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Functional Effects of Monoclonal Antibodies Directed Against the Tcr/Cd3 Complex of Various T Cell Phenotypes

Mortimer T. Alzona
Loyola University Chicago

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Loyola University Chicago

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The purpose of this project was to investigate the functional effects that different monoclonal antibodies (mAb) to the T cell antigen receptor/CD3 (TCR/CD3) complex have upon T cells of various phenotypes. The question of whether or not these different effects are due to the intrinsic properties of the mAb, the type of target cell, or the epitope (the site of the TCR/CD3 that the mAb binds to) in which the mAb recognize, was examined. The panel of mAb used included two anti-CD3 mAb and three pairs of anti-TCR mAb with each pair being specific for a different T cell tumor. Mab were tested on peripheral blood lymphocytes, normal human thymocytes, two acute lymphocytic leukemias, and one chronic lymphocytic leukemia. These cells were incubated with mAb and the occurence certain events were then measured. These events included: intracellular Ca^{++} flux, interleukin 2 surface receptor expression, cytokine secretion (interleukin 2, tumor necrosis factor α, and interferon γ), and proliferation. Occurence of these events indicates that the T cells have been activated by the binding of mAb to the TCR/CD3 complex. It was found that a pariticular mAb may cause different activation events in different target cells, even when the target cells have the

same phenotype and that there can be different effects when binding a mAb to the TCR as opposed to binding CD3. Furthermore, mAb that recognize the same epitope and are of the same isotype, seem to have identical effects on T cells. Anti-TCR mAb can induce apoptosis (programmed cell death) in some tumor cell lines but not in others. The finding that anti-TCR mAb may be anti-proliferative in some T cell leukemias is encouraging for mAb immunotherapy of these tumors. This work can be important when considering the use of mAb to treat T cell leukemias.

LOYOLA UNIVERSITY CHICAGO

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MORTIMER T. ALZONA

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CHAPTER I

INTRODUCTION

The human immune system is divided into two major branches: humoral immunity and cellular immunity. Humoral immunity consists of certain circulating factors in the serum that offer some form of resistance to infectious agents, or antigens (Ag). Such factors include antibodies (Ab) and complement proteins. Ab are serum glycoproteins that always initiate their biologic effects by binding to Ag. Complement is a system of functionally linked proteins that interact with one another in a highly regulated manner to provide many of the effector functions of humoral immunity and inflammation. These proteins "complement" Ab by providing a lytic function. Cellular immunity uses cytotoxic or phagocytic cells as the major form of resistance. These cells include lymphocytes and antigen presenting cells (APC). Lymphocytes have the remarkable ability to recognize antigens and to kill antigen-infected cells. The two major classes of antigen-reactive lymphocytes are the B cells and T cells. B cells were named for their discovery in the Bursa of Fabricius in chickens while T cells were discovered to originate in the thymus. B cells are primarily responsible for humoral, or antibody responses while T cells regulate and carry out cell-mediated responses (reviewed by Coleman et al., 1989).

B and T cells interact with antigens using a clone-specific receptor on their

surface membranes. On the surface of B cells, antibodies (also known as immunoglobulins (Ig)) are expressed. These surface Ig bind to Ag which then triggers a series of intracellular events that cause the B cell to become activated and start secreting Ig (reviewed by Abbas et al., 1991). The receptor on T cells is known as the T cell antigen receptor (TCR). The TCR has been well characterized since the development of monoclonal antibodies (mAb) designed to recognize the TCR in both mice (Haskins et al., 1983; Samelson et al., 1983) and humans (Bigler et al., 1983; Meuer et al., 1983). The TCR recognizes Ag in association with the major histocompatibility complex (MHC) found on APC and other cells. This binding of MHC + Ag to the TCR results in the activation of the T cell (Dembic et al., 1985; Yague et al., 1985; Marrack and Kappler, 1987).

The TCR is composed of at least seven transmembrane chains (Allison et al., 1982; Bigler et al., 1983; Clevers et al. 1988; Klausner et al., 1990; Weiss, 1990)) and these chains all possess N-terminal leader sequences and a single transmembrane domain with the amino terminus facing the outside of the cell (Klausner et al., 1990). The heterodimer of alpha and beta (TCR α/β) consists of disulfide linked chains which are the largest chains of the receptor (alpha is 47-54 kD, beta is 40-44 kD) (Weiss et al., 1986; Klausner et al., 1990; Weiss, 1990). They represent Ig-like glycoproteins containing both constant and variable regions (reviewed by Davis and Bjorkman, 1988). α/β heterodimers are found on about 96% of peripheral T cells, while the rest use a heterodimer of γ and δ chains. In addition to the TCR α/β and γ/δ chains, another set of invariant chains, known as CD3, is associated with the receptor. These chains and

their association with the TCR were also identified by mAb (Reinherz et al., 1982, 1983; Meuer et al., 1983; Weiss and Stobo, 1984). The CD3 chains are smaller and include gamma, delta, epsilon, and two zeta chains or zeta related chains (CD3γ, δ, ϵ, ζ_2) (Weiss et al., 1986; Klausner et al., 1990; Weiss, 1990). It is this integrated complex, known as the TCR/CD3 complex, that responds to antigen/MHC binding by transducing a signal which in turn causes the cell to become activated (Goverman et al., 1986; Weiss et al., 1986).

Many surface proteins and receptors are found on T cells. To keep the nomenclature simple, most have been given a cluster designation (e.g. CD25 is the designation given to the IL2 receptor). CD4, CD8, CD2, and CD5 are a few of the many surface proteins found on T cells. CD4 and CD8 are accessory molecules that are important as stabilizing elements and signal transducers in Ag-responding T cells (Swain, 1983; as reviewed by Paul, 1989; Finkel et al., 1991). CD4 is expressed on approximately 95% of thymocytes and 60% of peripheral T cells while CD8 is expressed on most thymocytes and on 40% of peripheral T lymphocytes. CD4 and CD8 function as adhesion molecules to stabilize interactions between the TCR and Ag/MHC association by binding to MHC class II and I, respectively (Swain, 1983). MHC is a surface protein involved in Ag presentation to T cells by APC. As signal transducers, some evidence suggests that CD4 and CD8 exert a regulatory function (which is inhibitory in nature) on signals transduced by the TCR (Bank and Chess, 1985; Wassmer et al., 1985; Moldwin et al., 1987; Owens et al., 1987). CD2 has been implicated in an "alternative pathway" model of T cell activation (Meuer et al., 1984). Mab generated to this accessory

molecule cause TCR/CD3-independent activation of T cells (O'Flynn et al., 1985a; Huet et al., 1986). CD5 is an accessory molecule that binds to a ligand on B cells, CD72. This CD5-CD72 interaction may be a way in which T cells communicate directly with B cells and may also contribute to T and B cell activation and proliferation (Van de Valde et al., 1991). However, soluble anti-CD5 mAb alone are not stimulatory to T cells (Vandenberghe and Ceuppens, 1991). It has recently been found that cross-linking of CD5 with an anti-CD5 mAb can provide a helper signal for T cell activation through the TCR/CD3 complex. By providing this additional activating stimulus, T cells can be induced to undergo full activation (Verwilghen et al., 1990; Vandenberghe and Ceuppens, 1991).

T cell activation occurs when antigen/MHC binds to the TCR/CD3 complex. Ag is first bound by APC. It is then internalized, processed, and re-expressed as peptides bound to surface MHC. Only when Ag peptides are associated with MHC can the TCR recognize and bind to them. This binding causes activation of the T cell (Braciale and Braciale, 1991). However, many researchers have found that mAb to the TCR/CD3 complex can mimic the role of Ag/MHC binding *in vitro*, thus also causing T cell activation (Meuer et al., 1983; Weiss et al., 1984b; Imboden and Stobo, 1985; MacDonald and Nabholz, 1986; Weiss et al., 1986; Nau et al., 1988; Schlitt et al., 1989). During activation of the T cell, four major events are known to occur: 1) there is an increase of cytoplasmic Ca^{++}; 2) lymphokines, such as interleukin-2 (IL2) and interferon-gamma (IFN-γ), are secreted by the cell; 3) proliferation (or clonal expansion) occurs, most notably in response to the IL2 secreted; and 4) there is an increase in the

expression of high affinity IL2 receptors (IL2R) on the cell surface, again in response to IL2 (Meuer et al., 1983; Weiss and Stobo, 1984; Imboden and Stobo, 1985; MacDonald and Nabholz, 1986).

The current model of T cell activation postulates that binding of Ag/MHC (or mAb) activates a phosphodiesterase that catalyzes the hydrolysis of phosphatidylinositol biphosphate to inositol triphosphate and diacylglycerol. Ca^{++} is released from cytoplasmic Ca^{++} stores in response to the formation of inositol triphosphate, in which inositol triphosphate acts as an intracellular Ca^{++} ionophore (Imboden and Stobo, 1985; Weiss and Imboden, 1987; Nel et al., 1991; Rothman et al., 1991). The release of Ca^{++} is a very early and rapid event, such that it can occur within three minutes of binding of a mAb to its ligand. The function of the released Ca^{++} is to stimulate other elements of the activation pathway. Such elements include protein kinase C (PKC), which is activated by diacylglycerol and Ca^{++}. The activation of PKC leads to secretion of IL2 and IFN-γ, through the activation of other proteins, which are involved in RNA and protein synthesis (Imboden and Stobo, 1985; MacDonald and Nabholz, 1986; Weiss et al., 1986). Along with IL2 and IFN-γ secretion, tumor necrosis factor alpha (TNF-α) is secreted.

The functions of the above cytokines are varied, with each having effects on many types of cells in the immune system. IL2 is an autocrine growth factor for T cells and also increases the cytolytic function of natural killer (NK) cells, producing lymphokine activated killer cells (reviewed by Abbas, 1991). IFN-γ promotes differentiation of T and B cells, activates macrophages, increases MHC class I and II expression, activates

neutrophils and NK cells, as well as activating vascular endothelial cells (Dianzani et al., 1990; Gresser, 1990; reviewed by Abbas, 1991). TNF-α is a costimulatory factor of T cell activation and B cell production of Ab. It can also activate inflammatory leukocytes to kill microbes as well as cause secretion of interleukin 1 and 6 (IL1 and IL6) by mononuclear phagocytes. TNF-α is also a natural pyrogen. It will cause fever in those individuals whose cells are secreting high amounts of the cytokine (Kunkel et al., 1989; Scheringa and Marquet, 1990; reviewed by Abbas, 1991).

In the process of activating T cells, there is an increase in the expression of high affinity IL2 receptors (IL2R). The high affinity IL2R is a combination of a 55 kD protein and a constitutively expressed 70 kD protein. The p55 receptor is expressed only during activation, therefore it is also referred to as the Tac (for T cell activation) antigen. Binding of IL2 to cells expressing only the p55 receptor is very low, while binding to cells expressing the p70 receptor is higher. However, during activation, when the p55 receptor is expressed and associates with the p70 receptor, p55 increases the affinity of the p70 receptor to bind IL2 and thereby allows a growth signal to be delivered at significantly lower concentrations of IL2 (Farrar and Ruscetti, 1986; Waldman, 1986; Smith, 1988; Smith, 1989).

The activation events thus described, lead to one of the last steps in T cell activation, proliferation. These events, Ca^{++} mobilization, cytokine secretion, and IL2R expression, either directly or indirectly play a role in clonal expansion of the activated T cell. When stimulated, for example by Ag in association with MHC, T cells will divide in order to create more T cells to combat the presence of the Ag. This becomes

a typical T cell immune response (Meuer et al., 1983; Weiss and Stobo, 1984; Imboden and Stobo, 1985; MacDonald and Nabholz, 1986).

Since the advent of the use of mAb to activate T cells, researchers have studied the effects of various mAb to different cell surface markers including the TCR/CD3 complex. In most cases they found that mAb generated against the TCR/CD3 complex had stimulatory effects on normal resting T cells. They observed proliferation of the T cells, secretion of IL2, and an increase in IL2R expression (Meuer et al., 1983; Weiss et al., 1984b; Weiss and Stobo, 1984; Bigler et al., 1985; Yssel et al., 1987). Others have also found that anti-TCR mAb stimulation of T cells can produce early events of T cell activation, such as an increase in cytoplasmic Ca^{++} (Weiss et al., 1984a; Imboden and Stobo, 1985; O'Flynn et al., 1985b; Shapiro et al., 1985; MacDonald and Nabholz, 1986; Weiss et al., 1986), release of IFN-γ (Weiss et al., 1986; Ambramowicz et al., 1989) and production of TNF-α (Ambramowicz et al., 1989). Anti-CD3 mAb can also give rise to intracellular Ca^{++} flux as seen with anti-TCR mAb (Sarkadi et al., 1991). Other events of activation seen with anti-CD3 were increased production of inositol phosphate and accumulation of CAMP (Bihoreau et al., 1991). However, some investigations have led to the conclusion that anti-TCR mAb do not cause activation of non-resting T cells (Breitmeyer et al., 1987; Takahashi et al., 1989). Evidence using two human T cell lines (both acute lymphocytic leukemias) showed that mAb to the TCR/CD3 complex can cause growth inhibition of T cells (Breitmeyer et al., 1987) and DNA fragmentation (apoptosis) followed by cell death (Takahashi et al., 1989). Thus, the functional effect of an anti-TCR/CD3 mAb may depend upon the type of target cell.

These results have lead to the current investigation of the functional effects of different mAb to the TCR/CD3 complex.

Another factor which may determine the functional effect of an anti-TCR/CD3 mAb may be the epitope recognized by the mAb. An epitope is that site on a receptor to which a particular mAb will bind. Research has suggested that the effects of mAb directed against surface receptors could be related to different epitopes recognized by each mAb (Lanier et al., 1986; Geppert et al., 1987; Rojo and Janeway, 1988; Finkel et al., 1989; Schlitt et al., 1989). Recent evidence has suggested that certain mAb directed at the TCR cause activation only under appropriate conditions, such as cross-linking of the mAb via a secondary antibody or immobilization on a solid phase. Binding of a mAb to a different epitope did not require cross-linking for activation to occur. This led to the conclusion that activation may depend on the epitope recognized (Schlitt et al., 1989). Whether specific epitopes of TCR/CD3 are responsible for activation, or whether other factors play a role, is yet unclear.

The isotype of the mAb used may also be a factor explaining these differential effects. Isotypic Ig determinants are amino acid residues that distinguish heavy (H) chain classes (IgG, IgM, IgA, IgD, IgE) and subclasses (IgG1, IgG2, IgG3, IgG4,and IgA1 and IgA2) (reviewed by Coleman et al., 1989). Mab of different isotypes can show different or similar effects (Schlitt et al., 1989; Schlitt et al., 1990; Frenken et al., 1991). Results showed that anti-TCR/CD3 IgG isotypes 2a and 3 cause high proliferation of peripheral blood lymphocytes (PBL) while IgG1 has moderate effects and IgG2b has low activity. Likewise, IgG2a and 3 also cause IFN-γ production while

IgG2b leads to little production (Frenken et al., 1991). These differences, however, can be attributed to cross-linking of certain isotypes of mAb by monocytes, a phenomenon which would not occur in clonal cell lines. Nevertheless, the differential effects caused by different mAb in non-clonal systems may strongly depend on their isotype as well as their epitope specificity (Schlitt et al., 1989; Schlitt et al., 1990).

This project was designed to investigate the possible differential effects caused by various mAb directed towards the TCR/CD3 complex of a panel of different target T cells. The design of the study would allow the determination of whether different functional effects were due to phenotype of the target cells and/or the binding specificity (epitope and/or affinity) of the mAb used in the assays. The effect of mAb isotype would be controlled for by comparing only mAb of the same isotype in any particular cell system.

This study differed from other work in that it compared a panel of mAb to different epitopes on the same T cell targets, as well as using those same mAb on other T cell targets. This allowed for an independent assessment of the contributions of epitope and target cell type to the functional effect of a mAb. Previous studies testing for mAb-induced activation have used either a T cell tumor line (with each researcher using a different phenotype) or normal PBL (Imboden and Stobo, 1985; Spits et al., 1985; Yssel et al., 1987; Schlitt et al., 1989; Takahashi et al., 1989; Schlitt et al., 1990; Frenken et al., 1991). The panel of T cells of various phenotypes was used for this project included a chronic lymphocytic leukemia, two acute lymphocytic leukemias, normal peripheral blood lymphocytes, and normal human thymocytes. The mAb used

were directed against multiple epitopes of the TCR, CD3, and CD5 (used as a negative control) surface proteins. By assaying for T cell activation events in each system, the epitope specificity of a mAb and the type of target cell could be related to a given effect.

The following parameters of activation were studied in this project: proliferation (or growth inhibition), cytokine release (specifically IL2, IFN-γ, and TNF-α), IL2R expression, and intracellular Ca^{++} mobilization. These parameters were chosen because it is well known that full activation of a T cell will entail these four major events. I wanted to study events that represent stages of activation; from the earliest event (intracellular Ca^{++} mobilization) to the latest event (proliferation).

Proliferation or growth inhibition was assayed by measuring the uptake of ^{3}H-thymidine. Another activation event, the secretion of cytokines (IL2, IFN-γ, and TNF-α) was measured by enzyme linked immunosorbent assay (ELISA). Finally, other events such as IL2R expression and Ca^{++} influx were measured by flow cytometry analysis.

Among the anti-TCR and anti-CD3 mAb specific for a given target cell, there were some differences in functional effects seen, and these effects were presumably due to the different epitopes recognized. Likewise, a comparison of target cells binding the same mAb revealed differences due only to the target cell phenotype. These investigations should provide further insights into the effects that mAb can have when used in immunotherapy against T cell leukemias or lymphomas.

CHAPTER II

MATERIALS AND METHODS

CELL POPULATIONS AND CELL LINES

Two human T acute lymphocytic leukemia cell lines were used in this project. SUP-T13 ($CD2^+$, $CD3^+$, $CD4^+$, $CD5^+$, $CD8^-$), a gift from Dr. Stephen Smith, University of Chicago, Chicago, IL, was grown in complete RPMI (RPMI-1640 medium (Irvine Scientific, Santa Ana, CA) supplemented with 10% newborn calf serum, penicillin, and streptomycin). HPB-ALL, the other acute lymphocytic leukemia cell line (also $CD2^+$, $CD3^+$, $CD4^+$, $CD5^+$, $CD8^+$), a gift of Dr. Bradley McIntyre, University of Texas, Houston, TX, was also grown in the same medium as SUP-T13. JE cells were obtained and prepared by Dr. Holden T. Maecker at Stanford University, Stanford, CA, from the spleen of a patient with chronic lymphocytic leukemia ($CD2^+$, $CD3^+$, $CD4^+$, $CD5^+$, $CD8^-$). JE cells were frozen in 45% FCS, 45% RPMI-1640, and 10% dimethyl sulfoxide (Baker, Phillipsburg, NJ), first at -70°C, then in liquid nitrogen (Maecker and Levy, 1989). These cells were thawed and assayed in the same medium as above. The normal thymocytes used in this project were obtained at Stanford University from thymectomized patients. Again these cells were grown in complete RPMI-1640.

MONOCLONAL ANTIBODIES

Mab used in this project were generated in mice against various T cell surface

markers such as the TCR, CD3, and CD5 molecules. mAb generated against the TCR of the HPB-ALL cell line are 4-9E and 5-10C (both IgG2a isotype). These were made by Drs. Shuji Takahashi and Holden T. Maecker at Stanford University, Stanford, CA and have been described elsewhere (Maecker et al., 1987). The mAb LC4 (IgG1) was generated against the TCR of SUP-T13 cells and was also made at Stanford University by Dr. H. T. Maecker (Maecker and Levy, 1989). JE5 and JE7 (both IgG1) are two mAb generated against the TCR of tumor cells from a patient with T chronic lymphocytic leukemia (T-CLL). These too have been made by Dr. H. T. Maecker at Stanford University and have been described elsewhere (Maecker and Levy, 1989). mAb generated against the CD3 molecule are not clone-specific as are those generated against the TCR. Thus an anti-CD3 mAb may bind to CD3 molecules of many different T cell lines. JE6 (IgG1) is an anti-CD3 mAb made by Dr. H. T. Maecker at Stanford University (Takahashi et al., 1989). OKT3 (IgG2a) is another anti-CD3 mAb and is a gift from Dr. Thomas Ellis, Loyola University of Chicago Stritch School of Medicine, Maywood, IL. As an irrelevant control, a mAb generated against CD5 was used. This mAb, Leu-1 (IgG1) was a gift from Dr. Ronald Levy, Stanford University.

PREPARATION OF WHOLE BLOOD

Assays in which normal peripheral blood lymphocytes were used required approximately 9.0 ml of blood to be drawn from a normal human donor into a heparin containing vaccutainer (Becton Dickinson, Rutherford, NJ). Blood was transferred to a 50 ml centrifuge tube and diluted with RPMI-1640 media (Sigma, St. Louis, MO) 1:1.

The sample was underlayed with 13 ml Ficoll-Histopaque (Sigma) and centrifuged at 400 x g for 15 min. The interface of lymphocytes was collected and diluted with media, and washed twice. Lymphocytes were resuspended in media to a concentration of 5 x 10^5 cells/ml.

PURIFICATION OF mAb

Each mAb was purified using affinity chromatography. One half ml of NH_4SO_4-precipitated and dialyzed ascites and 0.5 ml binding buffer (phosphate bufferred saline (PBS), pH 7.4) were added to 250 ul of Sepharose 4B beads conjugated to protein G (Pharmacia, Piscataway, New Jersey). This was rotated overnight at 4°C. The beads were spun at 15,000 x g for 3 minutes in a microfuge followed by removal of the supernatant. One ml PBS (pH 7.4) was added to the beads and then centrifuged again. This washing process was done an additional 4 times to ensure that all unbound Ab (specific and non-specific) and unwanted protein was removed from the beads. At this point, the beads should have contained only the specific mAb. Elution of the mAb was performed by adding 350 ul elution buffer (0.1 M glycine, pH 3.0) and incubation on ice for 2 min. Beads were then centrifuged for 2 min. at 4°C. Supernatant was collected and added to 350 ul neutralizing buffer (0.1 M PO_4, pH 9.0). The elution process was done an additional 5 times. Using a spectrophotometer, each of the 6 fractions was then measured for absorbance at 280 nm while using 350 ul elution buffer plus 350 ul neutralizing buffer as a blank. A rough estimate of concentration was calculated by the following equation:

$$\frac{\text{(Optical Density at 280 nm)(Dilution Factor)}}{1.4_{\text{(a constant for IgG)}}}$$

Those fractions exhibiting the highest estimated concentration of the mAb were dialyzed against 1.0 L PBS overnight at 4°C.

ASSESSMENT OF mAb ACTIVITY

To assess the binding activity of each purified mAb, an enzyme linked immunosorbent assay (ELISA) was used. Ninety-six well microtiter plates (ICN-Flow, Costa Mesa, CA) were coated with 50 ul of 10 ug/ml goat anti-mouse IgG (GAMIg) (Sigma) in phosphate bufferred saline (PBS). The plate was covered with parafilm and incubated for at least 18 hrs. at 4°C. The solution was washed out by dipping the plate in ELISA wash buffer (0.05% Triton X-100 in PBS) followed by gently shaking out the excess. This washing was done at least 3-5 times. A doubling dilution of each mAb was made as well as for the standard, OKT3 (8.0 ug/ml in media). The plate was covered, incubated for 45 min. at room temperature, and washed as before in ELISA wash buffer. A quantity of fifty ul of detector antibody (anti-mouse IgG conjugated to peroxidase (Sigma), 1:1000 dilution in PBS + 2% bovine serum albumin (BSA)) was added to each well. The plate was covered, incubated at room temperature for 45 min., and washed again in ELISA wash buffer. One hundred ul of substrate (0.1 ml 2,2'-Azino-bis-3-ethylbenzthiazoline sulfonic acid (ABTS) (Sigma) at 15 mg/ml, 3.3 ul H_2O_2, 10 ml of 0.036M citrate buffer, pH 4.0) were added to each well. The plate was incubated for 15-25 min. then read on an ELISA microtiter plate reader (Titertek Multiskan Plus

MKII; ICN-Flow) at an optical density of 405 nm. The results were plotted on a line graph and regression lines were generated along the slope of the lines. The horizontal distance measured between the lines and the standard at the midpoint of the dilution curves represented the dilution difference from the standard of 8 ug/ml in medium. The mAb were diluted in medium to achieve a final stock concentration of 8 ug/ml.

Ca^{++} FLUX ANALYSIS

To determine Ca^{++} flux, a dye that binds Ca^{++} combined with the technique of flow cytometry was used. Ten million cells were resuspended in 1.0 ml 5% BCS-HBSS (Hank's bufferred salt solution with 5% bovine calf serum). Fifty ug of FLUO-3/AM (Molecular Probes Inc., Eugene, OR) were dissolved in 43.8 ul DMSO. Twenty ul of the FLUO-3/AM and 5.0 ul of Pluronic F127 detergent were added to the cells, which were then rotated at room temperature for 1 hr. The cells were washed three times with 5% BCS-HBSS. Two-hundred ul of each mAb being tested were added to a 5 ml Falcon tube. Immediately before analysis on the flow cytometer, 200 ul of the dye-loaded cells were added to each mAb-containing tube. Shifts in fluorescence were monitored for 180 sec. for each sample on the flow cytometer using the Chronys program (Becton Dickinson, Mountain View, CA). To determine the effects of cross-linking with goat-anti mouse Ig, samples were washed with 5% BCS-HBSS after incubation with the first mAb, then resuspended with 200 ul BCS-HBSS. Ten ul of stock goat anti-mouse Ig (1.0 mg/ml, Sigma) were added to each sample. Shifts in fluorescence were again analyzed for 180 sec. on the flow cytometer.

IL2 RECEPTOR EXPRESSION

IL2 receptor expression, an indication of T cell activation, was quantified using fluorescence activated cell sorting (FACS or flow cytometry). Cells were incubated at 37°C in the presence of the mAb being tested (1 ug/ml of cells) for 3 days. Approximately 10^6 cells were then placed in a 5.0 ml test tube and centrifuged at 250 x g for 5 min.. The supernatant was removed and the pellets were resuspended in 100 ul of FITC conjugated anti-IL2R antibody (T Cell Sciences, Cambridge, MA) (1 ug/10^6 cells). Samples were incubated on ice for 30 min.. One wash in cold PBS + 2% BSA + 0.1% NaN_3 was done to ensure all unbound Ab was removed. The pellets were resuspended in 0.5-1.0 ml FACS Fixative (2% paraformaldehyde in PBS) and stored at 4°C until analysis. Flow cytometry analysis was done at Loyola University of Chicago Stritch School of Medicine, Maywood, IL using a FACStar Plus Cell Sorter (Becton Dickinson, Mountain View, CA). Statistical analysis was achieved using the Lysis program (Becton Dickinson) which utilizes the Kolmogorov-Smirnov test for the analysis of histograms (Young, 1977). Any comparison between mAb exhibiting a 99% probability of difference was deemed significant, while those comparisons exhibiting less than 90% probability of difference is valued as insignificant.

CYTOKINE SECRETION (ELISA)

IL2, IFN-γ, and TNF-α secretion, as indicators of T cell activation, were assayed using an enzyme linked immunosorbent assay. The supernatants from the cells incubated with mAb (as done in the proliferation assays) were used for analysis. The assay for IL2

had been commercially prepared in the form of a kit (Genzyme Corp., Cambridge, MA). The instructions received with the kit were followed. IFN-γ and TNF-α standards and mAb were a generous gift from Dr. Anita Chong, Rush Medical College, Chicago, IL. For the IFN-γ ELISA, each well of a 96 well flat-bottom plate (ICN-Flow) was coated with 50 ul anti-IFN-γ mAb (Olympus, Lake Success, NY) (7.5 ul of 1 mg/ml stock diluted in 5.0 ml of 1:1 PBS/carbonate buffer (0.795 g Na_2CO_3 + 1.465 g $NaHCO_3$ + 0.1 g NaN_3 + 450 ml dH_2O). Plates were incubated overnight at 4°C. Plates were washed twice in ELISA wash buffer (5 L PBS + 2.5 ml TWEEN 20 (Sigma)) and blotted dry on paper towels. IFN-γ standards were made by adding 20 ul of IFNγ (natural IFN-γ from Boehringer Mannheim, Indianapolis, IN) at a concentration of 10^6 U/ml (20 U/ng) to 5.0 ml PBS/TWEEN with bovine serum albumin (BSA) (2% w/v). This 200 ng/ml stock was serially diluted on the plate with complete RPMI to create a standard curve (20.0, 10.0, 5.0, 2.5, 1.25, 0.63, 0.31, 0 ng/ml). Fifty ul of each sample was added to the wells. Plates were incubated at room temperature for 90 min.. Plates were washed twice in wash buffer and blotted dry . Fifty ul of rabbit anti-human IFN-γ (prepared by adding 5.0 ml of PBS/TWEEN/BSA and 30 ul of rabbit anti-IFN-γ (anti-serum prepared by Phil Scuderi, Miles Laboratories, Brekeley, CA)) were added and the plate incubated for 1.5 hrs. at room temperature. The plate was then washed twice with wash buffer, blotted dry, and 50 ul goat anti-rabbit Ig (10 ml PBS/TWEEN + 1-1.5 ul goat anti-rabbit peroxidase (Boehringer Mannheim) were added to each well. The plate was incubated for 1.5 hrs. at room temperature and washed twice with wash buffer and blotted dry. One hundred ul ABTS substrate were added to each well and the

absorbance was read on an ELISA plate reader at 405 nm.

The TNF-α ELISA was done in a similar manner. Fifty ul of coat anti-TNF-α mAb were added to each well of a 96 well plate and incubated at 4°C overnight. Plates were washed twice in wash buffer and blotted dry on paper towels. TNF-α standards were made by diluting a 0.1 ug/ml stock to 20 ng/ml. Serial dilutions of this dilute stock were made in complete RPMI in order to create a standard curve (20, 10, 5, 2.5, 1.25, 0.63, 0.31, 0 ng/ml). Fifty ul of each sample was added to the wells. The plates were incubated for 90 min at 37°C. Plates were washed twice with wash buffer and blotted dry. Fifty ul of goat-anti-human-TNF-α (polyclonal anti-serum, Endogen) were added to each well and the plates were incubated for 1.5 hrs. The plates were then washed and blotted dry. Fifty ul of swine anti-goat peroxidase (Boehringer Mannheim) (1.5 ul stock + 10 ml PBS/TWEEN/BSA) was added to each well and incubated for 1.5 hrs. Plates were washed twice and blotted dry. One hundred ul of substrate was added to each well and the absorbance was read on an ELISA plate reader at 405 nm.

CELL PROLIFERATION ASSAYS

The proliferation of cells was assayed using a ^{3}H-thymidine assay. As cells divide, the available ^{3}H-thymidine is incorporated into the newly synthesized DNA. Thus the amount of incorporated radiation in a sample is proportional to the number of dividing cells present. Cells were diluted in medium to a concentration of 5.0×10^{5} cells/ml. For those cells that were tested with IL2 as the source for a second signal (PBL's and thymocytes), the volume was split in half and IL2 (Cetus Corp., Emeryville,

CA) (100 U of IL2/ml of cells) was added to one half. On a 96 well flat-bottom microtiter plate, half of the wells were plated with 175 ul of the IL2-containing cells, while the other half contained cells grown without IL2. To each well, 25 ul of the appropriate mAb (8.0 ug/ml in media) was added (final concentration in each well was 1.0 ug/ml). For those cells in which cross-linking of the test mAb was used as the source of a second signal instead of IL2, 12.5 ul goat anti-mouse IgG (Sigma) (8.0 ul/ml in media) was added to each well (final concentration in each well was 0.5 ug/ml). Plates were incubated for 3 days at 37°C. 15 ul of ^{3}H-thymidine (1 uCi/20 ul; NEN, Boston, Ma.) was then added to each well and the plate incubated for an additional 16 hrs. at 37°C. Cells were harvested onto filter strips (Brandel, Gathersburg, Md.) using a cell harvester (Brandel MH-12). Each filter disc was placed into a scintillation vial containing 5.0 ml scintillation cocktail (Packard Instruments, Downers Grove, IL) and counted using a gamma counter (Packard Tircarb 300).

STATISTICAL ANALYSIS

Means of triplicate or quadruplicate samples from cytokine or proliferation assays were compared using an analysis of variance. Where significant differences were found, a post-hoc test was performed by the method of Tukey or Newman-Keuls (Zar, 1984) to determine which pairs of means were significantly different. All statistics, except those used in IL2 receptor assays, were performed using Systat 4.1 statistical software at a 0.05 level of significance.

CHAPTER III

RESULTS

The following results were obtained from experiments chosen to assay for the presence or absence of a particular activation event. The earliest activation event tested was the release of Ca^{++} from intracellular Ca^{++} stores. The release of Ca^{++} is a critical event in the activation pathway of T cells. Therefore, any release of Ca^{++} after the addition of a mAb to the cells, would in turn, indicate triggering of this pathway. IL2 receptor expression was the second earliest event assayed. The expression of the high affinity receptor is a good way to test for any activation since a T cell will only express the high affinity form during activation. The secretion of certain cytokines, such as IFN-γ, TNF-α, and IL2, is also indicative of T cell activation. These cytokines play specific roles in further activating other T cells. Finally, clonal expansion of T cells was the last event analyzed. T cells may, depending upon their developmental stage, proliferate in response to triggering of the activation pathway.

To test the possible effects that may be caused by binding of mAb to different phenotypes, I chose to study mAb effects on the following cell types: normal peripheral blood lymphocytes (PBL), normal human thymocytes, two acute lymphocytic leukemias (ALL), and one chronic lymphocytic leukemia (CLL). Normal PBL were chosen as a baseline for comparison. Since none of the anti-TCR mAb used in this project were

specific for the PBL, any detectable effects seen by an anti-TCR mAb would be an indication of a non-specific effect. Thymocytes were chosen as a good candidate for studying mAb on immature T cells because they represent T cells at various developmental stages. However, the main goal of this project was to study mAb effects on leukemic cells. Therefore, two ALL cell lines were chosen, HPB-ALL and SUP-T13, as well as a CLL tumor, JE. Table 1 shows the phenotypes of the cells used as well as the mAb tested against each target cell. The T cell phenotypes chosen gave a somewhat broad spectrum to analyze. The choices also dictated which mAb were to be used in our panel. The anti-TCR mAb in the panel were not specific for the PBL used in this project nor were they specific for the thymocytes used. For the three leukemic cell types, a pair of anti-TCR mAb was chosen for each type. The anti-TCR mAb specific for HPB-ALL were 4-9E and 5-10C. Those anti-TCR mAb specific for SUP-T13 were LC4 and LC11. Anti-TCR mAb specific for JE cells were JE5 and JE7. OKT3 and JE6 were the two mAb binding to the invariant chains of CD3 in all cell types. Cross-blocking analysis of mAb (Table 2) showed OKT3 completely blocked JE6, 4-9E did not block 5-10C, and LC4 completely blocked LC11. Previous studies have shown JE5 to completely block JE7 (Maecker, 1989).

INTRACELLULAR Ca^{++} FLUX

Induction of intracellular Ca^{++} flux by mAb was the earliest activation event assayed. As stated in "Materials and Methods", cells were labelled with a Ca^{++} sensitive dye. Mab were then added prior to analysis on the flow cytometer. Any shifts in

fluorescence, indicating intracellular release of Ca^{++}, were then monitored for a time window of 180 sec. Figure 1 is a representative graph demonstrating such a shift in fluorescence in response to anti-CD3 mAb in SUP-T13 cells. Results of Ca^{++} flux experiments (Table 3) are depicted using a +/- system; those samples which exhibited a shift in fluorescence are represented with a "+" and those samples which exhibited no shift in fluorescence are represented with a "-".

In the PBL system, Ca^{++} mobilization was induced by both anti-CD3 mAb (OKT3 and JE6) in the presence and absence of IL2. On the other hand, none of the anti-TCR mAb induced any detectable Ca^{++} mobilization even in the presence of an additional stimulus, IL2. This data confirms our use of PBL as a baseline control. Their responsiveness to both anti-CD3 mAb was expected since the mAb binds to an invariant cell surface protein. The anti-TCR mAb may bind to, at the most, a very small population of PBL, so small that any Ca^{++} flux caused by these mAb would not be detected. Therefore the anti-TCR mAb are considered to be negative inducers of Ca^{++} mobilization in this system.

In assays using thymocytes, Ca^{++} flux could be induced using both of the anti-CD3 mAb, but only in the presence of IL2. These mAb used with cells not preincubated with IL2 caused no flux. None of the anti-TCR mAb, in the presence or absence of IL2, caused any detectable Ca^{++} mobilization. These results for both PBL and thymocytes treated with anti-TCR mAb were expected since none of the anti-TCR mAb should bind to these cells, or at the most, they would bind to a very small sub-population. Therefore, any effects caused by these anti-TCR mAb should be too small

to be detected. These experiments demonstrated that the anti-TCR mAb did not contain contaminants that might cause non-specific Ca^{++} flux. This was important to know before using these mAb in the cell lines for which they were specific.

In assaying HPB-ALL cells for Ca^{++} mobilization, Ca^{++} flux could not be induced with any of the mAb, either in the presence or absence of the cross-linking agent, goat-anti-mouse IgG (GAMIg). These results were confirmed by others who were not able to induce Ca^{++} mobilization with mAb to CD3 in these cells (Nel et al., 1991). HPB-ALL cells were tested with the Ca^{++} ionophore, ionomycin, which causes direct mobilization of Ca^{++} from intracellular Ca^{++} stores. There was a strong flux of Ca^{++} using ionomycin, indicating that these cells were properly loaded with dye, and that they have the ability to flux Ca^{++}. However, there appears to be an uncoupling of the Ca^{++} flux response from the TCR/CD3 stimulation pathway in these cells.

In the other ALL cell line, SUP-T13, the induction of Ca^{++} flux could be seen with both anti-CD3 mAb (OKT3 and JE6) and both anti-TCR mAb (LC4 and LC11). There appeared to be no differences in the magnitude of Ca^{++} flux induced between the anti-CD3 mAb and anti-TCR mAb. When these cells were tested with the mAb and the cross-linking agent, GAMIg, no additional flux could be induced. These results were in marked contrast to those found with the other ALL cell line, HPB-ALL.

In the only chronic lymphocytic leukemia cells that were tested, JE cells, Ca^{++} flux could not be induced with any of the mAb in the presence or absence of IL2. Furthermore, Ca^{++} mobilization could not be induced with ionomycin, indicating that either these cells were not able to flux Ca^{++}, or, most likely, these cells were not

properly loaded with the dye.

IL2 RECEPTOR EXPRESSION

The next earliest activation event assayed was surface expression of the IL2R. During T cell activation, a 55 kD protein associates with a constitutively expressed 70 kD protein, thus forming a high affinity IL2R. As described in "Materials and Methods", a fluorescently conjugated mAb to the 55 kD portion of the high affinity IL2R was used in flow cytometric analysis of IL2R expression. In all assays, the percent shift in fluorescence, which represents cells positive for IL2R, is divided by that seen with the negative control mAb, LEU1 (anti-CD5). Thus all results are indicated as a percent of control, in which the control represents 100%.

With normal PBL's (Fig. 2a), OKT3 in the presence of IL2 was the only mAb that induced an increase in the expression of the IL2R (189%) and this was found to have a 99% probability of difference from the negative control.

With thymocytes (Fig. 2b), again only OKT3, with and without IL2 present, showed a slight increase in IL2R expression (135% and 144%, respectively), however, as compared to LEU1, there was a less than 90% probability of difference. As before, these data indicate that our anti-TCR mAb do not cause any non-specific IL2R induction, as they would not be expected to bind to a significant population of thymocytes or PBL.

In assaying HPB-ALL cells (Fig. 2c), there were increases of IL2R expression with OKT3 and 4-9E with GAMIg present (659% and 238%, respectively). In comparing these two treatments with each other, there was a 99% probability of

difference. Comparing OKT3+GAMIg and 5-10C+GAMIg also yielded a 99% probability of difference. IL2R expression induced with other mAb, both in the presence and absence of GAMIg, was significantly less than that with OKT3+GAMIg and 4-9E+GAMIg. In treatments without GAMIg, there was less than a 90% probability of difference among the mAb. Thus, in this cell line, differences in IL2R expression could be seen both among and between anti-TCR and anti-CD3 mAb. In addition, cross-linking with GAMIg was necessary to induce significant IL2R expression.

IL2R analysis with SUP-T13 (Fig. 2d) showed extremely high increases of expression of IL2R with cells incubated with either of the anti-TCR mAb (LC4 and LC11) in the presence or absence of GAMIg. When comparing OKT3+GAMIg and JE6 with and without GAMIg to both LC4 and LC11 treatments, there was a 99% probability of difference. However, when comparing OKT3 in the absence of a cross-linker to either LC4 or LC11, the difference is marginal. Thus, mAb stimulation of SUP-T13 cells resulted in a very different profile of IL2R induction from that seen in HPB-ALL cells. In contrast to HPB-ALL cells, anti-TCR mAb caused the most significant Ca^{++} fluxes in SUP-T13, and no significant differences were noted among the two anti-TCR or anti-CD3 mAb.

Analysis of JE cells for IL2R (Fig. 2e) showed no significant increase in expression of this receptor using either anti-TCR or anti-CD3 mAb. The highest recorded increase was with OKT3+IL2 (120%). It is possible that JE cells exhibited a high constitutive level of IL2R expression, since they were grown in the presence of IL2. Alternately, they may not be readily activated with anti-TCR or anti-CD3 mAb.

CYTOKINE SECRETION

Secretion of certain cytokines as an indication of T cell activation was assayed using ELISA's. Comparison of absorbance readings to a standard curve allowed for conversion of all data to ng/ml of cytokine secreted. All data for IFN-γ secretion is shown in Table 4. In assays using PBL's, it was seen that both anti-CD3 mAb (OKT3 and JE6) in the presence of IL2 gave significantly higher levels of secretion as compared to other mAb with or without IL2 ($0.025 > P > .01$).

With the thymocytes, all mAb induced IFN-γ secretion at nearly the same level as the negative control, LEU1. These levels were on the range of approximately 0.12 to 0.75 ng/ml. Furthermore, there did not seem to be any significant differences in the ability of any of the mAb to secrete IFN-γ in either the presence or absence of IL2. Thus, as in previous experiments, thymocytes did not respond to anti-CD3 mAb stimulation nearly as strongly as did PBL. The data for both PBL and thymocytes again provide a baseline to which IFN-γ levels obtained with these mAb in the other cell lines can be compared.

Both anti-TCR and anti-CD3 mAb were not able to induce high levels of IFN-γ in the HPB-ALL cell line. Furthermore, mAb treatment with the cross-linking agent, GAMIg, also did not induce high levels of secretion. The concentrations ranged from 0.10 to 0.25 ng/ml. This was of similar magnitude to the background levels seen with thymocytes.

On the other hand, in SUP-T13, the other ALL, both anti-CD3 mAb (OKT3+GAMIg, JE6+GAMIg, and JE6 without GAMIg) were able to induce

significantly higher amounts of IFN-γ secretion as compared to the negative control, LEU1 ($P<0.001$). In comparing both anti-TCR mAb (LC4 and LC11), in the presence and absence of GAMIg, to LEU1, the anti-TCR mAb induced significantly higher amounts of IFN-γ secretion ($P<0.001$). The absorbance readings corresponding to these levels were found to be greater than the absorbance reading of the highest standard titer (>20 ng/ml). Anti-CD3 mAb treatment of SUP-T13 was not as potent in inducing IFN-γ secretion as the anti-TCR mAb. Without GAMIg, both anti-TCR mAb induced significantly higher amounts of IFN-γ than did anti-CD3 mAb ($P<0.001$). Although the levels achieved with anti-CD3 mAb stimulation were significant, they did not exceed the concentration of the highest standard titer, as did the anti-TCR mAb.

JE cells were found to be less inducible by any of the mAb. No detectable amounts of IFN-γ were obtained with any of the mAb in the presence of IL2 with the exception of the negative control; LEU1 yielded a detectable amount in only one out of the three wells. This is in accordance with the lack of Ca^{++} flux and IL2R expression seen in these cells.

TNF-α secretion (Table 5) was assayed in a similar manner as IFN-γ. In assays using PBL, there were no statistically significant differences observed among the mAb. Values ranged from 4.35 to 2.15 ng/ml. Unlike assays for IFN-γ, the anti-CD3 mAb did not induce high levels of TNF-α secretion.

In thymocytes, there were also no statistically significant differences seen among the mAb either in the presence or absence of IL2. Values ranged from 6.88 to 3.63 ng/ml. Although levels are higher than the levels found in IFN-γ assays, there was no

difference in the magnitude of secretion induced by any of the mAb. This is in accordance with the findings of IFN-γ experiments in which there was also no difference in the magnitude of IFN-γ induced secretion by any of the mAb.

HPB-ALL cells exhibited high amounts of TNF-α secretion which was induced by most of the mAb only in the presence of the cross-linking agent, GAMIg. All mAb, with the exception of JE6, were able to induce secretion of this cytokine to levels greater than 20 ng/ml (the highest titer of the standard). Using the raw absorbance values to generate statistical data, significant differences were found between cells treated with OKT3 versus cells treated with anti-TCR mAb, 4-9E or 5-10C, in the presence of GAMIg (OKT3 vs. 4-9E, $0.005 > P > 0.001$; OKT3 vs. 5-10C, $P < 0.001$). JE6, on the other hand, induced lower amounts of secretion than either of the anti-TCR mAb. These differences were deemed statistically significant via post-hoc student Newman-Keuls test. Likewise, statistically significant differences were found in the presence of GAMIg between 4-9E and 5-10C; cells treated with 4-9E had higher absorbance readings than cells treated with 5-10C, yet both absorbances were indicative of concentrations over 20 ng/ml. Without GAMIg, TNF-α secretion was relatively low (ranging from 3.75 to 4.50 ng/ml) and no statistical differences were found between any of the mAb. Thus, as with the IL2R induction data, significant differences both among and between anti-TCR and anti-CD3 mAb could be seen in HPB-ALL cells, and cross-linking with GAMIg was again important.

Analysis of TNF-α secretion in SUP-T13 cells showed a reverse pattern as compared to HPB-ALL cells. Relatively high amounts of cytokine secretion were seen

with all mAb in the absence, but not in the presence, of the cross-linking agent. Among the mAb with GAMIg present, there were no statistically significant differences found between them, but differences were found among the mAb when GAMIg was absent from the culture medium ($P < 0.001$). Statistical analysis showed OKT3 induced a higher level of secretion of TNF-α than either of the anti-TCR mAb ($P < 0.001$). JE6, on the other hand, induced lower amounts than LC11 and comparable levels to LC4. Differences were also seen between the two anti-TCR mAb with LC11 inducing higher levels of secretion than LC4. Thus, there are also significant differences among and between anti-TCR and anti-CD3 mAb in SUP-T13 cells, by the pattern and dependance on cross-linking is different from that seen with the other ALL cell line, HPB-ALL.

With JE cells, all levels of secretion were less than 20 ng/ml. Statistically significant differences were found between JE6 and both anti-TCR mAb (JE5 and JE7) in the presence of IL2; both JE5 and JE7 induced higher levels of secretion than JE6 ($0.025 > P > 0.01$ and $0.01 > P > 0.005$). OKT3 did not secrete significantly higher levels of TNF-α than JE5 or JE7. This is the first significant activation event to be assayed in JE cells, although it is of low magnitude. It does demonstrate, however, that activation differences between anti-TCR and anti-CD3 mAb can be seen in these (T-CLL) cells.

IL2 secretion was assayed using a commercial ELISA kit (Genzyme) with a sensitivity as accurate as 150 pg/ml. None of the cells assayed showed any detectable amounts of IL2 secretion. Whether or not the mAb were able to induce secretion is not known. As a positive control, PBL's were stimulated with phorbol myristate acetate (PMA). This incubation time was identical to mAb incubation times (3 days). The

presence of IL2 in the cell culture medium of PMA treated cells was not detected by this kit even though IL2 standards supplied with the kit did show positive results, thereby confirming the reliability of this ELISA.

PROLIFERATION

Proliferation of T cells was the latest activation event assayed. As described earlier, cells were incubated for three days with mAb, at which time, ^{3}H-thymidine was added for sixteen hours. Incorporation of ^{3}H-thymidine into DNA was counted on a scintillation counter and the values were then plotted as a percent of the control (LEU1).

With normal PBL's (Fig. 3a), OKT3 in the presence of IL2 induced a high amount of proliferation (364%) and this amount was found to be significantly different from all other mAb ($P<0.001$), with the exception of JE6. JE6 also induced high amounts of proliferation (311%) and like OKT3, was found to be significantly different from all the other anti-TCR mAb ($P<0.001$). In the absence of IL2, OKT3 again induced a high amount of proliferation (450%). This amount was found to be significantly different from all anti-TCR mAb as well as from JE6 ($P<0.001$). JE6 without IL2 did not induce proliferation and may have even inhibited expansion of these cells. This difference may be due to isotype differences between OKT3 and JE6.

In the ^{3}H-thymidine assay of normal thymocytes (Fig. 3b), OKT3 in the presence of IL2 seemed to have induced proliferation (381%), however due to the high variation among the replicates, this value was not statistically different from any of the other mAb. All other mAb, both in the presence and absence of IL2, showed no statistical difference

from each other. This, again, demonstrated a lack of non-specific stimulation by the mAb, and provided a baseline for comparison to the other cell lines.

Analysis of HPB-ALL (Fig. 3c) for proliferation showed that none of the mAb caused increased proliferation. In the presence of a cross-linking agent, levels ranged from 84% to 99.6% of the control. Likewise, in the absence of the cross-linking agent, levels ranged from 92% to 110% of the control. None of the mAb were found to be statistically different from each other. Thus, although HPB-ALL cells can be induced, with certain mAb, to express IL2R and to secrete IFN-γ and TNF-α, they can not be induced to flux Ca^{++} or to alter their proliferation rate with such mAb stimulation.

With the other ALL, SUP-T13 (Fig. 3d), ^{3}H-thymidine assays showed an inhibition of proliferation using both anti-TCR mAb in the presence and absence of GAMIg. Neither of the anti-CD3 mAb had any significant effect on proliferation. Even though a large difference in proliferation is seen between anti-CD3 and anti-TCR mAb, a post hoc Newman-Keuls test showed this difference not to be significant at an alpha level of 0.05. This can be attributed to the high amount of variation found in the replicates of each mAb sample.

To determine whether the difference between anti-TCR and anti-CD3 mAb in SUP-T13 cells was biologically significant, phase contrast microscopy was used to visually determine differences between cells treated with various mAb. Figure 4a shows cells pretreated with OKT3; an even "lawn" of cells was seen indicating that cells are healthy and alive. Similar results are shown in figures 4b and c, in which cells were treated with JE6 and LEU1, respectively. In figure 4d and e, cells treated with LC4 and

LC11, respectively, appear jagged and unhealthy as well as having much cellular debris in the surrounding media. This appearance is indicative of dying or dead cells. Figures 4f, g, and h are of cells treated with GAMIg and OKT3, JE6, and LEU1, respectively. There still appeared to be healthy live cells with more aggregation which was most likely due to the cross-linking nature of the GAMIg. Figures 4i and j are of cells treated with GAMIg and LC4 and LC11, respectively. As in figures 4d and e, cells in 4i and j appeared to be dying or dead. Work by previous researchers found similar results with LC4 treatment on SUP-T13 cells. They concluded that the killing proceeds by apoptosis (programmed cell death) (Takahashi et al., 1989). Thus, in SUP-T13 cells, a very dramatic difference can be seen in the outcome of activation by anti-CD3 versus anti-TCR mAb. While all mAb induce Ca^{++} fluxes, differences can be seen in the magnitude of IL2R induction and IFN-γ secretion. Yet, the most dramatic difference is that anti-TCR mAb induce apoptosis, while anti-CD3 mAb do not affect proliferation.

^{3}H-thymidine analysis of JE cells (Fig. 3e) showed a large increase in proliferation with OKT3+IL2 (409%), however, this value was not statistically different (at an alpha level of 0.05) from any other mAb, due to the high variation found within the OKT3 replicates. Yet results from an analysis of variance are suggestive of a significant difference being present between OKT3 and JE5 as well as between OKT3 and JE7 ($0.10 > P > 0.05$). Cells treated with JE5 and JE7 exhibited a slight inhibition of proliferation (68% and 57%, respectively) when compared to LEU1 (100%), yet, this was not deemed statistically significant. In the absence of IL2, JE5 and JE7 appear to inhibit proliferation more severely (36.2% and 28.1%, respectively) when compared to

the negative control. But again, due to the high variance among the replicates, the difference between JE5 and JE7 to LEU1, was not statistically significant at an alpha level of 0.05. If this apparent growth inhibition is biologically significant, it would still remain to be demonstrated whether this is a cytotoxic or merely a cytostatic effect; if it is cytotoxic, it is not known whether it proceeds via apoptosis, as in SUP-T13 cells. However, these results suggest that anti-TCR mAb can have different effects from anti-CD3 mAb in a ALL (SUP-T13) and possibly in a CLL (JE).

Table 1. Cell phenotypes and mAb.[1]

CELL	PHENOTYPE	CD3	TCR	CD5
PBL	normal resting T cells $TCR/CD3^+$, $CD4^+$ $CD8^-$ $TCR/CD3^+$, $CD4^-$ $CD8^+$	OKT3, JE6	4-9E, 510C, LC4 LC11, JE5, JE7	LEU1
THYMOCYTES	immature T cells $TCR/CD3^-$, $CD4^-$ $CD8^-$ $TCR/CD3^{lo}$, $CD4^+$ $CD8^+$ $TCR/CD3^{hi}$, $CD4^+$ $CD8^-$ $TCR/CD3^{hi}$, $CD4^-$ $CD8^+$	OKT3, JE6	4-9E, 510C, LC4 LC11, JE5, JE7	LEU1
HPB-ALL	acute lymphocytic leukemia $TCR/CD3^+$, $CD4^+$ $CD8^+$	OKT3, JE6	4-9E, 510C	LEU1
SUP-T13	acute lymphocytic leukemia $TCR/CD3^+$, $CD4^+$ $CD8^-$	OKT3, JE6	LC4, LC11	LEU1
JE	chronic lymphocytic leukemia $TCR/CD3^+$, $CD4^+$ $CD8^-$	OKT3, JE6	JE5, JE7	LEU1

[1]Phenotype of cells used and the mAb tested with each.

Table 2. Blocking Analysis and Isotypes of mAb.[1]

Ab1 (isotype)	Ab2 (isotype)	percent blocking
OKT3 (IgG_{2a})	JE6 (IgG_1)	95%
4-9E (IgG_{2a})	510C (IgG_{2a})	3.7%
LC4 (IgG_1)	LC11 (IgG_1)	98%
JE5 (IgG_1)	JE7 (IgG_1)	100%[2]
all anti-CD3 and anti-TCR mAb	LEU1 (IgG_1)	0%

[1]Blocking analysis of mAb. 2.5×10^5 Cells were incubated with an unconjugated mAb (1 ug/ml of cells) (Ab1) for 20 min., then challenged with a FITC-conjugated second mAb (Ab2) for 20 min. Samples were analyzed by flow cytometry.
[2](Maecker, 1989).

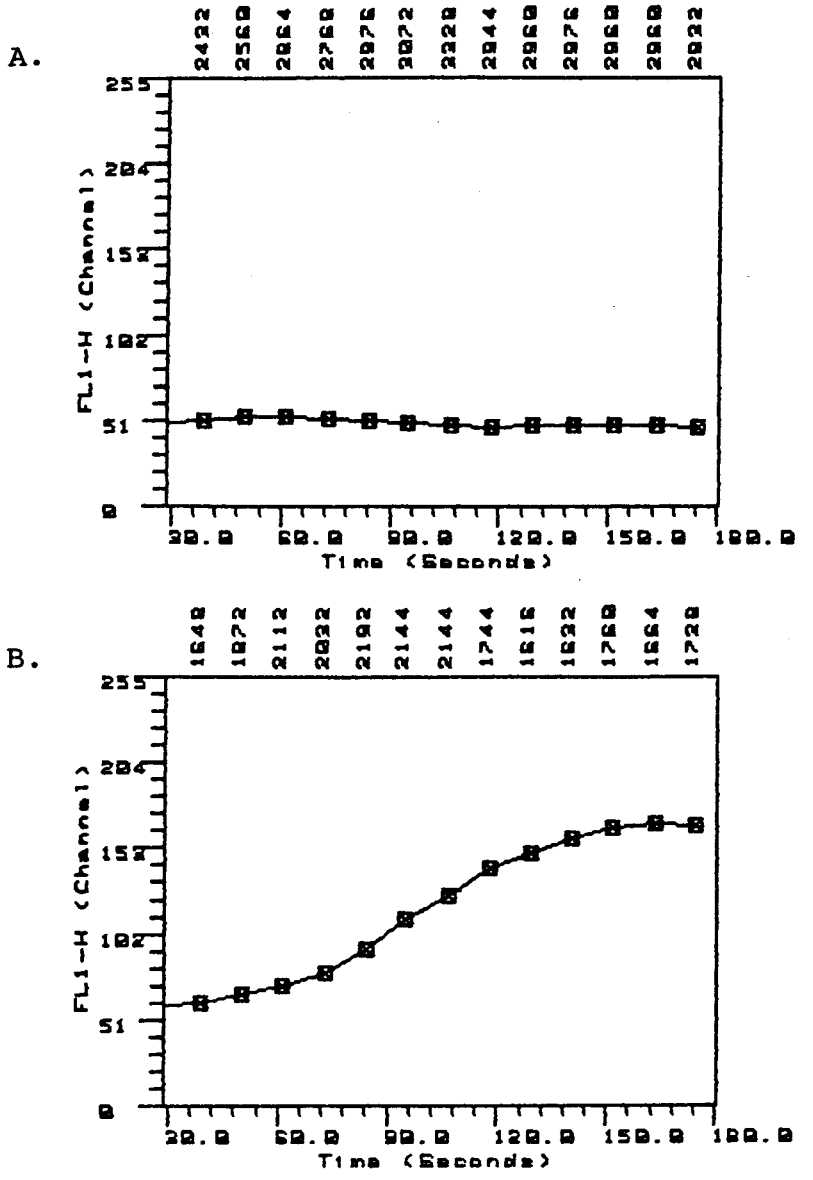


Figure 1. Analysis of Ca^{++} mobilization using FLUO-3 AM and flow cytometry (a time gate of 30.0-180.0 sec. is displayed). **a.** SUP-T13 cells with no mAb treatment (negative control), **b.** 1.0×10^6 SUP-T13 cells incubated with 1.6 ug of JE6.

Table 3. Ca^{++} Mobilization.[1]

CELL	OKT3		JE6		LEU1		49E		510C		LC4		LC11		JE5		JE7		IONO-MYCIN
	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	
PBL	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
THYMO-CYTES	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	ND[2]
HPB-ALL	-	-	-	-	-	-	-	-	-	-									+
SUP-T13	*[3]	+	*	+	-	-					*	+	*	+					+
JE	nc[4]		nc		nc										nc		nc		-

[1]Intracellular Ca^{++} flux using a Ca^{++} sensitive dye and flow cytometry. Cells were incubated with 1.6 ug of mAb then analyzed for 180 sec. The "+" columns indicate analysis in the presence of GAMIg (for HPB-ALL and SUP-T13), or analysis after overnight incubation with 100 U/ml IL2 (for PBL, thymocytes, and JE). The "-" columns indicates analysis in the absence of cross-linking (GAMIg) or a second signal (IL2). Samples exhibiting a shift in fluorescence (indicating Ca^{++} mobilization) are denoted with a "+" while those lacking shifts in fluorescence are denoted with a "-".

[2]ND=not determined.

[3]"*" denotes samples in which no additional flux was noted upon cross-linking with GAM.

[4]"nc" denotes samples in which results are not conclusive since no flux was observed with ionomycin.

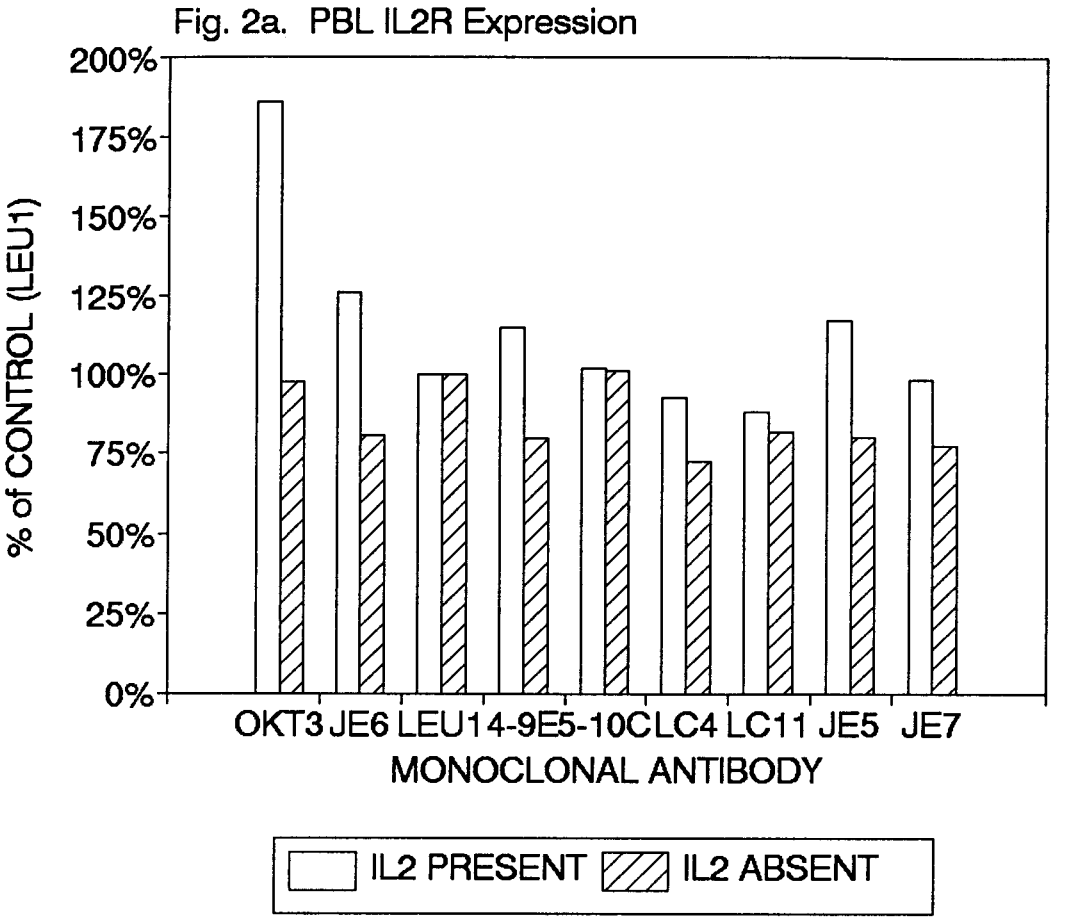


Figure 2. IL2 receptor expression. Cells were incubated with mAb in the presence (open bars) or absence (hatched bars) of IL2 or GAMIg. Results are shown as a percentage of the negative control (LEU1). **a.** PBL, **b.** thymocytes, **c.** HPB-ALL, **d.** SUP-T13, **e.** JE.

Fig. 2b. Thymocyte IL2R Expression

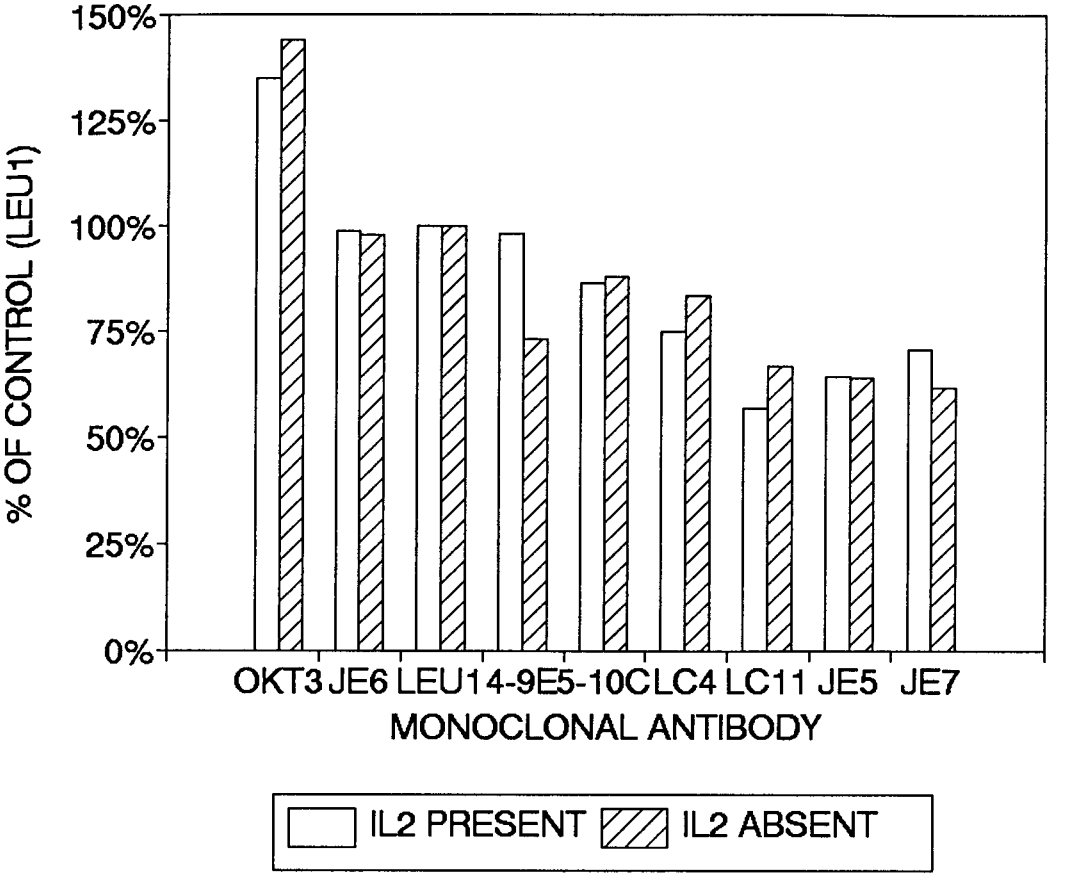


Fig. 2c. HPB-ALL IL2R Expression

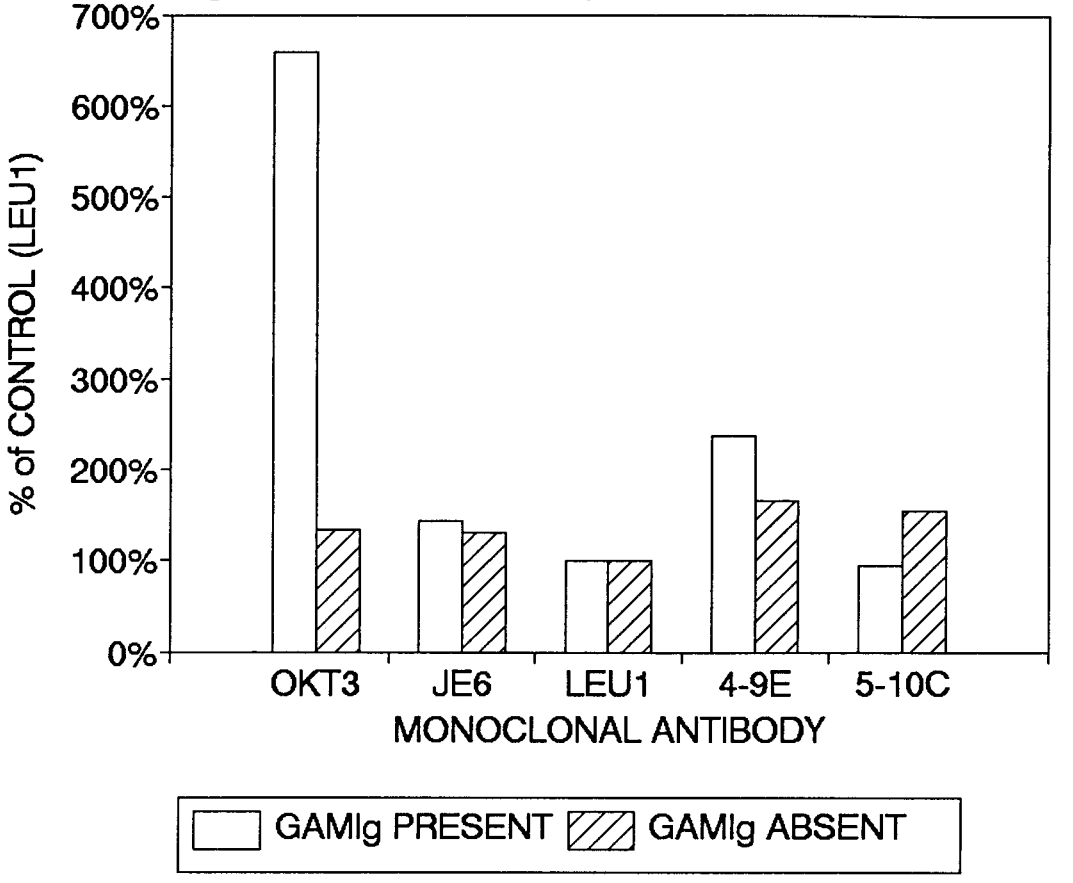


Fig. 2d. SUP-T13 IL2R Expression

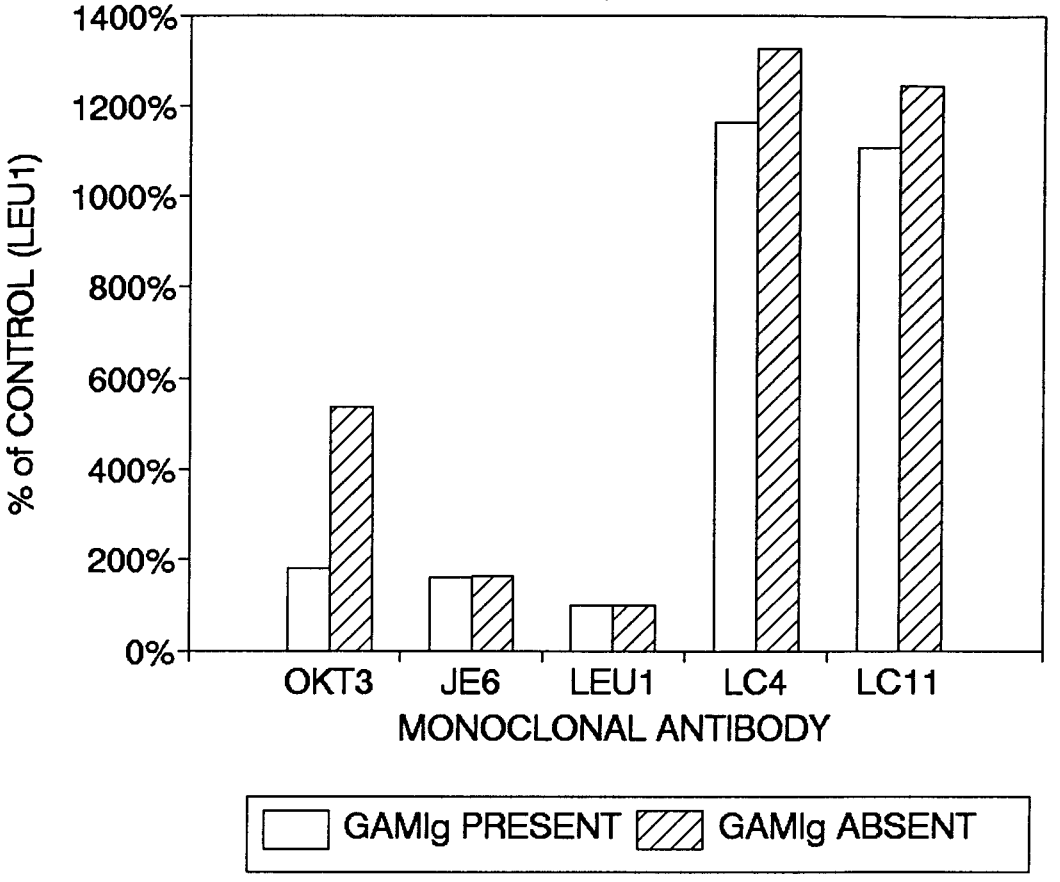


Fig. 2e. JE IL2R Expression

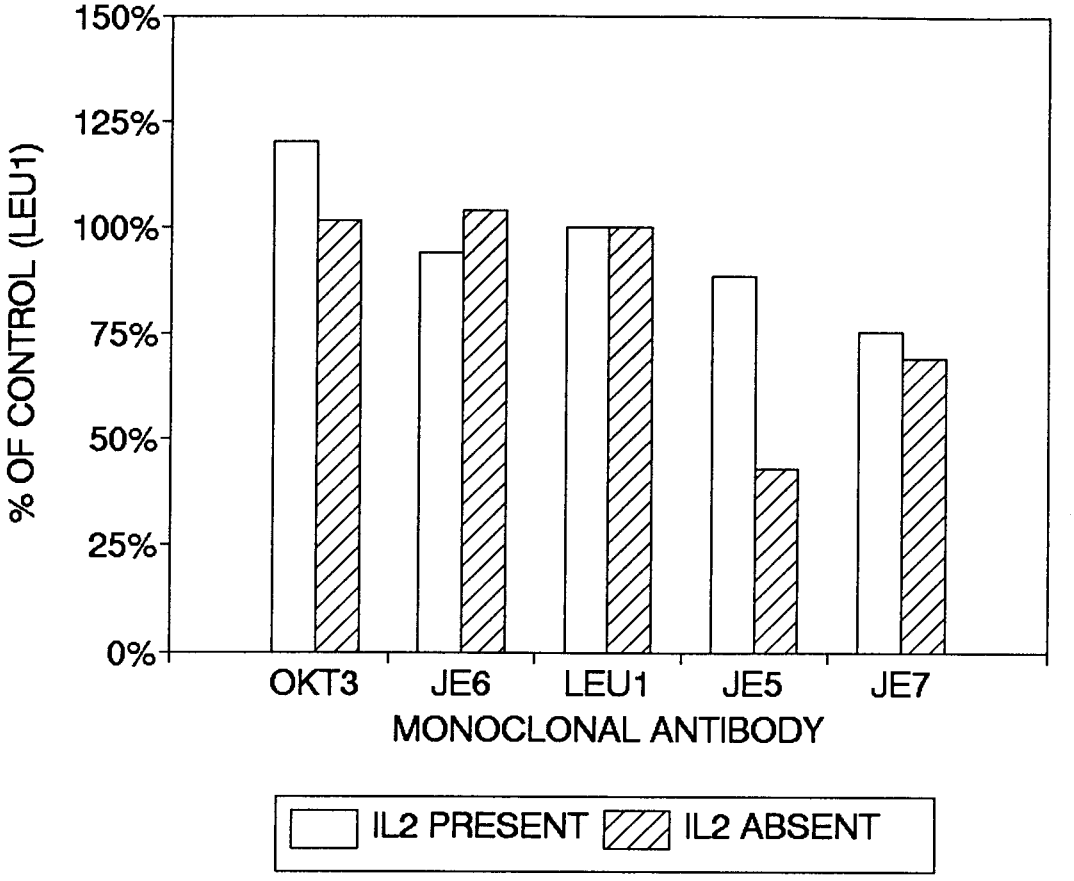


Table 4. IFN-γ Secretion.[1]

mAb		PBL	THYMO	HPB-ALL	SUP-T13	JE
OKT3	+	11.9	0.31	0.20	15.2	0
	-	6.75	0.15	0.15	0.20	0
JE6	+	14.2	0.65	0.10	13.6	0
	-	0	0.65	0.20	17.0	0
LEU1	+	1.90	0.25	0.10	0.07	1.25
	-	1.37	0.63	0.10	0.10	0
4-9E	+	1.80	0.38	0.10		
	-	1.60	0.63	0.25		
510C	+	0	0.31	0.10		
	-	0	0.31	0.25		
LC4	+	1.70	0.15		>20	
	-	0	0.25		>20	
LC11	+	2.35	0.75		>20	
	-	1.13	0.31		>20	
JE5	+	1.30	0.31			0
	-	1.50	0.12			0
JE7	+	1.90	0.75			0
	-	1.65	0.40			0

[1]IFN-γ secretion analysis using enzyme linked immunosorbent assays (ELISA). Each cell type was incubated with mAb in the presence (+) or absence (-) of either IL2 or GAMIg. Absorbance readings were assessed and converted into ng/ml.

Table 5. TNF-α Secretion.[1]

mAb		PBL	THYMO	HPB-ALL	SUP-T13	JE
OKT3	+	4.35	5.13	>20	4.55	18.6
	-	3.75	5.30	4.50	>20	16.9
JE6	+	4.25	4.58	9.65	6.20	15.9
	-	3.25	3.63	4.20	>20	18.2
LEU1	+	4.60	4.37	>20	4.20	16.8
	-	3.95	4.13	3.90	>20	16.7
4-9E	+	4.15	4.63	>20		
	-	2.75	4.37	3.75		
510C	+	3.25	6.13	>20		
	-	2.85	5.25	3.75		
LC4	+	3.15	6.13		4.00	
	-	3.00	4.63		>20	
LC11	+	3.10	6.88		3.20	
	-	3.10	5.88		>20	
JE5	+	2.70	5.13			19.0
	-	2.15	3.88			17.9
JE7	+	3.95	5.00			19.4
	-	4.15	4.38			18.2

[1]TNF-α secretion analysis using enzyme linked immunosorbent assays (ELISA). Each cell type was incubated with mAb in the presence (+) or absence (-) of either IL2 or GAMIg. Absorbance readings were assessed and converted into ng/ml.

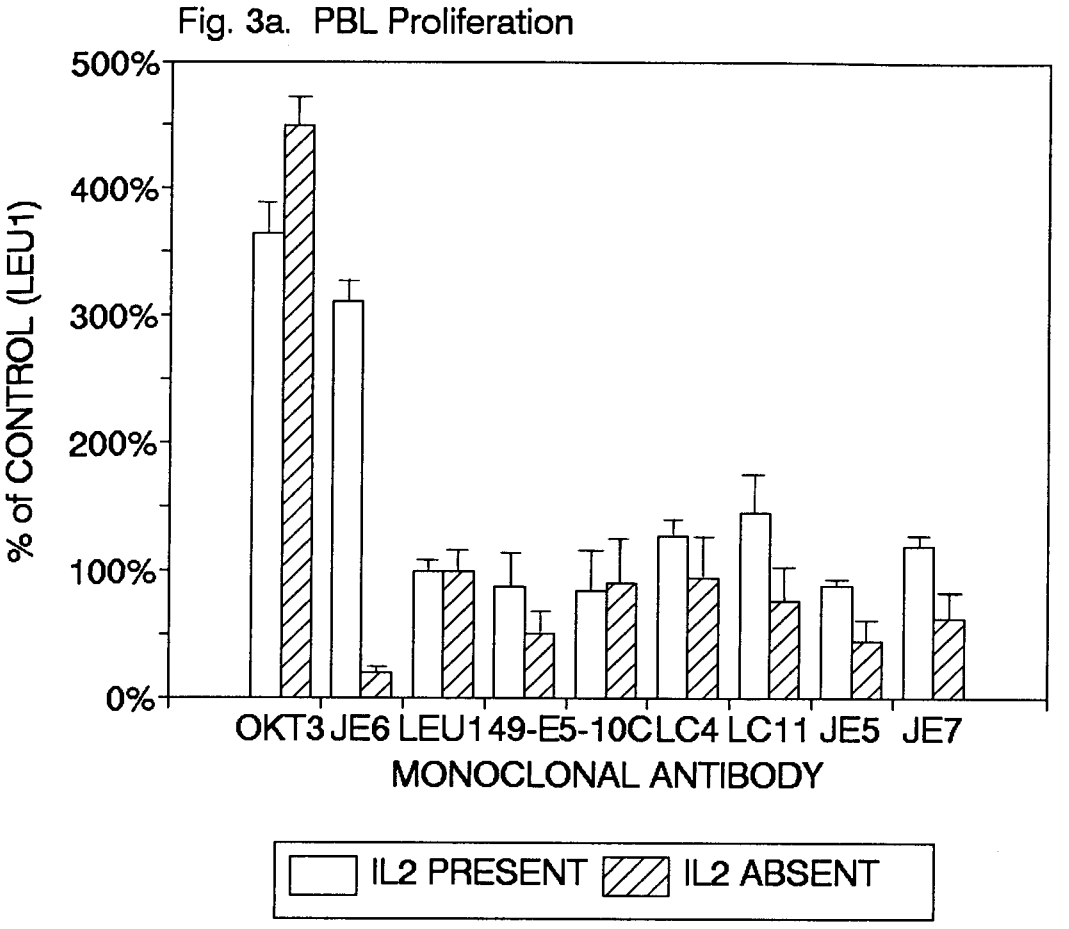


Figure 3. Proliferation assays using ^{3}H-thymidine. Cells were incubated with mAb in the presence (open bars) or absence (hatched bars) of IL2 or GAMIg. Results are shown as a percentage of the negative control (LEU1). **a.** PBL, **b.** thymocytes, **c.** HPB-ALL, **d.** SUP-T13, **e.** JE.

Fig. 3b. Thymocyte Proliferation

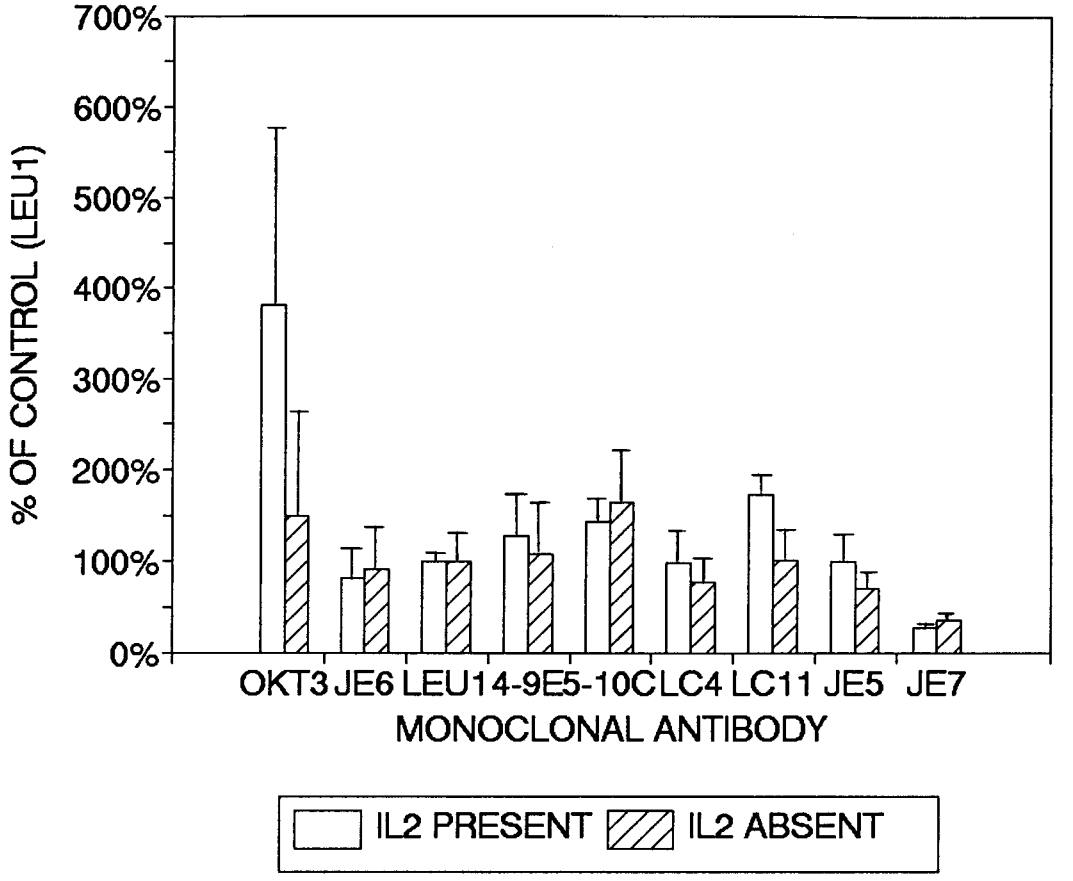


Fig. 3c. HPB-ALL Proliferation

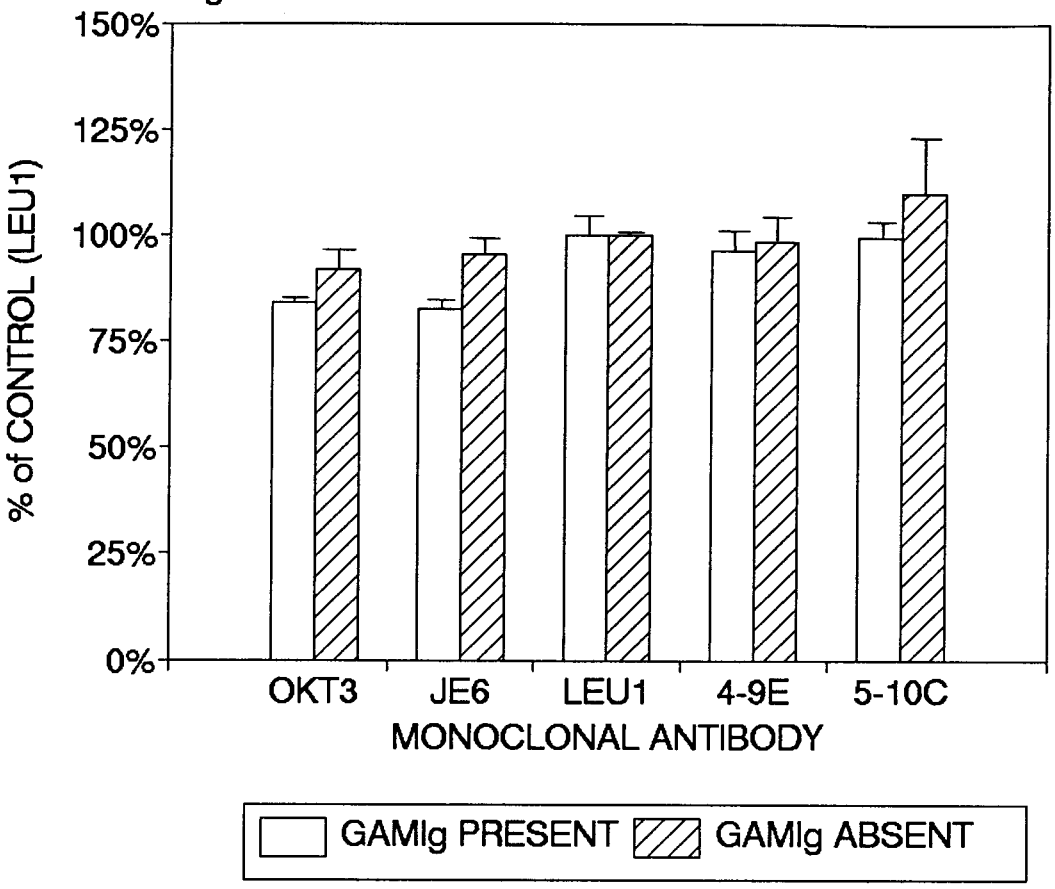


Fig. 3d. SUP-T13 Proliferation

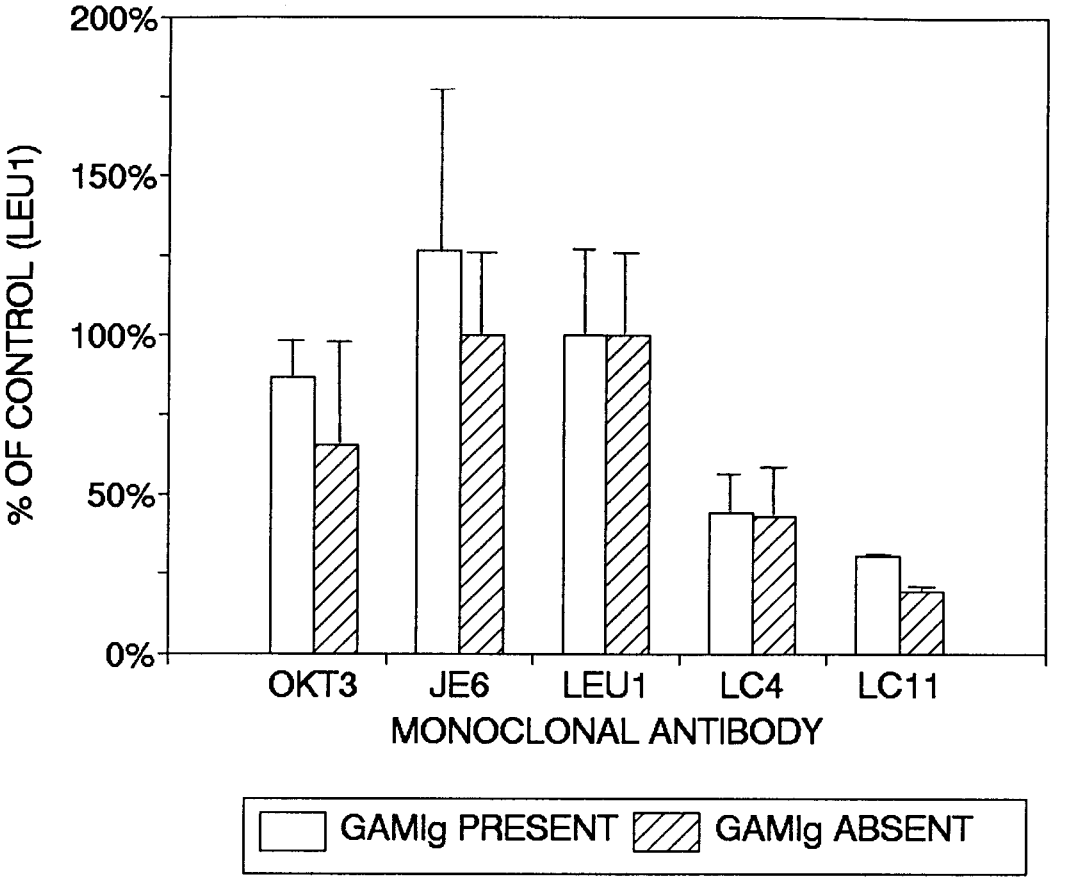
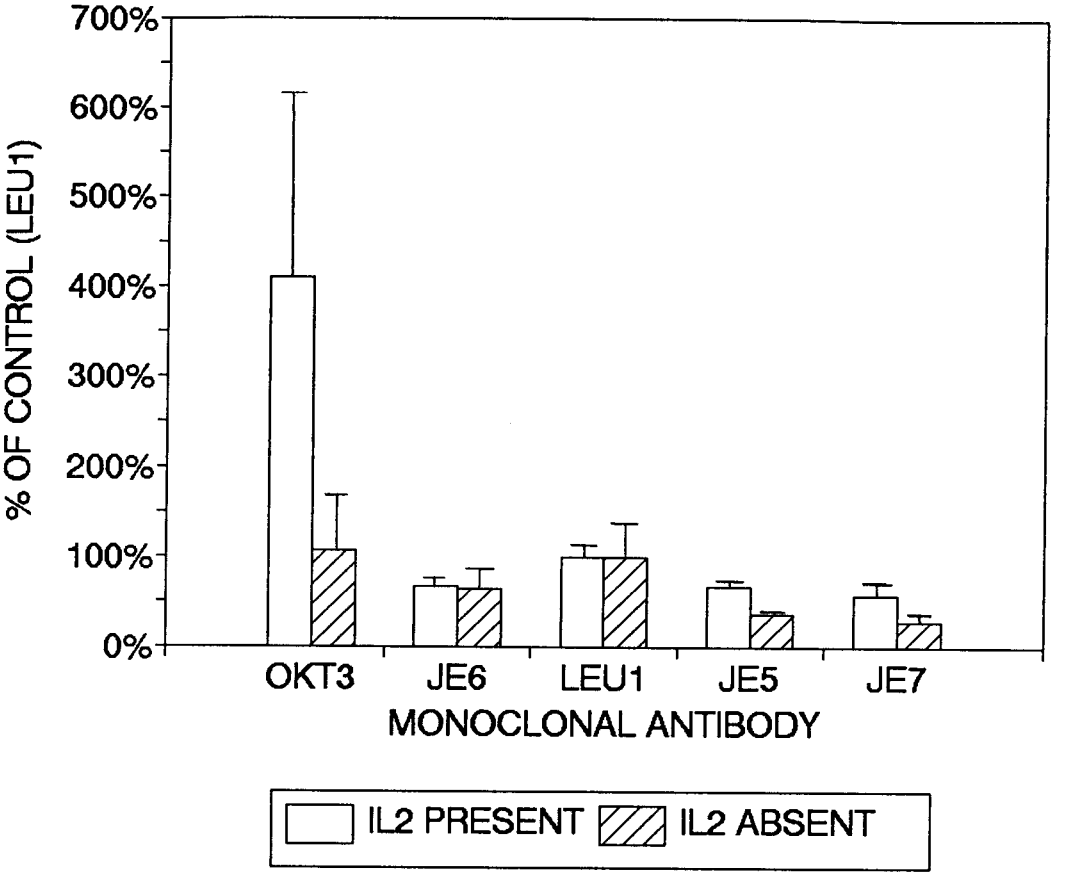


Fig. 3e. JE Proliferation



A.

B.

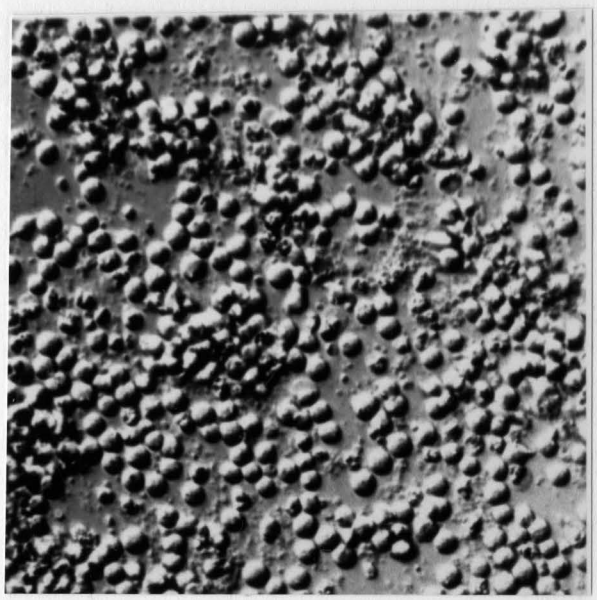
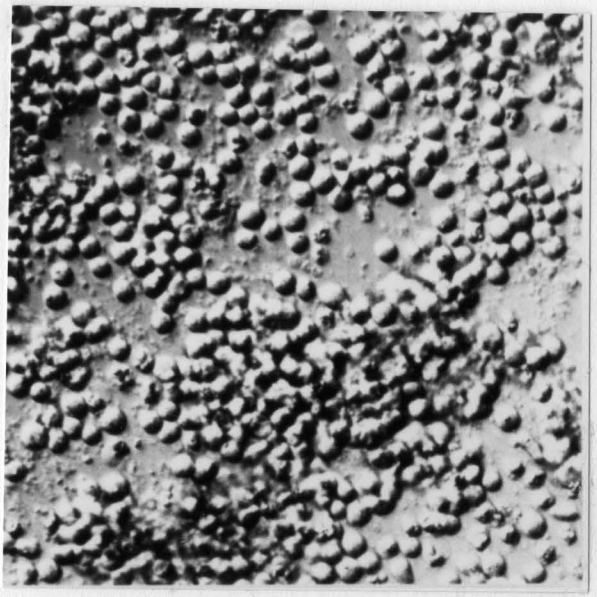
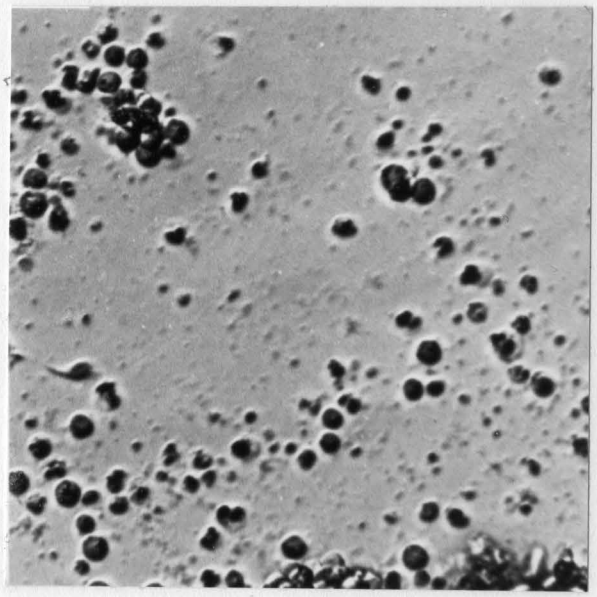


Figure 4. Phase contrast microscopy of SUP-T13 cells treated with mAb **a.** OKT3, **b.** JE6, **c.** LEU1, **d.** LC4, **e.** LC11, **f.** OKT3+GAMIg, **g.** JE6+GAMIg, **h.** LEU1+GAMIg, **i.** LC4+GAMIg, **j.** LC11+GAMIg.

C.



D.



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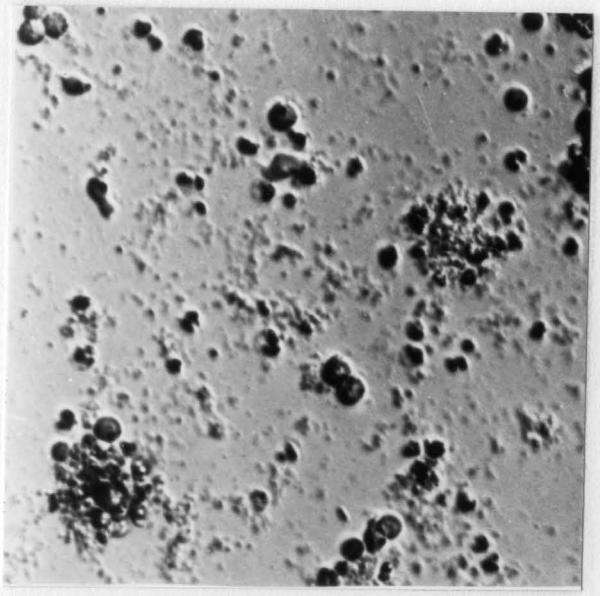

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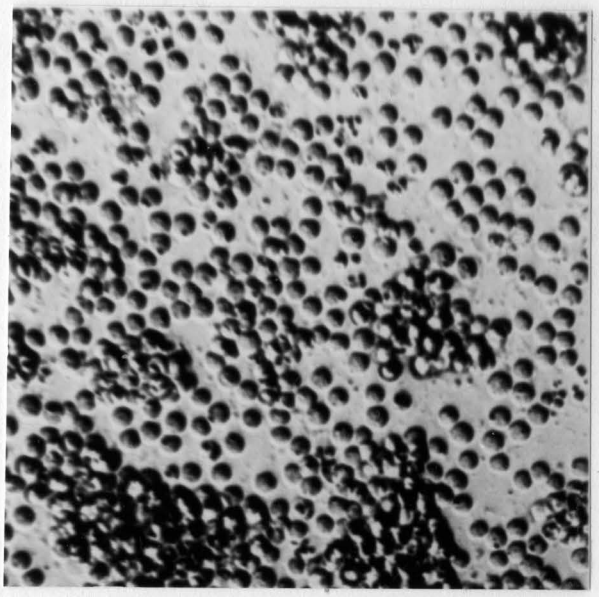
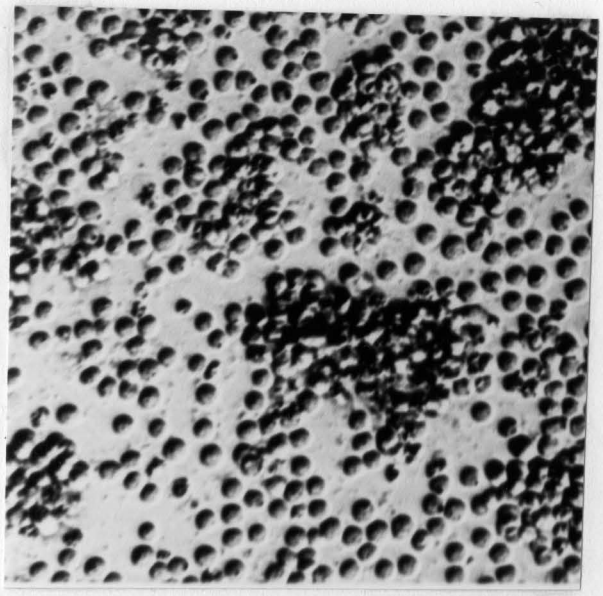
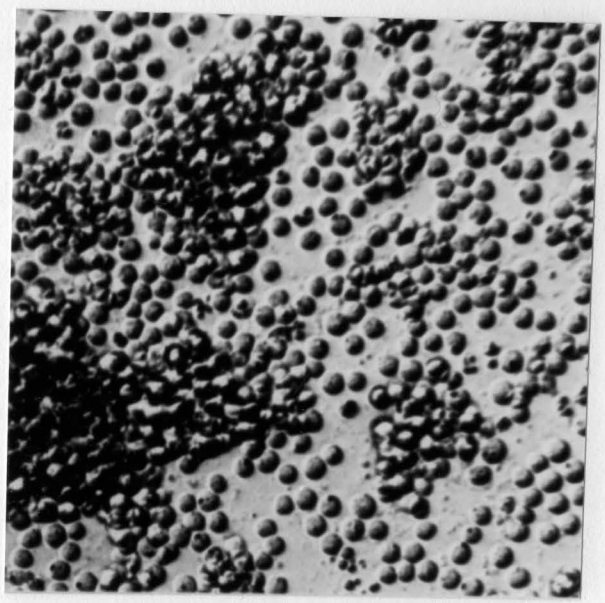


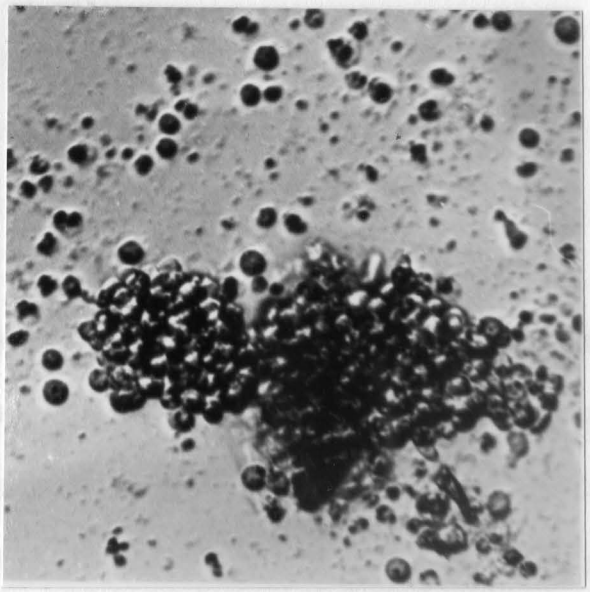
G.



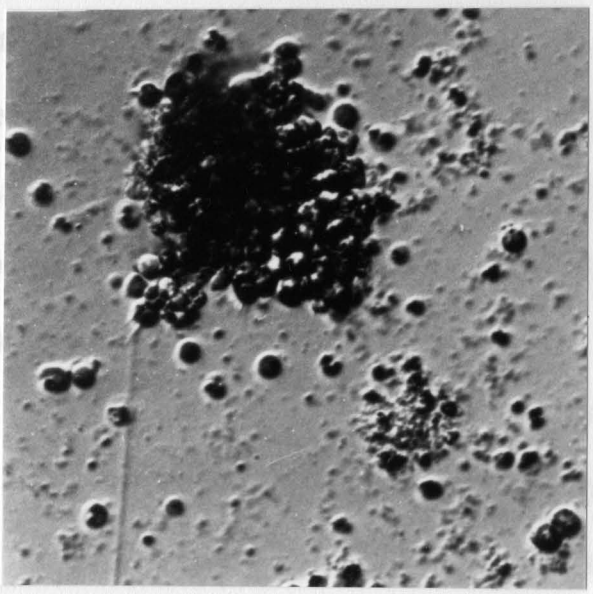
H.



I.



J.



CHAPTER IV

DISCUSSION

The purpose of this study was to provide additional insights into the biological effects that mAb can have on T cells. Since the development of technology to produce mAb, immunotherapy of certain diseases, such as leukemia, has gained more interest. Among the many biological applications of mAb, passive therapy of T cell tumors using mAb directed against the TCR is particularly attractive (Katz et al., 1989). This form of therapy was attempted on two patients; one with T-CLL (T chronic lymphocytic leukemia) and one with CTCL (cutaneous T cell lymphoma) (Janson, et al., 1989; Maloney et al., manuscript in preparation). Both studies suggested that potential benefits may be derived from this form of therapy, yet the advantages and disadvantages of mAb therapy are still unclear.

The goal of this project was to investigate the functional effects that different mAb to the TCR/CD3 complex have upon T cells of various phenotypes. The question of whether or not these different effects are due to the intrinsic properties of the mAb, the type of target cell, or the epitope which the mAb recognize, was examined. The panel of mAb used included two anti-CD3 mAb and three pairs of anti-TCR mAb with each pair being specific for a different T cell tumor. Mab were tested on peripheral blood lymphocytes (PBL), normal human thymocytes, two acute lymphocytic leukemias, and

one chronic lymphocytic leukemia. Various T cell activation events were then measured.

Mab, when bound to either the TCR or CD3 of T cells, can, in most cases, mimic Ag/MHC binding to the T cell. This mimicking by anti-TCR or anti-CD3 mAb will cause T cell activation (Meuer et al., 1983; Weiss et al., 1984b; Imboden and Stobo, 1985; MacDonald and Nabholz, 1986; Weiss et al., 1986: Nau et al., 1988; Schlitt et al., 1989). Furthermore, the form of presentation of the mAb can also determine whether or not a T cell is fully activated. Cross-linking of mAb by an anti-Ig provides a source of a secondary signal that may be required by the cell in order to achieve activation (Breitmeyer et al., 1987). After the TCR/CD3 complex is perturbed by either Ag/MHC or mAb, a cascade of events follows. Of this cascade, four major events are known to occur: 1) there is an increase in cytoplasmic Ca^{++}; 2) there is an increase in the expression of high affinity IL2 receptors on the cell surface; 3) lymphokines, such as IFN-γ, TNF-α, and IL2 are secreted by the cell; and 4) cell division and proliferation occurs, (Meuer et al., 1983; Weiss and Stobo, 1984, Imboden and Stobo, 1985; MacDonald and Nabholz, 1986; Weiss et al., 1986).

This project focused on the ability of mAb to induce or inhibit these four events as well as whether providing secondary signals (anti-Ig or IL2, depending on the phenotype of the cell) could enhance the effects induced by the mAb. The occurrence of these events could be disadvantageous for immunotherapy. For example, treatments with mAb that cause an increase in proliferation of tumor cells would be deemed counter-productive to their therapeutic role. Furthermore, high levels of cytokine secretion from T cells can cause side effects such as chills, fever, nausea, vomiting, diarrhea, dyspnea,

wheezing, and sterile meningitis (Goldstein et al., 1985). Therefore, by studying the effects that mAb can have on their target cells, unlikely mAb candidates for immunotherapy can be excluded before treatment. Furthermore, correlations may be drawn between certain activation events and particular mAb or particular tumors.

For the purpose of this project, PBL from a normal human donor were chosen as the baseline for comparison. None of the anti-TCR mAb were specific to more than a few percent of the cells from the donor. Although the anti-TCR mAb could bind to a very small subpopulation, any effects they would cause would be virtually undetectable. Therefore, if any gross effects were seen, we could assume that these effects were due to non-specific effects of the anti-TCR mAb on the population of PBL. Anti-CD3 mAb, however, would be expected to serve as a positive control, since they are known to stimulate resting PBL. Thymocytes were chosen as a second control population, one which represents immature T cells. This is significant since leukemic cells, particularly ALL's, resemble immature stages of T cell development (Weiss et al., 1983; reviewed by Devita et al., 1989). ALL may have the phenotype of early or common cortical thymocytes. On the other hand, T-CLL resemble more mature T cells that are activated (Reinherz et al., 1979; Pandolfi et al., 1982; reviewed by Devita et al., 1989).

INTRACELLULAR Ca^{++} MOBILIZATION

During activation of a T cell, Ca^{++} is released from cytoplasmic Ca^{++} stores in response to stimulation of the TCR/CD3 complex. This release of Ca^{++} was detected using a Ca^{++} sensitive dye and flow cytometry. As expected, PBL showed no detectable

release of Ca^{++} when treated with any of the anti-TCR mAb. However, both anti-CD3 mAb demonstrated the ability to induce Ca^{++} release. This was expected since anti-CD3 mAb bind to all T cells regardless of idiotypic differences, due to the invariant nature of the CD3 complex. The TCR on the other hand, varies from one T cell clone to the next, therefore not all anti-TCR specific mAb will bind to the TCR of different T cell clones. To test the ability of these cells to mobilize Ca^{++} as well as the loading of the cells with dye, ionomycin, a Ca^{++} ionophore, was used as a positive control. Treatment with ionomycin provided evidence that the cells did have the ability to flux Ca^{++} as well as demonstrating proper loading with the dye.

Thymocytes also showed signs of Ca^{++} release when treated with both anti-CD3 mAb (OKT3 and JE6), but like PBL, anti-TCR mAb were not able to induce flux as they did not bind to a large enough proportion of the cells. The reason for the lack of Ca^{++} mobilization by any of the anti-TCR mAb was analogous to that of PBL; none of the anti-TCR mAb in the panel were specific for the TCR of the thymocytes used.

Neither anti-TCR nor anti-CD3 mAb were able to induce Ca^{++} flux in HPB-ALL cells. However, treatment with the Ca^{++} ionophore, ionomycin, demonstrated mobilization of Ca^{++}. Other researchers have also found HPB-ALL cells to be deficient in the ability to mobilize Ca^{++} when stimulated with a mAb directed to CD3. They postulated that HPB-ALL cells were deficient in the ability to hydrolyze phosphatidylinositol biphosphate into its two products, inositol triphosphate and diacylglycerol (inositol triphosphate is needed to induce Ca^{++} mobilization) (Nel et al., 1991). This deficiency in phosphatidylinositol biphosphate turnover was attributed to

uncoupling of TCR/CD3 to phospholipase C activation (Brattsand et al., 1990). This may be the reason why HPB-ALL cells in these experiments failed to exhibit any Ca^{++} flux when stimulated with either anti-TCR or anti-CD3 mAb.

Ironically, the other ALL cell line, SUP-T13, exhibited Ca^{++} mobilization upon treatment with both of the anti-CD3 mAb as well as both anti-TCR mAb. Previous studies have shown SUP-T13 cells to flux Ca^{++} when treated with various anti-CD3 mAb (JE6, LEU4, EF3, EF6) as well as the anti-TCR mAb, LC4 (Takahashi et al., 1989). New to this panel of mAb being tested on SUP-T13 cells was LC11, an anti-TCR mAb which had similar effects as LC4 with regard to Ca^{++} mobilization. LC11, like LC4, is of the same isotype (an IgG1) and blocking analysis showed that LC11 could completely block the binding of LC4 to the TCR of SUP-T13 cells. Therefore, LC11 must recognize either the same epitope of the TCR as LC4 or an epitope in close proximity, and thus the similarity of its effects to LC4 was not unexpected.

JE was the only T-CLL examined in this project. Ca^{++} flux analysis of these cells was attempted. However there was no induction of Ca^{++} release by any of the mAb. Treatment with ionomycin did not produce any detectable fluxes. This suggests that the cells were not adequately loaded with the dye. However, due to the limited supply of these tumor cells, I was not able to repeat this experiment.

Overall, PBL provided a good baseline for comparison since there was no flux induced by any of the anti-TCR mAb while thymocytes demonstrated that Ca^{++} flux can be induced by anti-CD3 mAb in a non-tumor cell line. There was a difference with mAb stimulation between the two ALL cell lines. Ca^{++} flux was induced in SUP-T13, while

intracellular deficiencies did not allow HPB-ALL to flux Ca^{++} in response to mAb stimulation. Whether a T-CLL, like JE, is able to flux Ca^{++} in response to anti-CD3 or anti-TCR mAb is unclear.

IL2R EXPRESSION

During the activation of T lymphocytes, there is an increase in the expression of high affinity IL2 receptors (IL2R). This high affinity IL2R is a combination of a 55 kD protein and a constitutively expressed 70 kD protein. Tests for the expression of the p55 IL2R were performed since it is known that p55 expression is a good indicator of T cell activation. The lack of induction of IL2R expression among both PBL and thymocytes when treated with anti-TCR mAb confirmed the absence of non-specific effects of these mAb. Yet only one of the anti-CD3 mAb showed any significant induction, which was dependent upon the addition of IL2. The difference seen between OKT3 and JE6 can be attributed to isotypic differences between the antibodies. OKT3 is of the IgG_{2a} class of immunoglobulins while JE6 is of the IgG_1 class. Monocytes, which are present in the PBL population, contain Fc receptors. These receptors bind to the Fc portion of Ig, thus leading to cross-linking of mAb. However, the Fc receptor for IgG_1 is not found in all individuals. Therefore, an individual that does not have the IgG_1 Fc receptor on their monocytes, will not cross-link IgG_1 mAb, while those individuals that have the IgG_1 Fc receptor will cross-link these mAb thus enhancing the mitogenic stimulation of the mAb (Frenken et. al., 1991).

HPB-ALL cells could induce IL2R expression in response to OKT3 and 4-9E, yet

were not able to exhibit Ca^{++} flux in response to any of the mAb. It is unclear how IL2R expression could occur in the absence of any inducible Ca^{++} mobilization. It is possible that activation may occur via kinases other than PKC, and that these kinases are not Ca^{++} dependent. In addition, cross-linking of the mAb was needed to induce increases in IL2R expression.

In comparison to HPB-ALL, SUP-T13 cells demonstrated a very different IL2R expression profile. These cells exhibited extremely high increases in IL2R expression in response to one of the anti-CD3 mAb and to both of the anti-TCR mAb. Previous studies on SUP-T13 cells showed significant increases of IL2R expression by OKT3 and LC4, and to a lesser degree by JE6 (Takahashi et al., 1989). This project shows LC11 to induce a similar increase in IL2R expression as LC4. However, crosslinking with a secondary Ab is not a requirement for this ALL cell line since significant increase in IL2R expression were seen in the absence of GAMIg. This is evidence of the different effects these mAb can have on target cells of same phenotype.

The reason is unclear for the lack of any significant increases in IL2R expression in JE cells induced by any of the mAb in either the presence or absence of IL2. If JE cells do not flux Ca^{++} in response to anti-CD3 or anti-TCR mAb, then the lack of any increases in IL2R expression would coincide with that finding.

The IL2R data gives further evidence that PBL is a good baseline for comparison, since none of the anti-TCR mAb induced increases in IL2R expression. Furthermore, there are differences seen between the two ALL cell lines. In one cell line, HPB-ALL, IL2R expression was not increased in response to the mAb while in the other cell line,

SUP-T13, IL2R expression increased in response to anti-CD3 and anti-TCR mAb. Evidence that there can be differences between anti-CD3 and anti-TCR mAb is also demonstrated in SUP-T13 cells, in which anti-TCR mAb induced higher IL2R expression than anti-CD3 mAb.

CYTOKINE SECRETION

The general way to induce the synthesis of lymphokines by T cells is the stimulation through the TCR complex which results in activation of tyrosine kinases as well as phospholipase C. This activation of T lymphocytes via the TCR usually results in the production of a broad array of lymphokines (Germann et al., 1991). Such lymphokines would include IL2, IFN-γ, and TNF-α.

Normal PBL exhibited low amounts of IFN-γ secretion (0-2.4 ng/ml) with the anti-TCR mAb, however higher concentrations were induced by both anti-CD3 mAb in the presence of IL2. This is in accordance with the data from Ca^{++} mobilization experiments in which both OKT3 and JE6 (in the presence and absence of IL2) induced intracellular Ca^{++} flux. High levels of IFN-γ secretion were not detected with either of the anti-CD3 mAb in the absence of IL2. The reason for this may be that in resting T cells, high levels of IFN-γ may not be inducible without a second stimulus such as IL2.

Both thymocytes and HPB-ALL cells exhibited extremely low levels of IFN-γ secretion by both anti-TCR and anti-CD3 mAb. These two cell populations may require additional stimuli to secrete high levels of IFN-γ.

The secretion pattern of IFN-γ from SUP-T13 cells is similar to its pattern of

Ca^{++} mobilization and IL2R expression. Both anti-CD3 mAb induced high levels of IFN-γ secretion. However, LC4 and LC11 induced levels that exceeded the upper limit of the assay standard (20 ng/ml). This further confirmed the different effects induced by stimulating these cells with anti-CD3 mAb versus anti-TCR mAb. Furthermore, in comparing the two ALL cell lines, it is obvious that mAb can have quite different effects on cells of the same phenotype.

The lack of any detectable IFN-γ from JE cells coincides with the inability to induce Ca^{++} mobilization or further IL2R expression with any of the mAb. Therefore, it appears that JE cells are less readily activated by mAb even when provided with a secondary signal source, IL2.

Anti-CD5 mAb was shown to cause TNF-α production at levels equivalent with other mAb. I chose anti-CD5 as a negative control since soluble anti-CD5 mAb alone is not stimulatory to T cells (Vandenberghe and Ceuppens, 1991). However, recent evidence has shown that anti-CD5 mAb can cause certain activation events in PBL. This activation pathway is similar to but not identical to the TCR/CD3 activation pathway. Unlike the TCR\CD3 pathway, there is a lack of hydrolysis of inositol phosphate and an absence of intracellular Ca^{++} flux. Like the TCR\CD3 pathway, stimulation of CD5 with mAb induces activation of protein kinase C as well as tyrosine kinase activity; however, the substrate pattern of the tyrosine kinases differs from that induced after triggering T cells via TCR/CD3 (Alberola-Ila, et al., 1992). This activation of substrates by tyrosine kinases may lead to lymphokine production, specifically TNF-α.

Furthermore, in one cell population, HPB-ALL, stimulation via anti-TCR or anti-

CD3 mAb induced high levels of TNF-α secretion that was independent of Ca^{++} mobilization. There has been some evidence that an alternative pathway of induction of lymphokine production may be present in certain T cell clones (Dunn, et al., 1987; Dunn, et al., 1989). T cell clones stimulated with syngeneic or allogeneic accessory cells plus IL2 induced synthesis of lymphokines, such as IFN-γ. Additionally, it was shown that this pathway does not require intracellular Ca^{++} mobilization (Dunn et al., 1987). It may be possible that stimulation of HPB-ALL T cell clones via the TCR/CD3 complex may also activate a pathway for lymphokine secretion that is independent of Ca^{++} mobilization.

Although TNF-α secretion may have beneficial effects for tumor immunotherapy, induction of high quantities with a mAb can cause severe side effects. Therefore, for the purposes of immunotherapy, a mAb that induces low amounts of TNF-α may be desired. Unfortunately, high levels of TNF-α were obtained with mAb binding to the three tumor cell groups while lower levels were found with PBL and thymocytes. The high levels of TNF-α secretion may be attributed to the phenotype of the cell. Activated T cells may be more readily induced to secrete TNF-α than resting T cells (PBL and thymocytes).

Although no significant differences in TNF-α were seen among the panel of mAb in any given cell type, differences were observed between the presence and absence of a cross-linking agent. The role of anti-Ig is to link mAb together thus indirectly linking TCR or CD3 with each other. This cross-linking of TCR or CD3 enhances the mitogenic stimulation of the mAb to the cell (Breitmeyer et al., 1987). HPB-ALL cells

demonstrated enhanced TNF-α secretion when anti-Ig was present versus when absent from the media. However, high levels of TNF-α were induced only in the absence of anti-Ig in SUP-T13 cells. Previous work has been done on cytokine secretion in the presence and absence of a cross-linking agent. Researchers showed that cross-linking of an Ab to CD3 could cause IL2 secretion in some T cell clones but suppress it in other clones. The response was dependent on the clone, so that cross-linking of the TCR/CD3 complex is not necessarily required for T cell clone activation (Tamura and Nariuchi, 1992). Thus, cross-linking of mAb to TCR/CD3 in HPB-ALL cells may be needed to cause high level secretion of TNF-α, but such cross-linking may be inhibitory to SUP-T13. This may also explain the differences exhibited in IL2R induction by OKT3 between the two ALL populations when anti-Ig is present versus when it is absent.

Even though no significant differences were seen among the mAb used with JE cells, high levels of TNF-α were obtained. Again, this may be attributed to the non-specific activation of the alternative pathway for lymphokine production by the mAb. The low amounts of IFN-γ secreted from these cells may be in response to the relatively high amount of TNF-α produced. Previous work has shown TNF-α to play a regulatory role on IFN-γ production, such that TNF-α may inhibit the release of IFN-γ (Ferran et al., 1991).

It is becoming clearer that the effects mAb can have on a cell can differ among different T cell phenotypes as well as within the same T cell phenotype. Furthermore, binding of a mAb to the TCR may have a substantially different effect on the T cell than a mAb binding to the CD3 portion of the complex.

PROLIFERATION

One of the last steps in T cell activation is proliferation. When stimulated, for example by an Ag in association with MHC, T cells will respond by clonal expansion. Binding of a mAb to the TCR/CD3 complex can also cause proliferation of T cells. However, if mAb are to be used as a form of immunotherapy to leukemic cells, proliferation of these cells induced by the mAb would be contrary to its therapeutic role. It would be beneficial to be able to predict whether a mAb will cause further expansion of the target cells before the treatment is administered.

Proliferation of normal PBL was found to occur in response to treatment with both anti-CD3 mAb, with OKT3 being a stronger inducer of PBL proliferation than JE6. This difference can be attributed to isotype differences between the mAb, as stated previously. The analysis with anti-TCR mAb further confirms the absence of any non-specific effects.

In thymocytes, anti-CD3 mAb were less mitogenic than in PBL. Thymocytes represent a population of T cells from the thymus, therefore there can be subpopulations of T cells at different stages of development. There are precursor T cells that are $CD3^{lo}$ and negative for both CD4 and CD8; there are immature T cells that are also $CD3^{lo}$ but positive for both CD4 and CD8; and there are mature T cells that are $CD3^{hi}$ and single positive for either CD4 or CD8 (reviewed by Abbas, 1991). Previous work has shown the effects of anti-CD3 mAb to be dependent on the phenotype of the thymocyte (i.e. whether it was a precursor, immature, or mature T cell). Treatment of immature thymocytes in culture with anti-CD3 mAb, but not other mAb, induces cell death via

degradation of DNA to oligonucleosomal bands. DNA fragmentation is characteristic of apoptosis, or programmed cell death (Smith et al., 1989; Sancho et al., 1992). However, mature thymocytes can be induced to proliferate in response to anti-CD3 mAb + IL2 (Havran et al., 1987; Sancho et al., 1992). The marginal proliferation seen by anti-CD3 mAb in this experiment may be the result of the overall net effect induced by the mAb. The anti-CD3 mAb may have been inducing proliferation of the mature thymocytes in the population while at the same time inducing apoptosis of immature thymocytes. Because both types of T cells are present in the population, the overall net effect may be little or no proliferation. This may also explain the marginal differences seen in IL2R expression as well as the lack of any significant differences seen in cytokine secretion.

HPB-ALL cells did not proliferate in response to any of the anti-CD3 mAb or anti-TCR mAb although they could be induced to express IL2R and secrete cytokines. Because HPB-ALL cells are actively dividing T cells, mAb stimulation may not be able to increase the rate in which they divide. This rate may already be at its maximum limit.

Interestingly, the other ALL cell line, SUP-T13, had a much different proliferation profile than HPB-ALL cells. Anti-CD3 mAb did not increase proliferation of SUP-T13 cell, much like in HPB-ALL cells. However, the anti-TCR mAb, LC4 and LC11, caused a decrease in cell number. These mAb induced apoptosis in SUP-T13 cells, as shown by previous work on LC4 treatment of SUP-T13 (Takahashi et al., 1989). Adding to the work of Takahashi et al., are the results obtained from LC11 treatment. LC11 was found to be identical in every aspect to LC4. These findings

further imply that these mAb may be good candidates for immunotherapy of T cells recognized by LC4 and LC11 because of their anti-proliferative effect of inducing programmed cell death in SUP-T13 cells. Only additional experiments involving cells other than SUP-T13 that bind the two anti-TCR mAb will confirm whether or not the apoptosis seen in SUP-T13 cells can be attributed to the intrinsic property of the mAb or to the characteristics of the cell.

OKT3 induced proliferation of the CLL cells, JE. However, in comparing the effects of the anti-TCR mAb, JE5 and JE7 (in the absence of IL2) to the negative control (LEU1), the analysis suggests that both anti-TCR mAb may inhibit proliferation. This effect on proliferation would correlate with the low IL2R expression that is induced by these mAb. Furthermore, cross-blocking analysis shows JE5 to block JE7 completely. Therefore, both mAb must bind to either the same epitope or epitopes in close proximity to each other (Maecker, 1988). If they indeed inhibit proliferation, JE5 and JE7 may also be good candidates for immunotherapy, if other target CLL cells respond in a similar manner as JE.

CHAPTER V

CONCLUSION

The goal of this project was to bring some insight into the functional effects that mAb can have to T cells of various phenotypes. Six major conclusions can be made: 1) a particular mAb may cause different activation events in different target cells, even when those target cells have an identical phenotype (e.g., ALL). This was suggested by the differences found when the anti-CD3 framework mAb were used in PBL versus thymocytes versus ALL versus CLL. 2) There can be different effects when binding a mAb to the TCR as opposed to ligation of CD3. These findings were suggested by the differential effects seen among anti-CD3 mAb versus anti-TCR mAb when tested on the same cell. 3) Mab that recognize the same epitope and are of the same isotype seem to have identical effects on T cells. This was suggested by both LC4 and LC11 assays on SUP-T13 and JE5 and JE7 assays on JE cells. Even 4-9E and 510C, which recognize different TCR epitopes on HPB-ALL cells, had similar effects. 4) Cross linking of mAb has varying effects depending upon the target cell population. Some cells may exhibit an enhanced response when their TCR/CD3 complexes are cross-linked by mAb, while in other cells cross-linking of the TCR/CD3 complexes result in a suppressed response. In still other cells, cross-linking of TCR/CD3 complexes may not cause any change at all. Cross-linking of mAb in HPB-ALL cells enhanced the mAb induced effects, while

cross-linked mAb in SUP-T13 had a suppressive effect. 5) Intracellular Ca^{++} mobilization is not a necessary prerequisite for cytokine secretion or IL2R induction. Anti-CD3 mAb stimulation of HPB-ALL showed that IL2R expression and TNF-α secretion could be induced without any induction of Ca^{++} flux. 6) Anti-TCR mAb can induce apoptosis in some tumor cell lines, but not in others. However, the same anti-TCR mAb could not be tested in different tumors to conclusively determine whether this is a property of the tumor cell, or of the mAb.

These findings can be important when considering the use of mAb to treat T cell leukemias. Anti-TCR mAb generally did not cause proliferation of tumor T cells, which would be counter-productive to their intended effect (this is not necessarily true of anti-CD3 mAb). However, anti-TCR mAb can cause high amounts of cytokine secretion which lead to undesirable side effects. In conclusion, no single profile of activation events can be predicted for anti-CD3 or anti-TCR mAb, even in a given target cell population, such as T-ALL. Thus, the net effect of a mAb may be largely dependent on the individual target cell population. However, the finding that anti-TCR mAb may be anti-proliferative in some T-ALL as well as T-CLL tumors, is extremely encouraging for mAb immunotherapy of these tumors.

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VITA

The author, Mortimer T. Alzona, was born November 25, 1965, in Manila, Philippines. In 1983 he entered Indiana University in Bloomington, Indiana as a Biology major. He completed his undergraduate career in December 1987 with a Bachelor of Science in Biology. In August of 1990, he entered the graduate program in Biology at Loyola University and in 1991 was awarded a graduate assistantship.

APPROVAL SHEET

The thesis submitted by Mortimer T. Alzona has been read and approved by the following committee:

Dr. Holden T. Maecker, Director
Assistant Professor, Biology
Loyola University Chicago

Dr. Anthony J. Nappi
Professor, Biology
Loyola University Chicago

Dr. John Smarrelli
Associate Professor, Biology
Loyola University Chicago

The final copies have been examined by the director of the thesis and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the thesis is now given final approval by the Committee with reference to content and form.

The thesis is therefore accepted in partial fulfillment of the requirements for the degree of Masters of Science.

April 18, 1993
Date

Holden Maecker
Director's Signature