

In 1983 Meuer et. al. (39), and Reinherz et. al. (40) demonstrated that the heterodimer identified by the anti-Ti MAb was closely associated with, but distinct from another polypeptide expressed on the cell membrane, the T3 molecule. The T3 molecule had previously been determined to be expressed on the surface of all helper/inducer as well as cytotoxic/suppressor T cells (41). Co-modulation experiments showed that the T3 and Ti heterodimer both are modulated off of the cell surface when either of the molecules are modulated alone (39,40). The two determinants are distinct molecules however, as shown by sequential immunoprecipatation experiments (39). MAb reactive with the T3 determinant possess similar function properties compared to the anti-Ti MAb. Anti-T3 MAb are, in the presence of accessory cells (41,42) or phorbol esters (43) mitogenic for resting T cells. In addition, anti-T3 MAb can block both the induction and effector phase of cell mediated lympholysis (44). Anti-T3 MAb, in contrast to anti-Ti MAb, however react with virtually all T cells regardless of antigen specificity (41,42). In addition the T3 molecule does not display heterogeneity in structure when different T cell clones are compared (45). These results imply that the T3 molecule does not function in actual antigen recognition, but because of its important functional properties is likely to be involved in the activation process. Weiss et. al. (46) has hypothesized that the T3 molecule may be functioning as a signal for release of intracellular $CA^{++}$ following perturbation of either the T3 or Ti antigen receptor

complex. Increases in intracellular $Ca^{++}$ has been shown to occur following the activation process (46,47,48) and in fact agents which are capable of increasing the concentration such as $Ca^{++}$ ionophores are mitogenic for T cells (46).

Following the activation signal provided by antigen, anti-Ti or anti-T3 Mab, a number of intra- and extracellular events occur before the T cell is capable of undergoing proliferation. Anti-Ti or anti-T3 MAb in conjunction with phorbol myristate acetate (PMA) are capable of inducing T cells to secrete interleukin 2 (IL 2) and proliferate (40,49). This activation depends on the ability of the T3/Ti complex to increase free cytoplasmic $CA^{++}$. It was demonstrated by Imboden and Stobo (48) that the release of $Ca^{++}$ was a result of receptor-induced increases in inositol triphosphate ($IP_3$). $IP_3$ is known to increase intracellular $Ca^{++}$ in other hormone-receptor systems (50,51). PMA was necessary as a second signal for the ability of anti-Ti or anti-T3 MAb to activate the cells. It is known that PMA is capable of activating of protein kinase C (52), and it was hypothesized that this is its mode of action in the activation process. Protein phosphorylation is known to take place in T cells following activation which is consistent with this hypothesis (53,54).

In addition to these intracellular events, binding of IL 2 to specific receptors induced on the membrane following the activation event is necessary for cell proliferation and clonal expansion. The topic of IL 2 will be discussed in greater detail in subsequent sections of the discussion.

Although perturbation of the antigen receptor by interaction of antigen, in the context of MHC encoded determinants, anti-Ti MAb or anti-T3 MAb represents an important method of activating T cells, other alternative pathways of activation utilizing different determinants have been described. The T11 molecule is a 50,000 molecular weight structure that is expressed on all thymocytes and peripheral blood T cells of primate origin (55,56,57). The T11 determinant is the first known T-lineage-specific molecule to appear in human ontogeny (58,59), and is the molecule responsible for the sheep erythrocyte binding capability of T cells. Until recently little was known about the physiologic role of the determinant. To characterize the function of the T11 molecule, Meuer and Reinherz in 1984 produced a series of MAb which were reactive with various epitopes on the molecule (60). They then utilized the MAb in _in vitro_ assays to examine their effects on T cell proliferation. Utilizing two MAb (anti-$T11_2$ and anti-$T11_3$) reactive with two distinct epitopes on the T11 molecule they demonstrated that T cells could be activated and induced to proliferate by addition of the MAb to cultures of resting T cells. The mitogenic effect of the MAb was accessory cell independent which is in contrast to the anti-T3 MAb. As discussed earlier, anti-T3 MAb requires either the presence of accessory cells or phorbol esters for mitogeneity. In conjunction with the proliferation results, it was also determined by Meuer and Reinherz, and Fox et. al. (61) that anti-$T11_2$ and anti-$T11_3$ were capable of inducing secretion of IL 2 and expression of IL 2 receptors on resting T cells. Conversely, Tadmoori et. al. demonstrated that other anti-T11 MAb inhibited IL 2 secretion and IL 2

receptor expression further linking the determinant to the activation and proliferation process (62,63).

Because both T3/Ti antigen receptor and the T11 molecule are involved in T cell proliferation, Meuer and Reinherz were interested in the relationship between the two activation pathways. They demonstrated by use of modulation experiments that activation of T cells via the T11 molecule is highly dependent upon the presence of the T3/Ti complex on the surface of the cell. The reciprocal, however, was not observed. Anti-T11-modulated T cells were still capable of being activated by anti-T3 MAb. These results suggested that modulation of the T3/Ti complex is capable of regulating the T11-induced activation pathway. The authors hypothesize that this type of control of activation would inhibit non-specific cell activation once an antigen-specific activation event via the T3/Ti complex has occurred. Conversely, a pathway for activation of T cells independent of antigen may be desirable for cellular recruitment and lymphokine production, and this could be accomplished by utilizing the T11 pathway. Recently, Milanese, Richardson and Reinherz described a novel lymphokine, termed interleukin 4a (IL 4a) which is capable of stimulating resting T cells to proliferate (64). IL 4a is a 10,000-12,000 molecular weight glycoprotein and is produced only by T cells of the helper/inducer phenotype in response to stimulation with either anti-Ti or anti-T3 MAb. Preliminary results indicate that IL 4a may be inducing the proliferation via interaction with the T11 determinant. Adsorption experiments determined that a T cell line which is T11-positive was capable of adsorbing out IL 4a activity while a T11-negative mutant of

the same line was not. In addition, other cell types were used that were either T11-positive or negative and similar results were seen. These results indicate that the T11 molecule may be involved in binding of IL 4a and that the lymphokine may be the natural ligand for the receptor.

A third, determinant-mediated activation pathway has been described for T cells which utilizes a cell surface molecule designated T44 (65,66). Using a MAb (9.3), T44 has been defined as a T-specific di-sulfide-bonded dimer of approximately 88,000 molecular weight (65,67). Moretta and Moretta (66) demonstrated that the 9.3 MAb was capable of inducing peripheral blood T cells to proliferate if adherent cells were present in the culture system. This is similar to the requirement of anti-T3 and anti-Ti MAb which also require a second signal or stimulus for mitogenicity. In support of this observation, Hara and Hansen (65) determined that 9.3 MAb was highly mitogenic for monocyte-free peripheral T cells, but only in the presence of 12-O-tetra- deconyl phorbol-13-acetate (TPA), a phorbol ester. Phorbol esters can, in some situations, replace the requirement for adherent cells in the induction of proliferation of T cells(49,50). In addition to the mitogenic effect on T cells, as would be expected 9.3 MAb was capable of inducing IL 2 production by the cells (65). Interestingly, 9.3 MAb did not require the presence of adherent cells or TPA for the ability to induce JA3 cells (a variant of the IL 2-producing Jurkat leukemia cell line) to secrete IL 2, while anti T3 and anti Ti MAb did. In the same study it was determined that modulation of the T44

molecule from the surface of T cells had no effect on the expression of the T3/Ti complex, or on the ability of anti-T3 or anti-Ti MAb to induce cell proliferation and IL 2 production. As in the case of the T11 determinant, however, the reciprocal did not occur. Modulation of the T3/Ti receptor resulted in a cell which was not responsive to 9.3 (anti-T44) MAb induction of both proliferation and IL 2 production. These experiments served to demonstrate the functional, as well as physical relationship of the T44 and T3/Ti determinants. It was also determined that the T44 and T11 determinants did not co-modulate from the surface of T cells indicating that 1) they are distinct molecules and 2) the two determinants are not associated on the cell membrane. A functional relationship, however, was not established between the T11 and T44 determinants. Currently, no known natural ligand has been identified for the T44 determinant.

Following activation of T cells via each of these three determinant-mediated pathways, a second signal is required by the cells for subsequent proliferation and clonal expansion. As mentioned previously this signal is supplied by interaction of a growth hormone, IL 2, with specific receptors which have been induced on the cell membrane following the activation event. The following section will review the studies which have contributed to the elucidation of the IL 2 lymphokine system.

Historically, it has been well established that activated T cell cultures require a soluble mediator for their ability to proliferate _in_

*vitro* (68-71). The mediator could be generated by stimulating mononuclear cells with either mitogen or antigen (72); the growth activity being recovered in the cell-free supernatant. Various approaches have been utilized to purify the factor termed T cell growth factor (TCGF) including gel filtration and isoelectric focusing (73). Using these techniques, a glycoprotein of approximate molecular weight of 15,000 was purified to apparent homogeneity which was capable of inducing activated T cells to proliferate. The advent of human (74) neoplastic T cell lines which secreted high concentrations of the factor was essential for accumulating adequate amounts for purification. In addition, MAb were generated which were reactive with TCGF or interleukin 2, as it was subsequently renamed (81), allowing for a simplified and specific method of purifying the molecule (75,76). The primary amino acid sequence of purified IL 2 was deduced by Robb et. al. in 1984 (77), and in the same year two independent groups (78,79) cloned the gene encoding the lymphokine.

Functionally, IL 2 interacts with T cells in a hormone-like fashion exhibiting characteristics such as high affinity, target cell specificity, and ligand specificity. These properties were investigated by Robb, Munk and Smith using highly purified, radiolabelled IL 2, and a cloned IL 2-dependent T cell line (80). The experiments demonstrated that IL 2 bound specifically with IL 2 responsive cells expressing high affinity binding sites for the molecule. In addition it was shown that IL 2 binding curves, and growth curves for the IL 2-dependent T cells in response to varying

concentrations of IL 2, were remarkably similar indicating that the biological response is nearly proportional to the IL 2 binding site occupancy.

In a series of studies (82-86) conducted by Uchiyama et. al., a MAb reactive with a T cell membrane determinant was produced which appeared to identify the T cell IL 2 receptor. The MAb, termed anti-Tac, was capable of 1) blocking high affinity IL 2 binding to activated T cells (86), 2) inhibiting IL 2-induced cell proliferation (83,84), 3) immunoprecipitating a covalently-linked complex of labelled IL 2 and Tac protein (55,000 molecular weight) (87), and 4) utilizing solid supports, isolating the same solubilized membrane protein as IL 2 (88). Utilizing the anti-Tac MAb, a gene encoding the IL 2 receptor was isolated and cloned by various groups (89,90) confirming the specificity of the antibody. In addition, the use of the anti-Tac MAb has led to a greater understanding concerning the characteristics and function of IL 2 receptors.

IL 2 receptors are expressed at a low density on resting peripheral T cells (1,100/cell) (91). Within 24 hours following activation, both T and B lymphocytes are induced to express significantly greater numbers of receptors per cell (20,000-30,000/cell for T cells, 5,000-10,000/cell for B cells) (91,92). It was determined by Robb et. al. (91) and Wakasugi et. al. (93) that IL 2 receptors are expressed in at least two very different affinity forms on the membrane. By using different concentrations of labelled IL 2 in determining saturable binding to cells, it was found that IL 2

receptors existed on cells which exhibited dissociation constants that differed by as much as 4000-fold. The functional role of high and low affinity IL 2 receptors is not well defined. It has been established that resting T cells express predominantly low affinity receptors and will not proliferate until adequate numbers of high affinity receptors are induced following an activation stimulus (91,93). Wakasugi et. al. has proposed a role for monocytes in inducing a switch from low affinity to high affinity receptors on T cells (93). Stimulation of human purified T cells with Con-A or anti-T3 MAb alone resulted in increased IL 2 receptor expression predominantly of the low affinity type. These stimulated cells, however, were not capable of proliferating even with the addition of exogenous IL 2. When monocytes were added to the cultures the cells proliferated. Analysis of the IL 2 receptors expressed on the cells now indicated the presence of maximal numbers of high affinity receptors, implying that monocytes play an important role in the expression of high affinity IL 2 receptors on T cells.

Another well established role of monocytes in the proliferation of T cells is the production of interleukin 1 (IL 1), a monokine which is involved in the IL 2-producing capability of T cells. IL 1 is a multi-functional soluble mediator released by a number of different cell types including macrophages (94,95,96), dendritic cells (97), endothelial cells (98,99,100), fibroblasts (101), keratinocytes (102,103), and astrocytes (104). Although IL 1 possesses many diverse functional properties including endogenous fever mediation (105), bone resorption (106,107), and synthesis of acute-phase reactants (108), of

specific interest to this discussion is its ability to modulate the IL 2 lymphokine system. IL 1 is a polypeptide of approximately 15,000 molecular weight which can exist in at least four isoelectric point forms. Murine IL 1, initially purified from supernatants of phosbol myristate acetate (PMA)-stimulated P388D1 cells exhibited microcharge heterogeneity with isoelectric points (pI) of 4.9, 5.0 and 5.1 (109). In contrast human IL 1 purified from human peripheral blood monocytes exists in pI forms of 5.2, 5.7, 6.0 and 6.8 (110). The molecular basis of this charge heterogeneity remains unclear. The heterogeneity is likely not the result of glycosylation as there is no direct evidence that carbohydrate residues are present or essential for IL 1 activity (111,112). Alternate explanations for the heterogeneity include proteolytic cleavage of larger, precursor intracellular forms or the existence of a gene family each encoding a different pI form. Recent experiments have demonstrated that the mRNA transcript for IL 1 encodes a molecule that is at least twice the size (35 kilodaltons) of the IL 1 molecule found extracellularly (113,114). It was also determined that the larger precursor molecule is subsequently processed intracellularly into the smaller 15,000 molecular weight form. The heterogeneity displayed by IL 1 may be a result of variable cleavage of the precursor form of the molecule. Utilizing antisera that was specific for IL 1, an assay was developed that detected IL 1 mRNA which was subsequently used in the cloning of the genes encoding IL 1 (113,114). Two genes encoding IL 1 molecules were isolated, one which encoded a polypeptide which corresponded to the murine pI 5 forms termed IL 1 $\alpha$, and the

other encoding the human pI 7 form of IL 1 which was designated IL 1$\beta$. These results indicate that the heterogeneity of IL 1 molecules isolated is probably a result of both different gene products, as well as variable processing from the larger intracellular form to the smaller secrected form.

In 1972, Gery et. al. described a soluble mediator secreted by activated macrophages, which he termed lymphocyte activating factor (LAF) which was capable of strongly augmenting the proliferative response of murine thymocytes in response to suboptimal doses of mitogen (115). Since thymocyte proliferation is directly related to IL 2 concentration in the culture system, one explanation for the augmentation was that LAF was influencing the production of IL 2 by the thymocytes.

Using cloned T cell lymphomas, Gillis and Mizel investigated the specific role of LAF, subsequently renamed IL 1 (81) in inducing T cell proliferation (116). One of the cell lines (LBRM 33-5A4) secreted large concentrations of IL 2 following mitogen stimulation, while the second line (LBRM 33-1A5) was a non-secretor under the same conditions. Addition of purified IL 1 to the LBRM 33-1A5 non-producers resulted in the capability of the cells to secrete comparable amounts of IL 2 compared to the LBRM 33-5A4 producers following mitogen stimulation. This observation implied that IL 1 was functioning as a inducer of IL 2 production by T cells. Is IL 1, however, an essential component for the IL 2 production, and subsequently the proliferative capability of T cells? In attempting to answer this question, Smith

et. al. demonstrated that partially purified IL 1 could replace the presence of adherent cells in inducing splenic T cells to secrete IL 2 and proliferate in response to lectin stimulation (117). Durum and Gershon (118) utilized antigen-primed lymph node T cells and determined that if antigen presenting cells were depleted from the population using anti-IA antibody and complement the remaining T cells were not capable of responding to additional antigen stimulation. Addition of partially purified IL 1 to these depleted cultures restored the proliferative response. Scala and Oppenheim demonstrated that monocytes which were pulsed with antigen and subsequently treated with paraformaldehyde were not capable of stimulating T cell proliferation in contrast to non-paraformaldehyde treated, antigen-pulsed monocytes (119). The paraformaldehyde treatment was shown to prevent the monocytes from producing and secreting proteins including IL 1. When partially purified IL 1 was added to cultures of T cells and the paraformaldehyde-treated monocytes, the IL 2 producing and proliferative capability of the T cells was restored. In other studies (120,121) it was determined that IL 1 was not a necessary component in stimulating IL 2 release by T cells. In these studies, however, the T cell source used was T-T hybrids which may not reflect the requirements of normal T cells. T cell clones grown with antigen and feeder cells are capable of being stimulated with IL 1, however, following fusion with the thymoma fusion partner, the IL 1 responsive capability was frequently lost (121). These results imply that IL 1 can affect different IL 2-related T cell functions depending upon the particular T cell type or clone analyzed.

Although many studies have been conducted addressing the functional role of IL 1 in T cell proliferation, few reports have been published which have investigated IL 1-binding structures on cells. Dower et. al. (122,123) utilized radiolabelled recombinant IL 1 to demonstrate that an IL 1-responsive, IL 2 secreting T cell line (LBRM 33-1A5) was capable of specifically binding IL 1. Using a crosslinking protocol it was determined that the IL 1 bound to a surface protein of approximately 80,000 molecular weight. Analysis of a number of different cell types, established that the cells which specifically bound IL 1 were the types which have been reported to respond functionally to the molecule. In addition, it was determined that two diverse cell types known to respond to IL 1, LBRM 33-1A5 and 3T3, a murine fibroblast cell line were capable of specifically binding IL 1 via similar plasma membrane receptor molecules (123). This is based on the observations that the receptors on each cell type exhibit similar affinities for IL 1 and also a similar molecular size. Quantitation of receptors on the cells revealed that the LBRM cells expressed approximately 200 receptors per cell, compared to approximately 5,000 per cell for the 3T3 fibroblast line. Interestingly 3T3 fibroblasts require 30-40-fold higher concentrations of IL 1 for stimulation compared to the concentrations required to stimulate LBRM cells to secrete IL 2. The authors hypothesize that this concentration difference may reflect the different intracellular requirements for each cell type. LBRM cells may only require activation and transcription of the IL 2 gene, compared to the more complex

requirements of cellular proliferation for fibroblasts. These studies demonstrate the existence of specific membrane binding structures for IL 1, however, currently the IL 1 receptor has not been isolated and completely characterized.

To summarize, T lymphocytes are capable of being activated by triggering of certain receptors and determinants expressed on the surface of the cell. Of foremost physiological importance is activation via the T cell antigen receptor by nominal antigen in the context of haplocompatible MHC-encoded gene products or, in some cases, by MAb, which are reactive with the receptor complex. Following the initial activation signal a number of intracellular events occur including release of intracellular $CA^{++}$ stores, increase in $IP_3$ levels, protein phosphorylation and cyclic nucleotide changes. These intracellular changes are subsequently followed by the production of growth mediators such as IL 2 by the T cells which are required for proliferation of the cells.

In addition to the antigen receptor, other determinants expressed on the cell surface have been characterized, which when stimulated, are capable of inducing T cell proliferation. These include the T3 determinant, which is closely associated with the antigen receptor on the cell membrane, the T11 molecule which may function as an antigen-independent pathway of activation, and the T44 determinant which is capable of affecting IL-2 production by T cells.

Following activation of T cells via these determinant-mediated pathways, the cells require a second signal for subsequent proliferation to occur. This signal is provided by binding of IL 2 to specific receptors which have been induced on the cell membrane by the activation process. The receptor can exist on the membrane in at least two different affinity forms, the high affinity receptors being associated with the proliferative capability of the cell. IL 2 production by T cells is widely believed to be induced by binding of IL 1 to putative receptors expressed on the T cell. Depending upon the T cell source, however, IL 1 may not be required for IL 2 production but rather may play a role in inducing IL 2 receptor expression. Isolation and further characterization of the IL 1 receptor will help to clarify the role of IL 1 in affecting T cell function.

Utilization of hybridoma technology has been an important factor in the identification and characterization of the receptors and determinants which have been described in this introduction. The purpose of the research presented in this dissertation is to identify and characterize using MAb, membrane determinants that are involved in the ability of T cells to proliferate. We have utilized a human T cell lymphoma cell line which is OKT-3-negative/OKT-4-positive to produce MAb which are reactive with such determinants. Our rationale for using this cell line was that 1) we were interested in determinants that might be preferentially expressed on the T4-positive inducer/helper subpopulation of T cells, and 2) the T3-negative status of the cells

might be indicative of T cells existing in an early activation state thus providing a homogeneous population of cells expressing determinants associated with this stage. One such MAb designated TH5.2, which is highly reactive with the T cell lymphoma cell line, recognizes a determinant on normal human peripheral T cells that is involved in the proliferative, as well as IL 2-producing capability of the cell. In this dissertation we describe the functional and biochemical characterization of the TH5.2 determinant and also discuss its possible role in the T cell activation process.

# MATERIALS AND METHODS

CELL CULTURES. The HTL cell line used as the immunogen for producing the TH5.2 MAb hybridoma cell line is a HAT-sensitive human T cell lymphoma obtained from Dr. John Stobo (UCSF, San Francisco, CA). The HT-2 and CTLL cell lines used for quantitation of IL-2 and staining were obtained from Dr. David Hinrichs, WSU, Pullmam, WA). The P388D murine macrophage line, the LBRM 33-1A5 murine IL 1-responsive, IL 2-secreting T cell line, and the CRL-1510 human fibroblast line were obtained from the American Type Culture Collection (Rockville, MD). Staining of the human IL 2-dependent HUT-102 cells was performed by Dr. R. Robb. Peripheral blood mononuclear cells (PBMC) isolated from either buffy coats (drawn the same day) or fresh venous blood from healthy volunteers were isolated by centrifugation over Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, NJ), followed by three washes with RPMI. All cell cultures were maintained in RPMI 1640 media (Gibco, Grand Island, NY) supplemented with 10% fetal calf serum (Gibco) or human AB serum, 10 mM N-2 hydroxyethylpiperazine N'-2-ethanesulfonic acid (HEPES) buffer (Calbiochem, La Jolla, CA) L-glutamine (300 ug/ml), penicillin 100 units/ml, streptomyocin 100 ug/ml, and Fungizone (25 ug/ml) (all Gibco). Cultures of cells were incubated in a 37C humidifed incubator with 7% $CO_2$.

PRODUCTION OF TH5.2 MAb. Hybridoma production was accomplished using the protocol of Oi and Herzenberg (124). The spleen from a BALB/C mouse immunized with $10^7$ HTL cells intraperitoneally three times every two weeks was homogenized into a single cell suspension using a wire screen. Red blood cells were lysed from the suspension using a 0.85% $NH_4(Cl)_2$ solution. One hundred million spleen cells were fused to an equal number of NS-1 myeloma cells (from the American Type Culture Collection) using polyethylene glycol 1500 (BDH Ltd, Poole, England). Following the fusion, the cells were plated in microtiter plates at $10^6$ cells per well with the addition of thymocyte feeder cells in RPMI plus 10% fetal calf serum and 1X HAT (hypozanthine, aminopterin, thymidine). Approximately two weeks post-fusion, supernatants were collected and analyzed for reactivity using the cytoflurograf. Positive wells were subsequently clone two times by limiting dilution analysis.

CYTOFLUOROGRAPHIC ANALYSIS. Culture supernatants from hybridoma cells were screened initially by cytofluorometry. One million HTL or PBMC were stained with 100 ul of neat culture supernatant or, in some cases, a 1/50 dilution of ascites fluid for 30 min on ice. The cells were washed two times with ice-cold RPMI-1640 followed by a 30 min incubation on ice with a 1/40 dilution of a fluorescein isothyocyanate (FITC)-conjugated goat anti-mouse immunoglobulin (heavy and light chain specific) antibody ($Fab'_2$ Cappel, Cochranville, PA). After washing, the stained cells were analyzed for fluorescence intensity using an ORTHO systems 30H fluorescence activated cell sorter (FACS) (Ortho

Diagnostics, Westwood, MA). Dual-color fluorescence analysis was performed in other experiments. Phycoerythrin (PE)-conjugated MAb (Leu-2a, Leu-3a, Leu-M3)(Becton-Dickenson, Mountain View, CA) and FITC-conjugated goat anti-mouse IgM (u chain-specific, Cappel) in combination with TH5.2 MAb were utilized for the analysis. Resting PBMC were stained simultaneously with the PE-conjugated MAb and TH5.2 MAb on ice. The cells were then washed, and restained with a goat anti-mouse u chain FITC-conjugate. The cells were washed again and analyzed by dual-color fluorescence on a Becton Dickenson analyzer.

For experiments determining the effects of TH5.2 MAb on Tac antigen expression, PBMC were incubated with various concentrations of both phytohemmaglutinin (PHA) (Difco, Detroit, MI) and TH5.2 MAb and three days later stained with anti-Tac MAb (kindly supplied by Dr. T. Waldman) followed by a goat-anti mouse IgG, Fc fragment specific FITC-conjugated ($Fab'_2$, Cappel) second antibody.

PROLIFERATION ASSAYS. PBMC were set up in triplicate in 96 well culture plates (Linbro, McLean, VA) at a density of $10^5$ cells per well. PHA was added to some wells at a concentration ranging from 0.1 to 2 ug/ml. Hybridoma culture supernatants, or in some cases ascites fluid, were added to the wells at varying dilutions at the initiation of the culture period. Quantitation of IgM concentration was accomplished using a radial immunodiffusion assay; the suspensions adjusted to the same antibody concentration using RPMI medium. Control cultures contained cells plus media only, cells plus mitogen, but no TH5.2 MAb, or cells plus other IgM MAb (in the form of culture

supernatants or ascites fluid). Fifty-four hours after initiation of culture, the cells were pulsed for 18 hrs with tritiated thymidine ($^3$H-TdR, New England Nuclear, Boston, MA, 1 uci/well) harvested and subsequently counted in a liquid scintillation counter. In addition to PHA as a stimulant, various antigens were also used. These included keyhole limpet hemocyanin (KLH, VAMC, Portland, OR), streptokinase/streptodornase (SK-SD), and mumps antigen, (both M.A. Bioproducts, Walkersville, MD). All antigens were used at either a 1/10 or 1/100 final culture dilution except for KLH which was used at 10 ug/ml final culture concentration. The assays were identical to the PHA assays except the culture period was six days.

FACS SEPARATION OF T CELLS ON THE BASIS OF REACTIVITY TO TH5.2 MAb. Prior to the staining and sorting process normal PBMC were subjected to an adherent step using flasks coated for 1 hr at 4C with 10% FCS. PBMC were incubated in the coated flasks for 1 hr at 37C and the non-adherent population decanted and utilized for subsequent staining. Adherent cells were removed from the flasks by incubation for 10 min with 0.2% ethylenediaminetetraacetate (EDTA) and subsequently washed 2X with RPMI. Fifty million T cells were stained with TH5.2 MAb ( 1 ml of a 1/50 dilution of TH5.2 ascites fluid per $10^7$ cells in a 2 ml volume) and goat anti-mouse IgM (u chain specific) FITC-conjugate as described above. The stained cells were then sorted into two populations using an ORTHO systems 30H FACS. In order to further deplete the populations of accessory cells, the FACS was gated such that the monocyte population was not included in the sorted population. The first population consisted of PBMC which

reacted at high intensity with TH5.2 MAb. An arbitrary cut-off point was made on the channel selection for intensity on the FACS resulting in this population representing approximately 30 percent of the total T cell population. The second population of sorted cells consisted of T cells which reacted with low intensity to TH5.2 MAb. This subpopulation also represented approximately 30 percent of the total population of PBMC stained. These two sorted populations were then tested with and without the presence of adherent cells for their ability to proliferate and secrete IL 2 to mitogen stimulation in vitro , as well as for their cell surface phenotype.

CYTOTOXIC TREATMENT OF PBMC WITH TH5.2 MAb PLUS COMPLEMENT. One million PBMC were incubated with 100 ul of neat TH5.2 culture supernatant for 1 hr at room temperature. The cells were then washed two times with RPMI, followed by addition of 10-100 ul of rabbit low tox complement (Cedarlane, Hornby, Ontario, Canada). The cells were incubated for an additional 30 min at room temperature followed by two washes with RPMI. The viability of the treated cells ranged between 65-90 percent of the total PBMC treated as tested by total cell count and trypan blue exclusion staining. The depleted cells were then adjusted to $10^6$ cells per ml, set up in culture, stimulated with optimal doses of PHA (2 ug/ml), and assayed as described above. Control cultures for the experiment included PBMC treated with TH5.2 MAb only, complement only, or an irrelevent (non-reactive with PBMC) IgM MAb plus complement.

RECONSTITUTION EXPERIMENTS. Cultures of PBMC which had been treated with TH5.2 MAb plus complement (above) were set up in a proliferation assay with 2 ug/ml PHA as stimulant. To these cultures, various conditioned supernatants were added at the initiation of the culture period to assess their ability to effect the proliferative capability of the cytotoxically treated cells. Conditioned supernatants analyzed included 1) control supernatant generated by incubating PBMC ($10^6$/ml) with RPMI-FCS for 24 hrs, 2)PHA-conditioned supernatant, PBMC ($10^6$/ml) stimulated with 2 ug/ml PHA for 24 hrs, and 3) antigen-conditioned supernatants, PBMC ($2 \times 10^6$) from a mumps-sensitized donor stimulated with a 1/10 dilution of mumps antigen for 72 hrs. In addition, recombinant IL 2 (RIL 2) (kindly provided by Cetus Corp., Emeryville, CA) was tested. Supernatants or RIL 2 were added to the cultures at the initiation of the culture period and proliferation tested three days later by $^3$H-TdR uptake.

MEASUREMENT OF IL 2 CONCENTRATIONS IN SUPERNATANTS FROM PBMC STIMULATED WITH ANTIGEN AND TH5.2 MAb. PBMC at $2 \times 10^5$ cells per well were set up in 96 well microtiter plates with the addition of various antigens. In some wells TH5.2 MAb or control IgM MAb in the form of culture supernatants or ascites fluid were added at the beginning of the culture period. At 24, 48, and 72 hours, cell-free supernatants were collected and analyzed for IL 2 concentration as described in the next section.

MEASUREMENT OF IL-2 ACTIVITY. Assays for IL 2 activity utilized the CTLL murine IL 2-dependent cell line. The cells were used at a concentration of $4 \times 10^3$ cells per well. All cells were washed twice

with RPMI and set up in triplicate in microtiter plates with different concentrations of the various supernatants. The cultures were incubated for 24 hrs at 37 C with a 4 hr pulse of $^3$H-TdR (1 uci/well). The harvested cells were subsequently counted on a liquid scintillation counter and the results expressed as cpm $^3$H-TdR uptake by the cells.

BIOSYNTHETIC LABELLING OF TH5.2 ANTIGEN. Approximately $10^8$ mononuclear cells were isolated from peripheral blood and grown in 200 ml of RPMI 1640, 10% FCS, supplemented with Concanavalin A (Con A) (5ug/ml) for 4 days at 37C. These cells (or $10^8$ HTL cells) were washed in RPMI 1640 (deficient in glucose, supplemented with 2 mM sodium pyruvate). The cells were suspended in 10 ml of the same medium containing, in addition, 1% FCS and 0.5 mCi tritiated galactose (4,5-,$^3$H 50 Ci/mM). Labelling of $10^8$ cells was also accomplished with 0.5 mCi tritiated leucine (4,5- $^3$H, 50 Ci/mM) in leucine deficient RPMI 1640. Cells were grown for 4 hours at 37C with occasional shaking of the flask. The biosynthetically labelled cells were extracted with 8 ml of a 0.5 percent solution of the detergent 3-(3-chloamidopropyl) dimethyl-ammonio propanesulfonate (CHAPS) (Pierce Chemical Co.) in RPMI 1640 supplemented with the protease inhibitors phenylmethyl-sulfonyl fluoride (PMSF), L-a-p-tosyl-L-lysine chloromethyl ketone (TPCK), and N-a-p-tosyl-L-lysine chloromethyl ketone (TLCK) at 1 mM. Extraction was accomplished by intermittent pipetting of the cell suspension in detergent for 30 minutes on ice. Insoluble cell debris was removed by centrifugation at 30,000 x g for 30 minutes. A 0.5

percent NP-40 (Sigma, ST. Louis, MO) detergent solution was used for some extractions; an identical procedure was used with the CHAPS extraction.

CELL SURFACE RADIOIODINATION OF TH5.2 ANTIGEN. Cells were labeled with $^{125}I$ using the lactoperoxidase method described by Jones (125). Five millicuries of NaI (New England Nuclear) were used for $10^8$ cells.

CROSSLINKING OF CELL SURFACES. (Approximately 5 X $10^7$ radiolabelled cells were reacted with TH5.2 MAb or control IgM MAb for 30 minutes at 4C. Cells were washed 3 times and suspended in 10 ml of pH 7.5 phosphate buffered saline. Ten milligrams of the crosslinking agent dithiobis(succinimidyl proprionate), obtained from Pierce Chemical Co., were dissolved in 0.5 ml of dimethyl sulfoxide (Sigma) and added dropwise with mixing to the cell suspension on ice over a period of 30 minutes. The reaction was quenched by adding 4 ml of 50mM ethanolamine in phosphate buffered saline. Cells were washed and subsequently used for detergent extraction.

IMMUNOPRECIPITATION. Culture supernatants or ascites fluids containing the appropriate MAb were added to 2 to 4 ml aliquots of the detergent extracts. For a detergent extract derived from 2 x $10^7$ cells, the following amounts of MAb solutions were used: TH5.2 ascites, 30 ul; TH5.2 culture supernatant, 0.5 ml; control mouse IgM MAb culture supernatant, 0.5 ml; anti-Tac ascites, 5 ul (obtained from Dr. T. Waldman ) and OKT-4 MAb (Ortho Diagnostics, Boston, MA) 50 ul. Following overnight incubation at 4 C, 0.5 ml of goat anti-mouse IgM (u chain specific, Cappel) was added to IgM immunoprecipitates.

No additional antibody was used for Tac immunoprecipitation. After 4 hours of additional incubation at 4C, 0.2 ml of Protein A Sepharose (Sigma) was added with overnight shaking at 4C. The Protein A Sepharose was isolated and washed five times in a 0.25 percent CHAPS detergent solution and once in phosphate buffered saline. A 0.25 percent NP-40 detergent solution was used for washing if the original cell extract was made in NP-40. The immunoprecipitate was extracted from the Protein A Sepharose by suspension in 0.2 ml of electrophoresis sample buffer containing 3M mercaptoethanol (Sigma) with heating for 15 minutes in a boiling water bath.

SEQUENTIAL IMMUNOPRECIPITATION. The supernatants remaining after removal of the immunoprecipitates formed by TH5.2 and anti-Tac MAb were used for subsequent immunoprecipitation. Each supernatant was divided into two parts, and TH5.2 or anti-Tac MAb was added, followed by the immunoprecipitation procedure described above.

DISC GEL ELECTROPHORESIS. The extracted immunoprecipitate was assessed for radioactivity and applied to 10 percent sodium dodecyl sulfate (SDS) polyacrylamide tube gels for electrophoresis by the system of Laemmli (126). Gels were sliced into 1 mm discs and each slice was placed into a scintillation vial and counted for radioactivity.

MODULATION EXPERIMENTS. One million PBMC were incubated with either TH5.2, OKT-3, OKT-8, Leu-5 (10 ul), OKT-4 (20 ul) MAb or with RPMI 1640 plus 10% AB serum alone for 18 hrs in a 1 ml volume at 37C. Following the incubation period, the cells were washed two times with RPMI 1640

and stained for 30 min at 4 C with the appropriate MAb. The cells were then washed two times with ice-cold RPMI 1640 and re-incubated with either an FITC-conjugated goat anti-mouse IgM (u-chain specific) antibody, or an FITC-conjugated goat anti-mouse IgG (Fc-specific) antibody (both from Cappel Laboratories). After washing, the stained cells were analyzed for fluorescence intensity using the FACS.

# RESULTS

PRODUCTION OF TH5.2 MAb. Indirect immunofluorescent analysis using flow cytometry was used for the primary screen for reactivity of MAb produced by the hybrid cells. Approximately two weeks post-fusion, supernatants were collected and used to stain either HTL cells or normal resting PBMC. In order to reduce the number of test samples, every five wells on the plate were pooled and 100 ul used to stain $10^6$ cells. The supernatants from each positive pool were then re-analyzed for reactivity. Of approximately 350 wells showing hybrid growth, 30 were reactive against the immunizing HTL cell, as well as showing various reactivity patterns against normal resting PBMC. Positive supernatants from each reaction pattern group were then tested for their effect on the proliferation of mitogen-activated PBMC. One supernatant (reactivity pattern- 90% positive HTL, 50-60% positive PBMC) strongly affected the proliferation of the cells as will be described in detail in subsequent sections. The hybrid culture was cloned twice by limiting dilution and the resulting IgM MAb (by Ouchterlony and ELISA analysis) was used for subsequent experiments.

REACTIVITY AND SPECIFICITY OF TH5.2 MAb. As mentioned previously, TH5.2 reacted with virtually all HTL cells (90+%), as well as with resting and PHA-activated human PBMC (Table 1). The percentage of cells stained was approximately 60% for both populations. A human B cell myeloma line (LICR), as well as cell lines of murine origin (HT-2,

CTLL, SP-2, P388, LBRM 331A5B6) were not significantly reactive with TH5.2 MAb (Table 1). Plastic adherent cells obtained by incubating PBMC in fetal calf serum-coated flasks followed by eluting the adherent cells with EDTA, were minimally reactive with TH5.2 MAb. These cells were predominantly OKM-1-positive indicating that TH5.2 MAb did not significantly react with monocytes. A human skin fibroblast cell line, CRL-1510 was found to be reactive with TH5.2 MAb.

In order to further define which subpopulation of PBMC were reactive with TH5.2 MAb, dual-color fluorescence analysis was performed. The MAb utilized were phycoerythrin (PE)-conjugated Leu-3a, Leu-2a, and Leu-M3, and TH5.2 in conjunction with a goat anti-mouse u chain specific-FITC conjugate. As can be seen in Table 2, the majority (90%) of PBMC staining with Leu-3a MAb were reactive with TH5.2 MAb. Seventy-three percent of Leu 2a-positive T cells also were reactive with TH5.2 MAb. In contrast, none of the PBMC staining with LEU-M3 MAb expressed the TH5.2 determinant.

THE MOLECULAR WEIGHT OF TH5.2 ANTIGEN IS 55,000-60,000 DALTONS. Immunoprecipitates of TH5.2 MAb with CHAPS detergent extracts of HTL cells yielded a 55,000 to 60,000 dalton labeled molecule as revealed on SDS-PAGE analysis. Figure 1a shows the pattern obtained on gels using extracts from $^{3}$H-galactose labelled cells. Similar results were obtained with $^{3}$H-leucine labelled extracts except for an additional secondary peak occurring at 55,000 daltons (Fig 6). The apparent molecular weight was not significantly affected by the omission of reducing agent, during immunoprecipitation indicating that the antigen is occurring as a monomeric unit on the cell membrane. A control IgM MAb did not precipitate a significant amount of material

(Fig. 1b). The peak at 45,000 daltons, probably representing co-precipitated actin from the extract, was not always seen when comparing immunoprecipitation runs. With HTL cells as the source of antigen, a relatively narrow molecular weight range was seen for the principal peak. When these same HTL extracts or NP-40 detergent extracts were treated with anti-Tac MAb, no immunoprecipitate was measured (Fig. 1c).

Similar experiments were performed on normal human PBMC cultured for 4 days with Con A. Figure 2 shows the immunoprecipitation pattern obtained with TH5.2 MAb (Fig. 2a), control antibody (Fig. 2b) or anti-Tac MAb using a $^{3}$H-galactose label (Fig 2c). TH5.2 MAb precipitated two closely spaced peaks representing material of molecular weights 55,000 and 62,000 daltons. From the same extract, the immunoprecipitate formed with anti-Tac MAb gave a cell derived molecule of 54,000 daltons. This was similar to the pattern obtained with a $^{3}$H-Leucine label and HTL cells. Control IgM MAb immunoprecipitated no significant material from the cell extract.

Preliminary experiments with HTL extracts made with the non-ionic detergent NP-40 gave no immunoprecipitating cell material with TH5.2 MAb. In order to avoid potential detergent denaturing of the antibody or cell surface antigen, TH5.2 MAb was reacted with undisrupted HTL cells which had been surface iodinated with $^{125}$I. After removal of unbound antibody, cell surface molecules were crosslinked with the cleavable crosslinking agent dithiobis (succinimidyl propionate). Cells were then extracted using NP-40 with immunoprecipitation

proceeding as usual. Chemical crosslinks formed between antigen and antibody are cleaved in this procedure during the reductive solubilization of the immunoprecipitate from Protein A Sepharose (12). SDS-PAGE demonstrated a 55,000 dalton cell derived molecule in these immunoprecipitates (Fig. 3a). An additional peak at 45,000 daltons was found for both TH5.2 and control MAb (Fig. 3b). This probably represents actin, and indicates some labelling of internal cell proteins due to cell lysis.

PHENOTYPIC EXPRESSION OF SUBPOPULATIONS BASED ON TH5.2 MAb REACTIVITY. FACS analysis of adherent cell-depleted T cells stained with TH5.2 MAb allowed us to separate subsets of the population based on the intensity of fluorescence, and hence, density of the TH5.2 determinant on the surface of the cell. The cytofluorograph was arbitrarily gated so that approximately 30% of the T cell population fluorescing at the highest intensity was sorted into one subpopulation. Correspondingly, a second subpopulation comprising another 30% of the total population, and which fluoresced at low intensity with TH5.2 MAb was sorted. The remaining 40% of the population was not collected. Of the 40 X $10^6$ stained cells sorted, approximately 6 X $10^6$ cells were collected in each subpopulation. Following sorting of PBMC using the protocol described above, the sorted subpopulations were analyzed for their phenotypic expression.

Indirect immunofluorescence analysis of the subpopulation of cells expressing low densities of TH5.2 determinant showed that the T cells comprising this subpopulation were predominantly of the cytotoxic/suppressor phenotype (Leu-2a-positive) (Table 3). Forty-two percent of the cells reacted with Leu-2a MAb, 8% were Leu-3a-positive,

1% were OKM-1 positive, and 15% reacted with TH5.2 MAb. In contrast, 51% of the cells which fluoresced at high intensity with TH5.2 MAb also stained with Leu-3a MAb, while only 7% were Leu-2a positive.

THE EFFECT OF TH5.2 MAb ON THE PROLIFERATIVE RESPONSE OF PBMC TO SUBOPTIMAL DOSES OF MITOGEN. The effect of TH5.2 and control IgM MAb on mitogen-driven lymphocyte proliferation can be seen in Table 4. The data in Table 4 is representative of at least five separate experiments. The control MAb used in this experiment were an anti-HLA-B8 MAb which was not reactive with the cells, and also a MAb which reacted with all peripheral blood T cells ($S_{33}$). The isotype of both MAb is IgM. Addition of either anti-HLA-B8 or $S_{33}$ MAb to resting PBMC or PBMC stimulated with optimal or suboptimal concentrations of PHA, did not significantly affect the proliferative capability of the cells. Addition of TH5.2 MAb to some cultures also did not significantly affect $^{3}$H-TdR uptake of resting PBMC or PBMC stimulated with an optimal dose (2.0 ug/ml) of PHA. When the concentration of PHA was reduced to suboptimal levels, however, TH5.2 MAb was now capable of augmenting the proliferation of the cells in response to the mitogen. At a PHA concentration of 0.01 ug/ml, addition of TH5.2 MAb in the form of culture supernatant at a concentration of 6 ug/ml increased the $^{3}$H-TdR uptake from 6,200 cpm (medium only control) and 7,301-8,121 cpm (control IgM MAb) up to 35,217 cpm (Table 4). In other experiments TH5.2 MAb in the form of ascites fluid was also capable of augmenting the proliferative response of the cells stimulated with suboptimal doses of PHA (data not shown).

TH5.2 MAb by itself is not mitogenic for lymphocyte cultures as can be seen when no mitogen is added to the cultures (Table 4 ).

THE PROLIFERATIVE RESPONSE OF T CELLS TO ANTIGEN IS ENHANCED BY ADDITION OF TH5.2 MAb. Because TH5.2 MAb was able to effect the proliferative response of T cells to mitogen, we next were interested in the effects of the antibody on the ability of lymphocytes to respond to antigen stimulation. PBMC from donors were cultured with either SK-SD, mumps, or KLH antigen. TH5.2 MAb or control IgM MAb in the form of culture supernatants were added to the cultures at the same time as antigen and the proliferative response of the lymphocytes determined six days later. Results in Table 5 show that the addition of a 1/10 final culture dilution of TH5.2 MAb culture supernatant uniformly enhanced the proliferative response of the lymphocytes to all antigens tested. Utilizing a suboptimal dose of antigen (SK-SD, 1/100 dilution) for stimulation resulted in a greater augmentative effect when TH5.2 MAb was added to the cells compared to a 1/10 dilution (Table 5). This was similar to the pattern seen when PBMC were stimulated with PHA plus TH5.2 MAb (Table 4), in that a suboptimal dose of stimulation yielded higher augmentation. The augmentative effect of TH5.2 MAb was most dramatic when KLH was used as antigen with PBMC from a KLH immune donor. The proliferative response to KLH alone was 2,000 cpm; addition of TH5.2 MAb at a 1/10 dilution increased the $^{3}$H-TdR uptake to 18,800 cpm, a nine-fold increase in the response.

EFFECT OF CYTOTOXIC TREATMENT WITH TH5.2 MAb PLUS COMPLEMENT ON THE PROLIFERATIVE CAPABILITY OF PBMC. PBMC were treated with various concentrations of TH5.2 MAb and complement to test the effect of

removal of TH5.2-reactive cells from the population. The conditions for the cytotoxic treatment resulted in approximately 8-35% of the total cell population being lysed depending upon the concentration of antibody and complement used. These percentages were based on comparision of total cell count before and after treatment. Normal resting PBMC populations were subjected to the treatment and the depleted cells ability to respond to mitogen was tested. Results expressed in Table 6 represent experiments in which the concentration of TH5.2 MAb and complement used (100 ul of neat supernatant and 10-100 ul of complement) resulted in a decrease in the OKT-4-positive subpopulation by approximately eight percent of the total PBMC population. Lymphocytes subjected to no cytotoxic treatment responded to an optimal dose of PHA by incorporating 106,000 cpm. Addition of either TH5.2 MAb or rabbit complement to the cultures did not effect the ability of the lymphocytes to proliferate. Addition of TH5.2 MAb plus complement, however, reduced the proliferative response by 80% (Table 6). Using antigen-stimulated cultures a similar pattern was seen. Treatment with TH5.2 MAb plus complement resulted in reducing the proliferative response to background levels. TH5.2 MAb was seen to have an enhancing effect when added to the antigen-driven cultures indicating that the optimal response was not achieved by stimulation with antigen alone.

RECONSTITUTION OF THE PROLIFERATIVE RESPONSE OF PBMC TREATED WITH TH5.2 MAb PLUS COMPLEMENT. We have established that lymphocyte cultures depleted of a TH5.2-reactive subset of PBMC significantly lose their ability to proliferate in culture. We next investigated the

ability of various factors to reconstitute the proliferative response in these depleted cultures. Cytotoxically treated cells were cultured with either 2 ug/ml PHA or antigen for three and six days, respectively, with the addition of either 1) supernatants taken from 24 hr cultures of PHA-stimulated PBMC, 2) supernatants harvested from 24 hr cultures of non-stimulated PBMC, 3) 72 hr antigen-conditioned supernatants, or 4) RIL 2. Results of these experiments can be seen in Table 7. Cytotoxic removal of TH5.2-positive cells as observed previously abrogated the proliferative response of mitogen driven lymphocyte cultures. When mitogen-conditioned supernatants were added to wells containing the cytotoxicallly treated PBMC at a final culture concentration of a 1/25 dilution, the ability of the treated cells to proliferate in response to PHA was reconstituted back to pre-treatment levels. Supernatants from antigen-conditioned PBMC at a 1/2 dilution were capable of partially reconstituting the response. Analysis of both conditioned supernatants demonstrated the presence of IL 2 (data not shown). In order to determine if IL 2 alone was capable of reconstituting the response, RIL 2 was utilized in the same type of experiments. As can be seen in Table 8, the cloned IL 2 was able to reconstitute the proliferative response fully. The reconstitution was dose-dependent; at the lower concentrations of IL 2, the reconstitutive effect was lost or diminished.

COMPARISION OF PROLIFERATIVE CAPABILITY AND IL 2 PRODUCTION BETWEEN HIGH AND LOW TH5.2 INTENSITY PBMC. T cells sorted by FACS on the basis of staining intensity with TH5.2 MAb resulted in two populations of cells: one of which was predominantly Leu-3a-positive and a second

which predominantly expressed the Leu-2a determinant (Table 3). We next investigated two functional properties of the sorted populations; the ability to proliferate, and also secrete IL 2 in response to mitogen stimulation. Because the quantitative presence of monocytes can affect the ability of T cells to perform both functions (43,49,65), prior to the sorting procedure adherent cells (monocytes) were removed from the PBMC population and later added back to both sorted populations in equal number. The results of these experiments can be seen in Table 9. The sorted population of PBMC which fluoresce at high intensity with TH5.2 MAb were capable of greater proliferation (28,113 cpm vs. 5,941 cpm), as well as higher IL 2 production (supernatants yielded 14,114 cpm on CTLL cells vs. 2,335 cpm) compared to low TH5.2 intensity cells. These results were obtained when 10% adherent cells (which were predominantly monocytes, Table 9) were added back to the sorted populations. When the sorted populations were set up in culture without the addition of adherent cells and stimulated with PHA, it was found that the high TH5.2 intensity cells were incapable of responding to mitogen, while the low TH5.2 intensity population proliferated at approximately the same level as when adherent cells were added. IL 2 production was not significantly different in comparing the low TH5.2 intensity population with or without adherent cells added. The high TH5.2 intensity population, in contrast, was dependent upon adequate numbers of adherent cells to secrete optimal levels of IL 2.

TH5.2 MAb DOES NOT INDUCE INCREASED Tac ANTIGEN EXPRESSION ON T CELLS STIMULATED WITH MITOGEN. We next were interested in the mechanism by which TH5.2 MAb was capable of augmenting the

proliferative response of PBMC to either suboptimal doses of mitogen, or antigen. One possibility is that the antibody is inducing increased expression of IL 2 receptors on T cells. To test this hypothesis we incubated PBMC with various concentrations of PHA with and without TH5.2 MAb, and three days later analyzed the cells for expression of IL 2 receptors using anti-Tac MAb (Table 10). PBMC stimulated with 0.1 ug/ml PHA plus 5 ug/ml TH5.2 MAb in the form of ascites fluid were capable of incorporating approximately four-fold more $^{3}$H-TdR as compared to cells without TH5.2 MAb. When the cells were stained with anti-Tac MAb at 72 hours, it was found that there was no increase in the percentage of cells staining positive with anti-TAC MAb when comparing the two groups. Comparision of the mean fluorescence intensity between the two groups showed only a slight shift in higher intensity of the TH5.2-treated cells (data not shown). Using 1.0 ug/ml PHA, a similar pattern was observed, although with this concentration of mitogen the percentage of cells expressing Tac antigen was optimal.

EFFECT OF TH5.2 MAb ON THE IL 2-PRODUCING CAPABILITY OF T CELLS. In further analyzing the mechanism of augmentation of the proliferative response of stimulated T cells by TH5.2 MAb, we next investigated the ability of TH5.2 MAb to affect IL 2 production by T cells. PBMC were cultured with either antigen or suboptimal mitogen with the addition of TH5.2 MAb, control IgM MAb, or medium only in 96 well microtiter plates. Supernatant was collected at 24, 48, and 72 hour time periods and assayed on CTLL cells to determine the IL 2-producing capability of the treated T cells. In Table 11 are the results of these

experiments. Addition of TH5.2 MAb in the form of culture supernatant (1/16 final culture dilution), or ascites fluid 1/500 dilution) stimulated with either suboptimal doses of PHA or antigen, resulted in the previously described augmentation of the proliferative response. Supernatants from TH5.2-treated cells at all three time points consistently generated significantly higher $^{3}$H-TdR uptake levels by CTLL cells compared to supernatants from cells treated with control IgM MAb or medium only. TH5.2 MAb by itself did not have a stimulatory effect on CTLL cells (experiment 3), and control IgM MAb did not have a suppressive effect (data not shown) on CTLL cells.

EXOGENOUS RIL 2 IS CAPABLE OF REPLACING THE ABILITY OF TH5.2 MAb TO AUGMENT PROLIFERATION. It was established that TH5.2 MAb was capable of inducing increased IL 2 production by antigen-activated T cells. In order to investigate if this was the mechanism by which TH5.2 MAb was augmenting the proliferative response, antigen-activated T cells were supplemented with exogenous RIL 2 and the proliferative response measured six days later. In Table 12, it can be seen that the addition of the exogenous RIL 2 is capable of augmenting the proliferative response of either KLH or mumps-activated T cells. The magnitude of augmentation is approximately the same as induced by TH5.2 MAb when taking into account the observation that RIL 2 by itself is capable of inducing freshly isolated PBMC to proliferate in the absence of antigen

ANTIGENS RECOGNIZED BY TH5.2 AND ANTI-Tac MAb ARE ON DIFFERENT MOLECULES. Sequential immunoprecipitation was carried out with CHAPS detergent extracts of Con-A stimulated peripheral blood lymphocytes.

The first precipitate gave TH5.2 and anti-Tac MAb derived peaks as previously described. The second immunoprecipitation with anti-Tac MAb demonstrated that all of the Tac antigen had been removed during the first precipitation (Fig. 4d). The TH5.2 antigen however, could still be recovered from supernatants precleared with anti-Tac MAb (Fig. 4b). Similarly, preclearing with TH5.2 MAb still allowed for immunoprecipitation of the antigen recognized by anti-Tac MAb (Fig. 4e). These results are representative of at least five separate immunoprecipitation experiments. Additional evidence that TH5.2 and anti-Tac MAb recognize epitopes on separate molecules was obtained by combining immunoprecipitates obtained with TH5.2 and anti-Tac MAb (from $^{3}H$-galactose labeled PBMC) and co-electrophoresing them on the same gel. The TH5.2 precipitate gave two peaks of 64,000 and 60,000 molecular weight, while anti-Tac MAb gave a single peak of 54,000 molecular weight. When the two precipitates were run on the same gel, three peaks of 64,000, 60,000 and 54,000 were obtained (data not shown).

ANTIGENS RECOGNIZED BY TH5.2 AND OKT-4 MAb ARE ON DIFFERENT MOLECULES. Sequential immunoprecipitation was carried out with CHAPS detergent extracts of HTL cells. Molecular species of 62,000 daltons and 58,000 daltons were immunoprecipitated by TH5.2 and OKT-4 MAb respectively. When supernatants from each of these immunoprecipitates were divided into two equal parts and re-precipitated with the two antibodies, no additional antigen could be precipitated by successive treatment with the same antibody, while treatment with the alternate antibody gave the expected quantity of antigen (Figs. 5a and 5b).

TUNICAMYCIN TREATMENT DOES NOT SIGNIFICANTLY ALTER THE MOLECULAR WEIGHT OF THE ANTIGEN PRECIPITATED BY TH5.2. Tunicamycin inhibits the formation of asparagine-linked oligosaccharides in glycoproteins while leaving intact serine-linked oligosaccharides (128). This property is often used to test whether a protein contains a large amount of sugar residues, and it has been used for this purpose with the Tac IL-2 receptor (129). HTL cells were incubated with tunicamycin (10 ug/ml) for 18 hours and then labeled with $^{3}$H-leucine for 4 hours in the presence of tunicamycin. Control cells were treated in the same way but without tunicamycin. When the TH5.2 antigen was immunoprecipitated from detergent extracts of these cells and run on SDS-PAGE no major effects of tunicamycin on molecular weight were seen (Figure 6). Antigen from control cells gave a major peak at 59,000 daltons and a secondary peak at 55,000 daltons. Antigen from tunicamycin treated cells gave a peak at 58,000 daltons. A secondary peak at 53,000 daltons was larger than for antigen derived from control cells. A peak at 45,000 daltons for co-precipitated actin was seen in both cases.

THE TH5.2 ANTIGEN DOES NOT CO-MODULATE WITH CELL SURFACE ANTIGENS RECOGNIZED BY OKT-3, OKT-4, OKT-8 or LEU-5 MAb. In order to compare the cell surface determinant reactive with TH5.2 MAb with other defined molecules occurring on the surface of T cells, modulation experiments were performed using OKT-3, OKT-8, OKT-4, and Leu-5 MAb. As shown in Table 13, incubation of cells with either TH5.2, OKT-3, OKT-4, OKT-8, or Leu-5 MAb resulted in a markedly lower percentage of cells which restained with the respective monoclonal antibody. The treatment effectively modulated the appropriate determinant from the surface of

the cells. In contrast, modulation of the T3, T4, T8, or Leu-5 antigens from the cell surface did not significantly affect the ability of TH5.2 monoclonal antibody to stain the cells. In the reciprocal experiment, modulation of the TH5.2 determinant did not affect the ability of OKT-3, OKT-4, OKT-8, or Leu-5 MAb to stain the treated cells (Table 14).

# DISCUSSION

T lymphocytes are capable of being activated via interaction of the environment with receptors and determinants which are expressed on the cell surface. In the recent past, a number of these structures have been identified and characterized as was described in the Introduction of this dissertation. Crucial to the success of these studies has been the use of MAb in elucidating the nature of these structures. MAb offer the advantage of precise specificity as compared to a polyclonal antisera reagent. Antibody produced by a hybridoma clone will be specific not only with the relevant receptor molecule, but additionally with a specific epitope expressed on the structure. Of equal importance, the hybridoma process allows for selectivity of antibodies of the correct specificity. By utilization of a screening procedure or assay, the relevant MAb-producing clone can be isolated from the myriad of other antigen-specific hybridoma cells produced. The screening process can be of two forms: 1) physical- determination of reactivity with one cell type or structure and not another (i.e., reactivity with the antigen receptor on one antigen specific T cell clone and no others, and 2) functional- the ability of the MAb to affect a relevant function (i.e., anti-IL 2 receptor MAb inhibiting IL 2-induced T cell proliferation). Following this initial screening process, the MAb can then be used for further biochemical

characterization of the receptor, such as inhibition of ligand binding or determination of molecular weight.

We have utilized the hybridoma procedure to produce MAb reactive with determinants which might be involved in the proliferative capability of T cells. In this dissertation, we present data which supports the hypothesis that one MAb, designated TH5.2, recognizes a determinant expressed on T cells which is involved in the proliferative, and IL 2 producing capability of the cells.

First, addition of TH5.2 MAb to _in vitro_ cultures of PBMC strongly augmented the ability of the cells to proliferate in response to both suboptimal doses of mitogen as well as antigen. By using antigen stimulation we were able to demonstrate that the ability of TH5.2 MAb to augment proliferation of T cells involved a specific response which affected a smaller more defined population of T cells compared to mitogen stimulation. This point is emphasized when augmentation occurs using T cells from a donor specifically immunized with KLH which have been stimulated _in vitro_ with the same antigen, and in fact the largest augmentative response (nearly 10X) occurred under these conditions (Table 5).

In other studies, the effects of addition of MAb to cultures of T cells has been useful in determining the functional role of the receptor or determinant recognized by the antibody. Addition of anti-T11 MAb to cultures of T cells was capable of inducing the cells to proliferate, implying a stimulatory role for the molecule (60).

Recent investigations have described a lymphokine, IL 4a, which is mitogenic for resting T cells, and which appears to be triggering the cells via the T11 molecule (64). In another example, MAb which were capable of inducing antigen-specific T cell clones to become activated and proliferate in vitro were ultimately found to be reactive with the T cell antigen receptor (32,130). Lastly, addition of a MAb (anti Tac) reactive with activated T cells to culture of an IL 2-responsive T cell line was capable of inhibiting the proliferative capability of the cells to respond to IL 2 (83,84). Additional studies consequently determined that anti-Tac is reactive with the IL 2 receptor (89,90). These three receptors were initially characterized by observing the effect of addition of selective MAb on the proliferative capability of cultured T cells.

It has been determined that MAb reactive with the T3(41,42,43), Ti(32) or 9.3(66) antigens are capable of being mitogenic for resting PBMC by themselves. This is in contrast to TH5.2 MAb in that binding of the antibody to T cells by itself was not sufficient to induce proliferation. TH5.2 MAb required co-stimulation with either mitogen or antigen for the augumentative effect to be seen (Tables 4 and 5). Because of its non-mitogenic nature, TH5.2 MAb may not be reactive with a cell surface structure which is directly capable of inducing the activation event. It is possible, however, that TH5.2 MAb may be augmenting the proliferative response of mitogen or antigen-activated T cells by interacting with a determinant which influences the proliferative, rather than activating phase of the T cell activation/proliferation cycle.

As stated above, anti-T3 MAb are mitogenic for T cells in the presence of monocytes. The mitogenic capability, however, is dependent upon the isotype of the MAb. Hara and others (49,131,132) have shown that anti-T3 MAb of the IgG isotype are mitogenic, while interestingly, IgM isotype MAb are not. The mitogenic effect seems to be dependent upon interaction of IgG MAb with Fc receptors expressed on the surface of monocytes (49,133-135). The possibility exists that murine IgM MAb are not capable of interacting with monocytes in this fashion and this might be an alternative explanation for the non-mitogenic properties of TH5.2 MAb for PBMC.

Suboptimal doses of mitogen were necessary in order for TH5.2 MAb to augment the proliferative response (Table 4). Stimulation with optimal doses of mitogen result in maximal production of IL 2 by T cells, as well as induction of a high percentage of cells expressing IL 2 receptors (86). Conversely, when PBMC cultures are stimulated with suboptimal mitogen or antigen, a smaller percentage of cells are capable of expressing IL-2 receptors and producing IL 2 (86). Given this then, it was not surprising to us that the augmentation exhibited by the TH5.2 MAb was seen only when PBMC cultures were stimulated with either suboptimal mitogen or antigen (Tables 4 and 5).

A possible explanation for the ability of TH5.2 MAb to augment the proliferative response of activated T cells would be that the TH5.2 determinant is functioning as an IL 2 receptor, and that TH5.2 MAb is an IL 2 agonist. Support for this hypothesis comes from results (Figs. 2 and 3) which indicate that the molecular weight of the TH5.2 antigen

(55,000) is very close to the molecular weight of the IL 2 receptor as defined by the anti-Tac MAb. One major difference between the TH5.2 determinant and the IL 2 receptor, however, is that anti-Tac MAb reacts with low percentages of resting T cells by cytofluorographic analysis (82,86), while TH5.2 MAb stains the majority of the cells. Additionally, it was determined that TH5.2 MAb failed to inhibit binding of radiolabelled IL 2 to IL 2 receptor-positive cells (experiments performed by Dr. R. Robb). This is an indication, but not substantive proof that the Tac determinant and the TH5.2 antigen are not the same molecule. Definitive evidence that the molecules are not the same can be obtained from results of biochemical analysis. Although anti-Tac MAb which recognizes the IL 2 receptor does not bind to the HTL cell line, nor does it give an immunoprecipitate with HTL detergent extracts (Figure 1), it is possible that the epitope recognized by anti-Tac MAb is missing from the IL 2 receptor as it is expressed on HTL cells. As shown in Figure 2 both TH5.2 antigen and Tac antigen could be immunoprecipitated from Con-A-activated PBMC. In order to determine if the two molecules are, in fact, identical, sequential immunoprecipitation experiments were undertaken with CHAPS detergent extracts of activated peripheral blood mononuclear cells. The TH5.2 antigen was removed from the extract by immunoprecipitation with TH5.2 MAb. Its removal was complete as demonstrated when a second treatment with TH5.2 antibody yielded no immunoprecipitate. However, from this TH5.2-treated extract the expected amount of Tac antigen could be precipitated with anti-Tac MAb. When the Tac antigen was

removed first followed by TH5.2 immunoprecipitation, similar results were found (Figure 4). These experiments demonstrate that although TH5.2 and Tac antigens have similar molecular weights, they are distinct molecules. Additional evidence proving this point was obtained when TH5.2 and Tac immunoprecipitates were combined and electrophoresed on the same gel. The pattern obtained consisted of 3 major peaks, apparently representing the two peaks given by TH5.2 and the single peak given by anti-Tac MAb. Thus, the cell surface molecules immunoprecipiated by these two antibodies do not superimpose when electrophoresed under the same conditions, a fact which demonstrates that they are not identical.

Although the TH5.2 antigen can be biosynthetically labeled with both protein (leucine) and carbohydrate (galactose) radiolabels, the extent of its glycosylation was not known. The compound tunicamycin is reported to inhibit the formation of asparagine-linked oligosaccharides (128) and is often used to indicate the extent of protein glycosylation. Using this technique, Leonard et al. (129) have reported that the IL 2 receptor contains as much as 34 percent carbohydrate, since biosynthetic labeling of the Tac antigen with a protein label in the presence of tunicamycin yielded a molecular weight for the Tac antigen of 33,000 instead of 50,000 daltons. Using a protocol similar to that of Leonard et. al. with TH5.2 MAb, we did not find a marked effect of tunicamycin on the apparent molecular weight of TH5.2 antigen isolated from HTL cells (Fig. 6). Therefore, the carbohydrate content of the TH5.2 antigen must represent only a few

percent of the molecular weight rather than the one-third characteristic of the IL 2 receptor. We did, however, observe a shift in the quantity of immunoprecipitated material when comparing cells treated with, and without tunicamycin treatment. More antigen in the 59,000 dalton peak was immunoprecipitated in the absence of tunicamycin as compared to tunicamycin-treated cells, and the reciprocol was seen in the 55,000 dalton peak. These results are consistent with the hypothesis that the TH5.2 antigen is occurring as two species, one as an N-linked glycosylated molecule (60,000 daltons), and the second as an unglycosylated polypeptide structure. The major peak occurring on HTL cells is the 60,000 dalton peak (Figs. 1 and 6). Periperal blood T cells in contrast express both the 60,000 and 55,000 dalton peaks (Fig. 2). One explanation for these differences would be that, within a heterogeneous population of cells (peripheral T cells), both of the molecular species will be present in the total extract of cells in the population. In comparision, HTL cells represent a cloned lymphoma T cell line and it is possible that this homogeneous population of cells is processing the TH5.2 antigen in one manner resulting in a single species of antigen.

Expression and density of IL 2 receptors on T cells are important factors in determining the proliferative capability of the cells (82,91,93). Because stimulation with suboptimal doses of mitogen does not induce optimal percentages of IL 2 receptor-positive cells, the role of TH5.2 MAb in augmenting the proliferative response might be to induce increased IL 2 receptor expression. In Table 10 it can be seen

that the antibody does not have the ability, in conjunction with mitogen, to increase the number of cells expressing IL 2 (Tac) receptors. Also there was no significant change in the mean fluorescent intensity when comparing cells treated with or without TH5.2 MAb plus mitogen, indicating no quantitative difference in the density of IL 2 receptors expressed.

It has been shown that stimulation of T cells by either mitogen or OKT-3 MAb results in an increase in intracellular calcium stores in the cell, followed by subsequent proliferation (48,136). An alternative mechanism explaining the augmenting effect of TH5.2 MAb on T cell proliferation would be that the antibody is affecting intracellular calcium concentrations by interacting with a surface calcium channel allowing for influx of calcium. We feel this is not likely, because TH5.2 MAb is not mitogenic for resting cultures of PBMC. Treatment of resting PBMC cultures with a calcium ionophore results in increased intracellular concentrations of calcium and subsequent activation and proliferation of the cells (48,136).

In order to further investigate the possible functional role of the TH5.2 determinant, we utilized cytotoxic treatment with TH5.2 MAb plus complement to selectively remove cells expressing the TH5.2 antigen, and subsequently assessed the functional capability of the depleted cells. We observed that depending upon the concentration of both TH5.2 MAb and complement used in the experiments, the degree of cell lysis was greatly effected. By using high concentrations of both reagents, the majority of the T cells lysed; by utilizing lower

concentrations of MAb and complement correspondingly lower numbers of T cells were removed from the total PBMC population. By using concentrations which lysed a relatively small percentage of T cells (approximately ten percent of the total population predominately OKT-4-positive T cells), the proliferative response of the remaining cells to PHA stimulation was greatly decreased (Tables 6 and 7). Addition of either PHA or antigen- conditioned supernatants or RIL 2 was capable of restoring the proliferative response of the depleted cells to pre-treatment levels (Tables 7 and 8). These results indicated that 1) the depleted T cells following the cytotoxic treatment were functionally active and capable of responding to mitogen stimulation, and 2) a small percentage of T cells (predominately OKT-4-positive helper/inducer cells) which are preferentially lysed with TH5.2 MAb plus complement, play a critical role in T cell proliferation, and that this role might be one of IL 2 production.

We next wanted to more specifically define the relationship between TH5.2 determinant expression on T cells and the ability of the cells to secrete IL 2. Analysis of PBMC by FACS allowed us to separate T cells into two populations based on their reactivity with TH5.2 MAb. It was determined by cell sorting that T cells which stain at high intensity with TH5.2 MAb were able to produce higher concentrations of IL 2 compared to low TH5.2 intensity cells (Table 9). In other systems the intensity of the staining has been found to correlate with the density of the determinants expressed on the surface of the cells (91). This would imply that TH5.2 determinant density on T cells

correlates with the IL 2 producing capability of the cells. A precedent for this type of correlation exists with IL 2 receptors on T cells, as the density of IL 2 receptors on the surface of the cells is a critical factor in determining the function (in this case proliferative capability) of the cell (82,91,93). By FACS analysis the sorted population of T cells which expressed high densities of TH5.2 antigen were determined to be predominantly Leu-3a-positive helper/inducer cells (Table 3). It has been shown that helper/inducer T cells are capable of producing higher concentrations of IL 2 following mitogen stimulation compared to the cytotoxic/suppressor subpopulation (137,143). This observation is supportive of our results demonstrating that the high TH5.2 density T cells are the predominant IL 2 producing cells.

Malek (137), and Erard et. al. (138) have shown that murine L3T4+/Lyt-2- T cells upon Con-A stimulation require the presence of accessory cells for optimal IL 2 receptor expression, IL 2 production and proliferation compared to L3T4-/Lyt-2+ cells. Our results indicate that in the human system this may also be the case. By indirectly separating helper/inducer and suppressor/cytotoxic cells using TH5.2 MAb, we have demonstrated that the former subpopulation are significantly more dependent upon the presence of adherent cells for IL 2 production and proliferation compared to the suppressor/cytotoxic subpopulation (Table 9).

Adherent cells (monocytes) are the predominant Il 1 producing cells in PBMC populations (94-96). Because Il 1 is widely believed to

induce IL 2 production by IL 1 receptor-positive T cells (115-118), it can be speculated that in our experiments for IL 2 production, helper-inducer T cells require the presence of adherent cells, at least in part for their IL 1 producing capability. Following this line of reasoning, it is possible that the cytotoxic/suppressor subpopulation is less responsive to IL 1 induction of IL 2 production as compare to helper/inducer T cells. Alternatively, cytotoxic/suppressor T cells may require signal(s) in addition to IL 1 for maximal Il 2 production.

The above results suggest that the TH5.2 determinant is associated with the IL 2 producing capability of T cells. To determine if the antigen is directly involved in IL 2 production by the cells, we investigated the effect of addition of TH5.2 MAb to mitogen or antigen-stimulated T cells on the IL 2 producing capability of the cells. Results expressed in Table 11 show that TH5.2 MAb was capable of acting synergistically with antigen or suboptimal doses of mitogen in increasing the IL 2 concentration in supernatants collected from the stimulated cells. The possibilities exist that the antibody is capable of inducing de novo synthesis of IL 2, or that the increase in IL 2 concentration in the supernatants is a result of a higher secretion rate of the factor by the cells. Currently, we have no data which would support either possibility.

Data expressed in Table 11 and elsewhere show that augmented proliferation occurs when T cells were stimulated with antigen plus TH5.2 MAb. A high correlation exists between the proliferative capability of T cells in response to mitogen or antigen, and the

concentrations of IL 2 in the supernatants early in the culture period (65). It is likely that the increased IL 2 production seen when cells are co-stimulated with TH5.2 MAb plus antigen or mitogen is the mechanism by which the antibody augments the response. This is supported by data in Table 12 which shows that exogeneously added IL 2 is capable of replacing the augmenting effect of TH5.2 MAb.

Given the presented data, the TH5.2 antigen seems to be functioning as an inducer of IL 2 secretion by T cells. It is interesting that both TH5.2 MAb and IL 1 (117) are, by themselves, not mitogenic for resting peripheral T cells, however, both can act synergistically with mitogen to induce IL 2 production and proliferation by the cells. In addition, TH5.2 MAb was capable of reacting with a human fibroblast cell line; fibroblasts have been shown to express substantial numbers of IL 1 receptors (121,122). These observations are not inconsistent with the hypothesis that TH5.2 MAb is reactive with an IL 1 binding structure, however, we have no direct biochemical data supporting this hypothesis. Recently it has been proposed that the primary role of IL 1 in T cell proliferation is not induction of IL 2 production but rather stimulation of increased IL 2 receptor expression and possibly other activation determinants as well (139,140). If one hypothesizes that TH5.2 MAb is functioning as an IL 1 agonist these results would be contradictory to our data which show that TH5.2 MAb is capable of augmenting IL 2 production but has no affect on IL 2 receptor expression. One difference between our experiments and the aforementioned reports, however, is that in our

system normal, non-activated T cells were used compared to long term, antigen-activated T cell clones. We feel that caution must be used in comparing results utilizing cell populations which might differ in their functional requirements and characteristics.

We have discussed the fact that a number of defined determinants on the surface of T cells are involved in both IL 2 production, as well as the proliferative capability of the cell. It was necessary to differentiate these determinants from the TH5.2 antigen because 1) the functional properties of these determinants were similar to those of the TH5.2 antigen and 2) the molecular weights of the structures were similar.

The T4 molecule is a 62,000 molecular weight glycoprotein which is expressed predominately on the helper/inducer subpopulation of T cells, although cytotoxic T4-positive cells are known to occur (22). The T4-positive subpopulation are restricted in their functional response by MHC class II encoded gene products. The T4 molecule has been implicated in playing a role in the recognition of these class II gene products expressed on antigen-presenting monocytes (9,20,21).

The HTL cell line is a T cell lymphoma cell line of the helper/inducer phenotype. It was determined that the HTL cells are positive for the OKT-4 MAb and that the T4 molecule is expressed on HTL cells at approximately the same frequency and density as compared to the TH5.2 determinant. Sequential immunoprecipitation experiments (Fig. 5) however, determined that the epitope recognized by TH5.2 MAb and OKT-4 MAb do not co-precipitate on the same molecule in HTL cell

extracts establishing that the two structures are distinct and separate on the membrane surface.

Another technique that can be used to determine if two antigens are present on the same cell surface molecule is antibody-induced cell surface antigenic modulation. If cells are grown for 12-24 hours in the presence of excess amounts of an antibody binding to a cell surface determinant, the antigen-antibody complex will aggregate on the cell surface and be internalized or shed by the cell, leaving the cell surface devoid of the antigenic determinant. This phenomena occurs with many, but not all cell surface antigens (142,143). Utilizing this concept, we demonstrated that the TH5.2 determinant did no co-modulate, and therefore is distinct from the T3, T4, T8 and Len-5 (T11) T cell determinants (Tables 13 and 14). In addition, these results indicated that the TH5.2 determinant is not closely associated on the membrane with those other antigens, as is the case with the association between the T3 and Ti molecules. Thus, TH5.2 MAb reacts with a determinant which is distinguished from previously characterized receptors and determinants which are involved in T cell activation and proliferation.

As discussed earlier, our data demonstrates that the TH5.2 determinant is involved in the IL 2 producing capability of T cells. Specifically, triggering of the determinant via binding of TH5.2 MAb resulted in an enhancement of IL 2 production by the cells. In proposing a physiological role for the TH5.2 determinant, we speculate that either, 1) the determinant functions as an amplification signal

for T cell IL 2 production following activation of the cell, or 2) the TH5.2 determinant is directly involved in the capability of T cells to secrete IL 2. The latter hypothesis assumes that other signals might be required by T cells for IL 2 production, but that triggering of the TH5.2 determinant is a signal that is necessary for the production and secretion to occur. The IL 1 receptor on T cells would fit into this functional category. Alternatively, TH5.2 antigen may play a role in a determinant-mediated amplification pathway for IL 2 production by T cells. Other signals such as pertubation of the antigen receptor and binding of IL 1 to IL 1 receptors would be required for IL 2 production; triggering of the TH5.2 determinant would result in enhanced, optimal production of IL 2 by the cells. The TH5.2 molecule may act as a receptor for an uncharacterized lymphokine which is capable of inducing the amplification. Recently, a novel lymphokine, designated IL 4a, was identified which is thought to be involved in an amplification pathway for T cell activation via the T11 molecule. These results are indicative of the complexity of the T cell activation and proliferation pathway and imply that multiple growth mediators may be involved in the process. It must be emphasized that, although our data is consistent with these hypotheses, we do not have direct biochemical evidence that the TH5.2 determinant is functioning in these capacities. Our results, however, do indicate that the determinant is involved in the function of T cells and may play a role in determining the optimal IL 2 producing, and hence proliferative capability of T cells.

# SUMMARY AND CONCLUSIONS

In this dissertation we present data which characterizes both functionally and physically, a T cell membrane determinant which is involved in the proliferative capability of T lymphocytes. The characterization utilizes a MAb, designated TH5.2, which was produced by immunization with an OKT-3-negative, OKT-4-positive human T lymphoma cell line. In testing TH5.2 MAb against a variety of human and murine cell types , it was found that the determinant was expressed at high density only on human peripheral blood T lymphocytes, as well as a human fibroblast cell line. TH5.2 MAb, when added to in vitro cultures of human peripheral blood mononuclear cells is capable of specifically augmenting the proliferative capability of T cells to both suboptimal mitogen and antigen stimulation. It was determined that interaction of TH5.2 MAb with the cell surface antigen was capable of inducing the T cells to secrete higher concentrations of IL 2 following stimulation, and it is hypothesized that this is the mechanism of augmentation of the proliferative response induced by the antibody. The TH5.2 antigen is a 55,000-60,000 molecular weight glycoprotein which is expressed at higher densities on MHC class II-restricted T cells (Leu-3a/OKT-4-positive) as compared to class I-restricted (Leu-2a/OKT-8-positive) cells. T cells which express high densities of Th5.2 antigen are dependent upon the presence of accessory cells for

optimal IL 2 production and proliferation in contrast to low TH5.2 density cells. The determinant is a distinct molecule from the T3, T4, T8, Leu-5, or Tac T cell determinants, and is not closely associated with these structures on the cell membrane as determined by sequential immunoprecipitation and co-modulation experiments. We conclude that the TH5.2 cell surface determinant influences the IL 2 producing and proliferative capability of T cells. Perturbation of the determinant via TH5.2 MAb results in increased IL 2 production, followed by an enhanced proliferative response by the cells.

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TABLE 1. REACTIVITY OF TH5.2 MAb AGAINST VARIOUS CELL TYPES.

| CELL TYPE | PERCENTAGE STAINING POSITIVE WITH TH5.2 MAb[a] | | |
|---|---|---|---|
| | MEAN | RANGE | n |
| HTL | 91 | 80-99 | 10 |
| LICR | 1 | 0-4 | 5 |
| RESTING HUMAN PBMC | 52 | 50-61 | 10 |
| MITOGEN-ACTIVATED PBMC | 57 | 53-62 | 10 |
| PLASTIC ADHERENT POPULATION[b] | 4 | 1-6 | 3 |
| HT-2 | 3 | 0-7 | 4 |
| CTLL | 5 | 0-6 | 4 |
| HUT-102 | 1 | 0-4 | 4 |
| SP-2 | 2 | 0-3 | 3 |
| P388D | 4 | 1-9 | 3 |
| LBRM 33-1A5 | 7 | 5-10 | 3 |
| CRL 1510 | 58 | 55-62 | 2 |

[a] using a goat anti-mouse IgM (u chain specific) FITC-conjugated second antibody. Percentages are corrected for control IgM MAb and second antibody only staining.

[b] PBMC adhered to FCS-serum-coated flasks, adherent cells eluted with EDTA (70% OKM-1+, 3% OKT-3+).

TABLE 2. DUAL COLOR FLUORESCENCE ANALYSIS OF PBMC STAINING WITH TH5.2, AND LEU-3a, LEU-2a, OR LEU-M3 MAb.

| MAb USED FOR STAINING[a] | PERCENTAGE CELLS STAINING POSITIVE[b] WITH ONE MAb. | PERCENTAGE CELLS STAINING POSITIVE[b] WITH BOTH MAb. |
|---|---|---|
| OKT-3 | 55 | --- |
| TH5.2 | 52 | --- |
| Leu-3a | 35 | --- |
| Leu-3a+ TH5.2 | --- | 32 |
| Leu-2a | 15 | --- |
| Leu-2a+ TH5.2 | --- | 13 |
| Leu-M3 | 10 | --- |
| Leu-M3+ TH5.2 | --- | 1 |

[a] OKT-3 MAb + goat anti-mouse IgG (Fc specific) FITC conjugated antibody. TH5.2 MAb + goat anti-mouse IgM (u chain specific) FITC conjugated antibody. All Leu MAb phycoerythrin-conjugated.

[b] 10,000 cells analyzed per point by FACS. data representative of three experiments.

TABLE 3. PHENOTYPIC ANALYSIS OF TH5.2-POSITIVE CELLS[a] SORTED ON THE BASIS OF HIGH OR LOW TH5.2 INTENSITY FLUORESCENT STAINING.

| SORTED CELLS RESTAINED WITH: | PERCENTAGE STAINING POSITIVE WITH RESTAINING MAb. | |
|---|---|---|
| | HIGH TH5.2 INTENSITY POPULATION[b] | LOW TH5.2 INTENSITY POPULATION[c] |
| Leu-2a[d] | 7 | 42 |
| Leu-3a | 51 | 8 |
| Leu-M3 | 1 | 1 |
| TH5.2 | 65 | 15 |

[a] Normal PBMC stained with TH5.2 MAb plus FITC-conjugated goat anti-mouse IgM (u chain specific) second antibody (10,000 cells analyzed per point by FACS). Data representative of three experiments.

[b] the highest 30% intensity staining fraction of cells by FACS using TH5.2 MAb.

[c] the lowest 30% intensity staining fraction of cells by FACS.
[d] all Leu MAb phycoerythrin-conjugated.

TABLE 4. EFFECT OF TH5.2 MAb ON MITOGEN-INDUCED T CELL PROLIFERATION[a].

| MAb[b] | $^3$H-TdR UPTAKE (cpm) AT PHA CONCENTRATIONS (ug/ml) OF: | | | |
|---|---|---|---|---|
| | 0 | 2.0 | 0.25 | 0.10 |
| Medium | 3,100 ± 160 | 165,700 ± 11,710 | 29,580 ± 3,123 | 6,300 ± 1,021 |
| TH5.2 (1/10 dil) | 4,800 ± 520 | 144,800 ± 9,211 | 40,100 ± 3,711 | 35,217 ± 3,321 |
| TH5.2 (1/60 dil) | 2,900 ± 412 | 116,000 ± 25,120 | 38,300 ± 4,211 | 18,100 ± 1,821 |
| ANTI-HLA B8 (1/10 dil) | 2,722 ± 171 | 172,111 ± 20,201 | 33,213 ± 3,413 | 8,121 ± 1,021 |
| ANTI-HLA B8 (1/60 dil) | 3,213 ± 162 | 167,211 ± 21,211 | 27,211 ± 3,121 | 5,721 ± 1,521 |
| $S_{33}$ (1/10 dil) | 3,211 ± 272 | 171,211 ± 15,213 | 31,073 ± 2,121 | 7,301 ± 1,024 |
| $S_{33}$ (1/60 dil) | 3,712 ± 412 | 168,103 ± 14,211 | 32,111 ± 3,511 | 6,821 ± 1,251 |

[a] Normal PBMC incubated with PHA for 72 hours, pulsed at 18 hrs. with $^3$H-TdR.

[b] All MAb are IgM isotype in the form of culture supernatant, final culture dilution (1/10 dilution = 6 ug/ml). Data representative of 5 experiments

TABLE 5. EFFECT OF TH5.2 MAb ON THE PROLIFERATIVE CAPABILITY OF ANTIGEN-ACTIVATED T CELLS[a].

| MAb | $^{3}$H-TdR UPTAKE (CPM) FOLLOWING STIMULATION WITH: | | | | |
|---|---|---|---|---|---|
| | MEDIUM | SK-SD (1/10) | SK-SD (1/100) | MUMPS 1/10 | KLH[c] (10 ug/ml) |
| Medium | 1428 ± 102 | 13900 ± 1014 | 3400 ± 987 | 17800 ± 1824 | 2000 ± 297 |
| TH5.2 (1/10 dil) | 2900 ± 410 | 24400 ± 1424 | 9300 ± 724 | 44200 ± 3725 | 18800 ±1540 |
| TH5.2 (1/60 dil) | 1800 ± 85 | 11800 ± 2021 | 3923 ± 725 | 16200 ± 1424 | 8100 ± 722 |
| ANTI-HLA B8 (1/10 dil) | 4200 ± 783 | 15212 ± 1821 | 4521 ± 512 | 15213 ± 1724 | 4123 ± 522 |
| ANTI-HLA B8 (1/60 dil) | 3923 ± 318 | 11171 ± 2221 | 4211 ± 270 | 16813 ± 3214 | 2111 ± 178 |
| $S_{33}$ (1/10) | 5213 ± 1001 | 16211±2021 | 5211 ± 1059 | 22011 ± 3414 | 4521 ± 723 |
| $S_{33}$ (1/60) | 4811 ± 731 | 12108±1120 | 4110 ± 824 | 18103 ± 1541 | 1372 ±1021 |

[a] PBMC stimulated with antigen for 6 days. 18 hrs. prior to harvesting, cultures pulsed with $^{3}$H-TdR, representative of five experiments.

[b] All MAb are IgM isotype in the form of culture supernatant, final culture dilution. All supernatants were adjusted such that a 1/10 dilution = 6 ug/ml antibody.

[c] Using PBMC from a KLH immune donor.

TABLE 6. EFFECT OF CYTOTOXIC TREATMENT[a] WITH TH5.2 MAb PLUS COMPLEMENT ON THE PROLIFERATIVE CAPABILITY OF T CELLS.

| STIMULANT[b] | PROLIFERATIVE RESPONSE[c] FOLLOWING TREATMENT WITH | | | |
|---|---|---|---|---|
| | NONE | TH5.2 MAb | COMPLEMENT ALONE | TH5.2 MAb PLUS COMPLEMENT |
| NONE | 510 ± 152 | 1,090 ± 93 | 1,350 ± 142 | 480 ± 120 |
| PHA (2ug/ml) | 106,800 ± 12,010 | 126,580 ± 10,241 | 128,200 ±13,213 | 7,080 ± 1,021 |
| MUMPS (1/10 dilution) | 1,350 ± 272 | 3,520 ± 422 | 1,604 ± 72 | 601 ± 43 |
| VARICELLA (1/10 dilution) | 2,040 ± 420 | 5,160 ± 1121 | 1,290 ± 241 | 690 ± 57 |
| | PERCENTAGE STAINING POSITIVE | | | |
| CELLS STAINED WITH[d] | | | | |
| OKT-4 MAb | 33 | - | 33 | 25 |
| OKT-8 MAb | 29 | - | 28 | 35 |

a $10^6$ PBMC incubated with 100 ul of TH5.2 MAb culture supernatant for 1 hr/RT, washed 2X RPMI followed by addition of 10-100 ul rabbit complement for 30 min/RT.

b cells incubated with PHA for 3 days and antigen for 6 days.

c $^3$H-TdR uptake expressed as cpm following an 18 hr pulse, results representative of 5 separate experiments.

d PBMC treated appropriately followed by staining with either OKT-4 or OKT-8 MAb and goat anti-mouse IgG (Fc-specific) FITC second antibody (10,000 cells analyzed per point by FACS).

TABLE 7. ABILITY OF CONDITIONED SUPERNATANTS TO RECONSTITUTE THE PROLIFERATIVE CAPABILITY OF PBMC TREATED WITH TH5.2 MAb PLUS COMPLEMENT.

| TREATMENT | | PHA RESPONSE | PHA RESPONSE[b] WITH ADDITION OF: | | |
|---|---|---|---|---|---|
| | | | CONTROL SUP[c] | PHA-COND. SUP[d] | ANTIGEN-COND. SUP[e] |
| Expt 1 | CONTROL | 148,400±12,212 | 116,800±14,214 | 177,800±14,212 | n.d. |
| | TH5.2 + COMP.[a] | 12,100± 1,621 | 23,600± 1,471 | 67,100± 8,211 | n.d. |
| Expt 2 | CONTROL | 46,900± 5,024 | 46,300± 5,213 | 66,000± 7,201 | n.d. |
| | TH5.2 + COMP. | 8,060± 1,024 | 3,135± 624 | 50,400± 4,204 | n.d. |
| EXPT 3 | CONTROL | 46,400± 4,211 | 48,100± 4,211 | 61,800±7,312 | 40,900±5,099 |
| | TH5.2 COMP. | 4,700± 324 | 5,300± 624 | 48,500±5,328 | 26,600±2,122 |

[a] $10^6$ PBMC incubated with 100 ul of TH5.2 MAb culture supernatant for 1 hr/RT, washed 2X RPMI followed by addition of 10-100 ul rabbit complement for 30 min/RT.

[b] cells incubated with 2 ug/ml PHA plus appropriate supernatant for 3 days. CPM of $^3$H-TdR uptake, following an 18 hr. pulse. (n.d. = not done, representative of five experiments).

[c] supernatant from $10^6$ PBMC/ml incubated for 24 hours/37C.

[d] supernatant from $10^6$ PBMC/ml incubated with PHA, 2 ug/ml for 24 hrs/37C.

[e] supernatant from $2X10^6$ PBMC/ml incubated with mumps antigen (1/10 final culture dilution for 72 hrs/37C).

TABLE 8. ABILITY OF RIL 2 TO RECONSTITUTE THE PROLIFERATIVE CAPABILITY OF PBMC DEPLETED OF A TH5.2-REACTIVE SUBSET OF CELLS.

| TREATMENT[a] | PROLIFERATIVE RESPONSE[b] | |
|---|---|---|
| | EXPT. 1 | EXPT. 2 |
| NONE | 49,342 | 102,111 |
| COMPLEMENT ALONE | 48,209 | 98,213 |
| CONTROL MAb PLUS COMPLEMENT | 42,486 | 96,171 |
| TH5.2 MAb PLUS COMPLEMENT | 29,201 | 15,714 |
| TH5.2 MAb PLUS COMPLEMENT WITH ADDITION OF RIL 2: | | |
| 25 U/ml | 45,270 | 121,222 |
| 12 | 44,729 | 118,113 |
| 6 | 42,460 | 111,103 |
| 1 | 36,441 | 72,114 |
| 0.25 | 28,215 | 37,104 |

[a]$10^6$ PBMC incubated with 100 ul of TH5.2 MAb or control (non-reactive with PBMC) MAb culture supernatant for 1 hr./RT followed by addition of 10-100 ul rabbit complement for 30 min./RT.

[b]$10^5$ cells/well incubated with PHA, 2 ug/ml. Expressed as $^3$H-TdR uptake in cpm. Standard deviations of the mean were within 15%.

TABLE 9. FUNCTIONAL AND PHENOTYPIC CHARACTERISTICS OF T CELLS EXPRESSING HIGH AND LOW DENSITIES OF TH5.2 ANTIGEN.

| CELLS | PHENOTYPE | | PROLIFERATION[a] | IL 2 PRODUCTION[b] |
|---|---|---|---|---|
| UNTREATED PBMC | --- | | 35,231±1,790 | 18,201±2,022 |
| ADHERENT POPULATION[c] | OKT-3<br>Leu-M3 | 3%<br>70% | 2,901±770 | 272±121 |
| HIGH TH5.2-INTENSITY POPULATION[d] | Leu-3a<br>Leu-2a<br>Leu-M3 | 40%<br>7%<br>0-1% | 1,025±433 | 1,411±220 |
| LOW TH5.2 INTENSITY POPULATION[e] | Leu-3a<br>Leu-2a<br>Leu-M3 | 14%<br>51%<br>0-1% | 5,941±396 | 1,723±210 |
| HIGH TH5.2 INTENSITY POPULATION + 10% ADHERENT CELLS | --- | | 28,113±4,952 | 14,114±1,621 |
| LOW TH5.2 INTENSITY POPULATION + 10% ADHERENT CELLS | --- | | 7,308±35 | 2,335±211 |

[a] cells stimulated with 5 ug/ml PHA for 3 days. Expressed as cpm uptake of $^{3}$H-TdR following a 4 hr pulse. Results representative of three experiments.

[b] supernatants from appropriate cultures collected and tested on CTLL cells, $^{3}$H-TdR uptake in cpm following a four hr. pulse.

[c] cultures of PBMC adhered to FCS-coated flasks for 30 min. at 37C. Adherent cells removed by treatment with 0.2% EDTA with subsequent washing.

[d] high intensity= the highest 30% staining intensity fraction of cells by FACS.

[e] low intensity= the lowest 30% staining intensity fraction of cells by FACS.

TABLE 10. TH5.2 MAb DOES NOT INDUCE INCREASED Tac ANTIGEN EXPRESSION ON T CELLS STIMULATED WITH MITOGEN.

| TREATMENT[a] | | $^3$H-TdR UPTAKE[b] | % POSITIVE STAINING WITH ANTI-Tac MAb[c] |
|---|---|---|---|
| PHA (ug/ml) | TH5.2 (ug/ml) | | |
| 0 | 0 | 600± 201 | 0 |
| 0 | 1 | 527± 175 | 0 |
| 0 | 5 | 458± 426 | 0 |
| 0.1 | 0 | 8,764± 1,021 | 11 |
| 0.1 | 1 | 9,118± 872 | 12 |
| 0.1 | 5 | 39,177± 2,742 | 12 |
| 2.0 | 0 | 23,323± 1,921 | 60 |
| 2.0 | 1 | 33,559± 3,521 | 58 |
| 2.0 | 5 | 106,416± 12,001 | 57 |

[a] $10^5$ PBMC set up at initiation of cultures with PHA and/or TH5.2 MAb in the form of ascites fluid (1/200 final culture dilution).

[b] 72 hr assay/4 hr pulse with $^3$H-TdR. Results representative of two experiments.

[c] 1/10,000 dilution of anti-Tac ascites fluid plus goat anti-mouse IgG (Fc-chain specific) FITC-conjugated second antibody Analysis of 10,000 cells per point by FACS.

TABLE 11. EFFECT OF TH5.2 MAb ON THE ABILITY OF T CELLS TO SECRETE IL 2 IN RESPONSE TO ANTIGEN OR MITOGEN STIMULATION.

| EXPT. NO. | STIMULUS | TREATMENT | PROLIFERATION[a] | IL 2 PRODUCTION[b] | | |
|---|---|---|---|---|---|---|
| | | | | 24 hrs | 48 hrs | 72 hrs |
| 1 | mumps | medium | 14,655 | 698 | n.d. | n.d. |
| | mumps | TH5.2[d] | 38,367 | 2,518 | n.d. | n.d. |
| 2 | PHA[c] | medium | 2,041 | 102 | n.d. | n.d. |
| | PHA | TH5.2 | 9,978 | 1,253 | n.d. | n.d. |
| 3 | mumps | medium | 3,666 | n.d. | 2,245 | 3,005 |
| | mumps | TH5.2 | 8,999 | n.d. | 6,830 | 15,784 |
| | medium | TH5.2 | 1,027 | n.d. | 721 | 1,032 |
| | medium | medium | 672 | n.d. | 525 | 434 |
| 4 | mumps | control IgM | 6,241 | 1,427 | 4,404 | 2,211 |
| | mumps | TH5.2 | 24,938 | 3,023 | 12,709 | 8,608 |

[a] $^3$H-TdR uptake in cpm at 6 days, following an 18 hr. pulse. The standard deviation of the mean values were within 15%.

[b] supernatants from appropriate cultures collected at various time points and tested on CTLL cells. $^3$H-TdR uptake in cpm, standard deviations within 15% (n.d. = not done).

[c] 0.25 ug/ml PHA for 72 hrs.

[d] 1/16 final culture dilution of TH5.2 MAb or control IgM MAb (anti-HLA B8 or $S_{33}$) in the form of culture supernatant (expts. 1 and 2), or ascites fluid 1/200 dilution (expts.3 and 4).

TABLE 12. RIL 2 IS ABLE TO REPLACE TH5.2 MAb IN AUGMENTING THE PROLIFERATIVE RESPONSE OF ANTIGEN-ACTIVATED T CELLS.

| EXPT. NO. | ANTIGEN | LYMPHOCYTE PROLIFERATION[a] | | | | |
|---|---|---|---|---|---|---|
| | | +TH5.2 MAb | | | | +RIL 2 |
| | | NONE | 1/10[b] | 1/20 | 1/40 | 10U/WELL |
| 1 | KLH 50ug/ml[c] | 3,693 | 9,408 | 11,085 | 8,353 | 23,198 |
| | 25 | 1,021 | 8,738 | 12,579 | 3,245 | 21,211 |
| | 6 | 1,672 | 3,959 | 8,080 | 2,674 | 14,217 |
| | 0 | 1,428 | 1,279 | 1,744 | 1,110 | 10,231 |
| 2 | MUMPS 1/20 DIL. | 15,248 | 14,810 | 23,079 | 20,198 | 42,219 |
| | 1/160 | 3,169 | 13,137 | 8,634 | 5,583 | 31,602 |
| | 1/640 | 1,734 | 4,897 | 4,100 | 1,394 | 20,655 |
| | 0 | 278 | 678 | 963 | 1,153 | 17,333 |

[a] 3H-TdR uptake (cpm) measured at 6 days following an 18 hr. pulse. The standard deviation of the mean values were within 15%.

[b] final culture dilution of supernatant. A 1/10 dilution = 6 ug/ml antibody.

[c] using PBMC from a KLH-immune donor.

TABLE 13. EFFECT OF MODULATION[a] OF VARIOUS T CELL SURFACE ANTIGENS ON THE ABILITY OF TH5.2 MAb TO STAIN THE CELLS.

| INITIAL ANTIBODY[c] | BEFORE MODULATION, STAINED WITH INITIAL MAb | AFTER MODULATION WITH INITIAL MAb, RESTAINED WITH: | |
|---|---|---|---|
| | | INITIAL MAb[c] | TH5.2 MAb |
| TH5.2 | 60 | 37 | – |
| OKT-3 | 61 | 7 | 64 |
| OKT-4 | 35 | 9 | 51 |
| OKT-8 | 22 | 3 | 52 |
| Leu-5 | 63 | 2 | 54 |

[a] $10^6$ cells incubated with the appropriate MAb for 18 hrs at 37 C.

[b] results representative of three separate experiments. 10,000 cells analyzed per point by FACS.

[c] TH5.2 MAb plus goat anti-mouse IgM (u chain-specific) FITC conjugate. OKT-3,4,8, and Leu-5 MAb plus goat anti-mouse IgG (Fc-specific) FITC conjugate.

TABLE 14. MODULATION OF THE TH5.2 DETERMINANT FROM THE SURFACE OF T CELLS DID NOT AFFECT STAINING OF THE CELLS WITH OKT-3, OKT-4, OKT-8, or Leu-5 MAb.

| INITIAL MAb[b] | PERCENT POSITIVE STAINING[a]: BEFORE MODULATION, STAINED WITH INITIAL MAb | PERCENT POSITIVE STAINING[a]: AFTER MODULATION WITH TH5.2 MAb[c], RESTAINED WITH INITIAL MAb |
|---|---|---|
| TH5.2 | 60 | 37 |
| OKT-3 | 61 | 63 |
| OKT-4 | 31 | 32 |
| OKT-8 | 30 | 32 |
| Leu-5 | 63 | 69 |

[a] 10,000 cells analyzed per point by FACS, representative of two experiments.

[b] TH5.2 MAb plus goat anti-mouse IgM (u chain specific) FITC conjugate. OKT-3,4,8, and Leu-5 MAb plus goat anti-mouse IgG (Fc specific) FITC conjugate.

[c] $10^6$ cells incubated with TH5.2 Mab for 18 hrs at 37 C.

Figure 1. SDS-PAGE analysis of immunoprecipitates of detergent extracts of $^{3}$H-galactose-labelled HTL cells. Fig 1a, CHAPS detergent extract of HTL cells immunoprecipitated with TH5.2 ascites fluid. Fig. 1b, CHAPS detergent extract of HTL cells immunoprecipitated with a control IgM MAb. Fig. 1c, NP-40 detergent extract of HTL cells immunoprecipitated with anti-Tac MAb.

1a

58k

45k

2300
2200
2100
2000
1900
1800
1700
1600
1500
1400
1300
1200
1100
1000
900
800
700
600
500
400
300
200
100

Counts per minute

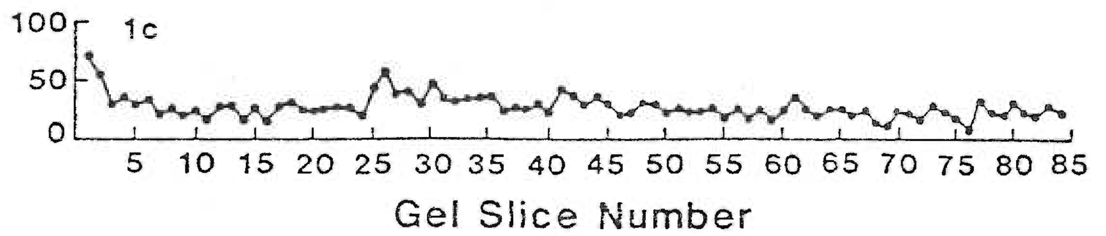


Figure 2. SDS-PAGE analysis of immunoprecipitates of CHAPS detergent extracts of $^{3}H$-galactose-labelled Con A blasts from human PBMC. Fig 2a, Immunoprecipitation with TH5.2 MAb. Fig. 2b, Immunoprecipitation with control IgM MAb. Fig. 2c, Immunoprecipitation with anti-Tac MAb.

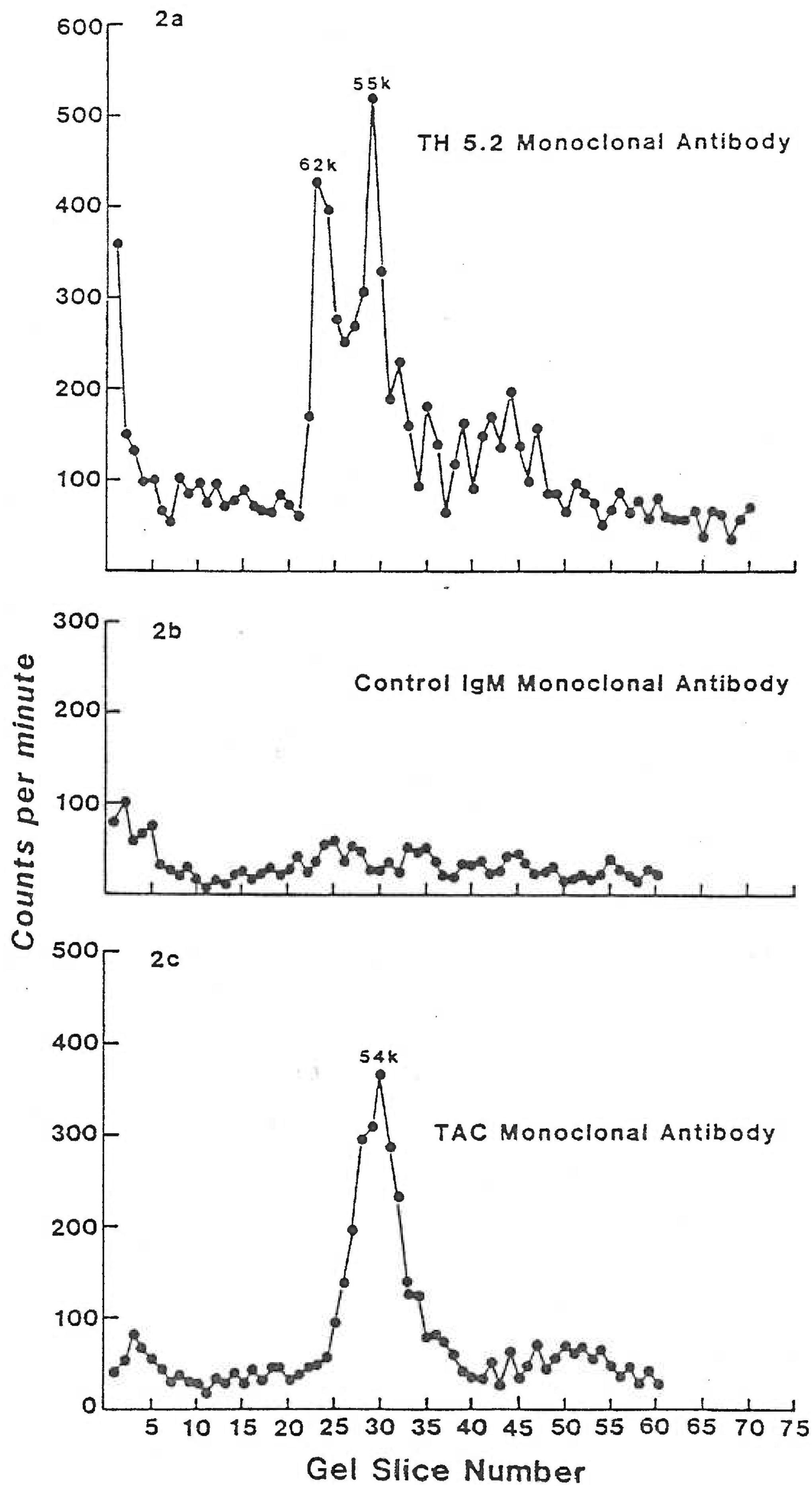
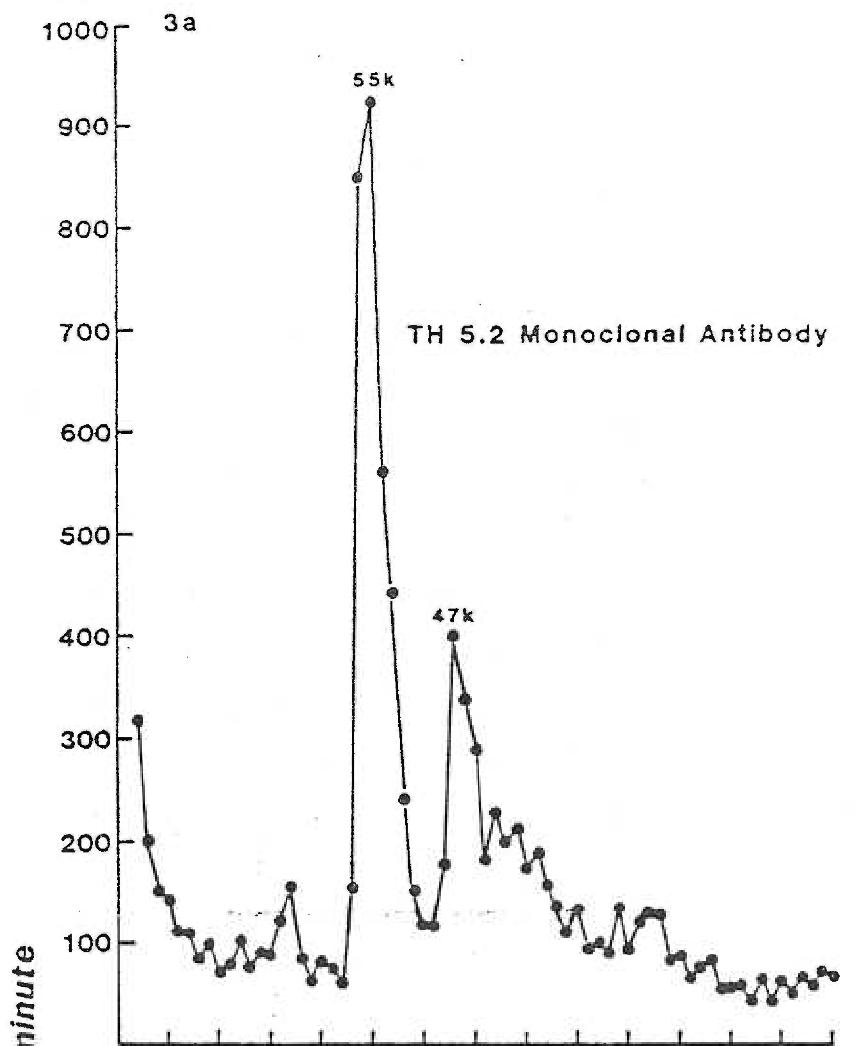

2a
TH 5.2 Monoclonal Antibody
62k
55k
600
500
400
300
200
100
2b
Control IgM Monoclonal Antibody
300
200
100
2c
TAC Monoclonal Antibody
54k
500
400
300
200
100
0
Counts per minute
5 10 15 20 25 30 35 40 45 50 55 60 65 70 75
Gel Slice Number

Figure 3. SDS-PAGE analysis of immunoprecipitates of NP-40 detergent extracts of $^{125}$I-labelled HTL cells. Cells were reacted with the antibody and washed. Antibody and cell surface antigens were chemically crosslinked as described in the MATERIALS AND METHODS section. NP-40 extracts of these cells were then used or immunoprecipitation with TH5.2 MAb (Fig. 3a) or control IgM MAb (Fig. 3b). The chemical crosslink was then cleaved by reduction with mercaptoethanol and SDS-PAGE analysis performed.


3a
55k
47k
TH 5.2 Monoclonal Antibody
1000
900
800
700
600
500
400
300
200
100
Counts per minute

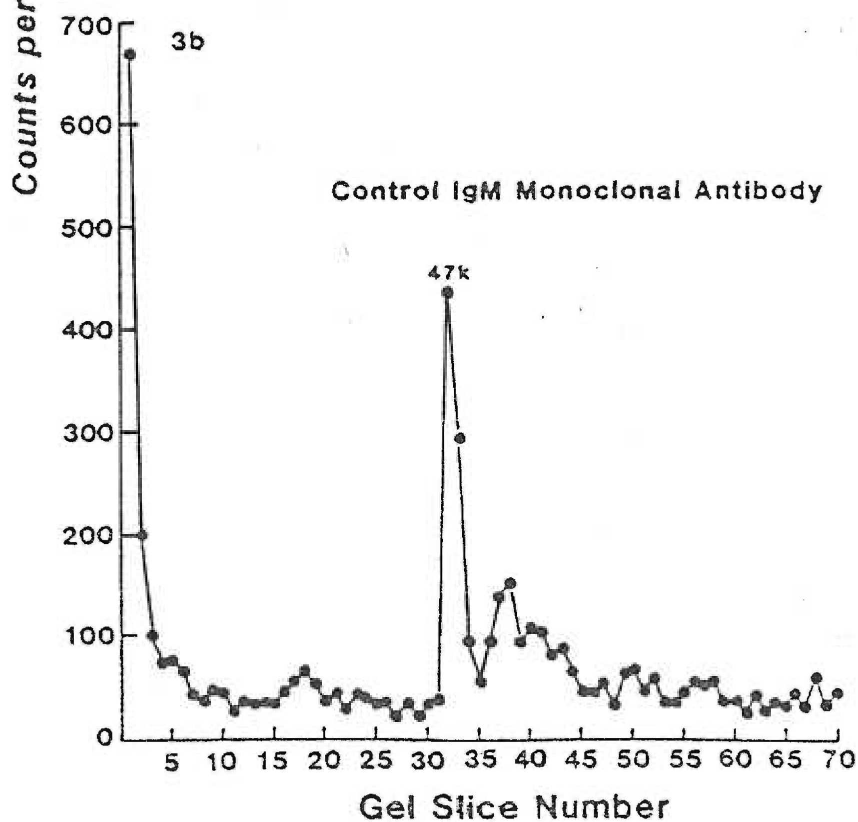
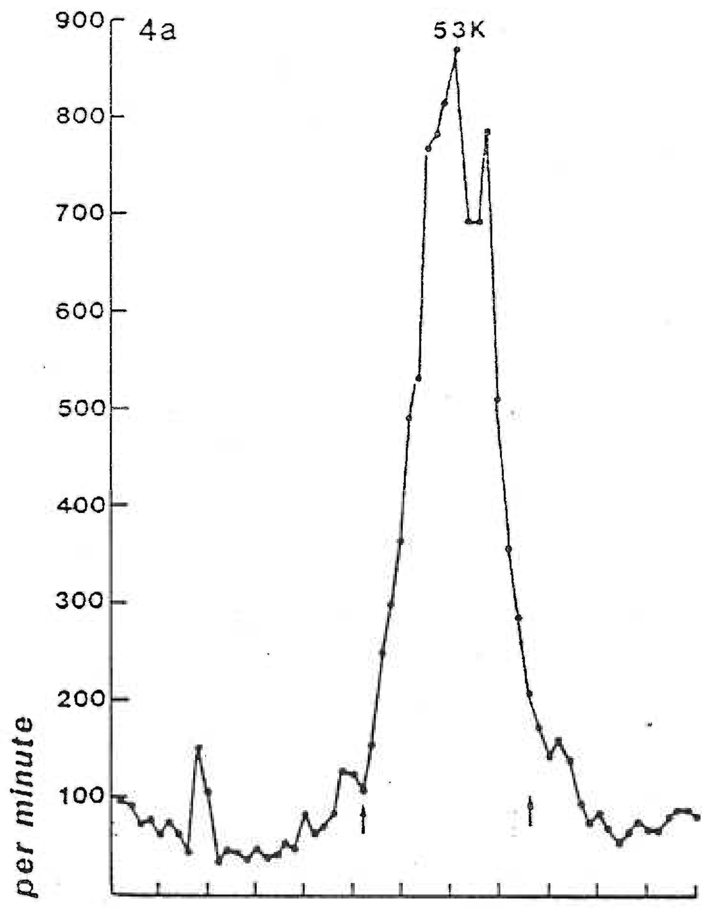
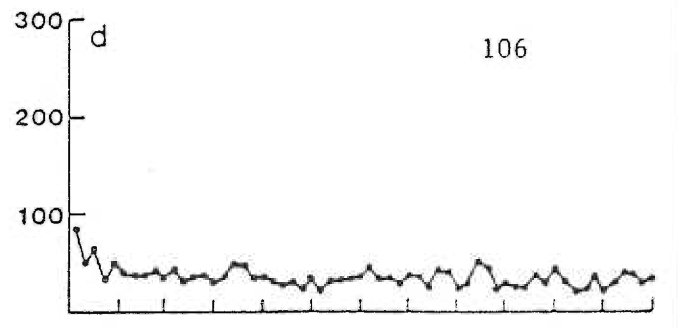
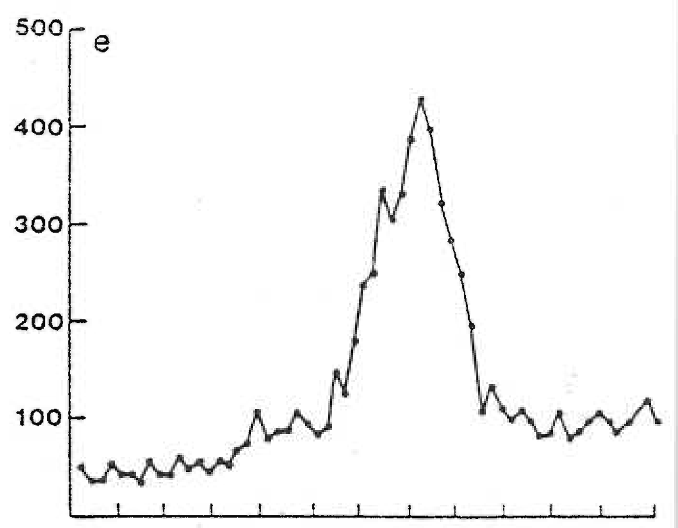
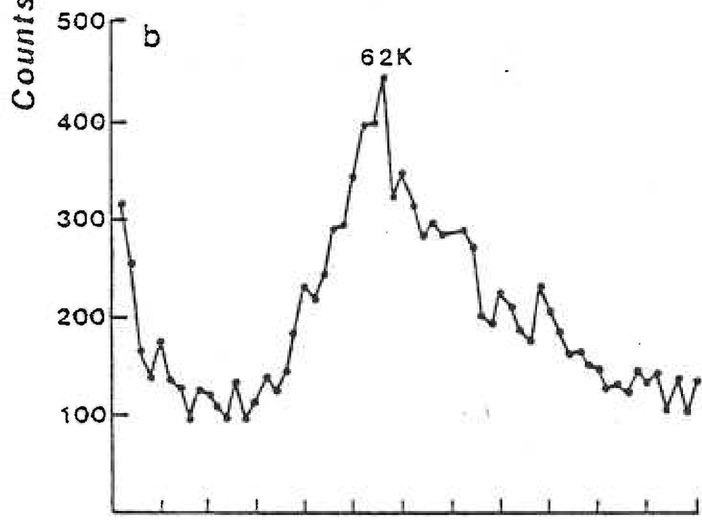

3b
Control IgM Monoclonal Antibody
47k
700
600
500
400
300
200
100
0
5 10 15 20 25 30 35 40 45 50 55 60 65 70
Gel Slice Number

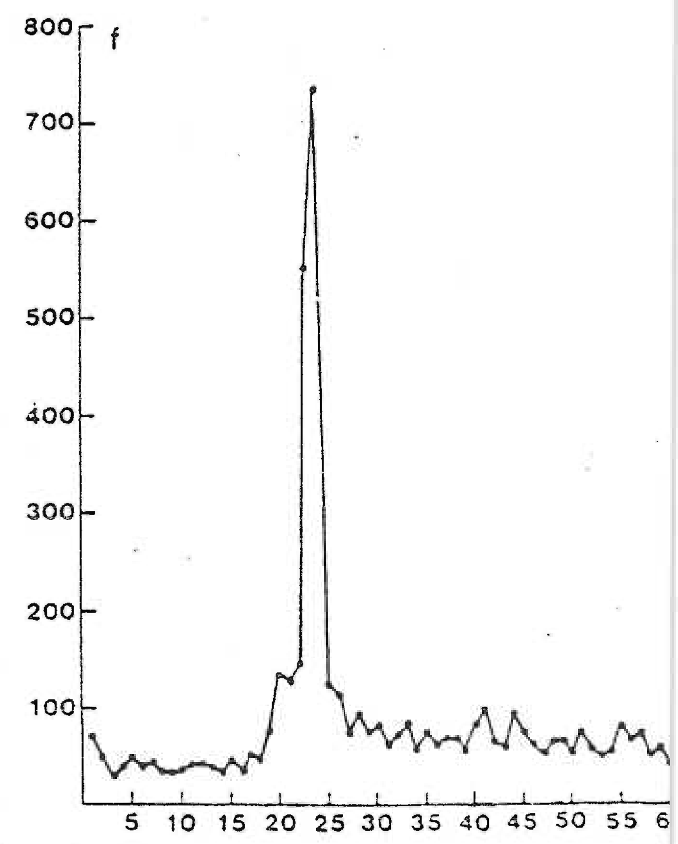
Figure 4. Sequential immunoprecipitation experiments using CHAPS detergent extracts of $10^8$ $^3$H-galactose-labelled Con A-stimulated PBMC with TH5.2 and anti-Tac MAb. Figure 4a shows the immunoprecipitate obtained with 5 ul anti-Tac ascites fluid; Figure 4b shows the immunoprecipitate obtained with 30 ul of TH5.2 ascites. The supernatant from the Tac precipitate was divided into two parts and re-precipitated with 5 ul of anti-Tac ascites fluid (Figure 4d) or with 30 ul of TH5.2 ascites fluid (Figure 4f). The supernatant from the TH5.2 immunoprecipitate was divided into two parts and re-precipitated with 30 ul of TH5.2 ascites fluid (Figure 4c) or 5 ul of anti-Tac ascites fluid (Figure 4e).

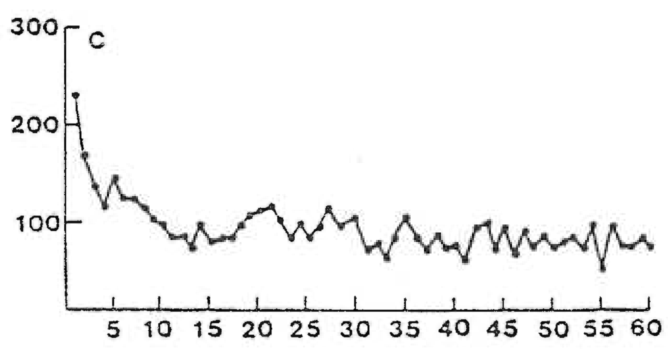


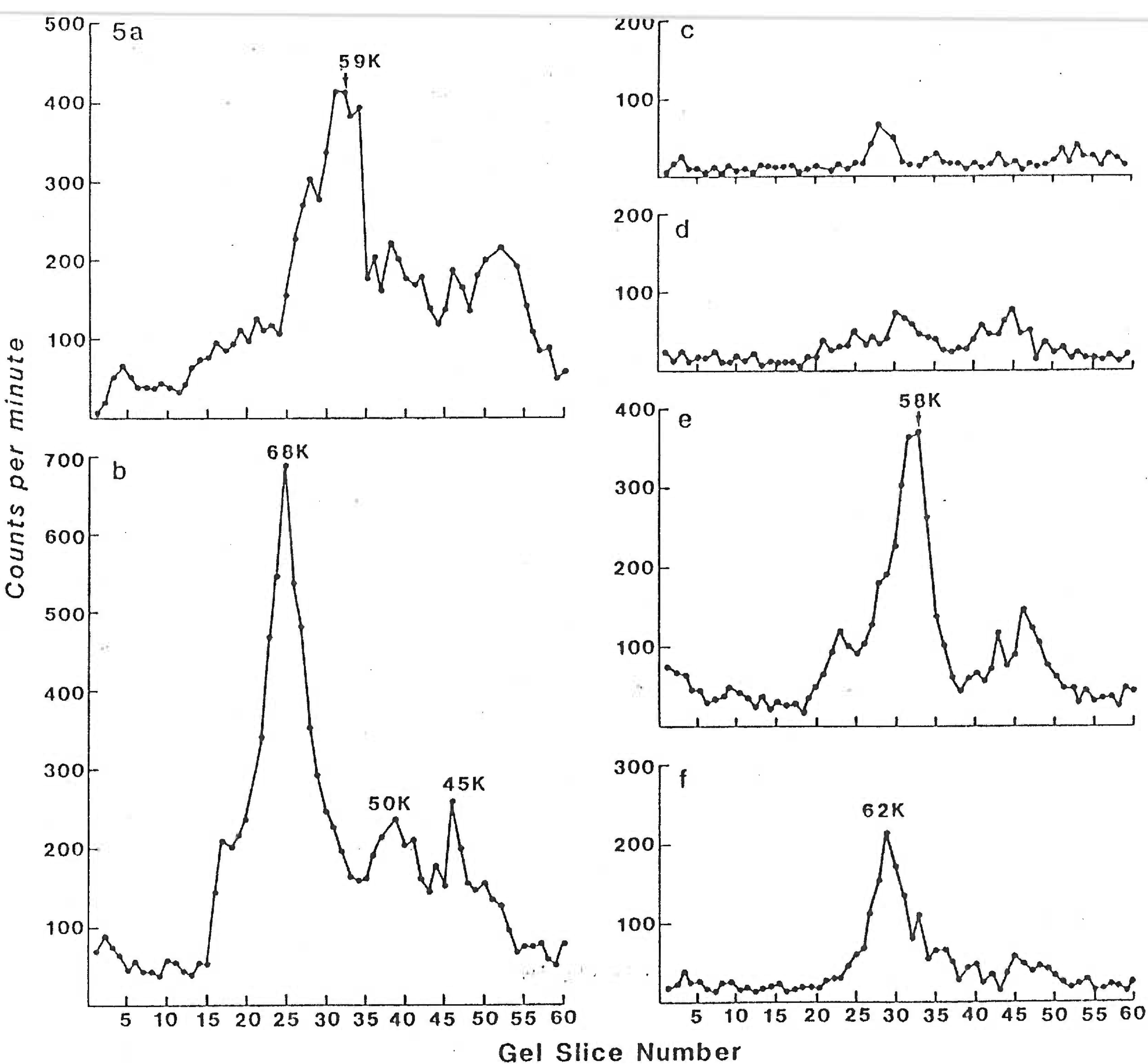



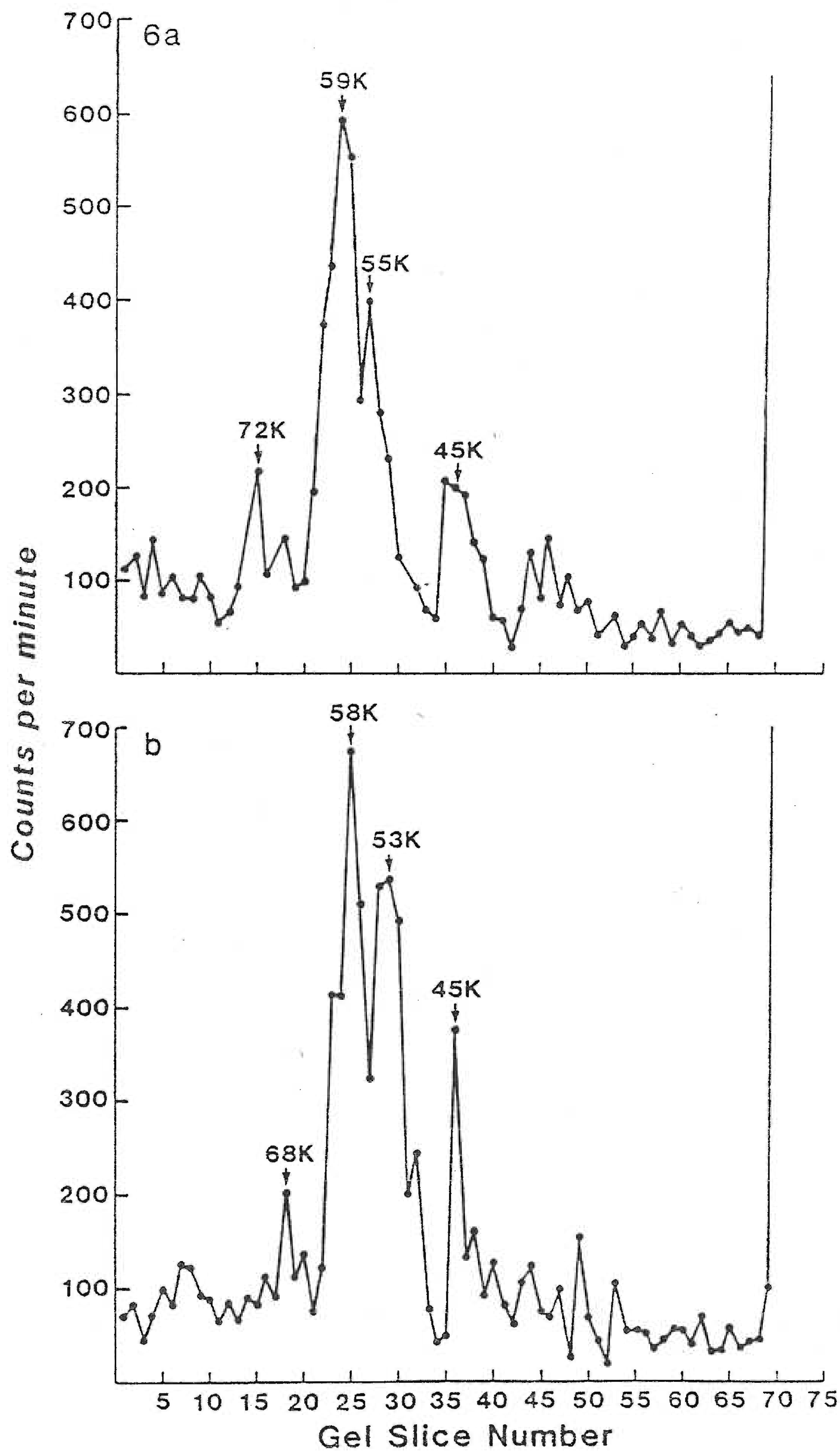

Gel Slice Number

Figure 5. Sequential immunoprecipitation experiments using CHAPS detergent extracts of $10^8$ $^3$H-galactose-labelled HTL cells with TH5.2 and OKT-4 MAb. Figure 5a shows the immunoprecipitate obtained with 50 ul of OKT-4 MAb; Figure 5b shows the immunoprecipitate obtained with 30 ul of TH5.2 ascites. The supernatant from the OKT-4 precipitation was divided into two parts and re-precipitated with 50 ul of OKT-4 (Figure 5d) or 30 ul of TH5.2 ascites fluid (Figure 5f). The supernatant from the TH5.2 precipitate was divided into two with 30 ul of TH5.2 ascites fluid (Figure 5c) or 50 ul of OKT-4 MAb (Figure 5e).



5a
59K
b
68K
50K
45K
c
d
e
58K
f
62K
Counts per minute
Gel Slice Number
5 10 15 20 25 30 35 40 45 50 55 60

Figure 6. SDS-PAGE analysis of immunoprecipitates of CHAPS detergent extracts of $10^8$ $^3H$-Leucine-labelled HTL cells in the presence and absence of tunicamycin. In Figure 6a cells were grown for 18 hours in RPMI 1640 with 10% FCS before labelling, detergent extraction, and immunoprecipitation with 30 ul of TH5.2 ascites. In Figure 6b cells were grown for 18 hours in the same medium supplemented with 10 ug/ml tunicamycin before labelling (tunicamycin also present), detergent extraction, and immunoprecipitation with 30 ul of TH5.2 ascites fluid.


6a
59K
55K
72K
45K
b
58K
53K
45K
68K
700
600
500
400
300
200
100
Counts per minute
5 10 15 20 25 30 35 40 45 50 55 60 65 70 75
Gel Slice Number

# ABBREVIATIONS

1) cDNA- complementary deoxyribose nucleic acid.

2) CHAPS- 3-(3-Cholamidopropyl) dimethyl-ammonio propanesulfonate.

3) Con-A- Concanavalin-A.

4) EDTA- ethylene diamine tetraacetate.

5) FACS- fluorescence activated cell sorter.

6) FCS- fetal calf serum.

7) FITC- fluorescein isothyocyanate.

8) IL 1- interleukin 1.

9) IL 2- interleukin 2.

10) IL 4a- interleukin 4a.

11) $IP_3$- inositol triphosphate.

12) KLH- keyhole limpet hemacyanin.

13) LAF- lymphocyte activating factor.

14) MAb- monoclonal antibody.

15) uCi- microcurie.

16) mCi.- millicurie.

17) MHC- major histocompatibility complex.

18) mRNA- messenger RNA.

19) NP-40- nonidet P40.

20) PBMC- peripheral blood mononuclear cell.

21) PE- phycoerythrin.

22) PHA- phytohemmaglutinin.

23) PMA- phorbol myristate acetate.

24) PMSF- phenylmethylsulfonyl fluoride.

25) RIL 2- recombinant IL 2.

26) SDS- sodium dodecyl sulfate.

27) SK-SD- streptokinase-streptodornase.

28) TCGF- T cell growth factor.

28) TLCK- N-a-p-tosyl-L-lysine chloromethyl ketone.

29) TPCK- L-1-tosylamide-2-phenylethylchloromethyl ketone.

30) TPA- 12-o-tetra decanoyl phorbol-13-acetate.

31) $^{3}$H-TdR- tritiated thymidine.