Role of Autocrine Growth Factors in the Tumorigenic Transformation of T Cells.

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(ABSTRACT)

In the current study we tested the hypothesis that tumorigenic transformation of T cells may result from aberrant regulation of autocrine growth factor production. We describe for the first time characterization of several normal T cell lines that underwent spontaneous transformation resulting from constitutive expression of interleukin-2 (IL-2) and interleukin 2 receptor (IL-2R) genes. Such cell lines could induce tumors *in vivo* and furthermore, the growth of such transformed cells both *ex vivo* and *in vivo* could be inhibited by monoclonal antibodies (mAbs) or antisense oligonucleotides specific for IL-2 or IL-2R. Interestingly, an *in vivo* originated T cell lymphoma, LSA, was also found to be dependent on constitutive production of IL-2 and Interleukin-4 (IL-4). Together, these data demonstrated that dysregulation in the production or respnsiveness to autocrine T cell growth factors, plays an important role in T cell transformation and tumorigenicity. Due to their ability to produce T cell growth factors, such tumor cells were found to be highly immunogenic. Thus it was surprising that some T cell lymphomas could still grow in an immunocompetent

host and kill the host. To this effect, we investigated additional mechanisms used by such tumor cell lines to grow in an immunocompetent host. It was noted that the tumor cells used mechanisms such as failure to express MHC and production of immunosuppressive cytokines, such as, transforming growth factor-β (TGF-β) and interleukin-10 (IL-10), to evade the action of the immune system. In addition, the T cell lymphomas constitutively expressed Fas-ligand and triggered apoptosis of host T cells that expressed Fas. Together, the current study suggests that cytokines produced by the tumor cells play an important role in tumorigenic transformation as well as maintenance of tumor growth in an immunocompetent host.

Dedication

I would like to dedicate this work to my parents, Rushdi and Suhailah Hassuneh, and to my sisters and brothers, Iman, Najiah (Maymoneh), Amin, Maa'moun and Saad.

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List of Abbreviations:

APC	Antigen Presenting cell
APO	Antisense phosphorothioate oligonucleotides
ATL	Adult T cell leukemia
bp	Base pair
BCNU	1,3-Bis (2-chloroethyl)-1-nitrosourea
CD	Cluster of differentiation
Con A	Concanavalin A
CsA	Cyclosporin A
CTL	Cytotoxic T lymphocyte
FITC	Fluorescein isothiocyanate
GM-CSF	Granulocyte-Macrophage colony stimulating factor
HPV	human papilloma viruse
HTLV	Human T cell lymphotropic viruses
IFN-γ	Interferon-γ
IL	Interleukin
IL-2R	Interleukin-2 receptor
IL-2R α	Interleukin-2 receptor α-chain

IL-2R β	Interleukin-2 receptor β-chain
IL-2R γ	Interleukin-2 receptor γ-chain
IL-4R	Interleukin-4 receptor
IL-15R	Interleukin-15 receptor
LAK	Lymphokine activated killer
LT	Lymphotoxin
mAbs	Monoclonal antibodies
MHC	Major histocopatibility complex
NF-AT	Nuclear factor for activated T cells
NK	Natural killer
PCR	Polymerase chain reaction
Rad LV	Radiation leukemia virus
RT-PCR	Reverse transcribed PCR
S. E.	Standard error
T_H1	T helper type 1
T_H2	T helper type 2
TCR	T cell receptor
TGF-β	Transforming growth factor-β
TNF-β	Tumor necrosis factor-β
UV	Ultra violet

Chapter 1: Background and Specific Aims

Introduction:

Immunity is a term derived from the Latin word *immunities,* referring to the exemption from various civic duties and legal prosecution offered to Roman senators during their tenure in office. The concept of immunity to infectious agents has existed as long ago as 5000 B. C., (Sigal and Ron, 1993) as evidenced by the ancient Chinese custom of making children inhale powders made from the crusts of skin lesions of patients recovering from smallpox. The first dramatic documentation of this immunologic phenomenon was recorded by the English physician Edward Jenner in 1798. Jenner noticed that milkmaids who recovered from cowpox never contracted the more serious smallpox. Based on this observation, he injected an 8-year old boy with material from a cowpox pustule and then intentionally infected the boy with smallpox, the boy did not develop smallpox (Abbas *et al.,* 1994). Pioneer immunologists such as Pasteur, Koch, Metchnikoff and Ehrlich, followed in Jenner's foot steps where they focused on the isolation of infectious agents and vaccination against them (Sigal and Ron, 1993). Since then, the horizons of the science of immunology have broadened to include: specific aspects of the immune response to self and non-self, self-tolerance and autoimmunity, tumor immunology, immune response to tissue transplants as well as the study of congenital and acquired immuno-deficiencies.

The immune system of healthy individuals can protect them from microbes in two different ways: innate and acquired immunity. Innate immunity includes the following: physical barriers, phagocytic cells such as macrophages and neutrophils, large granular lymphocytes known as natural killer (NK) cells and several blood-born molecules. Such defenses can distinguish self from nonself but, they can not discriminate one foreign substance from another. Thus, they are considered as non-specific defenses. While the innate defenses do not require prior exposure to the foreign material in order to be activated against it, the acquired immunity defenses do. Acquired immunity responds to foreign substances in a specific manner and foreign material that induces specific responses are called antigens. Specific immune responses are categorized into two main categories-humoral immunity and cellular immunity. Humoral immunity include macromolecules called antibodies which are capable of specifically recognizing a foreign antigen and neutralizing it. Such molecules are secreted by the terminally differentiated B lymphocytes known as plasma cells. Cellular immunity is mediated mainly by T lymphocytes (Abbas, *et al.*, 1994).

The immune response is usually directed against non-self antigens as well as transformed or altered self antigens. Non-self antigens are recognized when the foreign antigen is taken up by a special group of cells known as antigen presenting cells (APC)s which include mononuclear phagocytes or macrophages, B lymphocytes, dendritic cells, Langerhans cells in the skin and endothelial cells (Abbas, *et al.*, 1994). The engulfed invader is then digested and processed into smaller components consisting of: short peptides, nucleic acids, polysaccharides and lipids. T-cell mediated immune responses are

induced by protein antigens while humoral immune responses mediated by B lymphocytes can be induced by both protein as well as non-protein antigens (Abbas, *et al.,* 1994). Inasmuch as B lymphocytes can respond to soluble antigens while T cells can not, the activation of T cells requires the recognition of the foreign antigen in the context of major histocompatibility complex (MHC) molecules on the surface of APC.

Both B and T lymphocytes originate in the bone marrow, where B-cells undergo their early stages of maturation. Immature B lymphocytes are selected in the bone marrow so that only self tolerant cells can migrate from the bone marrow to the peripheral lymphoid tissues (Janeway and Travers, 1994). During this selection, a developing B cell that expresses receptors for self cell surface antigens such as self MHC is believed to undergo programmed cell death (apoptosis) (Hardy, *et al.,* 1991). Pre-T cells migrate from the bone marrow and mature in the thymus where they come into close physical contact with a variety of non-lymphoid cells. Such interactions are responsible for the selection of T cells that lead to the elimination of T cells with self-specificities (Janeway and Travers, 1994). Developing T cells in the thymus go through a series of stages that can be distinguished by the differential expression of surface molecules such as: the cluster of differentiation (CD)3, the T cell receptor (TCR) complex proteins and the co-receptor proteins CD4 and CD8. Mature T cells in the thymic medulla as well as those in the peripheral lymphoid organs can be categorized into two structurally and functionally distinct classes: helper T cells which are $CD3^+$, TCR-$\alpha\beta^+$, $CD4^+$ and $CD8^-$ and the cytotoxic T lymphocytes (CTL)s which are $CD3^+$, TCR-$\alpha\beta^+$, $CD4^-$ and $CD8^+$. The CD4 and CD8 molecules play an important role in the differential recognition of MHC class-II

and class-I molecules. For, CD4 binds to invariant parts of MHC class-II molecules and CD8 binds to invariant parts of MHC class-I molecules. Also, during antigen recognition, CD4 and CD8 molecules associate on the T cell surface with components of the TCR, which is why they are called co-receptors (Abbas, *et al.,* 1994). Because only professional APC express the MHC class-II molecules, while almost all cells express MHC class-I molecules, $CD4^+$ helper T cells can interact with only professional APC $CD8^+$ CTLs, on the other hand, can interact with all the cells in the body. Despite the structural similarity between class-I and class-II histocompatibility molecules, they bind peptides at different intracellular locations. MHC Class-I molecules present peptides that are derived from endogenous proteins , while MHC class-II molecules can present peptides from endocytosed (i.e., exogenous) antigens as well as endogenous antigens (Brodsky and Guagliardi, 1991).

Although antigen induced activation in both T cell subsets stimulates the same second messenger systems, nevertheless, the outcome of this activation is different in the two subsets. In helper T cells, antigen recognition brings about the production of a vast array of factors known as cytokines, which act to induce proliferation and differentiation of other T cells, B cells, macrophages and other inflammatory leukocytes. Following the TCR-CD3 mediated activation, helper T cells are induced to synthesize interferon γ (IFN-γ), IL-2, transforming growth factor-β (TGF-β), IL-2R, interleukin-3 (IL-3), interleukin-4 (IL-4), interleukin-5 (IL-5), interleukin-6 (IL-6), transferrin receptor and granulocyte-macrophage colony stimulating factor (GM-CSF) (Cabtree, 1989 and Kupfer and Singer, 1993). In addition, it is important to note that helper T cells have two distinct subsets known as helper T cell type 1 (T_H1) and helper T cell type 2 (T_H2). Upon

stimulation, the former secretes IL-2, IFN-γ and tumor necrosis factor- β (TNF-β) whereas, the latter secretes IL-4, IL-5, IL-6 and interleukin 10 (IL-10) (Mosmann and Coffman, 1989). T_H1 cells utilize IL-2 while T_H2 cells use IL-4 as autocrine growth factors *ex vivo*, However, following antigenic stimulation both subsets respond to both IL-2 and IL-4 (Fernandez-Dotran, *et al.,* 1988). $CD8^+$ CTLs on the other hand, when activated through the TCR-CD3 complex are induced to mediate target cell lysis by exocytosing their lytic granules, consisting of pore-forming protein (perforin) and granzymes. Also, CTLs in response to antigenic stimulation secrete IFN-γ, lymphotoxins (LT) or TNF-β and to a lesser degree IL-2 (Salgame, *et al.,* 1991). The secretion of perforin and granzymes by the CTL in the vicinity of the target cell induces osmotic swelling and lysis (necrosis). This mode of target cell killing is also shared by NK cells (Cohen and Duke, 1992). Furthermore, both NK cells and CTLs can kill their target by provoking programmed cell death or apoptosis (Zychlinsky, *et al,* 1991). Such a mechanism of cell death results when a special surface protein unique to T or NK cell, known as Fas ligand, binds to the Fas antigen present on target cells (Lowin, *et al.,* 1994). The binding of Fas ligand on CTL or NK cells to the Fas antigen on the target cell, triggers a series of events, which are still poorly characterized. Such events lead to the activation of endonucleases and thereby the digestion of nuclear DNA and eventual cell death (Abbas, *et al.,* 1994).

Cancer is one of the most abhorred diseases of the century, and it is among the leading causes of death in United States. This year only, about 554,740 people will die of cancer which comes to over 1,500 people a day (American Cancer Society, 1996). Cancer usually develops when a cell or a

group of cells proliferate in a way that defies the normal cellular growth controls. Normal cellular growth and differentiation is thought to be maintained by a balance between the activity of stimulating genes(proto-oncogenes) and inhibitory genes (anti-oncogenes) (Chiigira, *et al.,* 1990). Any imbalance in this regulatory mechanism would lead to uncontrolled cell growth or cell transformation and neoplasia. Disruption of this balance requires either the alteration of the proto-oncogenes in a way that hyper activates them (i.e., they become oncogenes) or the inactivation of the anti-oncogenes (Reddy, *et al.,* 1988).

Immortalization of a cell has been shown to occur in at least two steps: the first one is the initiation stage where a cell is subjected to certain carcinogenic factors that alter growth and differentiation machinery of the cell. Such alterations usually remain quiescent in resting cells (at G_0). However, during the next stage, the initiated cells are exposed to a stimulatory signal, which might not be mutagenic or carcinogenic, such stimulation will prompt the cells to divide continuously. The second stage is referred to as the promotion stage after which a cell becomes immortalized. The nature of the initiating or transforming factors range from chemicals, such as, polycyclic aromatic hydrocarbons (Miller and Miller, 1981); biological, such as, human papilloma viruses (HPV) (Howley, 1986) and human T cell lymphotropic viruses (HTLVs) (Yoshida and Seiki, 1987) or physical, such as, ultraviolet rays (UV) and ionizing radiation (Kohn and Fry, 1984). In order for immortalized cells to become neoplastic and tumorigenic, they have to go through a third stage known as the progression stage. During this stage the transformed cells will experience

successive cycles of mutations and natural selection that eventually lead to a full blown tumor (Cotran, 1989).

The abnormal growth behavior of cancer cells results from the expression of mutated or viral genes and/or dysregulated expression of normal genes. Such cells will express proteins that can appear as foreign to the tumor host, because they were never expressed in the host prior to tumor development or were expressed at such low levels that they did not induce tolerance (Abbas, *et al.,* 1994). Therefore, it is reasonable to assume that the immune system cells which screen normal tissues looking for the abnormal and the foreign, can mount a response against the transformed cells. However, it is obvious that in cancer patients the transformed cells escape this immune surveillance.

The evading of the immune system of healthy individuals by cancer cells may involve many factors. Such factors include the absence of certain surface molecules on the tumor cell which are essential for evoking an immune response against it. Also, cancer cells can evade the immune system if they produce immunosuppressive molecules or are capable of inducing a suppressor population of T cells that counteracts the effector T cells stimulated against it (Abbas, *et al.,* 1994). The different factors that may allow cancer cells to overcome the immune system will be discussed in more details in the coming chapters.

Cancer and Neoplastic Transformation:

As mentioned earlier, carcinogenic agents fall into the following categories: 1) chemical carcinogens, 2) radiation and 3) oncogenic viruses. Carcinogens generally initiate cancer and promote it by inducing genetic changes that increase cell proliferation. Such an increase in cell proliferation could be mediated by decreasing the dependence of cells on external growth factors, preventing cellular senescence or by inhibiting programmed cell death (Murry and Hunt, 1993). The induction of normal cellular proliferation starts by an external signal, such as the binding of a growth factor to its receptor. The receptor would then transfer the message through the membrane into the cytoplasm and ultimately to the nucleus. Proto-oncogenes have been found to function at each step of the mitotic cascade (Cotran, *et al.*, 1989). Upon activation, by either mutation or increased expression, the proto-oncogenes are converted to oncogenes that drive the cell to excessive or inappropriate proliferation. About 20% of known human tumors have been associated with activated oncogenes (Bishop, 1987 and Cotran, *et al.*, 1989). Alternatively, some cancers have been shown to arise due to the activation of genes that normally suppress cell proliferation (Friend, *et al.*, 1988). Thus, mutations that affect the normal functions of these genes would lead to the neoplastic transformation of cells.

Immune Response Against Tumor Cells:

Neoplastic transformation of normal cells results in significant phenotypic changes (Klein and Klein, 1985). Such changes may render them susceptible to be rejected by a healthy (competent) immune system. However, in many instances, transformed cells can escape the immune surveillance. In order for a transformed cell to instigate an immune response against it, has to fulfill the following conditions. First, it should express appropriate levels of the MHC class I molecules, as well as be able to process and present tumor specific antigens in the cleft of MHC molecules (Tonaka, *et al.,* 1988 and Urban, 1992). Also, the expression of molecules such as B7-1 or B7-2 on the tumor cells, which are ligands for CD28 and CTLA-4 on effector T cells, have been shown to be essential for the induction of immunity against the tumor (Chen, *et al.,* 1992; Matulonis, *et al.,* 1996; Townsend and Allison, 1993 and Zitvogel, *et al.,* 1996). Moreover, many tumors have been reported to secrete immunosuppressive cytokines (Janeway and Travers, 1994), some of which were reported to play an important role in the suppression of the immune response of the host against the tumor cells. Such cytokines include, TGF-β (Harada, *et al.,* 1995 and Torre-Amione, *et al.,*1990) and IL-10 (Suzuki, *et al.,* 1995). Furthermore, tumor cells that do not express molecules such as Fas have been reported to be more resistant to be killed by NK or CTLs (Gruss and Dower, 1995 and Rensing, *et al.,* 1995). Finally, some tumors may be inducing an immune response against transformed or foreign antigens on their surface, yet they evade the immune

system by continuously shedding these antigens or mutating them into new ones (Abbas, *et al.,* 1994).

Autocrine Growth Factors and Neoplasia:

Growth factors and growth factor receptors play a crucial role in cellular growth and proliferation to a degree that some of them are considered to be products of proto-oncogenes. Normally, growth factors are secreted by some cells to stimulate their targets which should express the growth factor receptor on their surface. The target cell may be at a distant site, neighboring cells or even the secreting cell itself. Thus, according to the location of the target cell, the effect of the growth factor is termed endocrine, paracrine, or autocrine respectively. Autonomous proliferation of the immortalized cells is presumed to be due to the following: auto- and paracrine growth stimulation, growth factor receptor abnormalities, abnormal signal transduction and self stimulation of an "initiator-replicon" system in DNA replication (Chiigira, *et al.,* 1990). In fact the role of autocrine growth in tumorigenic transformation is becoming increasingly evident (Chiigira, *et al.,* 1990; Lang and Burgess, 1990 and Walsh, *et al.,* 1991). In order for neoplastic growth to be associated with autocrine growth factor perturbation it must constitutively express the growth factor and/or the growth factor receptor (Jasmin, *et al.*,1990). Several myeloid and lymphocytic leukemias have been shown to have an autocrine growth factor involvement. Myeloma cells for instance, synthesize and respond to IL-6 and were shown to be inhibited *ex vivo* by anti-IL-6 mAbs (Kawano,*et al.*,1989). Also, IL-6 and GM-

CSF were reported to act as autocrine growth factors for chronic myelomonocytic leukemia cells (Everson, *et al.,* 1989). Moreover, adult T-cell leukemia (ATL) was shown to utilize interleukin-1 (IL-1)α as an autocrine growth factor to maintain its autonomous growth (Shirakawa, *et al.,* 1989). Also, a cell line derived from ATL patient was reported to secrete IL-2 and express IL-2Rs which stimulated autocrine growth and cellular proliferation *ex vivo.* Furthermore, the proliferation of these cells was inhibited by cyclosporin A (CsA) (Dautry-Varsat, *et al.,* 1988; Duprez, *et al.,* 1985; Hemar and Dautry-Varsat, 1990). In addition, human T-lymphotropic virus type 1 (HTLV-1), a provirus known to induce ATL, contains a unique sequence known as pX (containing the following regions $p40^{tax}$, $p27^{rex}$ and p21) which is thought to activate the IL-2 and IL-2R genes. The autocrine stimulation that results from the activation of these two genes is thought to play a role only in the initiation of the transformation. However, as the transformation progresses the cells lose the need for IL-2 expression yet they all seem to maintain the constitutive expression of IL-2R α chain (Nerenberg, *et al.,* 1987 and Yodoi and Uchiyama, 1992).

T Cell Autocrine Growth Factors:

Several cytokines have been shown to induce T cell proliferation including IL-2, IL-4 and interleukin 15 (IL-15) (Smith, 1988; Grabstein, *et al.,* 1994). The following is a review about their mechanism of action and their effect on other cells:

IL-2 and IL-2R:

IL-2 is considered the major T cell growth factor. It is a 14-17 KD glycoprotein produced mainly by $CD4^+$ cells and to a lesser extent by $CD8^+$ as mentioned earlier. It is the only factor capable of taking the T cell from early G1 phase into the S phase of the cell cycle. Also, IL-2 acts as an autocrine and paracrine growth factor for T cells. The activation of the IL-2 gene in T cells is triggered by signals that result from the binding of the TCR-CD3 complex to the foreign antigen in the context of self MHC as well as due to costimulatory signals initiated by the binding of CD28 on the T cell to B7 molecules on the APC (Abbas, *et al.,* 1994). As a result of these signals, a GTP binding protein is induced to activate an enzyme present in the inner face of the plasma membrane known as phospholipase C. Once activated, it can hydrolyze a plasma membrane phospholipid called phosphotidylinositol-4,5-biphosphate. As a result of this hydrolysis two compounds are produced: one is diacylglycerol and inositol-1,4,5-triphosphate. Diacylglycerol is believed to activate protein kinase C, while inositol-1,4,5-triphosphate acts to release Ca^{++} from its intracellular storage site, the endoplasmic reticulum. Ca^{++} binds to calmodulin which can then activate calmodulin dependent protein kinases (Smith, 1988 and Smith, 1989). The activation of protein kinases activates in turn several nuclear factors including: nuclear factor for activated T cells (NF-AT) (Rao, 1994), NF-κB (Thanos and Maniatis, 1995) and OCT-1 (De Grazia, *et al.,* 1994). In addition, activated protein kinases induce the transcription of c-*fos* proto-oncogene, the product of which binds to the product of c-*jun* proto-oncogene. The c-*jun* product is called AP-1. The binding of c-*fos* product to AP-1 results in the

formation of an active complex (Jain, *et al.,* 1992). Moreover, the costimulatory signals that result from the binding of CD28 to its ligand B7 molecule on the APC, lead to the activation of a unique nuclear factor , not well characterized yet, that binds to the IL-2 enhancer region (Umlauf, *et al.,* 1995). The activation of such nuclear factors allows them to bind to the enhancer region of the IL-2 gene, thus resulting in the transcription and the consequent translation of this cytokine (Ulman, *et al.,* 1990). The IL-2 mRNA appears 30 minutes following T cell stimulation and it keeps accumulating until it reaches a peak by 12 hours, after which it drastically declines. This decline results from the action of a specific ribonuclease that acts specifically on mRNA of the IL-2 gene, where it recognizes (AT) rich sequences at the 3' end of IL-2 mRNA (Hua, *et al.,* 1993). This rapid degradation of IL-2 message as well as the short half life of the translated protein *in vivo* (1-2 hours) ensures that the signal initiated by IL-2 remains transient (Smith, 1988 and Smith, 1989). It is also important to note that protein kinase activators such as phorbol myristate acetate (PMA), Ca^{++} ionophores (Smith, 1988 and Smith, 1989), and interactions through adhesion molecules such as MEL-14 and CD44 can mimic the activation through the TCR-CD3 complex (Seth, *et al.,* 1991).

Once translated and secreted outside the cell, IL-2 can bind to its receptors on the surface of the same cell that secretes it or other cells of the immune system. IL-2R consist of three distinct membrane components: α–chain (IL-2R α), β–chain (IL-2R β), and the γ–chain (IL-2R γ). IL-2R β and IL-2R γ but not IL-2R α belong to a newly identified super family of cytokine receptors. The binding of IL-2 to the different subunits of its receptor occurs at different affinities. The binding of IL-2 to IL-2R β and IL-2R γ is enough to trigger the

stimulation signals. Yet, in order to achieve the highest affinity binding, all three components must be present. The three subunits are termed according to their molecular weight IL-2R α (p55), IL-2R β (p75) and IL-2R γ (p64). All three subunits possess extracellular, transmembrane and cytoplasmic domains. The cytoplasmic domain of IL-2R β and IL-2R γ are thought to attribute to the IL-2 induced signaling, however, IL-2Rα cytoplasmic domain is not long enough to be involved in any signaling event (Minami, *et al.*,1993; Takeshita, *et al.*, 1992; Taniguchi and Minami 1993). Interestingly, the IL-2R β and IL-2R γ chains are shared by other cytokines. For example, the IL-2R β chain is utilized by both IL-2 and IL-15 while the IL-2R γ subunit is known as γ common (γc) chain since, it is shared by IL-2R, IL-4R, IL-7R, IL-9R and IL-15R (Keegan, *et al.*,1994; Giri, *et al.*, 1994 and Tangiguchi, 1995). The activation and proliferation signals that result from IL-2-IL-2R binding is mediated though the induction of several nuclear proto-oncogenes. This process occurs in two signal transduction pathways. One signal pathway is mediated via induction of the c-*myc* gene which is thought to be responsible for the cell division signal. The second pathway, results from the activation of $p21^{ras}$ and the induction of c-*fos*/c-*jun* genes. This is accomplished by the stimulation of the src-family kinases $p56^{lak}$ and $p59^{fyn}$ (Michiel, *et al.*, 1991; Minami, *et al.*, 1993 and Taniguchi, *et al.*, 1993).

These different signaling pathways which are activated as a result of IL-2 binding to its receptors reflect the potency of this growth factor. Therefore, besides its action on $CD4^+$ and $CD8^+$ T cells, IL-2 is known to activate natural killer (NK) cells and enhance their cytolytic functions. NK cells express IL-2R β similar to resting T cells but they do not express IL-2R α which necessitates the need for a high IL-2 concentration to stimulate through IL-2-IL-2R binding.

Therefore, IL-2 activation of NK cells to generate lymphokine activated killer cells (LAK), is usually done *ex vivo*. LAK cells have been used as a tool in adoptive immunotherapy of several cancers (Rosenberg and Lotze, 1986), but with limited success. Moreover, IL-2 is considered as a growth factor for B cells where it promotes immunoglobulin synthesis. Finally, IL-2 exerts its biological function on other IL-2R expressing cells like phagocytic macrophages, immature bone marrow cells as well as maturation of T cells in the thymus (Carding, *et al.*, 1991 and Smith, 1988).

IL-4 and IL-4R:

IL-4 is a 20 KD glycoprotein secreted mainly by Th2 cells and is used as an autocrine growth factor by these cells(Howard, *et al.*, 1982 and Smith, 1988). *Ex vivo*, however, it may be produced by some long term T cell lines when stimulated by antigens and APC or mitogens like concanavalin A (ConA). Moreover, a T cell lymphoma, EL-4, when stimulated with PMA will produce IL-4 (Paul and Ohard, 1987). The synthesis pathway of IL-4 is the same one as IL-2, since both of them can be induced by PMA, ConA and through the TCR (Fathman and Frelinger, 1983; Unanue, 1984 and Ziegler and Unanue, 1982). However, IL-4 appears later than IL-2, for IL-2 message starts appearing about 30 minutes after $CD4^+$ T cell stimulation (Lacour, *et al.*,1994) while the IL-4 message appears about 6 hours later (Cabtree, 1989). IL-4 receptor (IL-4R) expression has been shown to be augmented in a dose dependent fashion in response to IL-4 stimulation in murine T and B cells (Renz, 1991). The IL-4R is composed of three subunits 130KD, 75KD and 65KD (Achsah, *et al.*, 1994) the

latter being the (γc) of IL-2R. IL-4R is normally present at low density in resting cells. Also it has been shown that the expression of IL-2R is increased upon IL-4 stimulation (Dancescum, *et al.,* 1992). The details of the molecular events that follow the induction of the IL-4 signal are not fully understood. Thus far, such signals are reported to involve protein kinase C activation in B cells (Arruda and Ho, 1992). Also, It has been proposed that the IL-4-IL-4R mediated signal starts by the binding of IL-4 to the γc which could trigger kinase activation. Such activation is believed to result in the phosphorylation of tyrosine 497 in the IL-4R chain allowing it to interact with receptor-associated kinases (Keegan, 1994). Furthermore, it has been shown that IL-4 is incapable of taking the cell to the S phase unless it is already in late G1. This was evidenced by the fact that IL-4 synergizes with non-mitogenic concentrations of anti-immunoglobulin antibodies or phorbol esters to stimulate DNA synthesis and entry into the cell cycle (Klaus and Harnett, 1990).

IL-15 and IL-15R:

IL-15 is the most recently discovered cytokine which has been shown to exhibit IL-2 like activity. This growth factor was first isolated from a simian kidney epithelial cell line (Grabstein, *et al.,* 1994), and a human T cell leukemia cell line (Bamford, *et al.,* 1994 and Burton, *et al.,*1994). The activities shared by IL-2 and IL-15 are attributed to the fact that both growth factors interact with the same β and γ chains of the IL-2R (Bamford, *et al.,* 1994; Burton, *et al.,*1994; Giri, *et al.,* 1994 and Grabstein, *et al.,* 1994). Thus IL-15 can induce the same proliferation signal in T cells as IL-2. However, there is evidence for the

existence of a third subunit of the IL-15 receptor that is not shared by IL-2R. This suggests that there may be other biological activities of IL-15 that are not exhibited by IL-2 (Grabstein, *et al.,* 1994).

In conclusion, IL-2, IL-4 and IL-15 can be considered as progression factors since they can take the cell into the S-phase on the condition that the cell is already in the G1 phase. However, IL-2 is capable of acting on its target when the cell is in the early G1 phase and promoting it to late G1 and then S phase, while in order for IL-4 to activate, the cells have to be in late G1 phase. The potency of these growth factors made them the focus of several immunotherapeutic attempts to treat tumors. For example, IL-2 was used by Rosenberg's group to activate NK cells, and tumor infiltrating lymphocytes which were used in adoptive immunotherapy of several tumors but with partial success (Fearon, *et al.,* 1990; Foa, *et al.,* 1992 and Rosenberg and Lotze, 1986). In addition, when colon carcinoma cells were transfected with the IL-2 gene they were able to induce a protective CTL response against the tumor (Akashi, *et al.,* 1991) . Moreover, IL-4 treatment of a chronic myeloid monocytic leukemia inhibited the production of IL-6 and GM-CSF which led to suppression of spontaneous proliferation of these cells (Glombek, *et al.,* 1991). Also, IL-4 has been used on a human myeloid cell line where it upregulated the expression of human lymphocyte antigen DR (HLA-DR) belonging to class II MHC antigen (Rosenberg and Lotze, 1986). The enhanced expression of class II MHC on tumor cells makes them more immunogenic. Furthermore, in a study similar to IL-2 transfection, cells from a spontaneous murine renal tumor were transfected with IL-4 gene and reinjected into the tumor bearing mice. The parental tumor

was cured due to the enhanced $CD8^+$ T cell activity against the original tumor (Golumbek *et al.*, 1991).

Normally, T cell lines are difficult to establish. However, once established, they grow only when stimulated with antigen in the context of MHC and growth factors such as IL-2. Interestingly, among the many T cell lines grown in our lab, two were noticed to grow spontaneously without any further stimulation and addition of exogenous IL-2. These two cell lines which underwent spontaneous transformation *ex vivo* along with other *in vivo* originatedT cell lymphoma cell lines were the focus of this study.

Specific Aims:

Cancer development requires the simultaneous presence of several molecular perturbations and autocrine growth factor dysregulation is one such factor *ex vivo* In the current study we tested the hypothesis that dysregulation in the production and responsiveness to autocrine growth factors may be the sole cause for transformation and tumorigenesis of T lymphocytes. Using a variety of T cell lines transformed both *in vivo* and *ex vivo* we tested the above hypothesis. The specific aims were as follows:

1) To investigate whether the spontaneous *ex vivo* transformation of T cells observed in our lab resulted from the continuous production and responsiveness to autocrine growth factors. The role of T cell growth factors such as IL-2 was assessed by detecting constitutive expression of

these factors as well as by blocking the growth of these cell lines using growth factor-antagonists. It was also our aim, to test if such cells will induce tumors *in vivo* and whether they utilize autocrine growth factor, for their *in vivo* tumorigenesis.

2) To determine whether *in vivo* originated T cell lymphomas such as LSA and EL-4 employ autocrine growth factors (such as IL-2 or IL-4) for their growth and tumorigenesis.

3) To study the nature of the immune response against autocrine growth factor-induced lymphomas and examine the mechanism by which *in vivo* originating tumors can evade the immune system. This included delineating the role of CTL and NK cells in the rejection of such tumors as well as addressing whether such tumor cells produce any immunosuppressive cytokines such as TGF-β or IL-10. In addition, whether the expression of Fas-ligand on tumor cell would lead to apoptosis of activated T cells that come in contact with such tumor cells was investigated.

Chapter 2: Constitutive Activation of the Interleukin 2 Gene in the Induction of Spontaneous *Ex Vivo* Transformation and Tumorigenicity of T Cells.

Introduction:

The development of tumors is induced by several molecular perturbations, and it has become increasingly clear in recent years that dysregulation in autocrine growth factor production may constitute one such mechanism (reviewed in Lang and Burgess, 1991). In this process, a cell may continuously secrete and respond to a growth factor, resulting in tumorigenic transformation. Such a mechanism was first described for cells transformed by infection with transforming viruses (Todaro, *et al.*,1976 and Sporn and Todaro, 1980), in which the authors suggested that simultaneous and constitutive expression of a growth factor and its receptor leads to cell transformation through an autocrine mechanism.

Recently, several factors were shown to contribute to the tumorigenic transformation of cells. These factors included: platelet-derived growth factor (Clarke, *et al.*,1984), hemopoietic growth factors (Lang, *et al.*, 1985), epidermal growth factor/transforming growth factor α (Rosenthal, *et al.*, 1986 and Stern, *et al.*, 1987), fibroblast growth factor family (Rogeli, *et al.*, 1988), etc. In several instances, antibodies or antagonists to growth factors were shown to inhibit the

tumor cell proliferation *ex vivo* (Lang and Burgess, 1991). Such experimental models have suggested that autocrine stimulation can contribute to cell transformation and tumorigenesis.

In addition to the above-mentioned growth factors, it is not clear whether IL-2, a major T cell growth factor, is also involved in the tumorigenic transformation of T cells. In one study, IL-2-mediated autocrine growth was reported for a human T cell line isolated from a patient with malignant non-Hodgkin T cell lymphoma (Duprez, *et al.,* 1985). The IL-2 autocrine loop may also be involved in the development of T cell lymphomas or adult T cell leukemia by infection with HTLVs (Arima, *et al.,* 1986, Gootenberg, *et al.,* 1981, Maruyama, *et al.,* 1987 and Siekevitz, *et al.,* 1987). Furthermore, transfection with the IL-2 gene into T cells has been shown to result in autonomous growth, and such transformed cells are tumorigenic *in vivo* (Karasuyama, *et al.,* 1989 and Yamada, *et al.,* 1987).

However, despite the above examples, it is not proven that autocrine growth factor production is the sole cause for the origin or maintenance of neoplasia in animals (Lang and Burgess, 1991). In the current study, we made an interesting observation that T cells, upon prolonged *ex vivo* culture, can undergo spontaneous transformation resulting in autonomous growth and, furthermore, can induce tumors *in vivo*. Such spontaneous transformation resulted from continuous production of and responsiveness to autocrine IL-2. Furthermore, the growth of these tumors *in vivo* could be completely inhibited by administration of monoclonal antibodies against IL-2 or IL-2R. Our data demonstrate that dysregulation of IL-2 production can lead to spontaneous T cell transformation *ex vivo* and support the hypothesis that a similar pathway may

trigger some T cell transformation *in vivo*. The current study also suggests that extreme caution should be taken before performing clinical immunotherapy using *ex vivo*-cultured T cells to exclude any spontaneously transformed T cells.

Materials and Methods:

Mice: Adult, female, DBA/2, C57Bl/6, and nude mice were purchased from the National Institutes of Health (Bethesda, MD).

Antibodies: The mAbs against IL-2 were obtained from hybridoma S4B6; IL-2R, from PC61.5.3; CD3, from 145.2C11; and IL-4, from 11B11. The hybridomas were obtained from American Type Culture Collection (ATCC) and were grown *ex vivo* or *in vivo* as ascities. Ascities were induced in nude mice by intraperitoneal (i. p.) injection of 1 ml of pristane one week later, mice received i. p. injection of 50 x 10^6 hybridoma cells. Supernatant of hybridoma cells grown *ex vivo* was used after amicon concentration and precipitation with 50% ammonium sulfate and dialysis as described elsewhere (Kakkanaiah, *et al.,* 1990, Nagarkatti, *et al.*, 1990 and Nagarkatti, *et al.,* 1989).

Cell Lines: An autoreactive T cell line ($CD4^+$ $\alpha\beta TCR^+$) designated Auto D1.4 was established and characterized as described (Kakkanaiah, *et al.,* 1990 and Nagarkatti, *et al.*, 1989). This cell line was established and maintained by culturing $CD4^+$ T cells from DBA/2 mice with irradiated syngeneic splenic adherent cells in the presence of recombinant human IL-2 (kindly provided by Hoffmann-La Roche) at 50 units/ml. The cell line was maintained by

subculturing the cells twice a week. The cell line underwent spontaneous transformation approximately 1 year after establishment and started growing autonomously in culture in the absence of exogenous IL-2 or accessory cells. Such a transformed cell line was designated Auto D1.4T.

PE-9 is a $CD8^+$ $\alpha\beta TCR^+$ cytotoxic T cell line that was established from LSA tumor-bearing mice (Seth, *et al.,* 1991). The establishment of this cell line was achieved by purifying T cells from spleens of 1,3-Bis (2-chloroethyl)-1-nitrosourea (BCNU) -cured LSA C57BL/6 mice. Such cells, were grown in the presence of mitomycin (Sigma Chemical Co., St. Louis, MO)-treated syngeneic APCs, LSA and 7.5% T cell growth factor (TCGF) (Selvan *et al.,* 1991). After a few months, this cell line was characterized to be a cytotoxic T cell line specific to LSA tumor and was maintained in culture by addition of exogenous IL-2 (50 units/ml) and occasional stimulation with irradiated LSA tumor cells (Seth, *et al.,* 1991). This cell line also underwent spontaneous transformation 6 months after its establishment and started growing autonomously in the absence of exogenous IL-2 and was designated PE-9T. When tested for adhesion molecule expression, Auto D1.4 was found to express low densities of CD4 but not CD3- or $\alpha\beta TCR$, whereas PE-9T cells expressed CD44, CD45R, and Mel-14 as described (Seth, *et al.,* 1991) but failed to express J11d, CD8, and CD3- $\alpha\beta TCR$. The transformed cell lines were maintained by culturing them in tissue culture flasks in RPMI-1640 supplemented with 10% (vol/vol) fetal bovine serum (Atlanta Biologicals, Norcross, GA), 10 mM Hepes, 1 mM glutamine, gentamicin sulfate at 40 μg/ml, and 50 μM 2-mercaptoethanol at a cell density of 2×10^5 cells/ml.

Cell Proliferation: The proliferation of cells was measured by the uptake of [^{3}H]thymidine (Nagarkatti, *et al.,* 1989) as well as by counting viable cells using trypan blue dye exclusion. For the [^{3}H]thymidine uptake assay, the cells were cultured in 96 well tissue culture plates as described above and were pulse labled with 0.1 μCi of [^{3}H]-thymidine followed by cell harvesting 8 hours later. The radioactivity was measured using a liquid scintillation counter. In experiments studying the effect of cyclosporin A (CsA) (kindly supplied by Sandoz Pharmaceutical) on cell proliferation, CsA was prepared by dissolving 1 mg of CsA in 0.1 ml of ethanol and 0.02 ml of tween 80, followed by the addition of 1 ml of RPMI-1640. The medium used for dissolving the highest concentration of CsA was used as a vehicle control.

PCR Analysis of IL-2 Gene Expression: The PCR method was employed to study whether the transformed cells constitutively expressed the IL-2 gene as described (Hammond, *et al.,* 1993). Total RNA was extracted from the cells at various time intervals after fresh subculture and reverse transcribed. The resulting cDNA samples were subjected to PCR specific amplification using synthetic oligonucleotide primers for IL-2 or β-actin as an internal control. The primers for β-actin were 5'-TATCCTGACCCTGAAGTACCCCATT-3' and 5'-AGCACAGCTTCTCTTTGATGTCACG-3', and the primers for IL-2 were 5'-ATGTACAGCATGCAGCTCGCATC-3' and 5'-GGCTTGTTGAGATGATGCTTT-ACA-3'. The PCR product was electrophoresed through a 1.5% agarose gel containing ethidium bromide. The demonstration of a single 464- and 502-bp band was considered to be indicative of the expression of β-actin and IL-2 genes respectively.

IL-2 Antisense Oligonucleotide: Antisense oligonucleotide was synthesized in our department facility using the sequence 5'-GACA-AGGAGCACAAG-3', which was complementary to the nucleotides of IL-2 mRNA, 35 bases downstream of the initiation codon. The lack of thymidine was chosen to avoid interference with [^{3}H]thymidine incorporation assay used to measure cell proliferation.

***In Vivo* Tumor Induction:** The transformed cells (10^6) were injected into mice subcutaneously, and the tumor growth was monitored in groups of untreated or those treated with mAbs or CsA by surgically removing the tumor and measuring the weight. In all experiments, groups of three to four mice were used.

Statistical Analysis: The inhibition of cell growth or tumor growth caused by treatment with mAbs or drug was compared to the control groups using Student's *t* test, and *p* values <0.05 were considered to be statistically significant.

Results:

Aberrant production of and responsiveness to IL-2 is responsible for inducing the *ex vivo* proliferation of Auto D1.4T cell line:

The transformed T cell line, Auto D1.4T, was derived from a well-characterized $CD4^+$ $\alpha\beta TCR^+$ autoreactive T cell line, Auto D1.4 (Kakkanaiah, *et al.*, 1990 and Nagarkatti, *et al.*, 1989). This cell line had been established by culturing $CD4^+$ T cells from DBA/2 mice with irradiated syngeneic splenic adherent cells in the presence of IL-2 (Kakkanaiah, *et al.*, 1990 and Nagarkatti, *et al.*, 1989). When the Auto D1.4 line underwent spontaneous transformation and started growing autonomously in culture, we first addressed whether this resulted from aberrant production of and responsiveness to IL-2. As shown in Figure 2.1, the addition of mAbs against IL-2 or IL-2R, but not normal IgG, significantly inhibited the *ex vivo* proliferation of Auto D1.4T as measured by [^{3}H]-Thymidine incorporation assay. Also, addition of CsA to cultures , which is known to inhibit lymphokine secretion by T cells, caused a dose-dependent inhibition of cell proliferation *ex vivo* (Figure 2.2). Furthermore, when antisense oligonucleotides specific for IL-2 were added to *ex vivo* cultures, there was inhibition of specific proliferation of Auto D1.4T but not that of a nonspecific mastocytoma tumor cell line, P815, in which IL-2 did not play any role in cell proliferation (Figure 2.3). It was found that IL-2 antisense oligonucleotides at 50-100 μM concentrations inhibited cell proliferation at 48 hr, and by 72 hr all cells died, whereas similar concentrations of antisense oligonucleotide had no effect on the proliferation of P815 cells. These data together suggested that IL-2 acted as an autocrine growth factor and was responsible for the *ex vivo* transformation of Auto D1.4T.

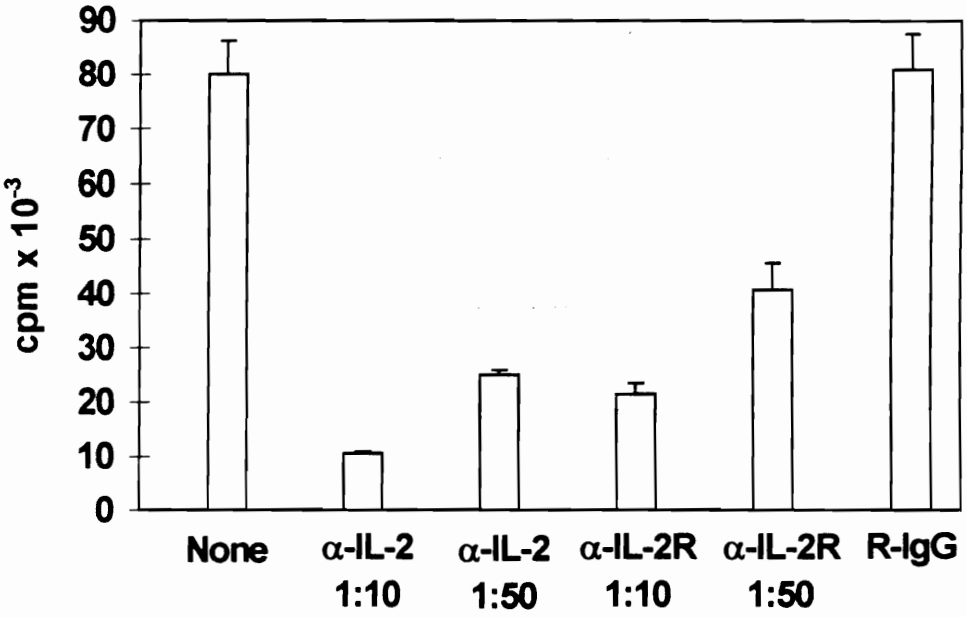

Fig 2.1. Inhibition of *ex vivo* proliferation of Auto D1.4 cells by antibodies against IL-2 and IL-2R. Cells were cultured in 96-well tissue culture plates at concentration of 5 x 10^4 cells / well in 0.2 ml of complete RPMI-1640 medium supplemented with 10% fetal calf serum. To these cultures, mAbs against IL-2 (αIL-2) or IL-2R (αIL-2R) were added at 1:10 or 1:50 final dilution. Normal rat IgG (RIgG) was used at a concentration equivalent to the lowest dilution 50 μg/ml as a negative control. The cells were cultured for 40 hr, pulsed with 0.1 μCi (1 Ci =37 Gbq) of [^{3}H]thymidine, followed by cell harvesting 8 hr later. The cultures were carried out in triplicate and means ± standard error (SE) were plotted. The results are from one representative experiment out of three.

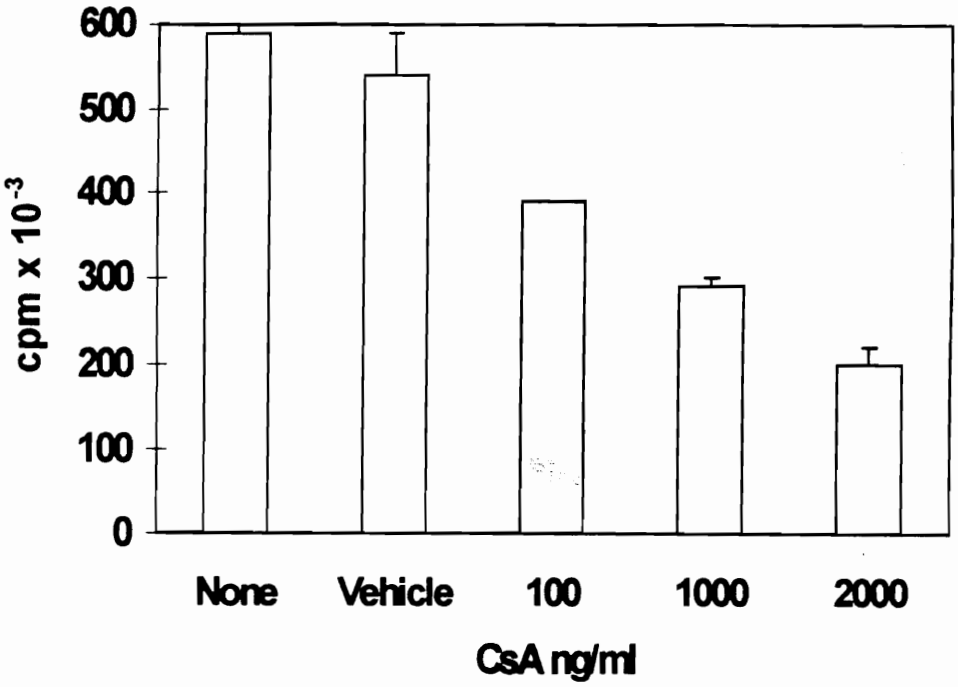

Fig. 2.2. Inhibition of *ex vivo* proliferation of Auto D1.4T cell in the presence of CsA. Cells were cultured as described in Fig. 3.1, to these cultures, CsA at 100, 1000, 2000 ng/ml concentration was added. Also, a group of cultures was left untreated and another group was treated with the vehicle for CsA at the highest concentration. 40 hrs later, cells were pulsed with 0.1 μCi (1 Ci =37 Gbq) of [^{3}H]thymidine, followed by cell harvesting 8 hr later. The cultures were carried out in triplicate and means ± standard error (SE) were plotted. The results are from one representative experiment out of three.

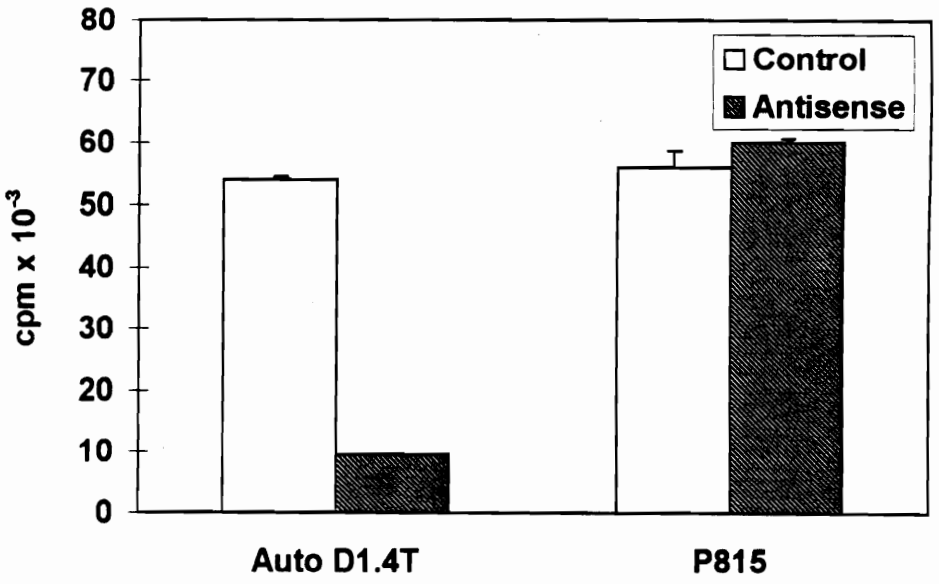

Fig. 2.3. Inhibition of *ex vivo* proliferation of Auto D1.4T but not P815 cells in the presence of IL-2 antisense oligonucleotides. Auto D1.4T and P815 cells were cultured as described in Fig. 2.1, in the presence or absence of antisense oligonucleotide (50 μM) directed against the IL-2 mRNA. After 40 hrs incubation, cells were pulsed with 0.1 μCi (1 Ci =37 Gbq) of [^{3}H]thymidine, followed by cell harvesting 8 hr later. The cultures were carried out in triplicate and means ± standard error (SE) were plotted. The results are from one representative experiment out of three. The results are from one representative experiment out of three.

Constitutive expression of the IL-2 mRNA in the transformed cell line Auto D1.4T:

We also addressed whether the transformed Auto D1.4T cell line constitutively expressed the IL-2 gene by using PCR analysis. The data shown in Figure 2.4 indicated that Auto D1.4T expressed the mRNA for the IL-2 gene at all time intervals tested such as at 12, 24, 36 and 48 hr of culture. In contrast, naive splenic T cells failed to express IL-2 gene and did so only upon activation through CD3.

It should be noted that when attempts were made to detect IL-2 in the culture supernatants of the Auto D1.4T cell line, we were unable to detect IL-2. However, upon irradiation of the cells at 2000 rads (1 rad = 0.01 Gy), to prevent the cells from dividing, we were able to detect IL-2 within 12 hr in the culture supernatants. These data suggested that the cell line was utilizing the IL-2 that was being produced. The constitutive expression of the IL-2 gene further supported the autocrine hypothesis of T cell transformation.

The *In vivo* tumorigenecity of Auto D1.4T transformed cells in nude mice:

Next, we tested whether Auto D1.4T could induce tumors *in vivo*. Injection of Auto D1.4T into normal syngeneic mice by any route failed to induce tumors. Considering that Auto D1.4T may be immunogenic, we injected it into immunodeficient nude mice. Interestingly, subcutaneous injection of Auto D1.4T induced a solid tumor at the site of the inoculation (Figure 2.5). Furthermore, intravenous or intraperitoneal injections of Auto D1.4T also led to development of tumors. It should be noted that Auto D1.4T is highly immunogenic in normal mice and triggers cytotoxic T-cell responses (chapter 4).

This may also be facilitated by the constitutive secretion of IL-2 by Auto D1.4T cells *in vivo*. Earlier studies using T-cell lines transfected with IL-2 gene have demonstrated that such cells can grow only in nude mice (Karasuyama, *et al.*, 1989) similar to the current study or in both normal syngeneic or nude mice (Yamada, *et al.*, 1987).

The *in vivo* growth of Auto D1.4T cells was inhibited by IL-2 autocrine loop antagonists:

To investigate whether the *in vivo* tumorigenesis of Auto D1.4T cells was also mediated by autocrine growth factor production, we injected mAbs against IL-2 and IL-2R, a combination of mAbs against IL-2 and IL-2R, and CsA. The data shown in Figure 2.6 (*A* and *B*) demonstrate that injections of antibodies (60 μg per mouse) against IL-2 and IL-2R significantly inhibited the growth of Auto D1.4T-induced tumors in nude mice. Furthermore, a combination of antibodies against IL-2 and IL-2R induced synergistic inhibition of tumor growth when compared to injections with antibodies against IL-2 or IL-2R alone. Also, injections of higher concentrations (120 μg per mouse) of mAbs against IL-2 completely prevented the growth of Auto D1.4T *in vivo* (Figure 2.7). These data clearly suggest that IL-2 is indeed used by Auto D1.4T to grow and induce tumors *in vivo* and that it is the primary cause of tumorigenesis. It was also noted that CsA (50 μg/kg) could inhibit the growth of the tumor *in vivo* and furthermore, when given along with anti-IL-2 antibodies (60 μg per mouse), completely prevented the tumor cells from growing (Figure 2.7).

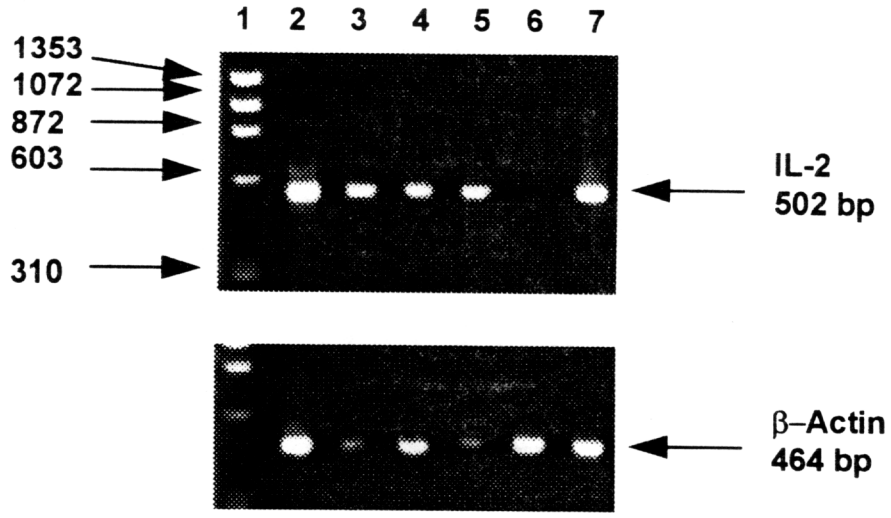

Fig. 2.4. Constitutive IL-2 gene expression in Auto D1.4T cells. Total RNA was extracted from the Auto D1.4T cells at 12, 24, 36, or 48 hr of *ex vivo* culture and reverse transcribed, and cDNA samples were subjected to PCR amplification using synthetic oligonucleotide primers for IL-2 or β-actin as an internal control. The PCR product was electrophoresed through a 1.5 % agarose gel containing ethidium bromide. Lane 1, ΦX 174 *Hea* III digest *(*molecular size marker); lane 2-5, Auto D1.4T cells cultured *ex vivo* for 12, 24, 36, and 48 hrs, respectively, and screened for IL-2 expression; lane 6, normal unstimulated spleen cells from C57BL/6 mice as a negative control; lane 7, spleen cells stimulated for 8 hr with anti-CD3 mAbs as a positive control. The demonstration of a single464- and 502-bp band was considered to be indicative of the expression of β-actin and IL-2 genes, respectively.

Fig. 2.5. *Ex vivo*-transformed Auto D1.4T cells can induce tumors when injected into nude mice. Mice were injected with 1x 10^6 transformed viable Auto D1.4T cells, resulting in tumor growth *(Right)* or the normal Auto D1.4 cells *(Left)* as a control, which failed to induce tumors. The figure depicts tumor growth 60 days after tumor cell inoculation.

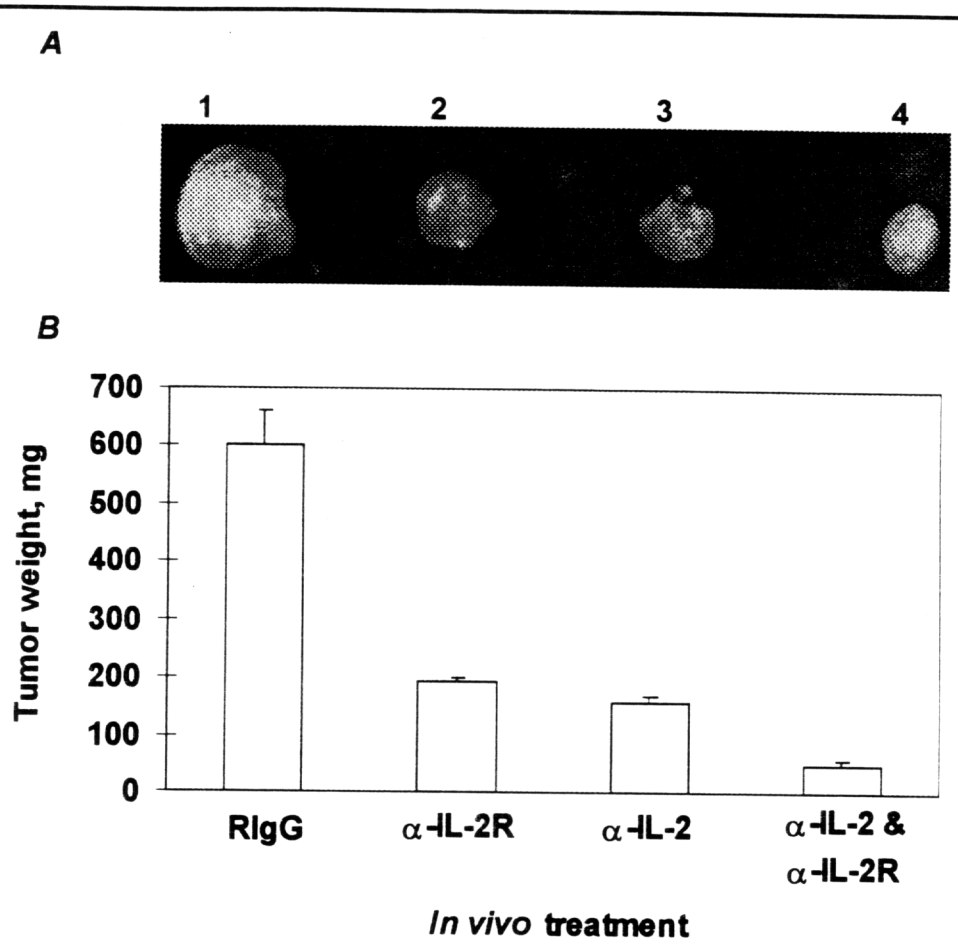

Fig. 2.6. Auto D1.4T cells induce tumors in nude mice, that can be inhibited by mAbs against IL-2 and IL-2R. *(A)* Representative experiment showing the size of subcutaneous tumor nodule in control nude mice (tumor 1) or in nude mice treated with antibodies against IL-2R (2), IL-2 (3) or a combination of IL-2R plus IL-2 (4) as described below. *(B)* Groups of three mice injected with auto D1.4T cells were treated with daily intraperitoneal injections of normal rat IgG (RIgG) as a control or with mAbs against IL-2 and IL-2R. All antibodies were injected once a day for 14 days at a dose of 60 μg per mouse, after which the solid tumor was excised and weighed.

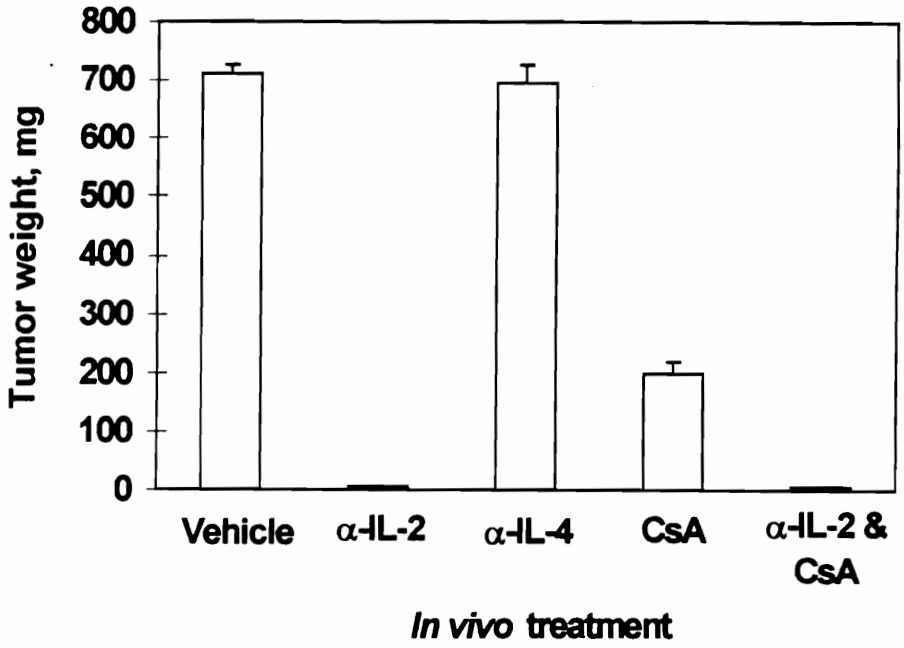

Fig 2.7. Auto D1.4T cell- induced tumors can be inhibited with anti-IL-2 antibodies and CsA. Nude mice injected with the transformed Auto D1.4T cells were treated as describe in Fig. 3.6 with mAbs against IL-2 (120 μg per mouse), IL-4 (60 μg per mouse), CsA (50 mg/kg), or a combination of CsA (50 mg/kg) and anti-IL-2 mAbs (60 μg per mouse). CsA was dissolved in olive oil (vehicle) and injected once a day for 14 days by intraperitoneal route.

Discussion:

Two distinct mechanisms of autocrine stimulation have been proposed (Lang and Burgess, 1991). First, the cell may secrete the growth factor, which may bind to the external receptor expressed on the cell membrane, leading to growth induction. Second, the growth factor may interact with an internal receptor expressed within the cell, leading to cell division. In the first mechanism, therefore, mAbs against the growth factor or its receptor should be able to inhibit cell proliferation, while, in the second mechanism, such an inhibition would not be effective. Based on the current study, we suggest that the transformed cells used the first mechanism described above. However, it is possible that in other instances of T cell transformation, the second mechanism may be operative, which can be detected only by looking at constitutive expression of the IL-2 gene or the IL-2 receptor gene and the ability of antisense oligonucleotides to block both *ex vivo* and *in vivo* cell proliferation.

Recently, we noted that another well characterized $CD8^+$ cytotoxic T cell line , PE-9 (Seth, *et al.,* 1991), also underwent spontaneous transformation *ex vivo* resulting in autonomous growth. The transformation of this cell line also resulted from autocrine IL-2-mediated self-stimulation as evidenced by the fact that this transformed cell line was inhibited from growing *ex vivo* in the presence mAbs against IL-2, IL-2R, or antisense oligonucleotides specific for IL-2 . Also, the transformed cells induced tumors *in vivo* when injected into nude mice. Thus, isolation of T cell lines which underwent spontaneous transformation *ex*

vivo, on two independent occasions, suggests that such an occurrence may not be uncommon during *ex vivo* culture.

It is becoming increasingly clear that cells can escape normal growth regulation and became transformed by constitutively producing growth factors as well as responding to the same. Such a mechanism, designated "autocrine growth stimulation", has been tested *ex vivo*, and several growth factors has been characterized that appear to be involved in cell transformation (Clarke, *et al.,* 1984; Lang, *et al.,*1985; Rosenthal, *et al.,* 1986 and Stern, *et al.,* 1987). The possibility that IL-2 may be involved in autocrine cell transformation was suggested using a human T cell lymphoma line derived from a patient with adult T cell leukemia (Duprez, *et al.,*1985). Further studies in adult T cell leukemia patients as well as T cell lines infected with HTLV have suggested that autocrine IL-2-induced self-stimulation, at least in some instances, appears to play an important role in T cell transformation (Arima, *et al.,* 1986; Gootenberg, *et al.,* 1981; Maruyama, *et al.,* 1987 and Siekevitz, et al., 1987). Also, a recent study suggested that IL-2 transcript and protein were detectable at the single-cell level in most cells of HTLV-1-infected T cell lines (Farcet, *et al.,* 1991). Interestingly, transfection of T cell lines with the IL-2 gene has also shown to result in autonomous growth, cell transformation and tumorigenicity *in vivo* (Karasuyama, *et al.,* 1989 and Yamada, *et al.,* 1987). Such studies have suggested that autocrine IL-2-mediated self-stimulation may play a crucial role in T cell transformation.

In the current study, the exact mechanism that led to constitutive production of IL-2 in transformed cells is not clear. It should be noted that the mutants do not express CD3-$\alpha\beta$TCR, thereby ruling out that activation through

other adhesion molecules may not be involved. It is possible that the constitutive production of IL-2 may result from mutations or hyper-expression of cellular proto-oncogenes, which may act as transcription factors regulating IL-2 gene expression. Alternatively, it may result from retroviral infection, particularly because HTLV is believed to trigger autocrine IL-2 stimulation (Arima, *et al.,* 1986; Gootenberg, *et al.,* 1981; Maruyama, *et al.,* 1987; Siekevitz, et al., 1987 and Farcet, *et al.,* 1991).

Despite examples in which IL-2 has been shown to be involved in cell transformation, it is not proven that IL-2 or other growth factor production is totally responsible for the maintenance of neoplastic transformation in animals (Lang and Burgess, *et al.,* 1990). To the best of our knowledge, the current study demonstrates for the first time two important facts about the role of autocrine growth factors in tumorigenic transformation of T cells. First, our studies demonstrate that T cell lines can undergo spontaneous transformation during long-term *ex vivo* culture and divide autonomously resulting from constitutive production of and responsiveness to autocrine IL-2. Furthermore, such cells can induce tumors in an immunodeficient host, which can be completely inhibited by mAbs against IL-2, suggesting that the autocrine IL-2 circuit is sufficient for the *in vivo* growth and maintenance of tumorigenicity. Our data are also important because they stress the need to impose caution when employing immunotherapy currently being used or recommended against cancer (Rosenberg, *et al.*, 1986) and viral infections (Riddell, *et al.,* 1992) particularly in immunodeficient patients, using *ex vivo* cultured T cells to ensure that the adoptively transferred T cells are devoid of spontaneously transformed mutants. On the other hand, our studies demonstrating that the transformed T

cells would not grow in immunocompetent hosts suggest that immunotherapy may be safe and that such spontaneously originating tumors *in vivo* may be rejected in an immunocompetent host. Further studies on T cell malignancies originating spontaneously *in vivo* should yield useful information on the role of IL-2 or other T cell growth factors in the spontaneous T cell transformation *in vivo* and possible approaches to treat such malignancies using antibodies, drugs, or antisense oligonucleotides specific to the growth factor.

Chapter 3: Evidence for the Participation of Interleukin-2 and Interleukin-4 in the Regulation of Autonomous Growth and Tumorigenesis of Transformed Cells of Lymphoid Origin.

Introduction:

There is increasing evidence to suggest that tumorigenic transformation of cells may result from aberrant regulation of autocrine growth factor production (reviewed in Lang and Burgess,1990). In this autocrine mechanism, a cell may constitutively secrete and respond to the growth factor, resulting in tumorigenic transformation. Such a mechanism was first described for cells transformed by infection with transforming viruses (Sporn and Todaro,1980; Todaro and De Larco, 1976). Recently, several factors were shown to contribute to the tumorigenicity of cells, these factors include: platelet derived growth factor (Clarke *et al.*, 1984), epidermal growth factor/transforming growth factor–α (Normanno *et al.*, 1994; Rosenthal *et al.*, 1986; Stern *et al.*, 1987), fibroblast growth factor family (Rogelj *et al.*,1988), IL-11 (Kobayashi *et al.*, 1993) ,IL-6 (Goto *et al.*, 1995), TNF (O'Connell *et al.*, 1995) etc. In addition to the above, IL-2 a major T cell growth factor has also been implicated in the tumorigenic transformation of T cells (Duperz *et al.*, 1985). The IL-2 autocrine loop may

particularly play an important role in the development of T cell lymphomas or adult T cell leukemias by infection with human T cell lymphotrophic viruses (HTLVs) (Arima *et al.*, 1986; Gootenberg *et al.*, 1981; Maruyama *et al.*, 1987). Moreover, transfection of the IL-2 gene into T cells has also been shown to result in autonomous growth and tumorigenesis *in vivo* (Karasuyama *et al.*, 1989; Yamada *et al.*, 1987). In an earlier study, we reported that some normal T cell lines, upon long term culture, underwent spontaneous transformation *ex vivo* and started growing autonomously (Seth *et al.*, 1991). Such cells constitutively expressed IL-2 and IL-2R genes and could be inhibited from growing *in vivo* and *ex vivo* by agents that blocked IL-2 production or IL-2R expression, thereby suggesting that dysregulation in IL-2 production was sufficient to trigger transformation and tumorigenesis *in vivo* (Seth *et al.*, 1991).

IL-2R has been shown to be expressed not only by lymphoid cells but also by non-lymphoid cells (Hicks *et al.*, 1991; Plaisance *et al.*, 1992; Saneto *et al.*, 1986; Weidmann *et al.*, 1992). In fact, in a recent study which screened a variety of human solid tumor cell lines, it was reported that in 36 of the 38 malignant tumors examined, IL-2R was constitutively expressed (McMillan *et al.*, 1995). These data suggested that IL-2R expression may be characteristic of most human malignant cells and that IL-2 may play a role in malignant cell proliferation through IL-2R (McMillan *et al.*, 1995). Although, IL-2R seems to be expressed on a wide variety of tumors, whether such tumor cells also constitutively express IL-2 or other cytokines which can act through the IL-2R, such as IL-4, IL-7, IL-9 and IL-15 and be responsible for autocrine stimulation and growth, is not clear . In the current study, we systematically examined four murine transformed cell lines of both T and non-T cell origin which constitutively

expressed IL-2R and delineated whether these cell lines expressed IL-2 and IL-4 genes constitutively and whether such cells were dependent on IL-2 or IL-4 for autocrine growth. Our data demonstrate that all four cell lines screened expressed IL-2R and IL-4R genes. However, only one of the *in vivo* originated tumor cell lines of T cell origin, was dependent on IL-2 and IL-4 for *ex vivo* autocrine growth as well as *in vivo* tumorigenicity. These studies demonstrate that dysregulation in T cell growth factor production and responsiveness can lead to cell transformation and tumorigenesis.

Materials and Methods:

Mice: Adult male athymic Nu/Nu nude mice were purchased from the National Institutes of Health (Bethesda, MD).

Tumor Cell Lines: LSA, is a radiation leukemia virus (Rad LV) induced T cell lymphoma syngeneic to C57BL/6 mouse (Silini *et al.*, 1965). This cell line has been extensively characterized and used in our previous studies (Nagarkatti *et al.*, 1990). EL-4, is a chemically induced lymphoma also syngeneic to C57BL/6 mouse (Gorer, 1950). P815 is a mastocytoma syngeneic to DBA/2 mouse (Lundak *et al.*, 1973). PE-3 is a $CD8^+$ $\alpha\beta TCR^+$ cytotoxic T cell line that was established from LSA tumor bearing mice (Hammond-McKibben *et al.*, 1995). This cytotoxic T cell line was specific to LSA tumor and was originally maintained in culture by addition of exogenous IL-2 (50u/ml) and occasional stimulation with X-irradiated LSA tumor cells (Hammond-McKibben *et al.*, 1995).

About 6 months after its establishment, this cell line underwent spontaneous transformation and started to grow autonomously in the absence of exogenous IL-2 and was designated PE-3T. The cell line was tumorigenic *in vivo* when injected into nude mice. All tumor cell lines were maintained by culturing them in tissue culture flasks, in RPMI 1640 medium with additional supplements and 10% fetal bovine serum as described (Nagarkatti *et al.*, 1994).

Antibodies: The mAbs used were in culture supernatants as described in detail elsewhere (Hammond *et al.*, 1993; Nagarkatti *et al.*, 1994; Seth *et al.*, 1991 and in chapter 2). The mAbs against IL-2 were obtained from hybridoma S4B6; anti-IL-2R from PC61.5.3 and 7D4; and anti-IL-4 from 11B11. The 11B11 hybridoma was kindly provided by Dr. William Paul, National Institutes of Health (NIH), Bethesda. MD. All other hybridomas were purchased from American Type Culture Collection (ATCC). All mAbs were concentrated using Amicon filtration as described (Nagarkatti *et al.*, 1994).

Flow cytometric analysis of IL-2R expression: The expression of IL-2R by tumor cells was determined by immuno-fluorescence (Hammond-McKibben *et al.*, 1995). To this effect, $1x10^6$ tumor cells were washed in PBS containing 0.1 % sodium azide and incubated at 4° C for 30 minutes in a test tube with Abs directed against IL-2R (7D4). After washing the cells twice, fluorescein isothiocyanate-(FITC)-conjugated secondary Ab consisting of $F(ab')_2$ fragments of anti-rat IgM (Cappel, Durham, NC) was added and the cells were incubated on ice for 30 minutes. The cells were washed twice and analyzed using a flow cytometer (Epics V, model 752: Coulter, Hialeah, FL). The negative controls

consisted of cells incubated with normal rat IgM followed by FITC conjugated secondary Abs.

Cell Proliferation: The proliferation of the cells in culture was studied by enumerating viable cells using a trypan blue dye exclusion assay (Nagarkatti *et al.*, 1994). The cells were cultured in 96-well tissue culture plates at a concentration of $5x10^4$ cells/well in 0.2 ml complete RPMI-1640 medium supplemented with 10% fetal calf serum. To these cultures, various concentrations of mAbs against the growth factors or their receptors or antisense oligonucleotides were added. The anti-IL-2, anti-IL-4 and anti-IL-2R mAbs were tested at 1:2, 1:4 and 1:10 dilutions of concentrated culture supernatants and antisense oligonucleotides at 25, 50 and 100 μM concentrations. These concentrations were based on their ability to inhibit the IL-2 and IL-4 induced proliferation of HT-2 cells and autonomous growth of IL-2 autocrine-dependent proliferation of Auto D1.4T cells (Nagarkatti *et al.*, 1990; Nagarkatti *et al.*, 1994). The cultures were incubated at 37° C for 1-3 days and every 24 hours the cells were enumerated for viability and growth. To accommodate for the growth, the cultures were split every 24 hours and all cultures received fresh medium and additional growth factor antagonists.

In addition, in some experiments, cell proliferation was also measured by a [^{3}H]-thymidine incorporation assay as described (Nagarkatti *et al.*, 1994). Briefly, the cells were cultured in 96 well tissue culture plates as described above and were pulse labled with 0.1 μC of [^{3}H]-thymidine followed by cell harvesting 8 hours later. The radioactivity was measured using a liquid scintillation counter. In experiments studying the effect of CsA (kindly provided

by Sandoz Pharmaceutical) on cell proliferation, CsA was prepared by dissolving 1 mg of CsA in 0.1 ml ethanol and 0.02 ml of Tween-80 followed by addition of 1 ml of RPMI-1640. The medium used for dissolving the highest concentration of CsA was used as a vehicle control (Nagarkatti *et al.,* 1994).

RT-PCR analysis of cytokine and cytokine receptor gene expression: The reverse transcribed PCR (RT-PCR) method was employed to study whether the transformed cells constitutively express IL-2, IL-4, IL-2R, IL-4R genes as described in detail else where (Nagarkatti *et al.,* 1994; Hammond *et al.*, 1993). To this effect, total RNA was extracted from the cells at various time intervals of cell culture and reverse transcribed. The cDNA samples were subjected to PCR amplification using synthetic oligonucleotide primers for various cytokine and their receptor genes, with β-actin serving as an internal control. The oligonucleotide primers used to amplify the IL-2 gene were: 5'-TTCAAGCTCCACTTCAAGCTCTACAGCGGAAG-3' and 5'-GACAGAAGGCTA-TCATCTCCTCAGAAAGTCC-3'; for the IL-4 gene, 5'-CCAGCTAGTTGTCAT-CCTGCTCTTCTTTCTCG-3' and the 5'-CAGTGATGTGGACTTGGACTCATT-CATGGTGC; for the IL-4R gene, 5'-GCTTCTCTGACTACATCCGCACT-TCCACG-3' and the 5'-TTGACTCCTGGCTTCGGGTCTGC-TTATCC-3'; for β–actin gene, 5'-TATCCTGACCCTGAAGTACCCCATT-3' and 5'- AGCAC-AGCTTCTCTTTGATGTCACG-3' and for IL-2R β-chain p75, 5'-TGGAGAA-CAGGATGACTACTGTGC-3' and 5'-GACCAGGAAGTAGCAAATGGAGTTC-3'. The primers for IL-2R and β–actin were synthesized by Oligos etc (Wilsonville, OR) and for IL-2, IL-4 and IL-4R were purchased from Clontech (Palo Alto, CA). The PCR product was electrophoresed through a 1.5% agarose gel containing

ethidium bromide. The demonstration of a single 464-, 413-, 384-, 545-, 792-bp band was considered to be indicative of the expression of β–Actin, IL-2, IL-4, IL-2R β-chain p75 and IL-4R genes respectively.

Antisense Oligonucleotides: The antisense oligonucleotides specific for IL-2, IL-4, IL-2R β-chain p75 and IL-2R γ-chain p64 genes were designed to hybridize at sequences immediately down stream from the initiation codon of the mRNA of different genes. Since different cell lines may have different exonuclease or endonuclease activities, the selected oligonucleotides were modified to be nuclease-resistant by making them 100% phosphorothioated (Crooke *et al.*, 1991; Iversen *et al.*, 1991). Such a modification is important especially when the antisense is used on different cell lines because, they might have varying nuclease activity. The antisense oligonucleotide sequences for IL-2 gene was 5' GAGCTGCATGCTGTA 3'; for IL-4 gene, 5' CTGGGGGTTGAGACC 3'; for IL-2Rβ gene, 5' AAGAGCTATGGTAGC 3' and for IL-2Rγ, gene 5' CAATAATAGTTTCAA 3'. The oligonucleotides were dissolved in Tris-EDTA buffer at pH 7.4 and added directly at different concentrations to cultures of tumor cells. In addition several control oligonucleotides were used in the current study. One such control consisted of a 15 base pair oligomer (mer) having the same base composition as the IL-2 antisense oligonucleotide but a randomized sequence. The other control was an 18-mer, which had non-specific base composition. The sequences for these control oligonucleotides are: 5' CTGAGATGGTCTAGC 3' and 5' TGGATCCGACATGTCAGA 3' respectively.

***In Vivo* Tumor Induction:** The LSA or P815 tumor cells (0.5×10^6) were injected into nude mice subcutaneously. These mice received 650 μg of anti-IL-2 and 650 μg of anti-IL-4 mAbs once every other day intraperitoneally for 16 days. The control mice received similar concentrations of normal rat-IgG. The tumor growth was monitored daily and after 17 days the mice were sacrificed and the tumor growth was assessed by surgically removing the tumors and measuring the weight. In all experiments, groups of four mice were used. The tumor size in the experimental group was compared to the controls using Student's *t* test and deferences with $p < 0.05$ were considered to be statistically significant.

Results:

Detection of IL-2 receptor on the tumor cell lines:

In the current study we used four tumor cell lines, two of which were previously characterized as T cell lymphoma lines originated *in vivo* (LSA and EL-4), a third cell line (PE-3T) also of T cell origin but transformed *ex vivo*, and a fourth cell line that was characterized as a mastocytoma (P815) which originated *in vivo*. Initially, we screened these cell lines for phenotypic expression of IL-2R and as shown in Figures 3.1 and 3.2, all the four cell lines screened, expressed the IL-2R and therefore these cell lines were further used in our study for the analysis of the constitutive expression of IL-2, IL-4 and their receptor gene expression.

RT-PCR analysis of the expression of IL-2R and IL-4R genes:

Using PCR analysis, it was observed (Figures 3.3 and 3.4) that the LSA, PE-3T, EL-4 and P815 tumor cell lines expressed IL-2R and IL-4R at all time intervals tested for example at 12, 24, 36 and 48 hours of culture, thereby demonstrating that these cell lines constitutively expressed the IL-2R and IL-4R genes. In this experiment, β-actin served as a positive internal control. In addition, unstimulated spleen cells from C57BL/6 mice served as a negative control for the growth factor receptors and spleen cells stimulated with ConA served as a positive control for the expression of the growth factor receptors.

Constitutive expression of IL-2 and IL-4 genes in tumor cells:

Using RT-PCR analysis we further addressed whether the tumor cell lines under investigation constitutively expressed IL-2 and IL-4 mRNA. The data shown in Figures 3.5 and 3.6, indicated that LSA and PE-3T tumor cells expressed IL-2 and IL-4 mRNA, whereas, EL-4 and P815 tumor cells expressed only IL-4 gene but not IL-2. These data suggested that tumor cell lines, including those of T cell origin, despite expressing IL-2R, do not always express the IL-2 gene. Also, it was interesting to note that all tumor cell lines screened constitutively expressed IL-4 gene, thereby suggesting the possibility that IL-4 may serve as an autocrine growth factor.

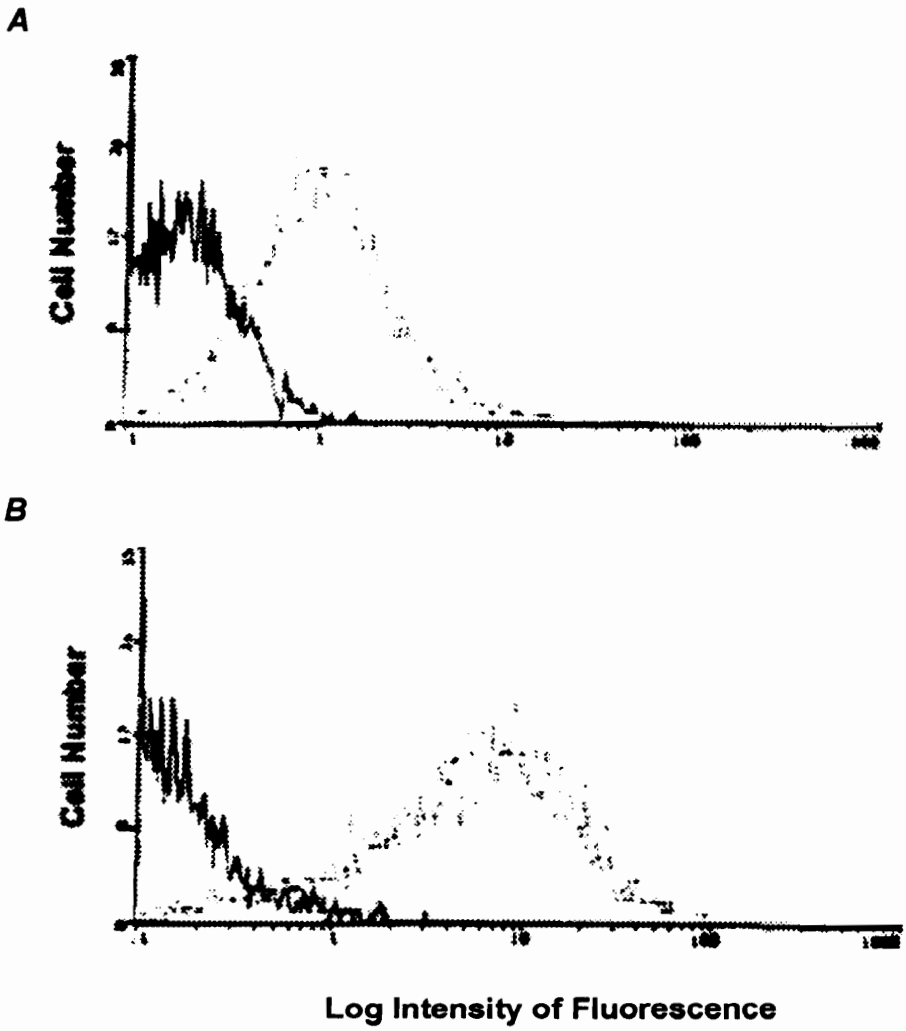

Fig. 3.1. Flow cytometric analysis of IL-2R expression on LSA and PE-3T tumor cell lines. The LSA (***A***) and the PE-3T (***B***) tumor cell lines were incubated with anti-IL-2R mAbs (7D4) followed by FITC-conjugated secondary antibodies as described in materials and methods. The cells were analyzed using a flow cytometer. The bold histogram represents the negative control and the broken histogram depicts cells stained for IL-2R expression.

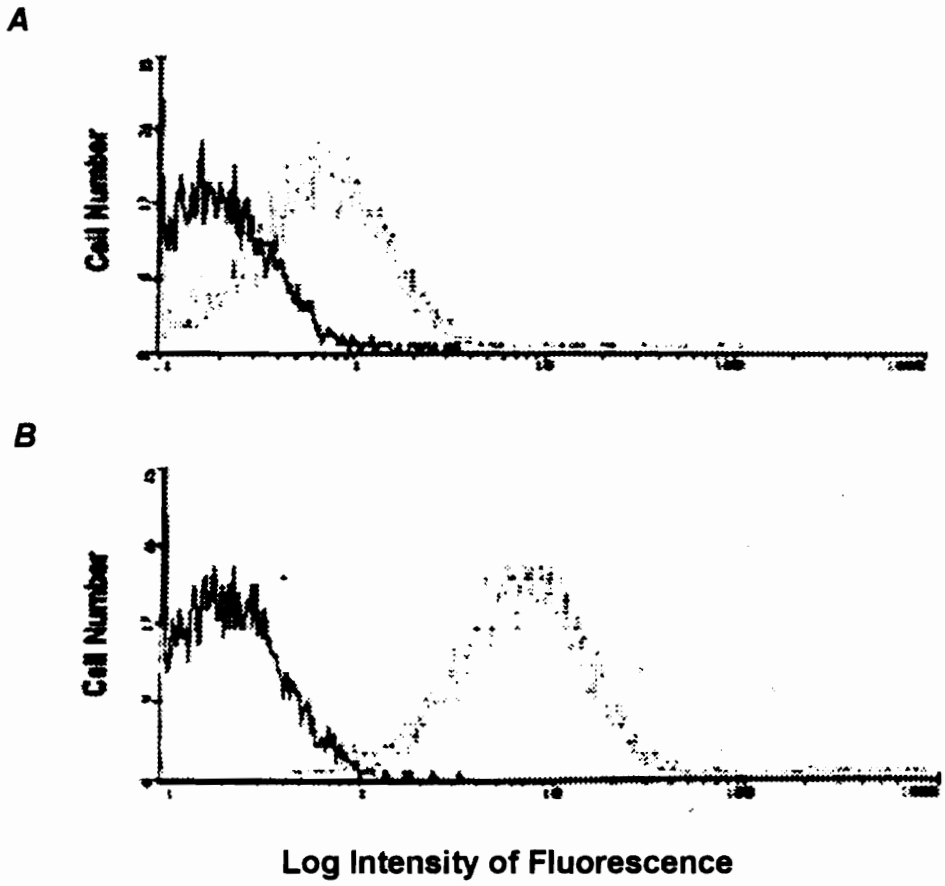

Fig. 3.2. Flow cytometric analysis of IL-2R expression on EL-4 and P815 tumor cell lines. The EL-4 (***A***) and the P815 (***B***) tumor cell lines were incubated with anti-IL-2R mAbs (7D4) followed by FITC-conjugated secondary antibodies as described in materials and methods. The cells were analyzed using a flow cytometer. The bold histogram represents the negative control and the broken histogram depicts cells stained for IL-2R expression.

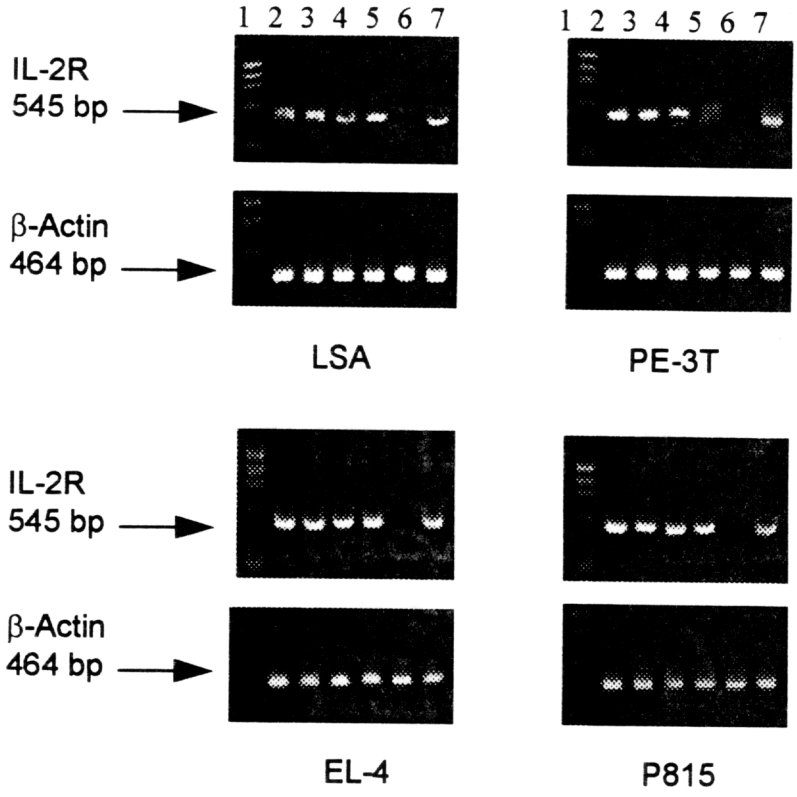

Fig. 3.3. Expression of IL-2R mRNA in tumor cell lines as detected by RT-PCR. Total RNA was extracted from various cell lines at 12, 24, 36 or 48 hours of *ex vivo* culture, reverse transcribed and cDNA samples were subjected to PCR amplification using synthetic oligonucleotide primers for IL-2R and β-actin as an internal control. The PCR product was electrophoresed through a 1.5% agarose gel containing ethidium bromide. Lane 1, molecular size standard (ΦX 174 *Hea* III digest), Lane 2-5 cytokine and β-actin genes expression at 12, 24, 36 and 48 hours of culture respectively; Lane 6, negative control consisting of normal unstimulated spleen cells from C57BL/6 mice and Lane 7, spleen cells cultured with ConA for 8 hours, used as a positive control.

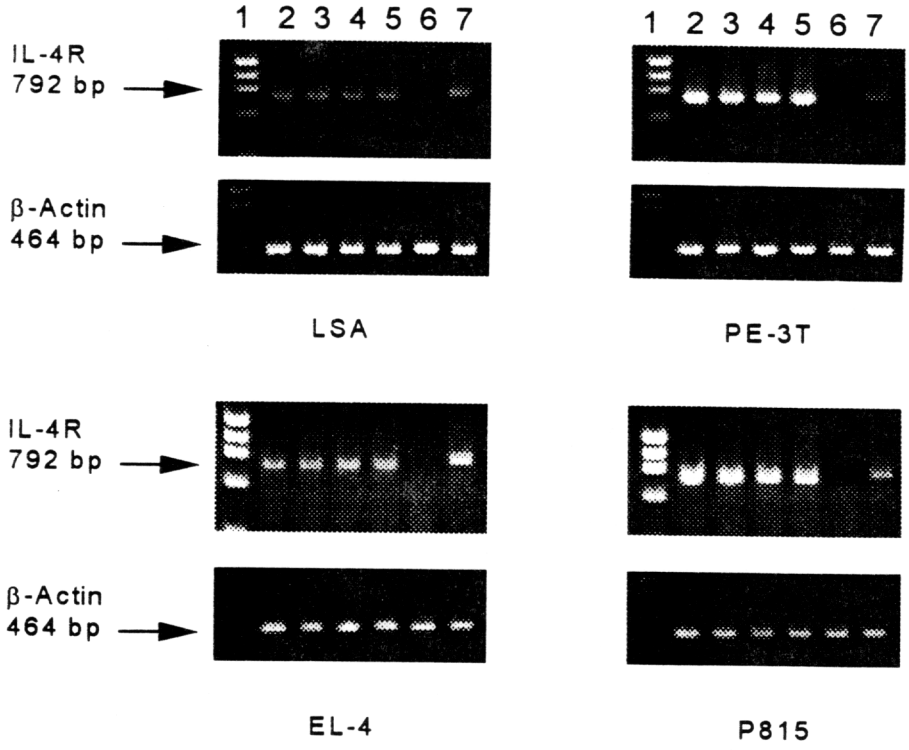

Fig. 3.4. Expression of IL-4R mRNA in tumor cell lines as detected by RT-PCR. Total RNA was extracted from various cell lines at 12, 24, 36 or 48 hours of *ex vivo* culture, reverse transcribed and cDNA samples were subjected to PCR amplification using synthetic oligonucleotide primers for IL-4R and β-actin as an internal control. The PCR product was electrophoresed through a 1.5% agarose gel containing ethidium bromide. Lane 1, molecular size standard (ΦX 174 *Hea* III digest), Lane 2-5 cytokine and β-actin genes expression at 12, 24, 36 and 48 hours of culture respectively; Lane 6, negative control consisting of normal unstimulated spleen cells from C57BL/6 mice and Lane 7, spleen cells cultured with ConA for 8 hours, used as a positive control.

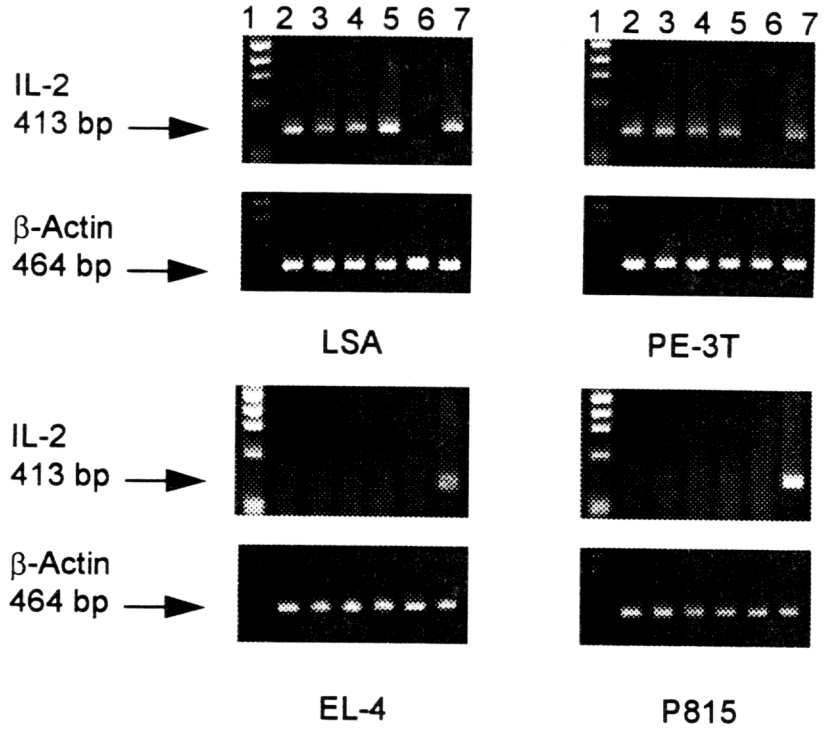

Fig. 3.5. Expression of IL-2 mRNA in tumor cell lines as detected by RT-PCR. Total RNA was extracted from various cell lines at 12, 24, 36 or 48 hours of *ex vivo* culture, reverse transcribed and cDNA samples were subjected to PCR amplification using synthetic oligonucleotide primers for IL-2 and β-actin as an internal control. The PCR product was electrophoresed through a 1.5% agarose gel containing ethidium bromide. Lane 1, molecular size standard (ΦX 174 *Hea* III digest), Lane 2-5 cytokine and β-actin genes expression at 12, 24, 36 and 48 hours of culture respectively; Lane 6, negative control consisting of normal unstimulated spleen cells from C57BL/6 mice and Lane 7, spleen cells cultured with ConA for 8 hours, used as a positive control.

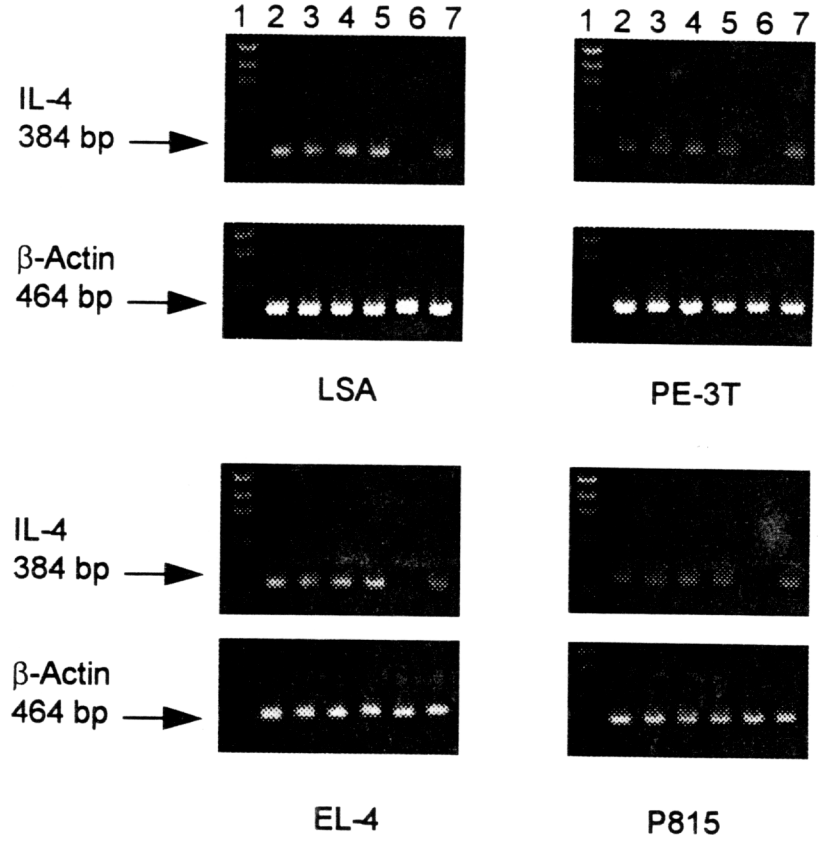

Fig. 3.6. Expression of IL-4 mRNA in tumor cell lines as detected by RT-PCR. Total RNA was extracted from various cell lines at 12, 24, 36 or 48 hours of *ex vivo* culture, reverse transcribed and cDNA samples were subjected to PCR amplification using synthetic oligonucleotide primers for IL-4 and β-actin as an internal control. The PCR product was electrophoresed through 1.5% agarose gel containing ethidium bromide. Lane 1, molecular size standard (ΦX 174 *Hea* III digest), Lane 2-5 cytokine and β-actin genes expression at 12, 24, 36 and 48 hours of culture respectively; Lane 6, negative control consisting of normal unstimulated spleen cells from C57BL/6 mice and Lane 7, spleen cells cultured with ConA for 8 hours, used as a positive control.

Effect of addition of monoclonal antibodies (mAbs) against growth factors or their receptors on tumor cell growth *ex vivo*:

Having established that some of the tumor cell lines screened constitutively expressed IL-2, IL-4 and their receptors, we next determined whether addition of mAbs against these growth factors or their receptors would inhibit the autonomous growth *ex vivo*. To this effect, various concentrations of anti-IL-2, anti-IL-2R or anti-IL-4 mAbs or a combination of antibodies against IL-2 and IL-4 were used in cultures and a representative experiment using one of the concentrations of these antibodies has been depicted in Figures 3.7-3.10. As a control, in these studies, normal Rat IgG was used, in addition to the medium control. The concentrations of antibodies against IL-2, IL-2R and IL-4, were previously shown to inhibit the IL-2 or IL-4-induced growth of HT-2 cells. The mAbs added did not have a significant inhibitory effect on the growth of tumor cells when screened for a period of 72 hours on PE-3T, EL-4 or P815 cells (Fig 3.8, 3.9 and 3.10 respectively). Interestingly however, antibodies against IL-2, IL-2R, IL-4 or a combination of antibodies against IL-2 and IL-4 dramatically inhibited the *ex vivo* growth of LSA tumor cell line particularly by 72 hours of culture (Fig 3.7). Similar results were obtained when cell proliferation was measured by [^{3}H]-thymidine uptake assay. These data demonstrated that IL-2 and IL-4 may serve as autocrine growth factors regulating LSA tumor cell proliferation but not that of other tumor cell lines screened, despite the fact that other tumors constitutively expressed certain growth factors or growth factor receptors. The fact that combination of anti-IL-2 and anti-IL-4 Abs could inhibit

the LSA tumor cell proliferation better than either of the Abs alone suggested that both IL-2 and IL-4 were involved in growth regulation.

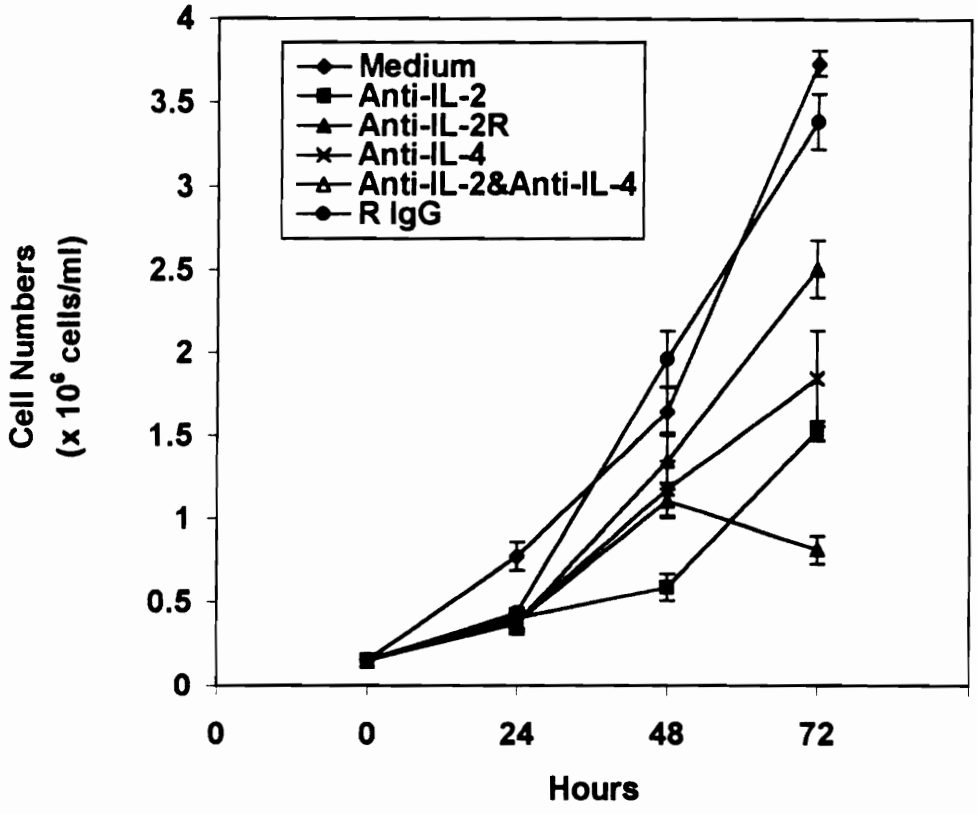

Fig. 3.7. Effect of addition of antibodies against T cell growth factors or their receptors on LSA tumor cell line proliferation. The LSA tumor cell line was cultured in the presence of mAbs against IL-2, IL-2R, IL-4 or a combination of anti-IL-2 and anti-IL-4 at 1:4 dilution of purified antibodies, for 0-72 hours. Incubation of cells with media alone or with rat IgG was used as a control. Cell proliferation was measured by counting the number of viable cells using trypan blue dye exclusion.

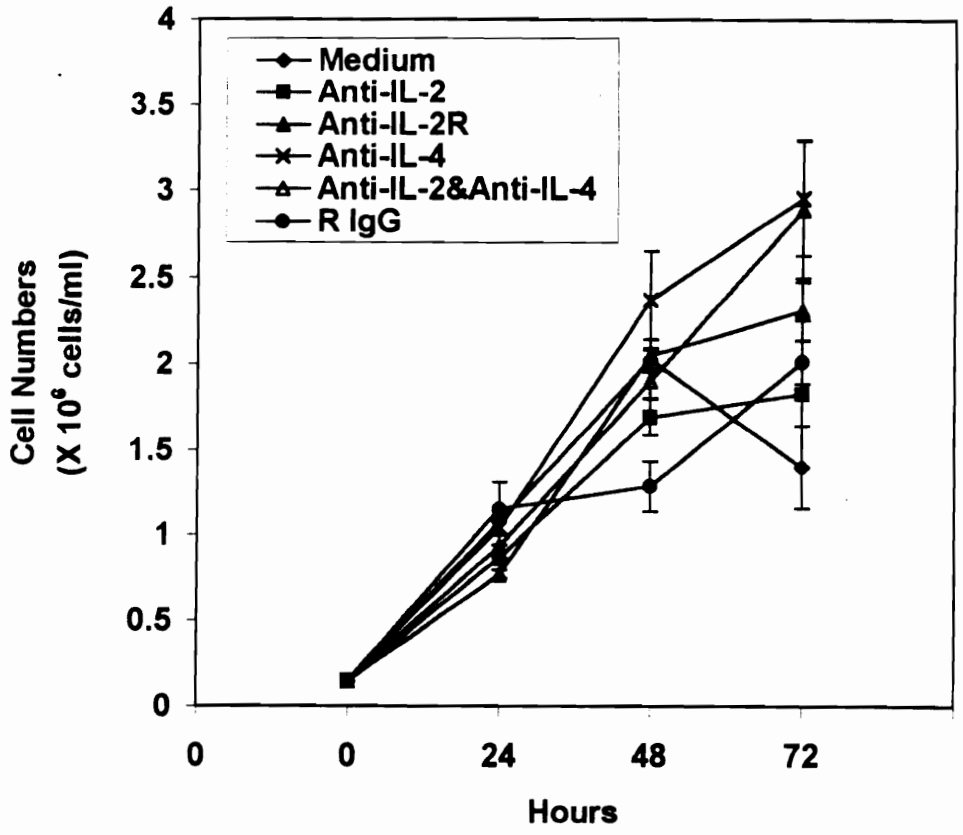

Fig. 3.8. Effect of addition of antibodies against T cell growth factors or their receptors on PE-3T transformed cell line proliferation. The PE-3T transformed cell line was cultured in the presence of mAbs against IL-2, IL-2R, IL-4 or a combination of anti-IL-2 and anti-IL-4 at 1:4 dilution of purified antibodies, for 0-72 hours. Incubation of cells with media alone or with rat IgG was used as a control. The cell proliferation was measured by counting the number of viable cells using trypan blue dye exclusion.

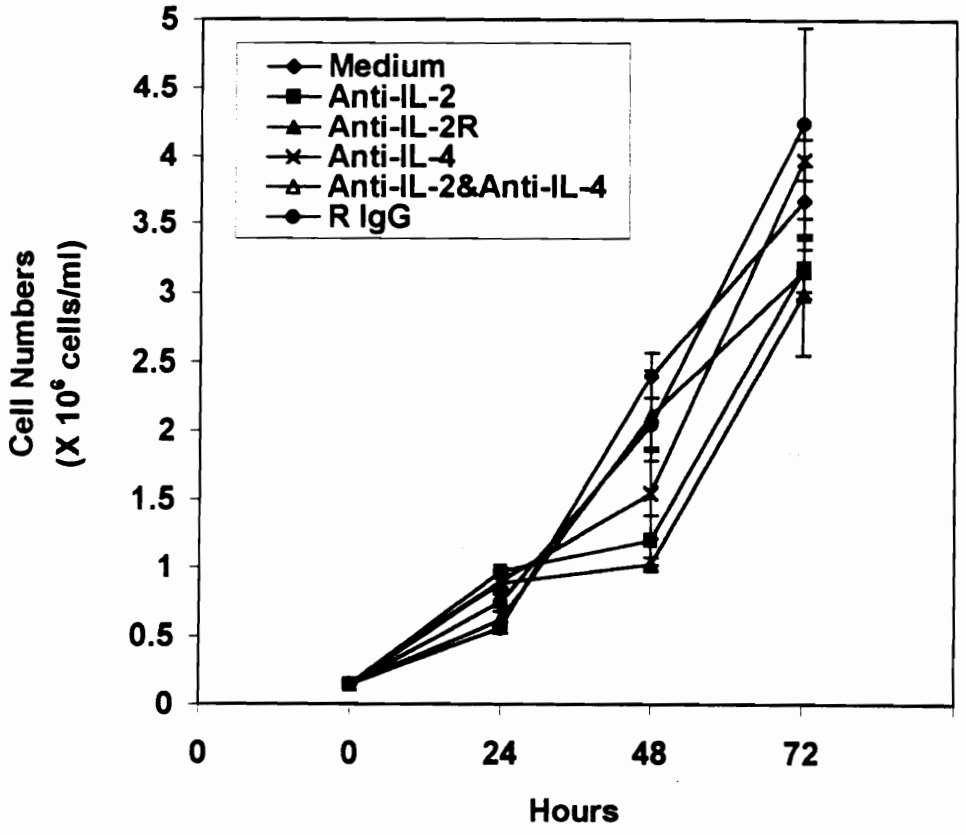

Fig. 3.9. Effect of addition of antibodies against T cell growth factors or their receptors on EL-4 tumor cell line proliferation. The EL-4 tumor cell line was cultured in the presence of mAbs against IL-2, IL-2R, IL-4 or a combination of anti-IL-2 and anti-IL-4 at 1:4 dilution of purified antibodies, for 0-72 hours. Incubation of cells with media alone or with rat IgG was used as a control. The cell proliferation was measured by counting the nuber of viable cells using trypan blue dye exclusion.

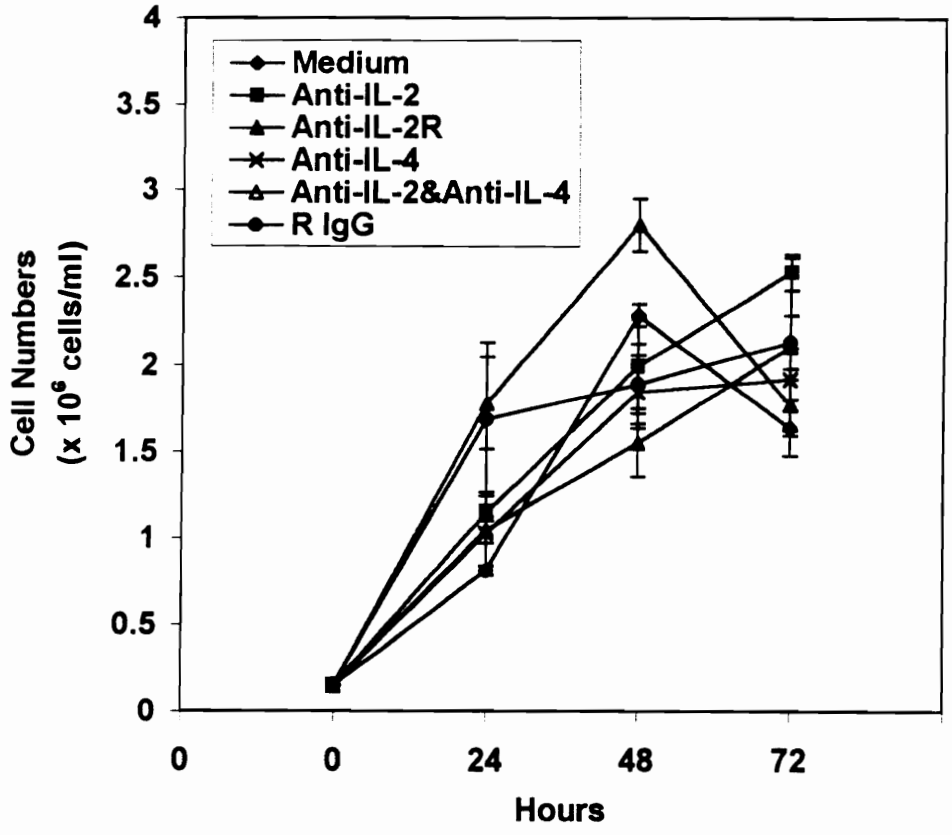

Fig. 3.10. Effect of addition of antibodies against T cell growth factors or their receptors on P815 tumor cell line proliferation. The P815 tumor cell line was cultured in the presence of mAbs against IL-2, IL-2R, IL-4 or a combination of anti-IL-2 and anti-IL-4 at 1:4 dilution of purified antibodies, for 0-72 hours. Incubation of cells with media alone or with rat IgG was used as a control. The cell proliferation was measured by counting the nuber of viable cells using trypan blue dye exclusion.

The effect of addition of antisense phosphorothioate oligonucleotides (APO) specific for the growth factors or their receptors on cell proliferation *ex vivo*:

It was possible that the reason why mAbs against the growth factors or their receptor may not have inhibited the proliferation of PE-3T, EL-4 and P815 cells was that in these cell lines, the growth factor may not be actively secreted outside the cell but that the growth factor may interact with its receptor within the cell which may then lead to autocrine stimulation and proliferation (Lang and Burgess,1990). To address this possibility, we used various concentrations of APO, specific to various growth factors and their receptors which would specifically inhibit the endogenous gene activation. The representative data using 20μM of APO has been depicted in Figures 3.11-3.14 which demonstrated that the APO specific for IL-2, IL-4, combination of IL-2 and IL-4 or against the IL-2R β or γ chains failed to inhibit the proliferation of the PE-3T, EL-4 and P815 cells (Fig 3.12, 3.13 and 3.14). However, when used against LSA cells, the APO specific for IL-2, IL-4 or combination of these as well as those against IL-2R β or IL-2R γ chains could markedly inhibit the growth of the LSA tumor cell line (Fig 3.11). These data corroborated the results obtained using mAbs against the growth factors or their receptors and demonstrated that of the four cell lines screened, only the LSA tumor cells were using both IL-2 and IL-4 as autocrine growth factors for the *ex vivo* cell proliferation. In addition, we have used control oligonucleotides as described in materials and methods. However, these controls inhibited the proliferation of one or the other of the tested cell lines. The results from a representative experiment has been shown in Figure 3.15.

The data suggest that the 18-mer non-specific oligonucleotide inhibited the growth of PE-3T cell line, but no significant inhibition was noticed for the other cell lines. Because, in these experiments we were detecting the growth of the cells, which is the final out come of complicated and ill-defined processes, it would be hard to point out at which level such control oligonucleotides acted, in order to cause this inhibition. Moreover, similar results have been observed by other researchers who noticed that such control oligonucleotides often produce biological effects that are indistinguishable from the antisense oligonucleotides (Stein *et al.*, 1993). For this purpose, we considered the use of IL-2 specific antisense in an IL-2-independent cell line, such as P815, to be the more appropriate control.

Effect of CsA on tumor cell growth:

To address the possibility that PE-3T, EL-4 and P815 tumor cells used some other cytokines for autonomous growth, we tested the effect of CsA on these cell lines inasmuch as, CsA is known to block the synthesis of a variety of cytokines (reviewed in Bierer *et al.*, 1993). The data shown in Figures 3.16-3.19 demonstrated that CsA caused a dose dependent inhibition of the proliferation of all the tumor cell lines tested.

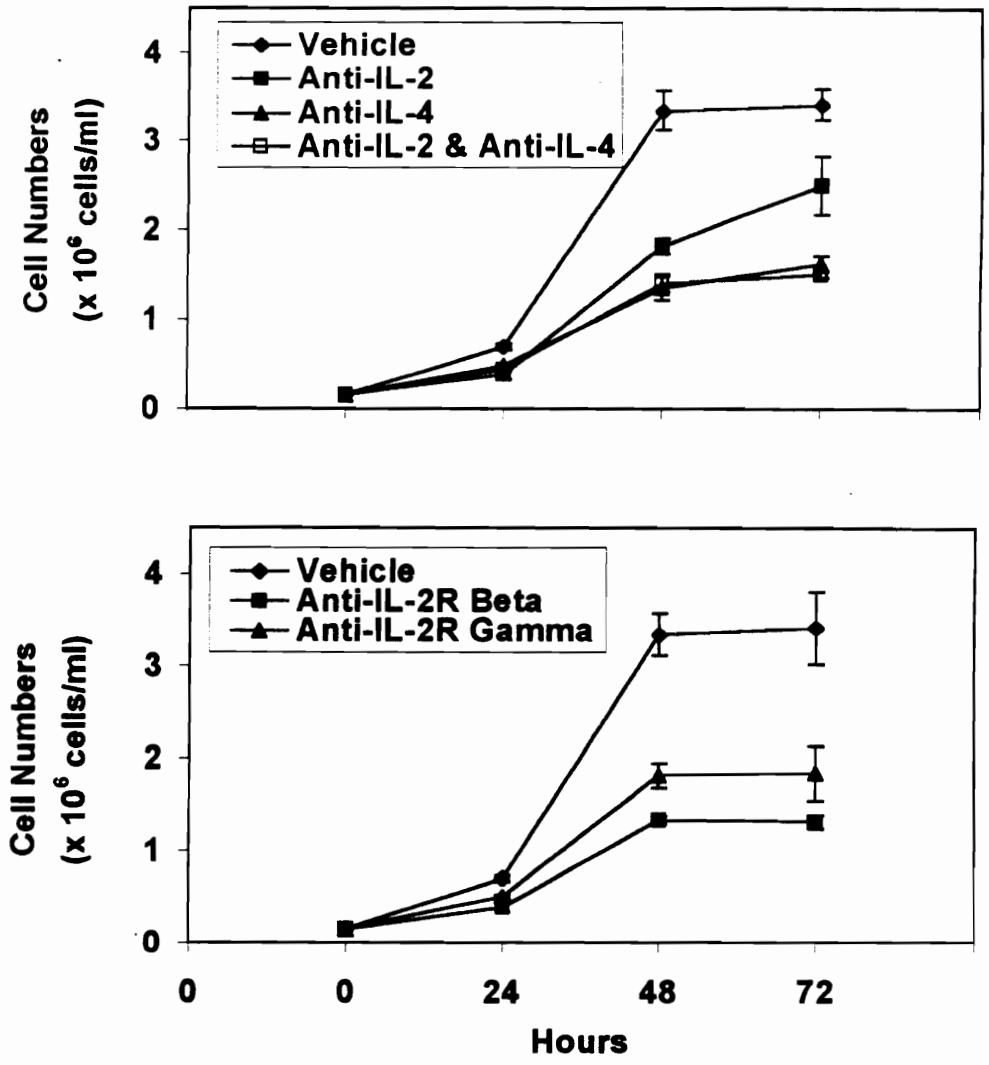

Fig. 3.11. Effect of antisense phosphorothioate oligonucleotides (APO) on LSA tumor cell line proliferation. Tumor cell line LSA was incubated with 20 μM APO for 0-72 hours. APO include anti-IL-2, anti-IL-4 and a combination of anti-IL-2 and anti-IL-4 or APO against the IL-2R β or γ chains. The viable cell number was calculated as described in Fig. 3.7-3.10.

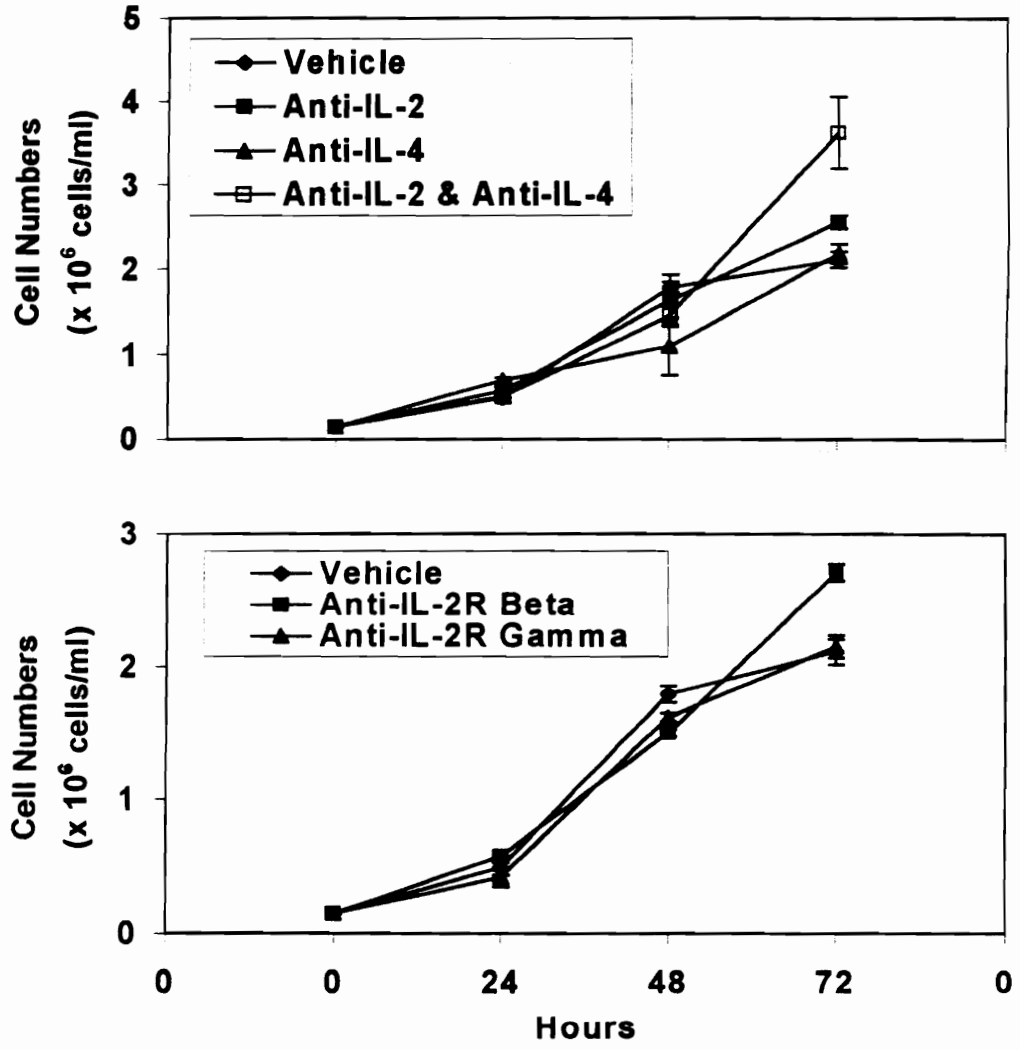

Fig. 3.12. Effect of antisense phosphorothioate oligonucleotides (APO) on PE-3T tumor cell line proliferation. Transformed cell line PE-3T was incubated with 20 μM APO for 0-72 hours. APO include anti-IL-2, anti-IL-4 and a combination of anti-IL-2 and anti-IL-4 or APO against the IL-2R β or γ chains. The viable cell number was calculated as described in Fig. 3.7-3.10.

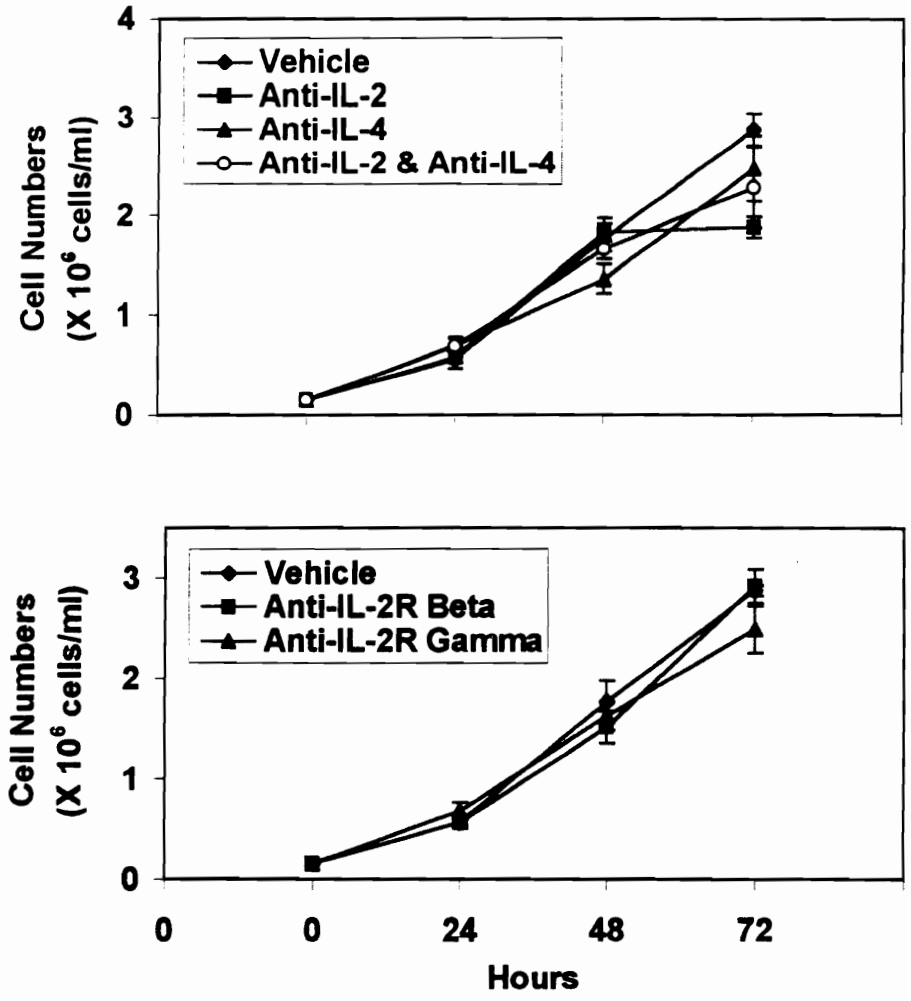

Fig. 3.13. Effect of antisense phosphorothioate oligonucleotides (APO) on EL-4 tumor cell line proliferation. Tumor cell line EL-4 was incubated with 20 μM APO for 0-72 hours. APO include anti-IL-2, anti-IL-4 and a combination of anti-IL-2 and anti-IL-4 or APO against the IL-2R β or γ chains. The viable cell number was calculated as described in Fig. 3.7-3.10.

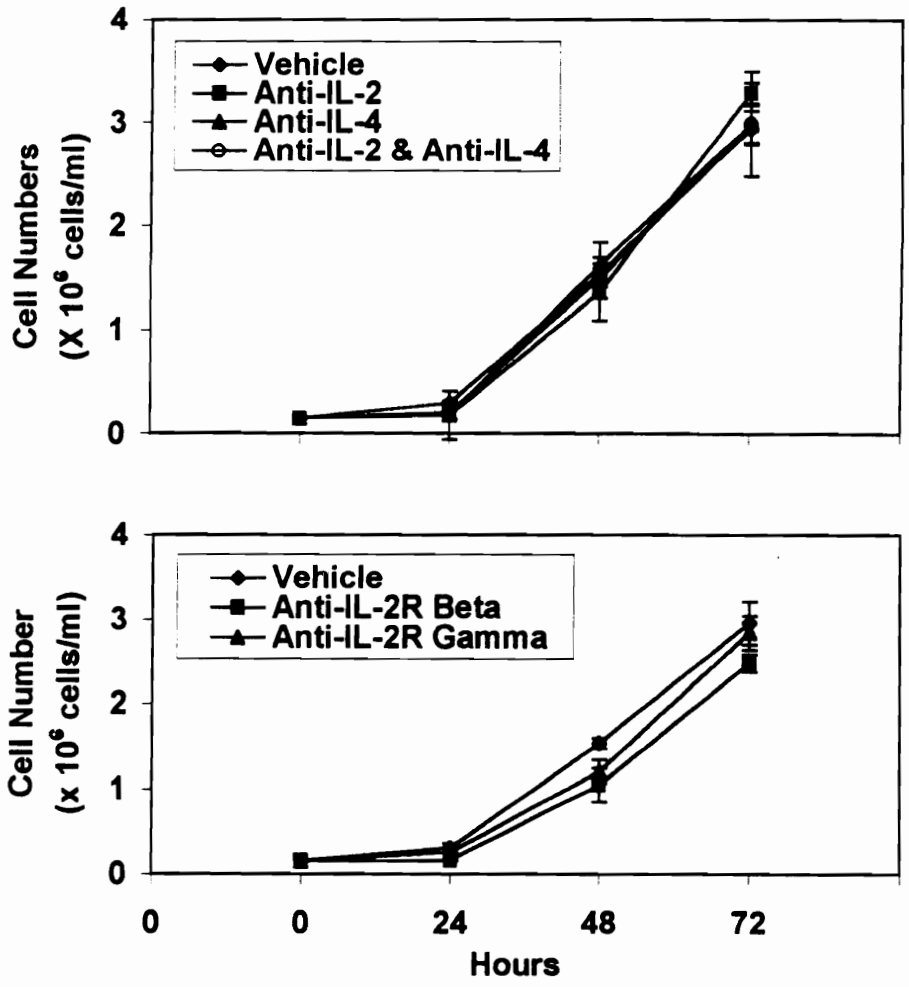

Fig. 3.14. Effect of antisense phosphorothioate oligonucleotides (APO) on P815 tumor cell line proliferation. Tumor cell line P815 was incubated with 20 μM APO for 0-72 hours. APO include anti-IL-2, anti-IL-4 and a combination of anti-IL-2 and anti-IL-4 or APO against the IL-2R β or γ chains. The viable cell number was calculated as described in Fig. 3.7-3.10.

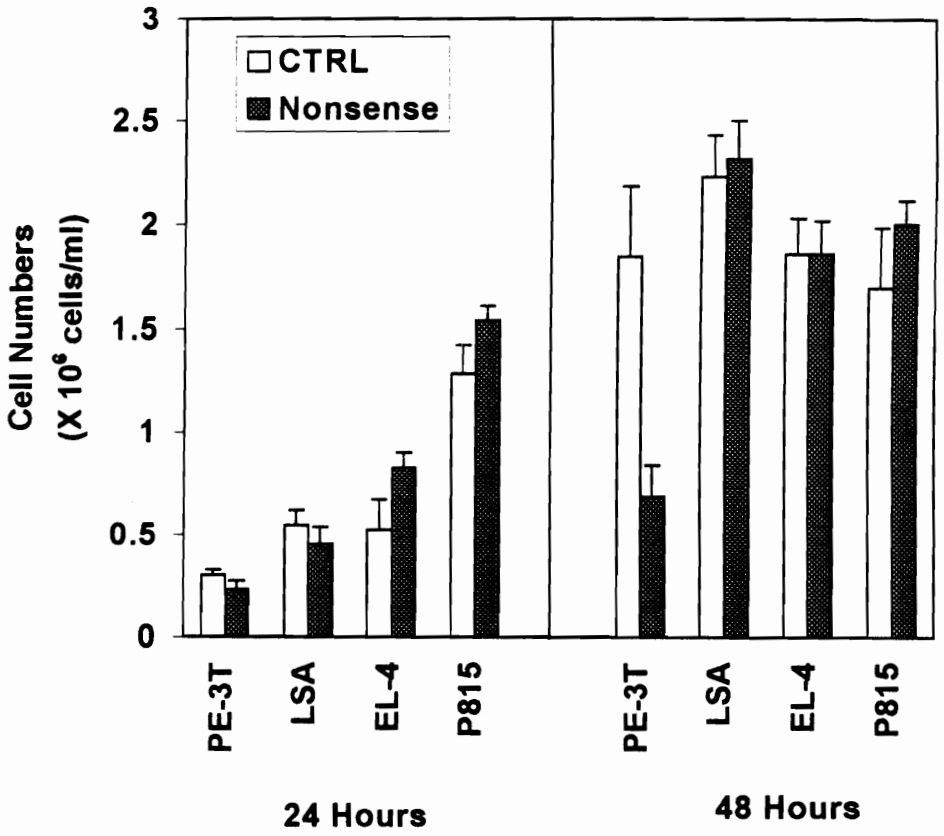

Fig. 3.15. The effect of addition of a non-specific (nonsense) oligonucleotide, as a control, on the growth of the tumor cell lines *ex vivo*. Tumor cell lines PE-3T, LSA, EL-4 and P815 was incubated in the presence or absence of 20 μM of control nonsense oligonucleotide for 24 and 48 hours. The viable cell number was calculated as described in Fig. 3.7-3.10.

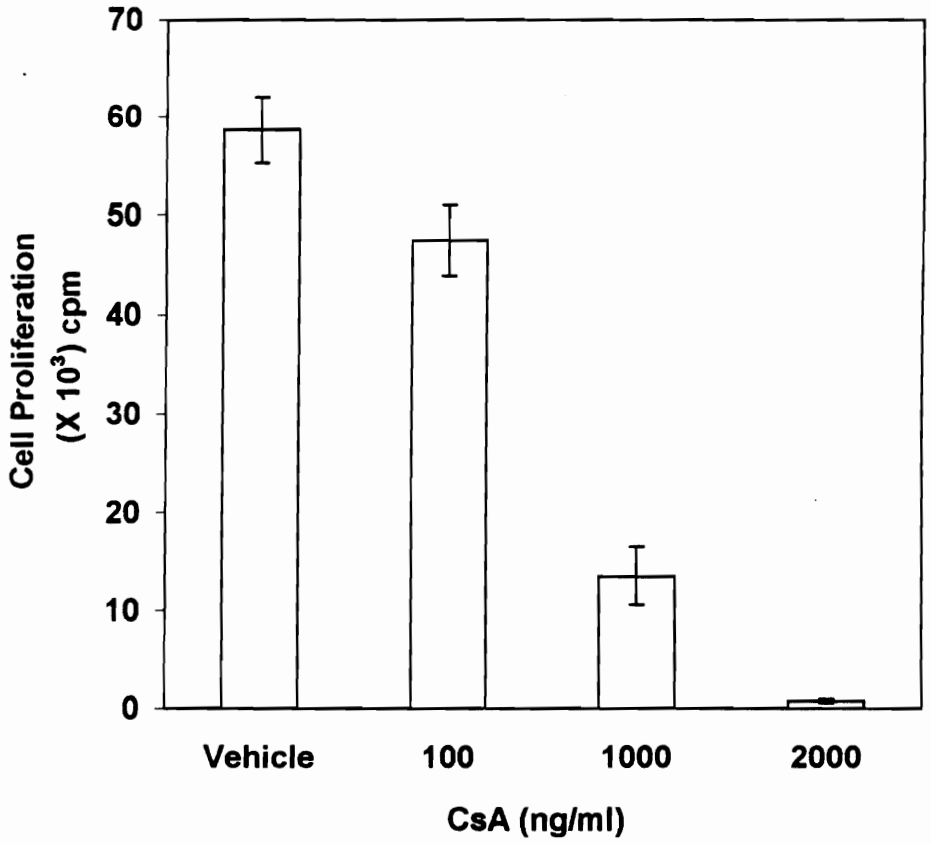

Fig. 3.16. Cyclosporin A can inhibit the proliferation of the LSA tumor cell line *ex vivo*. Tumor cell line LSA was incubated with various concentrations of CsA and the cell proliferation was studied by a [^{3}H]-thymidine incorporation assay 48 hours after cell culture.

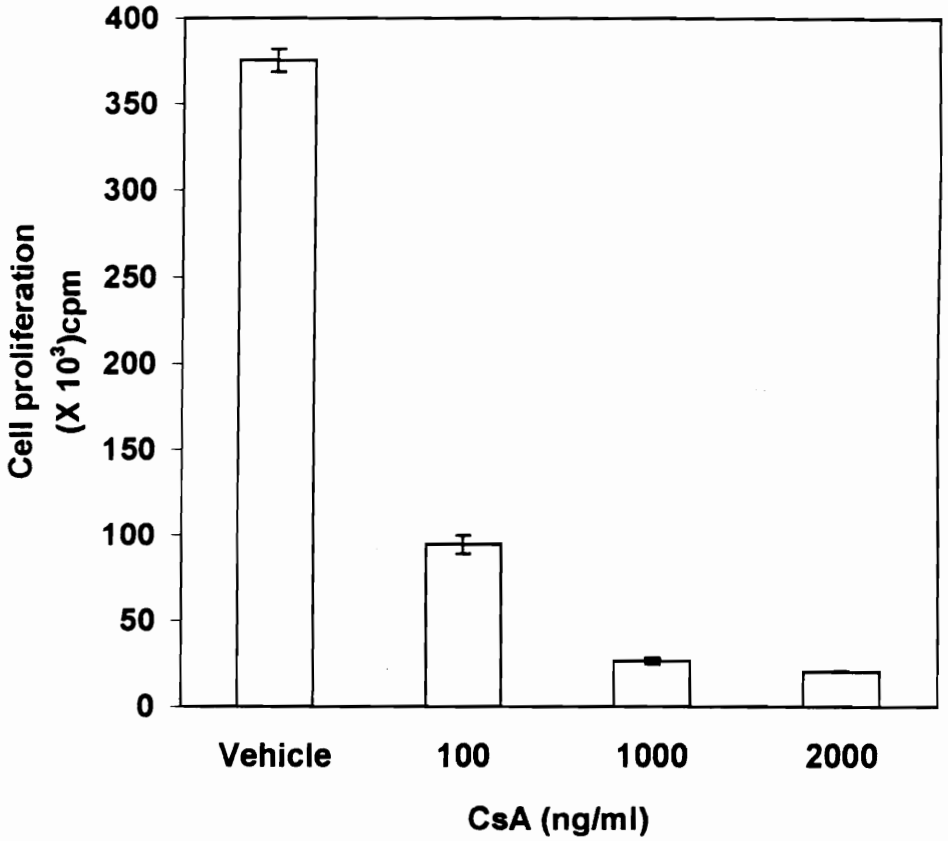

Fig. 3.17. Cyclosporin A can inhibit the proliferation of PE-3T tumor cell line *ex vivo*. Transformed cell line PE-3T was incubated with various concentrations of CsA and cell proliferation was studied by a [^{3}H]-thymidine incorporation assay 48 hours after cell culture.

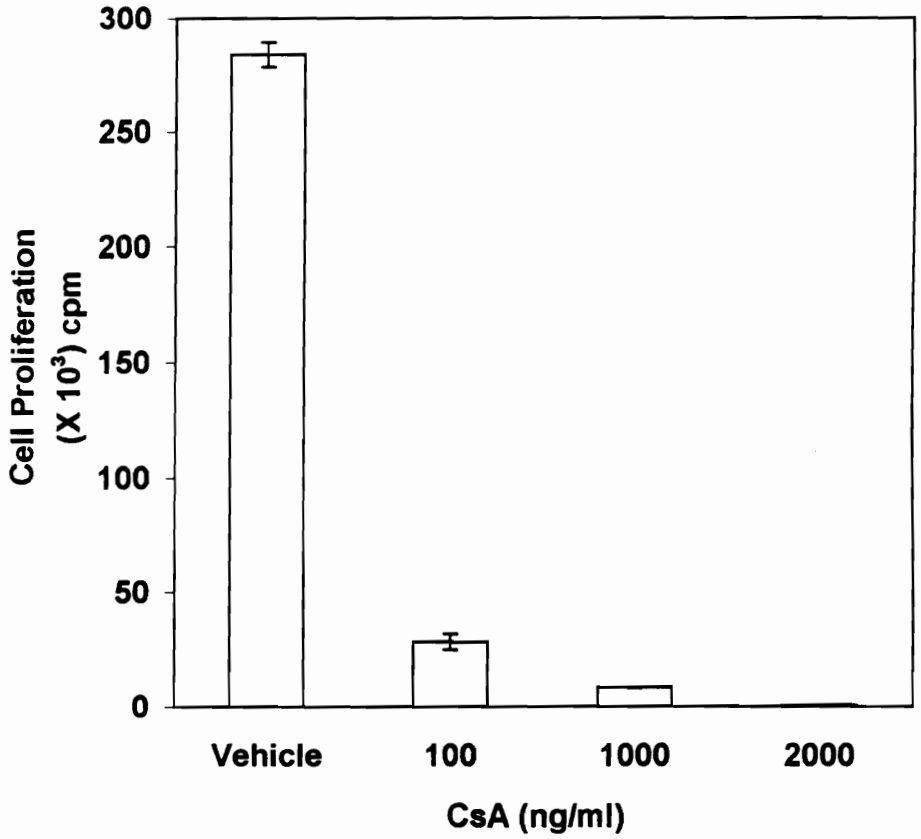

Fig. 3.18. Cyclosporin A can inhibit the proliferation of EL-4 tumor cell line *ex vivo*. Tumor cell line EL-4 was incubated with various concentrations of CsA and cell proliferation was studied by a [^{3}H]-thymidine incorporation assay 48 hours after cell culture.

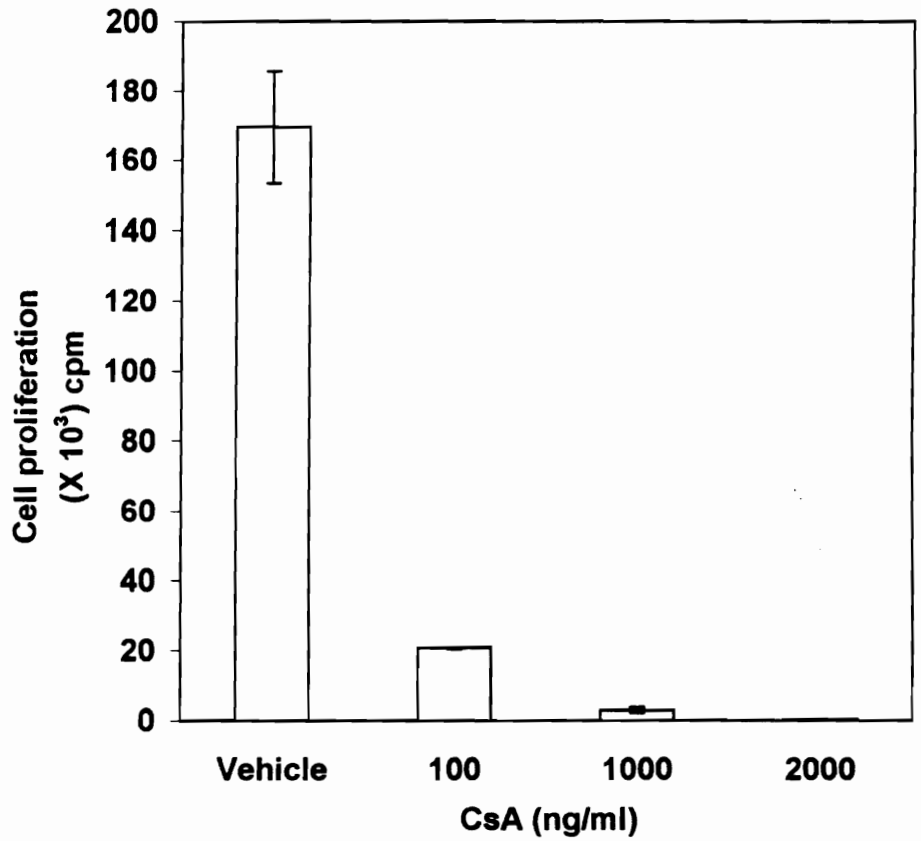

Fig. 3.19. Cyclosporin A can inhibit the proliferation of P815 tumor cell line *ex vivo*. Tumor cell line P815 was incubated with various concentrations of CsA and cell proliferation was studied by a [^{3}H]-thymidine incorporation assay 48 hours after cell culture.

Inhibition of in vivo tumorigenicity of LSA using mAbs against IL-2 and IL-4.

After showing that the *ex vivo* growth of LSA is mediated by the autocrine stimulation of both IL-2 and IL-4, we investigated whether the *in vivo* tumorigenicity of LSA was also mediated by production of autocrine growth factors. Since treatments that antagonize IL-2 and IL-4 would interfere with anti-tumor immunity of the immunocompetent host, this possibility was tested in immunodeficient, nude mice. To accomplish this, we induced subcutaneous LSA tumors into nude mice and on the same day as tumor challenge, these mice were injected with normal rat-IgG as a control or a combination of equal amounts of mAbs against IL-2 and IL-4 as described in methods. The subcutaneously injected tumors grew as localized solid tumors without undergoing metastasis. As demonstrated in Figure 3.20, a dramatic inhibition of LSA tumor growth in the nude mice treated with the combination of mAbs against the growth factors was observed. Interestingly, one of the mice treated with antibodies against IL-2 and IL-4 did not display any tumor growth. These data were repeated with consistent results. Also similar treatment of P815 tumor cell in nude mice failed to cause significant inhibition of *in vivo* growth (Figure 3.21). These results demonstrate that LSA tumor cells depended on IL-2 and IL-4 for tumorigenesis even *in vivo*.

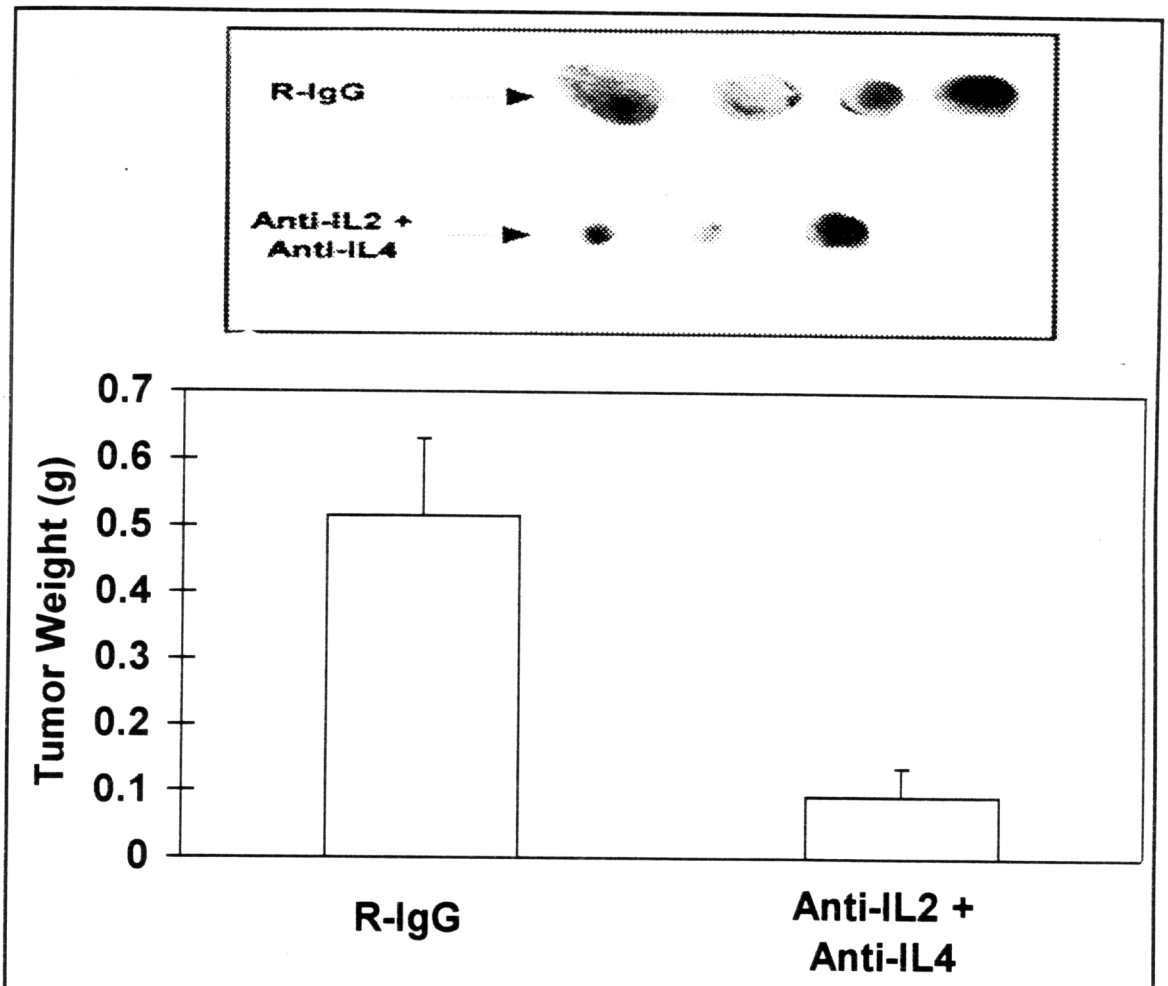

Fig. 3.20. Antibodies to IL-2 and IL-4 can inhibit the *in vivo* growth of LSA in nude mice. Groups of four nude mice injected with LSA subcutaneously, were treated on the same day and thereafter for 16 days with intraperitoneal injections of normal R-IgG (control) or with a combination of mAbs against IL-2 and IL-4, on alternate days. On the seventeenth day, tumors were surgically removed and weighed. The vertical bars represent the means ± S. E. values of tumor weight. It should be noted that in one nude mouse treated with anti-IL-2 and anti-IL-4 Abs , the tumor completely failed to grow and the mouse survived indefinitely.

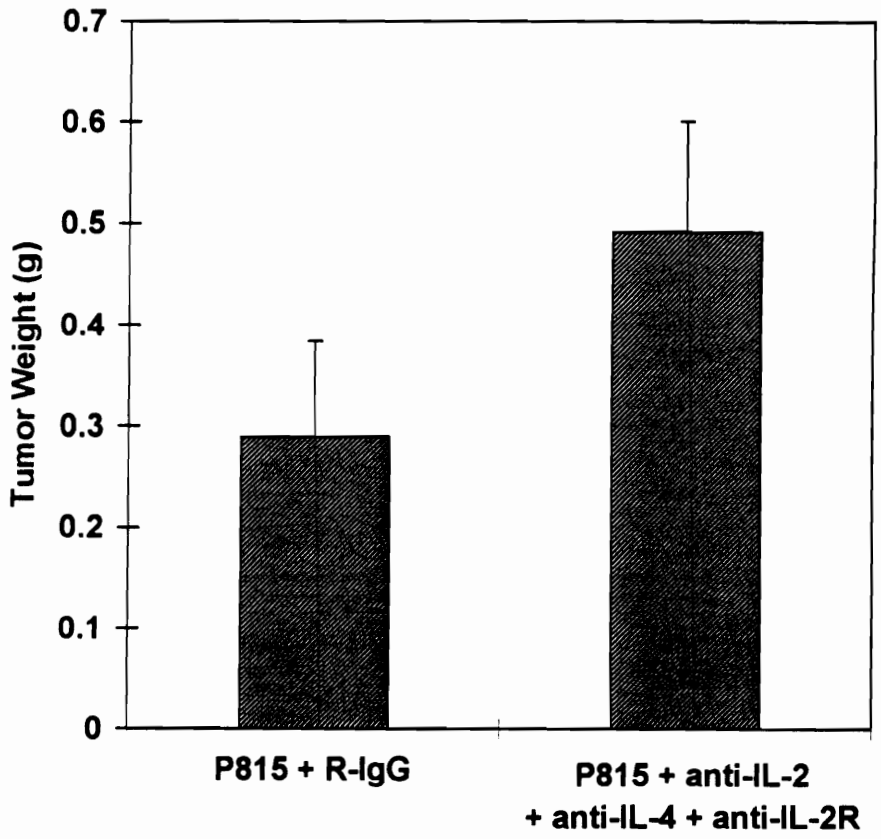

Fig 3.21. Antibodies to IL-2, IL-2R and IL-4 can not inhibit the *in vivo* growth of P815 in nude mice. Groups of four nude mice injected with P815 subcutaneously, were treated on the same day and thereafter for 16 days with intraperitoneal injections of normal R-IgG (control) or with a combination of mAbs against IL-2, IL-2R and IL-4, on alternate days. On the seventeenth day, tumors were surgically removed and weighed. The vertical bars represent the means ± S. E. values of tumor weight.

Discussion:

In the current study, we investigated four murine cell lines of different origins for their ability to grow *ex vivo* using IL-2 and IL-4 as autocrine growth factors. It was interesting to note that all four cell lines screened constitutively expressed both IL-2R and IL-4R genes. However, when expression for the growth factors was screened, it was observed that LSA and PE-3T but not EL-4 and P815 cells constitutively expressed the IL-2 gene. In contrast, IL-4 gene was expressed constitutively by all the four cell lines screened. Despite this, it was noted that the cell lines markedly differed in their ability to demonstrate IL-2 or IL-4-dependent autocrine growth. For example, of the four cell lines screened, only LSA was dependent on IL-2 and IL-4 for autonomous growth *ex vivo,* whereas, none of the other cell lines screened could be inhibited by either mAbs against the growth factors, IL-2 and IL-4 or their receptors.

The reason why some tumor cells despite expressing the growth factor or their receptor genes constitutively, fail to exhibit autocrine pattern of growth is not clear. It can be speculated that there may be other growth factors and their receptors not screened in this study, which may be involved in the autonomous growth of these cells. Secondly, it is possible that these cell lines are independent of autocrine growth factors for their proliferation. Thirdly, multiple cytokines such as IL-1, IL-6, TNF-α and TNF-β have earlier been shown to act synergistically to maintain the growth of transformed cell lines (Abken *et al.*, 1992). Thus, blocking a few cytokines may not be sufficient to inhibit tumor cell growth. In this context, it is interesting to note that in an earlier study we

observed that a spontaneously *ex vivo* transformed T cell clone similar to PE-3T cells expressed constitutively IL-2 and IL-2R genes and was completely inhibited from growing *ex vivo* as well as inducing tumors *in* vivo in the presence of IL-2 or IL-2R antagonists (Nagarkatti *et al.*, 1994). Recently, a number of studies have demonstrated that IL-2R is expressed on a wide variety of human lymphoid as well as non-lymphoid tumor cells (Mcmillan *et al.*, 1995). Furthermore, secretion of IL-2 by such malignant cells has also been used to suggest that IL-2 may play an important role as an autocrine growth factor in the proliferation of malignant cells *in vivo* (Mcmillan *et al.*, 1995). However, in addition to IL-2, a variety of other cytokines may share a common receptor thereby making it difficult to treat such tumors *in vivo* if the tumor cell growth is dependent on multiple cytokines. For example, IL-2R consists of three subunits the α, β, and γ chains (reviewed in Taniguchi, 1995). IL-2Rβ is a subunit of IL-15 receptor and IL-2Rγ is a subunit for IL-4, IL-7, IL-9 and IL-15 receptors (Giri *et al.*, 1994; Grabstein *et al.*, 1994; Kondo *et al.* 1993; Russell *et al.*, 1993). Thus, such redundancy in the cytokine function may make it difficult to identify and treat tumors which are dependent on multiple cytokines for autonomous growth *in vivo*. However, in the current study, we observed that APO against IL-2R β or γ subunits blocked LSA proliferation but not that of other tumor cell lines. Since these subunits are also a component of receptors for IL-4, IL-7, IL-9 and IL-15 (reviewed in Karnitz and Abraham, 1995), we can rule out the possible involvement of these cytokines. However, the ability of CsA to inhibit the growth of all such cell lines supports the notion that cytokines other than those mentioned above may participate in the autocrine regulation.

It is becoming increasingly clear that IL-2 may serve as an autocrine growth factor responsible for maintenance and proliferation of human T cells *in vivo*. The possibility that IL-2 may be involved in the autocrine growth factor-dependent cell transformation was suggested in a human T cell lymphoma line derived from a patient with adult T cell leukemia (Duperz *et al.*, 1985). Further studies in adult T cell leukemia patients as well as T cell lines infected with human T cell lymphotropic virus (HTLV) have suggested that autocrine -IL-2-induced self-stimulation may play an important role in T cell transformation (Arima *et al.*, 1986; Gootenberg *et al.*, 1981; Maruyama *et al.*, 1987). Furthermore, IL-2 transcript and protein were detected at the single cell level in most cells of HTLV-1 infected T cell lines (Farcet *et al.*, 1991). HTLV-1 contains a region called pX which encodes for at least two regulatory proteins, tax and rex (Felber *et al.*, 1985; Sodrowski *et al.*, 1984). Tax is a potent transcriptional transactivator of the provirus LTR and furthermore, it can transactivate and induce the expression of a number of heterologous cellular genes some of which are also involved in T cell growth, proliferation and possibly transformation (reviewed in Hall, 1994). The cellular genes which are known to be transactivated by tax include: IL-2, IL-2R α, IL-3, IL-6, GM-CSF and TNF-β. Tax has also been shown to transactivate the promoters of a number of oncogenes. Thus, the transactivation of IL-2 and IL-2R has led to the hypothesis that constitutive production and responsiveness to autocrine IL-2 may be responsible for HTLV-1 induced T cell transformation and the development of adult T cell leukemia. In this context, it is interesting to note that the LSA tumor cell line which originated *in vivo* following low dose radiation treatment, is a murine retrovirus induced tumor and therefore, may share similar characteristics

with HTLV in being able to activate cytokine genes and trigger autonomous growth.

Despite examples in which IL-2 or other growth factors have been shown to be involved in cell transformation, it is not proven that such growth factor production is totally responsible for the maintenance of neoplastic transformation *in vivo* (Lang and Burgess, 1990). However, in a previous study we demonstrated that the induction of tumors *in vivo* caused by an *ex vivo* transformed tumor cell line Auto D1.4T, was completely inhibited by injection of mAbs against IL-2 and IL-2R thereby demonstrating that IL-2 can serve as a primary growth factor responsible for the maintenance of neoplasia *in vivo* (Nagarkatti *et al.*, 1994). The present study extends our earlier observation and demonstrates for the first time that an *in vivo* originated lymphoma cell line is also dependent on T cell growth factors, IL-2 and IL-4 for *in vivo* growth. The fact that in one out of the four mice studied, the LSA tumor cells failed to grow, suggests that IL-2 and IL-4 may be the sole factors responsible for maintenance of tumor growth *in vivo*.

The data from the current and previous studies (Nagarkatti *et al.*, 1994), have significant implications in immunotherapy of cancer and viral infections. It has been suggested that administration of tumor- or virus-specific T cells may offer an effective way to treat cancer and certain viral infections (Riddell *et al.*, 1992; Rosenberg *et al.*, 1986). However, studies from our lab have demonstrated that during *ex vivo* culture, mutants may originate which use autocrine growth factors for cell transformation, capable of inducing tumors in

immunodeficient host (Nagarkatti *et al.*, 1994). In a recent study by Treisman *et al.* (1995), the authors demonstrated that a T cell line retroviraly transduced with cDNA for IL-2 was able to grow in an autocrine fashion and this cell line was able to maintain antigen-specific responsiveness and failed to induce tumors in normal syngeneic mice. The authors suggested that such cell lines can be used for immunotherapy thereby obviating the need to administer IL-2 for the maintenance of the T cell lines *in vivo*. In these studies, however, the authors did not address whether the IL-2 transduced lymphocytes would induce tumors *in vivo* in immunodeficient mice. For example, we noted that the *ex vivo* transformed T cell lines, namely the Auto D1.4T and PE-3T were not able to grow in normal mice but were able to induce tumors in immunodeficient, nude mice (Nagarkatti *et al.*, 1994). Thus, careful consideration should be given to immunotheraputic approaches which use T cells transfected with growth factor genes, particularly in immunodeficient host.

The reason why *ex vivo* transformed T cell lines (such as Auto D1.4T and PE-3T) which produce IL-2 and IL-4 constitutively, fail to induce tumors in normal syngeneic host but do so in immunodeficient host is not clear. However, preliminary studies revealed that the *ex vivo* transformed cell lines can trigger a strong immune response mediated both by NK cells and cytotoxic T cells thereby preventing them from growing in vivo (Nagarkatti *et al.*, 1994 and unpublished data). The strong anti-tumor immunity induced by the *ex vivo* transformed cell lines may have resulted from the active secretion of IL-2 , IL-4 or other cytokines by these cells when injected into normal mice. Such an observation has also been made by other investigators who have tried to transfect tumor cell

lines with IL-2 and other growth factor genes (reviewed in Paradoll, 1995). These findings raise the question of why, tumors originated *in vivo*, such as LSA or HTLV-induced T cell tumors, known to depend on IL-2 autocrine stimulation, fail to trigger a strong immune response in the patient and succeed in inducing malignancies. There could be multiple explanations for this phenomenon and one important factor could be that the *in vivo* transformed T cell lines which constitutively express IL-2 and depend on IL-2 for malignancy may also produce certain immunosuppressive cytokines which may suppress the anti-tumor immunity and facilitate the growth of these tumor cells *in vivo*. Preliminary studies carried out in our lab have demonstrated that while the *in vivo* transformed cell line, LSA, produces constitutively TGF-β and IL-10, the *ex vivo* transformed cell line Auto D1.4T and PE-3T do not produce TGF-β and express low levels of IL-10. This may provide one possible explanation as to how a cell may use IL-2 for autocrine growth and transformation and simultaneously use immunosuppressive cytokines to shield from the anticancer immunity and therefore be able to induce malignancy. Further studies on the nature of cytokines produced by transformed cells in regulating autocrine growth and the antitumor immunity of the host should provide useful information on tumorigenesis and possible approaches to treat cancer.

Chapter 4: Nature of the Immune Response Against Autocrine Growth Factor Induced Lymphomas: Role of Cell Mediated Immunity, Immunosuppressive Cytokines and Fas-Fas- Ligand Interactions.

Introduction:

Neoplastic transformation occurs when a cell or a group of cells undergo simultaneous molecular changes that allow them to grow in defiance of the normal cellular growth controls. Factors that lead to these molecular changes vary from chemical carcinogens or physical such as ionizing radiation to biological such as oncogenic viruses. The transformation process itself, results in a wide variety of molecular changes (Cotran *et al.*, 1989). Such changes in

turn can lead to new expression of cell surface and/or secreted antigenic molecules (reviewed in Tanaka *et al.,* 1988 and Urban *et al.,* 1992). Thus, the immune system may be invoked against the transformed cells. In fact, there is physical evidence for the presence of an active immune response against tumor cells. Such evidence is perceived in the following phenomena: the accumulation of tumor infiltrating lymphocytes, which have been used in adoptive immunotherapy of certain malignancies (Roseberg *et al.,* 1986). Also, the notable hyperplasia in the lymph nodes draining the site of the tumor indicates the increased stimulation of immune cells due to the presence of the tumor. In addition, the presence of MHC-restricted and autologous CTL activity against certain tumors such as melanomas (Herlyn *et al.,* 1988), breast cancer (Baxevanis *et al.,* 1994) and ovarian cancer (Ioannides *et al.,* 1991). Moreover, the idea of the constant immunosurveillance against neoplastically transformed cells is supported by the increased cancer incidence in immunodeficient individuals (Penn, 1994 and van Gorp, 1994).

In earlier studies, we described several transformed tumor cell lines, two of which (Auto D1.4T and PE-3T) were originally normal T cell clones that underwent spontaneous transformation *ex vivo*. The remaining were two *in vivo* originated T cell lymphomas LSA and EL-4 as well as a mastocytoma cell line, P815. In these studies, we noticed that the *ex vivo* originated transformed T

cells could grow in an immunodeficient host but not in the normal host, whereas, the *in vivo* originated tumor cells could grow both in normal and immunodeficient syngeneic host. Also, we reported that two of the tumor cell lines Auto D1.4T and LSA exhibited dependence on autocrine growth factors for their *ex vivo* as well as the *in vivo* growth.

In the current study, we have investigated the nature of the immune response against these tumor cell lines in the normal host. Furthermore, we addressed the question of why the one autocrine growth factor-induced tumor cell line, which originated *in vivo*, was capable of growing in immunocompetent host while the other, which originated *ex vivo*, could grow only in an immunodeficient host. In order to address this question, we examined the expression of MHC molecules on the surface of the tumor cell lines as indication of the of ability to present antigens to host T cells. In addition, we tested the induction of CTL or NK activity by the different tumor cell lines. Moreover, the possibility of the involvement of immunosuppressive factors in the escape of the *in vivo* originating tumors from the anti-tumor immune responses of the normal host was also addressed. This was accomplished, by screening the tumor cell lines for the expression of the immunosuppressive cytokines IL-10 and TGF-β. Lastly, we tested the hypothesis that the tumor cells of T cell origin may express Fas-ligand and therefore mediate apoptosis in Fas^+ T cells that come in contact with them.

Our studies, demonstrated that tumor cells expressing high levels of MHC induced high levels of CTL activity. However, those that did not express sufficient MHC molecules on their surface elicited significant NK activity. All tumor cell lines screened, with exception the of EL-4, demonstrated constitutive expression of mRNA for the immuno-suppressive cytokine TGF-β. The constitutive expression of another immunosuppressive cytokine IL-10, on the other hand, was only detected in the LSA T cell lymphoma. Interestingly, we also detected the expression of Fas-ligand on two *in vivo* originated T cell lymphomas, LSA and EL-4. Also, the ability of these tumor cell lines to mediate apoptosis in Fas-expressing activated T cells from syngeneic mice was demonstrated. Together, these studies suggest that the growth of some tumor cells *in vivo* could be facilitated by the induction of immune suppressive molecules. In addition, we demonstrated a novel mechanism employed by tumor cells for attacking activated immune T cells using Fas-Ligand-Fas interactions. Such mechanisms could facilitate the growth of the tumors *in vivo* by evading the action of the immune system.

Materials and Methods:

Mice: Female, adult mice of the DBA/2, C57BL/6- +/+ and C57BL/6-*lpr/lpr* strains were originally obtained from the Jackson Laboratories (Bas Harbor, Maine) and bred in our animal facilities.

Antibodies: Anti-H-2^d (DBA) and anti-H-2^b (C57BL/6) polyclonal antibodies were kindly provided by NIH (Bethesda, MD). Anti-Fas mAbs were purchased from Pharmingen (San Diego, CA). Fas-Ig fusion protein kindly provided by Dr. Carl F. Ware (University of California, Riverside, CA). FITC-conjugated $F(ab')_2$ anti-mouse IgG and anti-rat IgG were obtained from Cappel Laboratories (Malvern, PA). Affinity purified normal rat serum was purchased from Jackson Immune Laboratories (West Grove, PA). Anti-IL-10 SXC.1 hybridoma (kindly provided by Dr. Tim Mossman, Alberta University, Alberta, Canada) was grown *ex vivo* and purified as described in chapter 3.

Cell Lines: Auto D1.4T syngeneic to H-2^d and PE-3T syngeneic to H-2^b were *ex vivo* originated tumor cell lines of T cell origin LSA, a thymic lymphoma syngeneic to H-2^b; EL-4 a chemically induced leukemia syngeneic to H-2^b and P815 a mastocytoma syngeneic to H-2^d were all maintained *ex vivo* as described in earlier chapters.

Flow cytometric analysis: The phenotypic expression of MHC molecules, Fas and Fas-ligand was determined by an immunofluorescence technique (Seth *et al.*, 1991). Briefly, 10^6 cells were washed in PBS containing 0.1% (w/v) sodium azide and incubated for 30 min at 4° C in the presence of anti-H-2^b, anti-H-2^d, anti-Fas (1.0 μg) or Fas-Ig fusion protein (1.0 μg). The cells were then washed twice with sodium azide containing PBS and incubated for another 30 min at 4° C with the FITC-conjugated secondary antibodies. After the second incubation period the cells were washed thrice and fixed with 0.1% (w/v) paraformaldehyde in PBS. The cells were analyzed using a flow cytometer (Epics V, model 752: Coulter, Hialeah, FL). The negative controls consisted of cells incubated with normal rat IgG or normal mouse IgG followed by FITC conjugated secondary Abs.

^{51}Cr-release assay for CTL and NK activity: CTL mediated lysis of the tumor cell lines was determined using ^{51}Cr-release assay (Nagarkatti *et al.*, 1985). Briefly, effector cells were induced *in vivo* by injecting DBA/2 or C57BL/6 mice with 10^8 X-Irradiated tumor cells intraperitoneally once a week. One to two weeks after the initial injection, mice were sacrificed and the splenocytes were extracted for T cell purification. To enrich for T cells, the spleen cells were plated in sterile 100 mm petri dishes and incubated at 37° C for 1.0 hour. The

non--adherent cells were then passed over nylon wool columns, the recovered cells were enriched for T cells. Next, 5 x 10^6 T cells were activated *ex vivo* by incubating them with 5 x 10^4 X-Irradiated syngeneic tumor cells at 37° C, 5% CO_2 /95% air humidified incubator for 4-5 days. At the end of the incubation, viable cells were isolated via density gradient centrifugation over histopaque (Sigma Chemical Co., St. Louis, MO) and used in the ^{51}Cr-release assay. Target cells (Auto D1.4T and PE-3T) were labeled with 200 μCi of $Na_2{}^{51}CrO_4$ by incubating at 37° C for 1.0 hour then washed thrice. Varying ratios of effector:target cells in triplicate were mixed in 96-well round-bottom plates (Falcon 3910, Becton Dickinson and Company, Lincoln Park, NJ) and incubated for 4 hours at 37° C for 4 hours. The spontaneous release was measured by incubating ^{51}Cr-labeled targets alone and the total release was determined by incubating the targets with 0.1 M sodium dodecyl sulfate. At the end of the incubation period, the supernatants were harvested with a Titertech collecting system (Skatron Inc., Sterling, VA) and radioactivity was measured with a gamma counter (TmAnalytic, Elk Grove Village, IL). The mean percentage of specific cytotoxicity ± S. E. M. was calculated from triplicate cultures using the formula: % Specific Cytotoxicity = 100 x (Experimental Release - Spontaneous Release)/ (Total release - Spontaneous release)

Assessing cytotoxicity using the JAM test. To demonstrate the reverse lysis phenomenon where tumor cells mediate lysis of activated T cells through apoptosis, we used the JAM test (Matzinger, 1991). Briefly, 5 x 10^6 spleen cells from C57BL/6 +/+ and C57BL/6 *lpr/lpr* were stimulated with Con A at 2.5 μg/ml and incubated at 37° C, 5% CO_2/ 95% air humidified incubator. After 36 hours, the T cells were pulsed with 8 μCi/ml [^{3}H]-thymidine and incubated for an additional 12 hours, harvested and washed once. The effector tumor cells Auto D1.4T, PE-3T, LSA, EL-4 and P815 were grown *ex vivo* as described earlier. Varying ratios of effector:target cells in triplicate were mixed in 96-well round-bottom plates (Falcon 3910, Becton Dickinson and Company, Lincoln Park, NJ) and incubated for 4 hours at 37° C for 4 hours . The spontaneous cell death was measured by incubating [^{3}H]-thymidine-labeled targets alone. Cells were then washed with deionized H_2O and harvested on glass-fiber filters with a semiautomatic cell harvester (Skatron, Sterling, VA) and the radioactivity remaining in the intact nuclear DNA was determined in a liquid scintillation counter (Betatrac 6895, TM Analytic, Inc., Elk Village, IL). The % cytotoxicity was determined according to the formula: % cytotoxicity= 100x (Spontaneous Death - Experimental Death)/ Spontaneous Death.

RT-PCR analysis of TGF-β, IL-10, Fas-Ligand and perforin gene expression in the tumor cell lines: The reverse transcribed PCR (RT-PCR)

method was employed to study whether the transformed cells constitutively expressed TGF-β, IL-10, Fas-Ligand and perforin genes as described in detail elsewhere (Nagarkatti *et al.,* 1994; Hammond *et al.*, 1993). Total RNA was extracted from the cells at various time intervals of cell culture and reverse transcribed. The cDNA samples were subjected to PCR amplification using synthetic oligonucleotide primers for various cytokines and their receptor genes, with β-actin serving as an internal control. The oligonucleotide primers used to amplify the IL-10 gene were: 5'-ATGCAGGACTTTAAGGGTTACTTGGGTT-3' and 5'-ATTTCGGAGAGGTACA-AACGAGGTTT-3'; for the TGF-β gene, 5'-TGGACCGCAACAACGCCATCTAT-GAAAACC-3' and 5'-TGGAGCTGAAGCAA-TAGTTGGTATCCAGGGCT-3'; for the Fas-Ligand gene were 5'-GAGAA-GGAAACCCTTTCCTG-3' and the 5'-ATATTC-CGGTGCCCATGAT-3'; for the perforin gene, 5'-GGCTTGTTGAGATGAGATGCTTTGACA-3' and 5'-TTGAAG-TCAAGGTGGAGTGGAGGAGGTT-3'; and for the β–Actin gene, 5'-TATCC-TGACCCTGAAGTACCCCATT-3' and 5'-AGCACAGCTTCTCTTTGATGTCACG-3'. The primers for β–Actin were synthesized by Oligos etc (Wilsonville, OR) and for IL-10 and TGF-β were purchased from Clontech (Palo Alto, CA). The PCR product was electrophoresed through a 1.5% agarose gel containing ethidium bromide. The demonstration of a single 464-, 455-, 525-, 940- and 499-bp band was considered to be indicative of the expression of β–Actin, IL-10, TGF-β, Fas-ligand and perforin genes respectively.

***In Vivo* Tumor Induction:** The LSA tumor cells (1.0×10^6) were injected into C57BL/6 mice intraparitoneally. These mice received 1.5 mg of anti-IL-10 mAbs, once every other day, intraparitoneally, for 16 days. The control mice received similar concentrations of normal rat-serum. In all experiments, groups of four mice were used and the survival of mice was monitored daily. The mean survival time in the experimental group was compared to the controls using the Chi-Square test and defferences with $p < 0.05$ were considered to be statistically significant.

Results and Discussion:

CTL activity induced by tumor cell lines:

Inasmuch as the *ex vivo* originating tumor cell lines Auto D1.4T and PE-3T were tumorigenic only in an immunodeficient host and that both cell lines were shown to constitutively express T cell growth factors, we wanted to assess the immunogenicity of these cell lines and compare it with *in vivo* originating T cell lymphomas. In order to do that, we tested the induction of CTL activity by Auto D1.4T and PE-3T. Mice were immunized with syngeneic tumor cells as described in material and methods. After *in vivo* stimulation, T cells from these

mice were isolated and subjected to further *ex vivo* stimulation by incubating them in the presence of X-irradiated tumor cells. The resulting autologous MHC-restricted activated T cells were tested in ^{51}Cr-release assay.

We noticed that the PE-3T cell line induced very high levels of specific toxicity (Figure 4.1). Also, the cytotoxic activity increased following secondary challenge thereby suggesting the involvement of T cells. The Auto D1.4T tumor cell line also induced significant lysis, although not as high as PE-3T (Figure 4.2). These results suggested that both cell lines were capable of inducing CTL activity. This may have resulted from the fact that these tumor cells were not only expressing tumor associated antigens in the context of their MHC, but also, secreting T cell growth factor that activated the CTL activity in the normal host against the tumor. This finding corroborated our earlier studies and explained why such tumor cells fail to grow *in vivo* in normal syngeneic mice. However, it should be noted that the *in vivo* originated tumor cell lines, LSA, EL-4 and P815 have been shown earlier to invoke a strong CTL activity in a syngeneic host (Nagarkatti *et al.,* 1988 and Selvan *et al.,* 1991). Despite that, such tumor cells grow and kill a normal host. Thus there may be other factors which could favor the growth of these tumor cells in the normal host.

Expression of MHC molecules on the surface of the tumor cell lines:

The fact that, Auto D1.4T did not elicit an equivalently strong CTL activity indicated that this transformed cell line is not presenting tumor specific antigens. This could be a result of several factors, one reason could be that it is down-regulating the MHC molecules on its surface while the other tumor cell lines are not. To test this hypothesis, we stained the tumor cell lines with polyclonal antibodies against MHC molecules as described in materials and methods. Interestingly, we noticed that Auto D1.4T tumor cells did not express the syngeneic H-2^d MHC molecules on their surface, while P815 expressed the H-2^d MHC molecules as shown in Figure 4.3. Furthermore, LSA, EL-4 and PE-3T tumor cell lines expressed high levels of the syngeneic H-2^b MHC molecules on their surface (Figure 4.4).

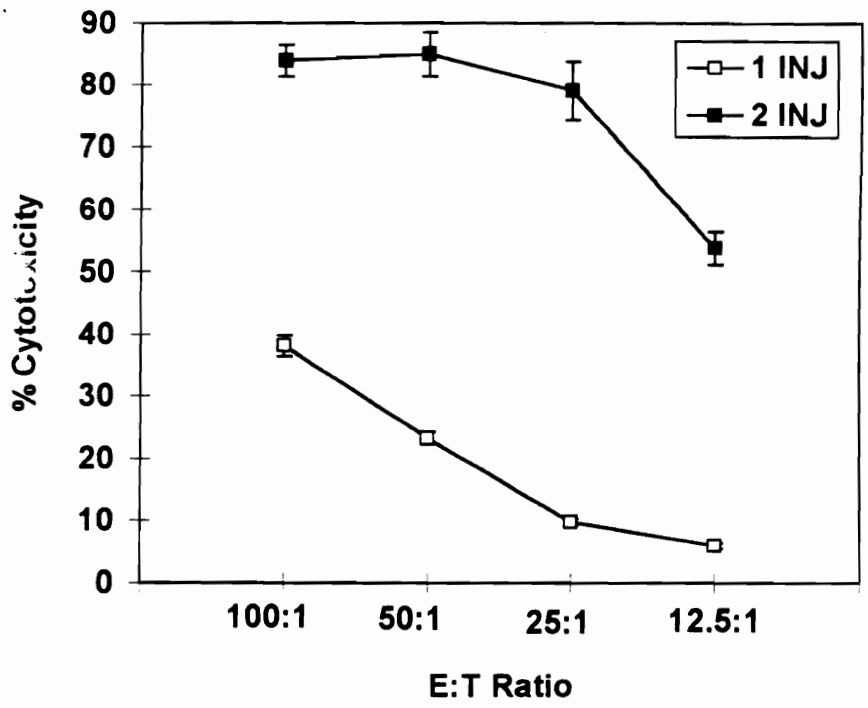

Fig. 4.1. Induction of CTL activity by PE-3T transformed cell line in syngeneic C57BL/6 mice. T cells from C57BL/6 mice immunized by i. p. injection of X-Irradiated PE-3T cells were further stimulated *ex vivo* and used as effectors in the ^{51}Cr-release assay. The cytotoxicity against PE-3T targets was assessed when the effectors were obtained from mice that were immunized once (1 INJ) or from mice immunized twice (2 INJ). The data are expressed as mean percent cytotoxicity ± S. E. at various effector:target (E:T) cell ratios.

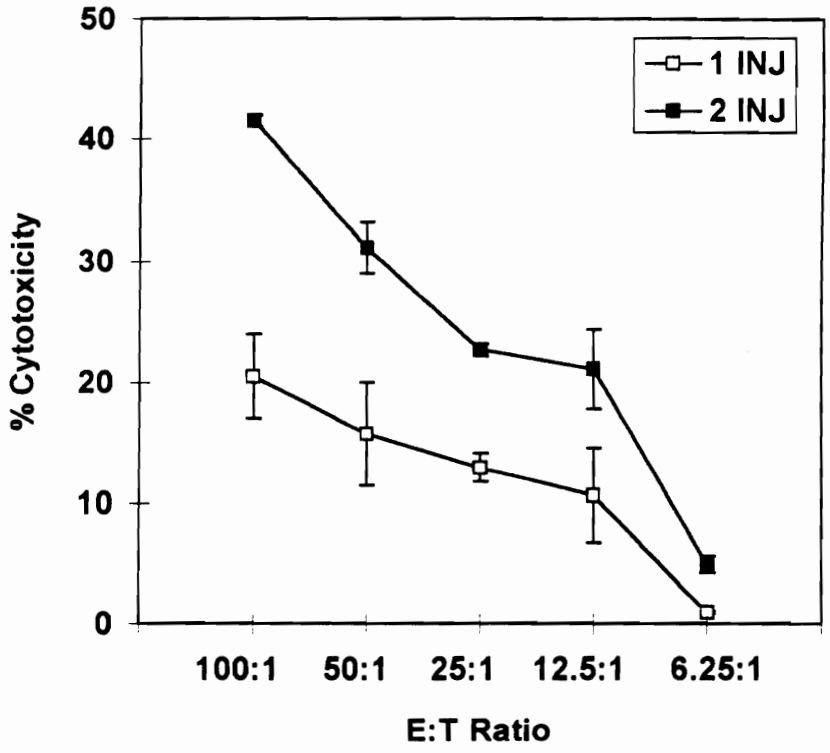

Fig. 4.2. The induction of CTL activity by Auto D1.4T transformed cell line in syngeneic DBA/2 mice. T cells from DBA/2 mice immunized by i. p. injection of X-Irradiated Auto D1.4T cells were further stimulated *ex vivo* and used as effectors in the ^{51}Cr-release assay. The cytotoxicity against Auto D1.4T targets was assessed when the effectors were obtained from mice that were immunized once (1 INJ) or from mice immunized twice (2 INJ). The data is expressed as mean percent cytotoxicity ± S. E. at various effector:target (E:T) cell ratios.

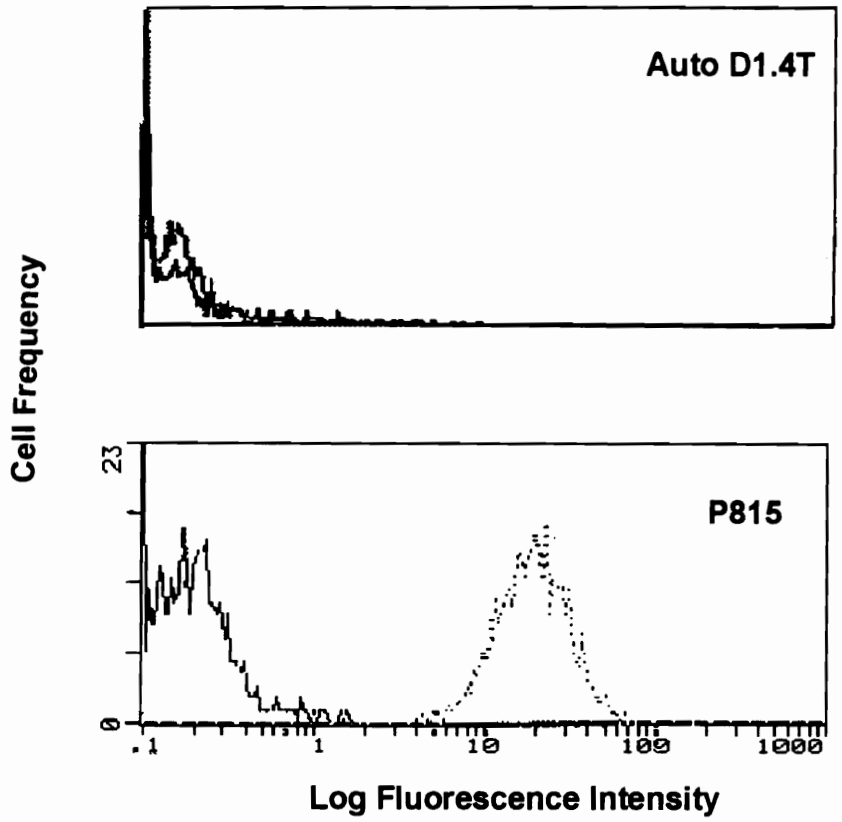

Fig. 4.3. Flow cytometric analysis of H-2^d MHC molecules expression on Auto D1.4T and P815 tumor cell lines. The Auto D1.4T and the P815 tumor cell lines were incubated with anti- H-2^d polyclonal antibodies followed by FITC-conjugated secondary antibodies as described in materials and methods. The cells were analyzed using a flow cytometer. The bold histogram represents the negative control and the broken histogram depicts cells stained for H-2^d expression.

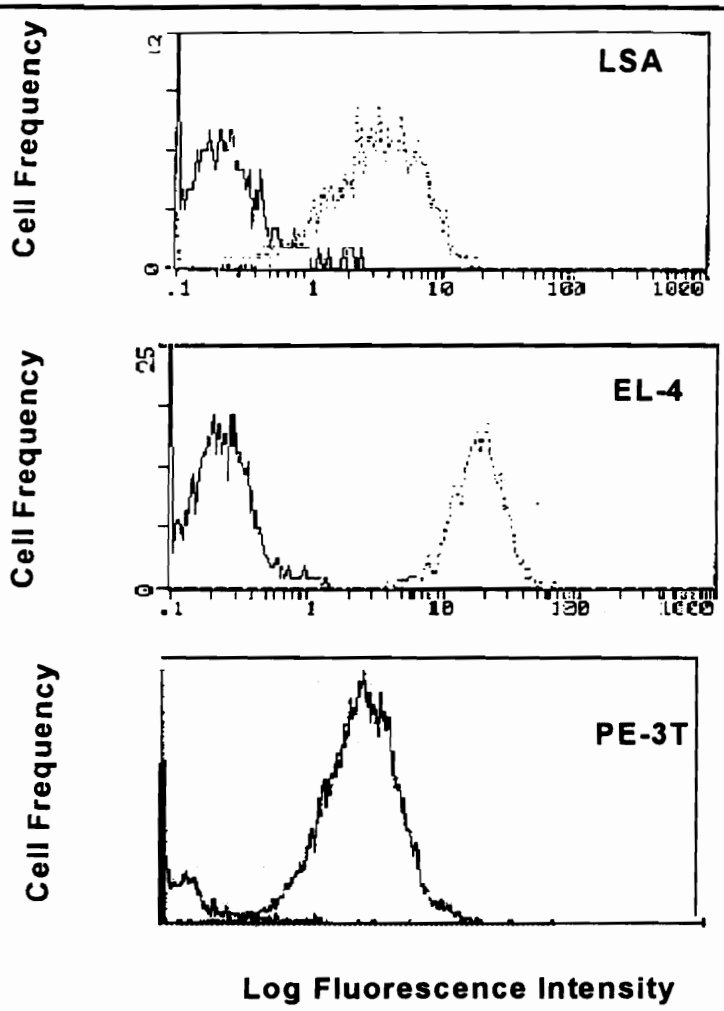

Fig. 4.4. Flow cytometric analysis of H-2^b MHC molecule expression on LSA, EL-4 and PE-3T tumor cell lines. The LSA, EL-4 and PE-3T tumor cell lines were incubated with anti- H-2^d polyclonal antibodies followed by FITC-conjugated secondary antibodies as described in materials and methods. The cells were analyzed using a flow cytometer. The bold histogram represents the negative control and the broken histogram depicts cells stained for H-2^b expression.

NK activity induced by the Auto D1.4T tumor cell line:

Inasmuch as the Auto D1.4T cell line did not express MHC molecules on its surface and induced lower levels of CTL activity, it was still not clear why it could not grow in immunocompetent host. One reason that could resolve this discrepancy was that it was capable of inducing NK activity which is not MHC-restricted. To investigate this possibility, we incubated ^{51}Cr-labeled Auto D1.4T in the presence of freshly isolated splenic cells from DBA/2 mice at different effector:target ratios. As a result, we noticed that the naive splenic cells were able to mediate significant cell lysis (Fig 4.5). Also, a similar test was performed using X-irradiated splenic cells from DBA/2 mice, since NK cells are known to be radio-resistant. Comparable levels of cytotoxicity were obtained when X-irradiated spleen cells were used as effectors (Fig 4.5). Such results indicated that the Auto D1.4T cell line is NK-sensitive, which explains why this tumor cell line is rejected in immunocompetent mice. However, these results did not clarify the reasons why highly immunogenic tumor cell lines such as LSA, EL-4 and P815 can evade the immune system (Nagarkatti *et al.,* 1988). Thus, we investigated the role of immunosuppressive cytokines in the tumorigenicity of these cell lines.

Constitutive expression of the TGF-β and IL-10 genes:

In earlier studies, we reported that both Auto D1.4T and LSA tumor cell lines utilized autocrine growth factors for their *ex vivo* growth and *in vivo* tumorigenicity (Hassuneh *et al.,* 1995 and Nagarkatti *et al.,* 1994). Also, both cell lines are highly immunogenic. However, Auto D1.4T could not grow in an immunocompetent host, while LSA could grow and kill the normal host.

Recently, it has been shown that certain tumor cells produce immunosuppressive cytokines such as TGF-β (Hsu and Hsu, 1994 and Fischer, *et al.,* 1994) or IL-10 (Suzuki *et al.,* 1995) which could in turn, suppress the anti-tumor immunity of the host thereby facilitating the growth of the tumor. Therefore, we screened Auto D1.4, PE-3T, LSA, EL-4 and P815 tumor cell lines for the constitutive expression of TGF-β and IL-10. This was achieved by performing RT-PCR using specific primers for the two cytokines as well as β-actin as an internal control.

Total cytoplasmic RNA was extracted from tumor cells grown for various periods of time including 12, 24 and 36 hours followed by RT-PCR for TGF-β, IL-10 and β-actin. Figure 5.6, demonstrates that all screened tumor cell lines with the exception of EL-4 expressed TGF-β mRNA constitutively. The IL-10 expression depicted in Figure 4.7, indicates that among the screened cell lines

only the LSA tumor cell line expressed the mRNA for the immunosuppressive cytokine, IL-10. These results suggest that the immunosuppression by TGF-β produced by the screened cell lines might not play a critical role in the *in vivo* tumorigenicity of these tumor cells because this cytokine was produced by tumor cells which could either grow or not grow in a normal host. However, the fact that only the LSA tumor cell line constitutively expressed IL-10 mRNA suggested that IL-10 may be inducing immunosuppression in the normal host and thereby allowing the growth of the highly immunogenic cell line LSA.

Such finding, also suggests that blocking such immunosuppressive cytokines may render the immunogenic tumor cells susceptible to rejection by the competent immune system of the host. Therefore, we tested whether injecting mAbs to IL-10 into LSA tumor bearing mice would lead to prolonged survival of these mice. The results depicted in Figure 4.8, indicated a slight increase in mean survival time in mice treated with anti-IL-10 mAbs, however this increase was not significant ($p>0.05$). It should be noted that the failure of anti-IL-10 mAbs to induce tumor rejection does not conclusively exclude the role of IL-10 in immunosuppression. It is possible that the amount of Abs used may not be sufficient to neutralize all IL-10 produced by LSA tumor cells. We have noticed earlier that LSA tumor cells can grow up to $200x10^6$ cells/mouse prior to death. Thus the IL-10 produced by LSA may not be neutralized by the mAbs.

The role of IL-10 can be further tested by transfecting the LSA tumor cells with IL-10 antisense and studying whether blocking of endogenous production of IL-10 would prevent tumorigenicity of LSA in normal mice.

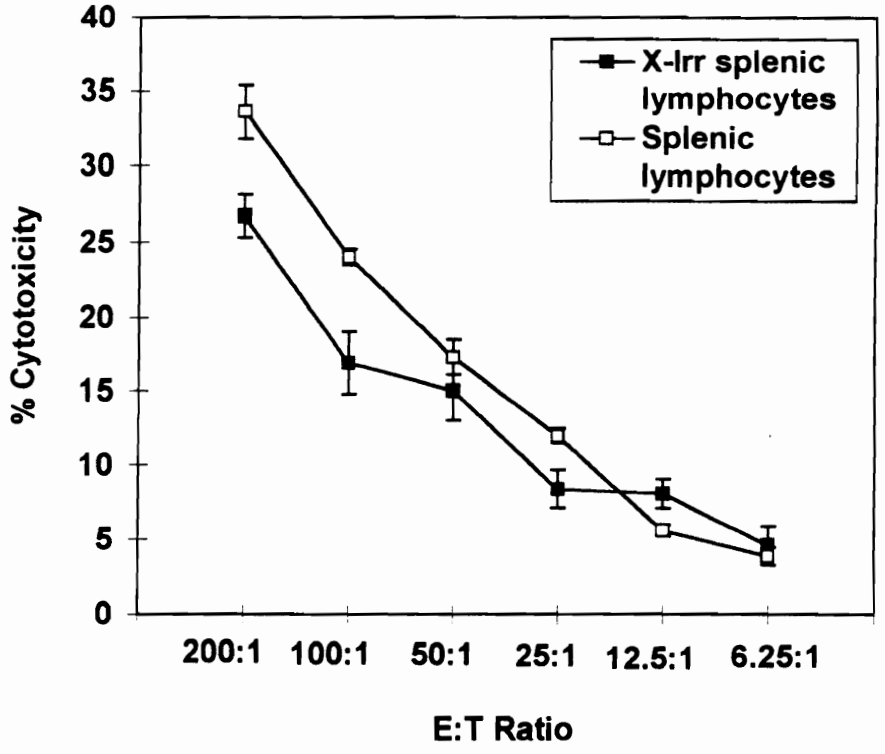

Fig. 4.5. NK activity induced by Auto D1.4T tumor cell line. The lytic activity of naive spleen cells from DBA/2 mice against the ^{51}Cr-labeled Auto D1.4T tumor cell line was tested using a ^{51}Cr-release assay. Effectors were freshly isolated splenic cell or X-irradiated splenic cells as described in materials and methods. The data is expressed as mean percent cytotoxicity ± S. E. at various effector:target (E:T) cell ratios.

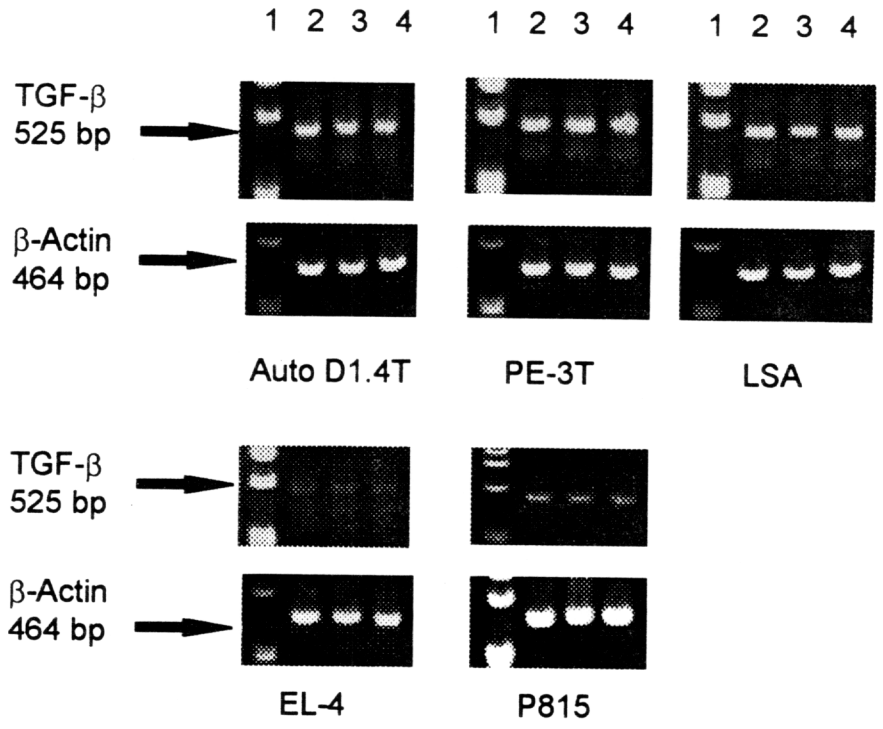

Fig. 4.6. Constitutive expression of the TGF-β gene in tumor cell lines. Total RNA was extracted from various cell lines at 12, 24, or 36 hours of *ex vivo* culture, reverse transcribed and cDNA samples were subjected to PCR amplification using synthetic oligonucleotide primers for TGF-β and β-actin as an internal control. The PCR product was electrophoresed through a 1.5% agarose gel containing ethidium bromide. Lane 1, molecular size standard (ΦX 174 *Hea* III digest), Lane 2-4 cytokine and β-actin genes expression at 12, 24 or 36 hours of culture respectively.

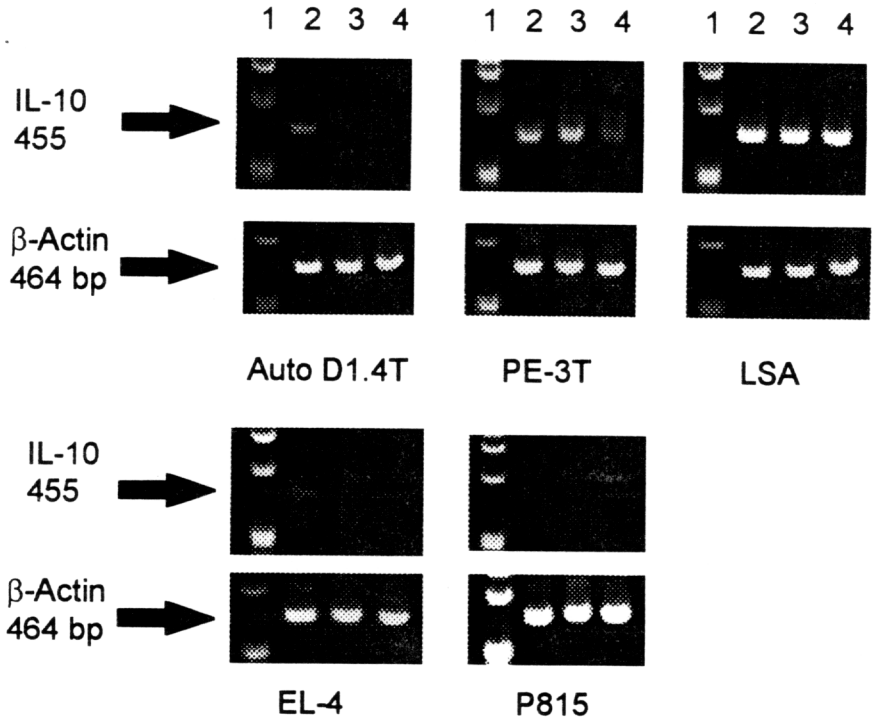

Fig. 4.7. Constitutive expression of the IL-10 gene in tumor cell lines. Total RNA was extracted from various cell lines at 12, 24, or 36 hours of *ex vivo* culture, reverse transcribed and cDNA samples were subjected to PCR amplification using synthetic oligonucleotide primers for IL-10 and β-actin as an internal control. The PCR product was electrophoresed through a 1.5% agarose gel containing ethidium bromide. Lane 1, molecular size standard (ΦX 174 *Hea* III digest), Lane 2-4 cytokine and β-actin genes expression at 12, 24 or 36 hours of culture respectively.

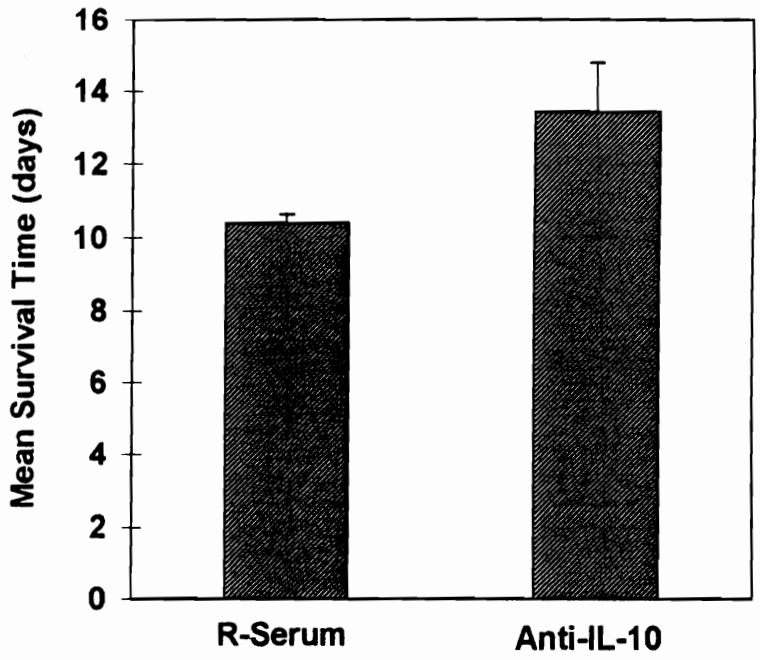

Fig. 4.8. The effect of anti-IL-10 mAbs on the survival of LSA-bearing C57BL/6 mice. Two groups of C57BL/6 mice were injected with LSA as described in materials and methods and received 1.5 mg of mAb against IL-10 (Anti-IL-10) or normal rat serum (R-serum). The survival of the tumor bearing mice was monitored and mean survival time in the experimental group and the control ± S. E. was plotted.

The role of Fas-Fas-ligand interaction in the immunosuppression induced by T cell lymphomas:

One tempting hypothesis on how tumor cells can escape anti-tumor immune response of the host involves the induction of programmed cell death (apoptosis) via Fas-Fas-ligand interactions. Apoptotic cell death can be induced in target tumor cells by NK cells and CTLs (Kagi *et al.,* 1994, Hanabuchi *et al.,* 1994 and Zychlinsky, *et al,* 1991). Such a mechanism of cell death is different from necrotic cell death, since necrotic cell death induced in target cells involves perforation of the cell membrane. Any damage to the integrity to the cell membrane leads to osmotic imbalance and swelling of cellular compartment and the eventual cell death. In apoptosis, the cell membrane remains intact however, the message triggered by the Fas-Fas ligand interactions leads to the fragmentation of the nuclear DNA. The fragmented DNA that results during apoptosis has the characteristic length of 180-200 bp and appear as a ladder when run on an agarose gel. The fragmented DNA from a cell that has undergone necrotic death appears as a smear when run on agarose gel (Wyllie *et al.,* 1980).

Recently, It has been reported that the apoptosis inducing Fas or (APO-1) molecules are directly involved in T cell-mediated cytotoxicity (Kagi *et al.,* 1994 and Hanabuchi *et al.,* 1994). Fas is a type-I membrane protein that belongs to

the TNF/nerve growth factor receptor family (Itoh *et al.,* 1991). The Fas protein is expressed on a great variety of cells including liver, heart and ovary (Watanabe-Fukunaga *et al.,* 1992). Fas-ligand on the other hand, is type-II membrane protein of the TNF family (Suda *et al.,* 1993 and Takahashi *et al.,* 1994). It's expression is restricted to activated mature T lymphocytes, except in the testis (Suda *et al.,* 1993). The binding of the Fas protein to its ligand triggers activation of DNA endonucleases resulting in cell death.

Based on these facts, we proposed that if a tumor cell line of T cell lineage retained expression of Fas-ligand, it may trigger apoptosis in Fas^+ T cells that come in contact with it . Considering that the tumor cell lines under investigation were of T cell origin except for P815, we tested whether the transformed cells have indeed expressed Fas-ligand.

This was achieved by determining the phenotypic expression via flow cytometric staining. Figure 4.9 *A* and *B* , demonstrated that the *in vivo* originated T cell lymphomas, LSA and EL-4 expressed Fas-ligand while the mastocytoma tumor cell line P815 and the *ex vivo* originating T cell tumor cell line (Auto D1.4T) did not. In order to determine whether Fas-ligand expressing tumor cell lines would be able to induce apopotosis in activated T cells, we used ConA stimulated splenic T cells from C57BL/6 +/+ and C57BL/6 lpr/lpr. The C57BL/6 lpr/lpr mice carry the classical *lpr* mutation which leads to severely reduced

expression of intact Fas mRNA (Adachi *et al.,* 1993; Kobayashi *et al.,* and Wu *et al.,* 1993). As shown in Figure 4.10, Con A blast cells from C57BL/6 lpr/lpr mice did not express the Fas protein while ConA activated cells from C57BL/6 +/+ expressed it.

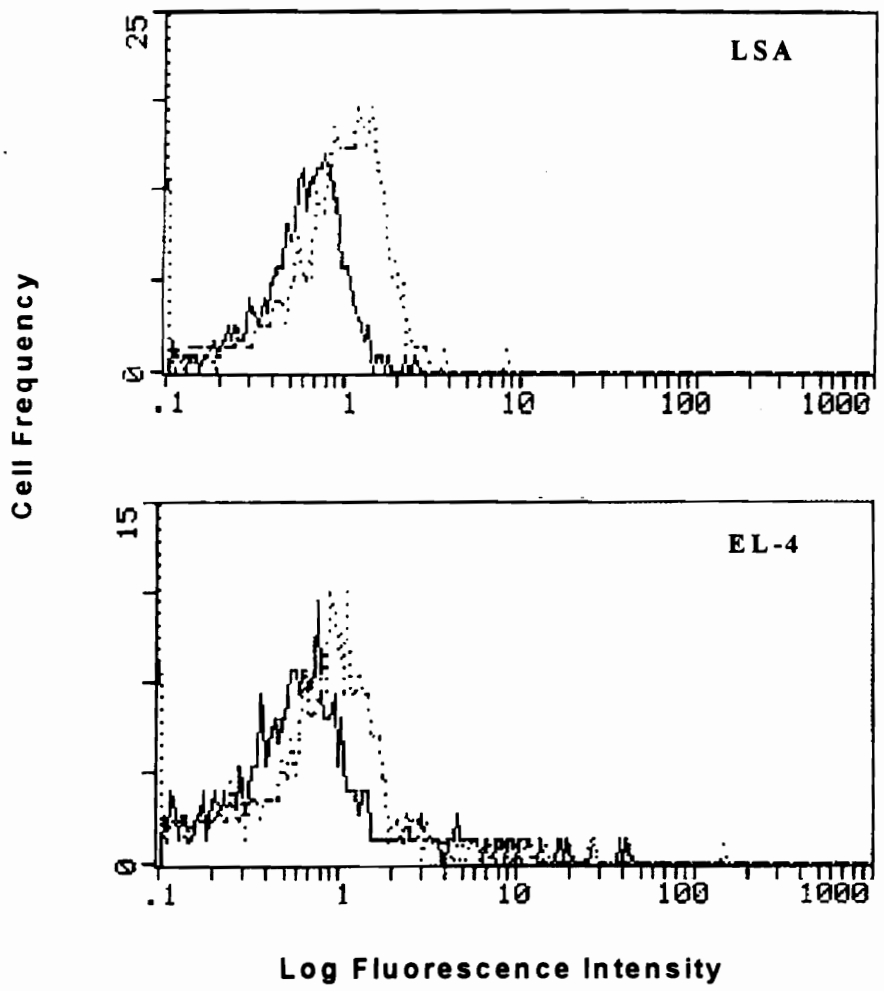

Fig. 4.9. ***A)*** **Flow cytometric analysis of the Fas-Ligand expression on LSA and EL-4 tumor cell lines.** The LSA and EL-4 tumor cell lines were incubated with Fas-Ig fusion protein followed by FITC-conjugated secondary antibodies as described in materials and methods. The cells were analyzed using a flow cytometer. The bold histogram represents the negative control and the broken histogram depicts cells stained for Fas-ligand expression.

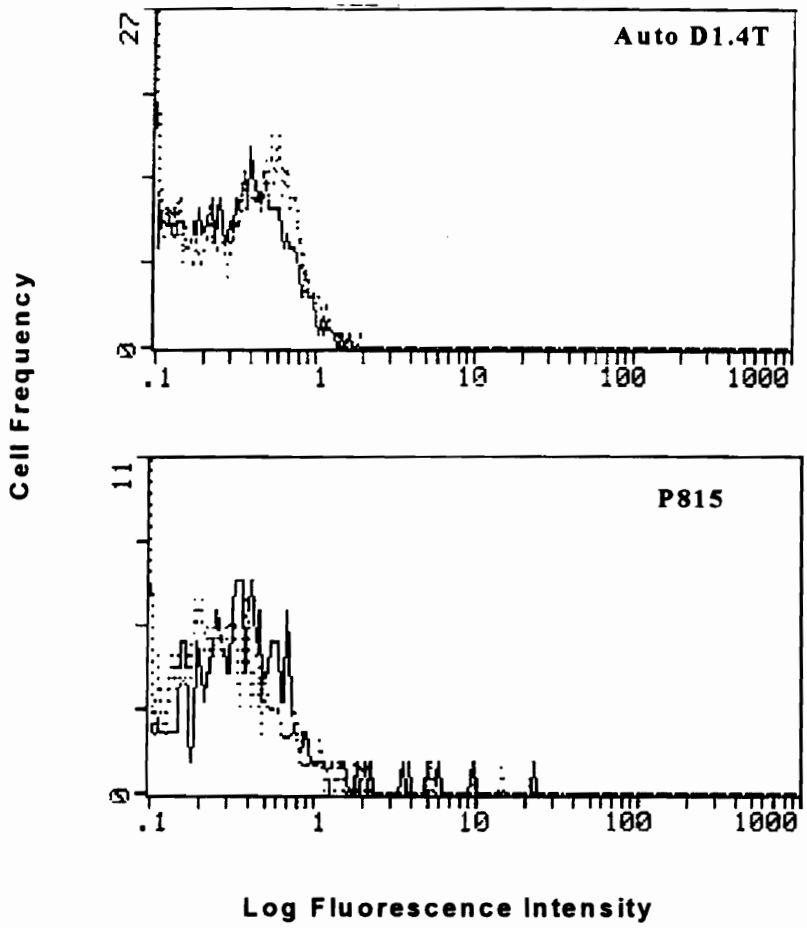

Fig. 4.9. *B)* Flow cytometric analysis of the Fas-Ligand expression on Auto D1.4T and P815 tumor cell lines. The Auto D1.4T and P815 tumor cell lines were incubated with Fas-Ig fusion protein followed by FITC-conjugated secondary antibodies as described in materials and methods. The cells were analyzed using a flow cytometer. The bold histogram represents the negative control and the broken histogram depicts cells stained for Fas-ligand expression.

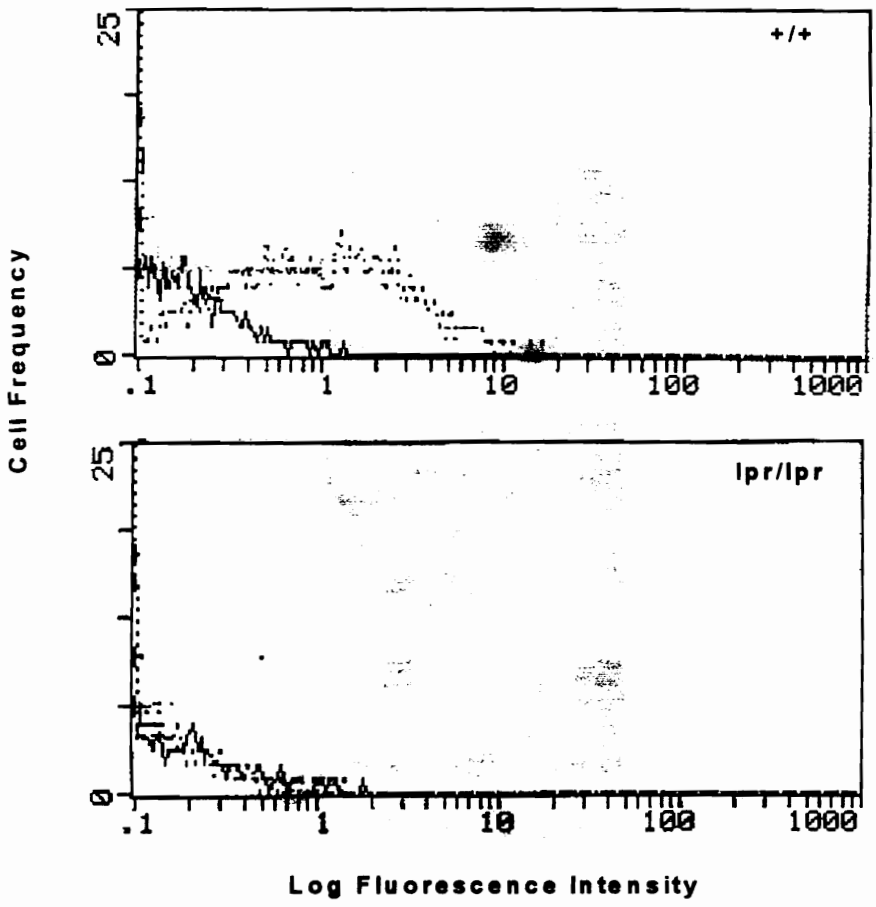

Fig. 4.10. Flow cytometric analysis of the Fas expression on Con A blast cells from C57BL/6 +/+ and C57BL/6 lpr/lpr. The Con A blast cells were incubated with anti-Fas mAbs followed by FITC-conjugated secondary antibodies as described in materials and methods. The cells were analyzed using a flow cytometer. The bold histogram represents the negative control and the broken histogram depicts cells stained for Fas expression.

To test whether the tumor cell lines expressing the Fas-ligand would be able to induce apoptosis in Con A blast cells we used the JAM test. This test was chosen over the ^{51}Cr-release assay, which can detect cell death, because the JAM test directly measures DNA fragmentation. Also, labeling of normal activated cells is more efficient when using ^{3}H-thymidine than with $^{51}Na_2CrO_4$. The results in Figure 4.11, demonstrate that the *in vivo* originated T cell lymphomas, LSA and EL-4, mediated significant killing of Con A blast cells from C57BL/6 +/+ but not from C57BL/6 lpr/lpr mice. The Auto D1.4T and the P815 tumor cell lines on the other hand, failed to trigger apoptosis in T cells (Figure 4.12). The fact that the LSA and EL-4 tumor cell lines were able to kill Con A blasts from +/+ mice and not the Con A blast cells from the lpr/lpr mice, indicated that the tumor cell-induced T cell death was mediated through Fas-Fas-ligand interactions. Furthermore, in order to exclude the involvement of other killing mechanisms such as exocytosis of perforin by the tumor cells, we tested these cell lines for expression of perforin. In addition to perforin, we also screened the cell lines for the expression of Fas-ligand and β-actin as an internal control using RT-PCR. Interestingly, both LSA and EL-4 expressed mRNA for Fas-ligand but not for perforin, while the Auto D1.4T and P815 tumor cell lines expressed mRNA for perforin and not Fas-ligand (Figure 4.13). These data demonstrated that perforin was not responsible for the induction of lysis of T cells and that Fas-ligand played a critical role. Together, these results demonstrated for the first

time, that some *in vivo* originating tumors of T cell lineage could be mediating apoptosis of tumor-specific T cells of the host and this may contribute to an important mechanism by which tumor cells could evade the action of the immune system.

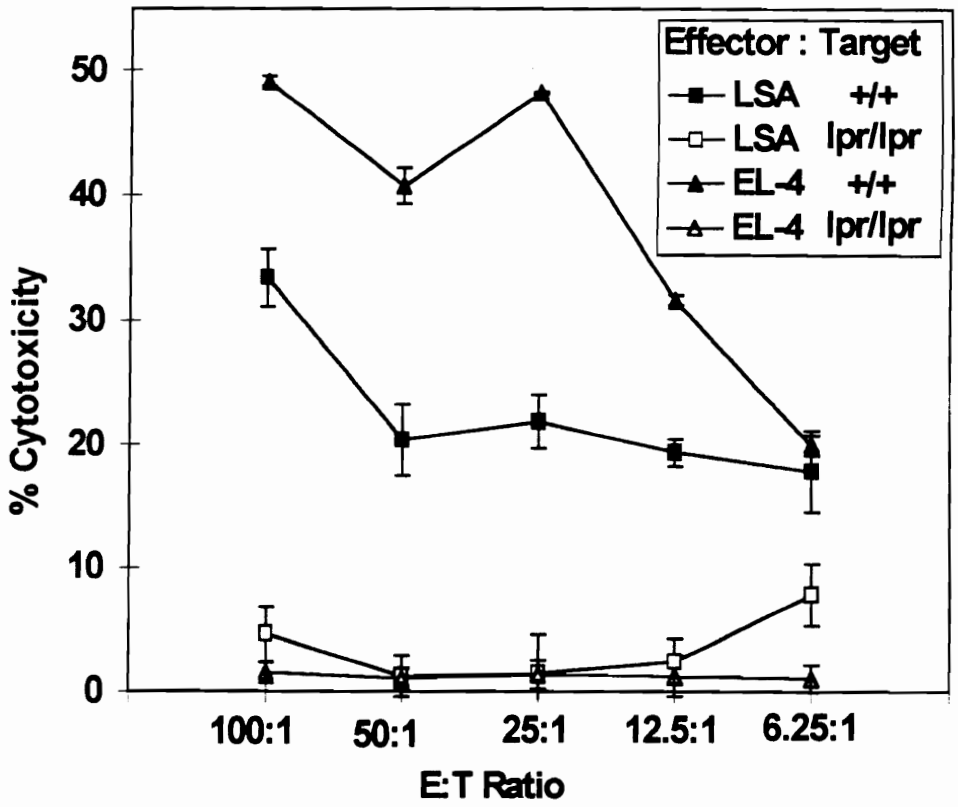

Fig 4.11. The induction of apoptosis in Con A induced T cell blasts by T cell lymphomas. The JAM test was used to test whether LSA and EL-4 tumor cell lines would be able to mediate apoptosis in Con A induced T cell blasts from C57BL/6 +/+ or C57BL/6 lpr/lpr mice. The Con A blast targets were labeled with [^{3}H]-thymidine and mixed with effector tumor cells as described in materials and methods. The data are expressed as mean percent cytotoxicity ± S. E. at various effector:target (E:T) cell ratios.

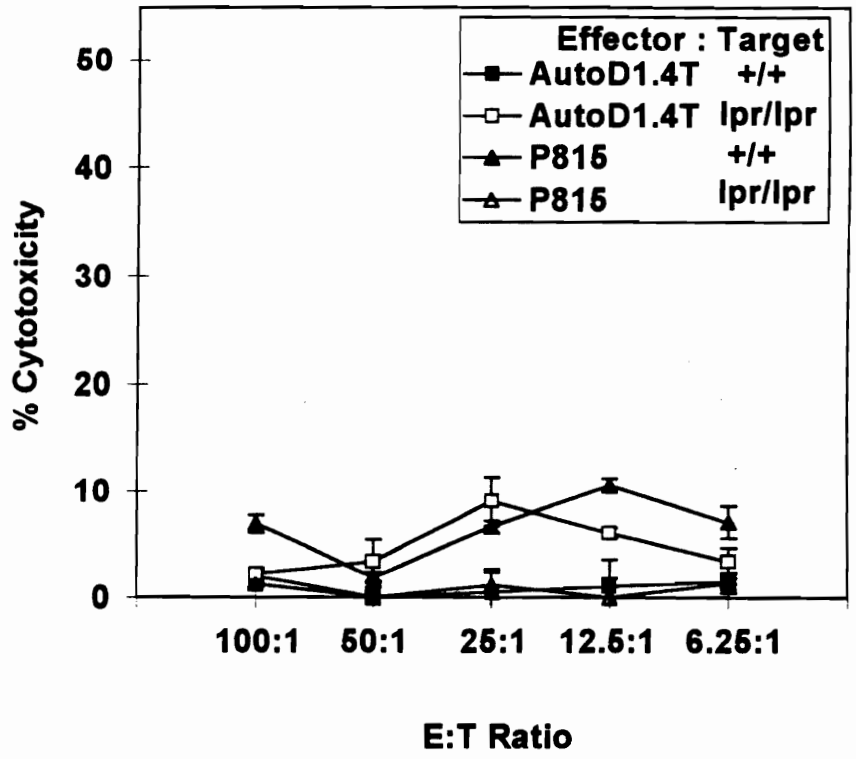

Fig 4.12. The induction of apoptosis in Con A induced T cell blasts by tumor cells. The JAM test was used to test whether LSA and EL-4 tumor cell lines would be able to mediate apoptosis in Con A induced T cell blasts from C57BL/6 +/+ or C57BL/6 lpr/lpr mice. The Con A blast targets were labeled with [^{3}H]-thymidine and mixed with effector tumor cells as described in materials and methods. The data is expressed as mean percent cytotoxicity ± S. E. at various effector:target (E:T) cell ratios.

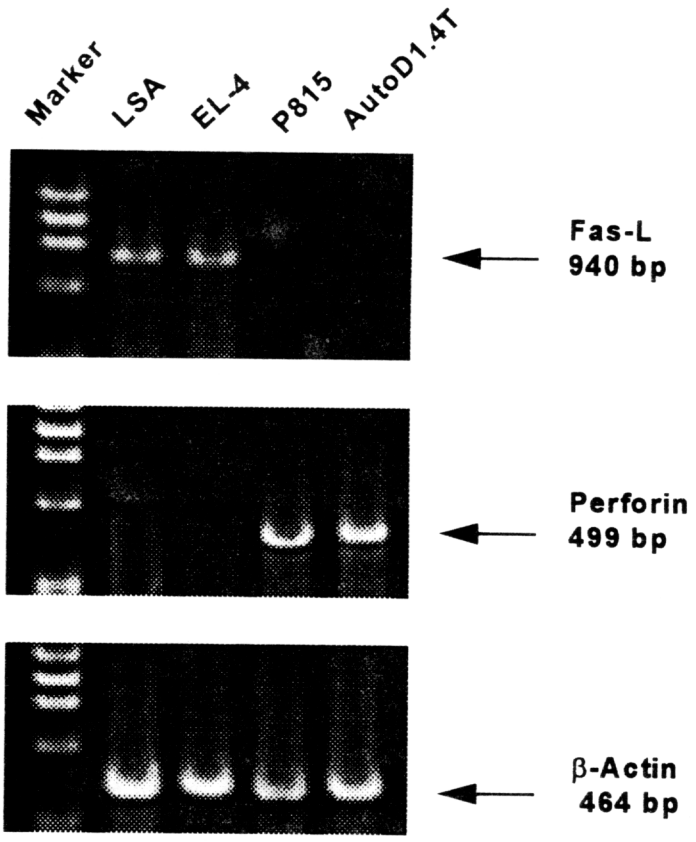

Fig 4.13. The expression of Fas-ligand and perforin mRNA in the tumor cell lines. Total cytoplasmic RNA was extracted from LSA, EL-4, P815 and Auto D1.4T tumor cell lines grown in fresh media for 36 hours. The resulting RNA was subjected to RT-PCR as described in materials and methods. The PCR product waselectrophoresed through a 1.5% agarose gel containing ethidium bromide.

The *in vivo* tumorigenicity of a tumor cell line depends on many factors. Such factors include: the capability of the tumor cell line to process and present tumor specific antigens in the context of MHC molecules (reviewed in Tanaka *et al.*, 1988 and Urban *et al.*, 1992). Also, whether the tumor cells express costimulatory molecules such as B7-1 or B7-2 is thought to play an important role in the induction of anti-cancer immunity (Matulonis *et al.*, 1996). Moreover, the tumor cells could elude the immune system by shedding antigens or receptors for growth factor essential for an active immune response. Another way by which tumor a cell can evade the immune system is by producing factors that suppress the cells of the immune system (Torre-Amion *et al.*, 1990). In the current study, we addressed some of these factors as well as other novel mechanisms and demonstrated that many varied factors may participate in the survival of cancer in the host. The ability of tumor cells to express Fas-Ligand and trigger apoptosis in T cells is an exciting finding, which may account for the escape of tumor cells from destruction caused by tumor-specific T cells. If this mechanism plays an important role, it should be possible to treat such cancers with soluble Fas. Also, the tumor cells could produce soluble Fas-Ligand which may cause significant toxicity in the host. Thus further research in this area should provide new and useful information on treatment of cancer.

Chapter 5: Summary.

Neoplastic transformation results from the simultaneous presence of several molecular changes. There is increasing evidence to suggest that constitutive production and responsiveness to autocrine growth factors may represent one such perturbation. This study was launched, when two of the several T cell clones maintained routinely in our lab, underwent spontaneous transformation. One of the transformed clones was a $CD4^+$ autoreactive T cell clone designated Auto D1.4, which was grown prior to transformation in the presence of rIL-2 and syngeneic APC. The other clone was a $CD8^+$ CTL clone, designated PE-9, maintained in culture, prior to transformation in the presence of rIL-2 and X-irradiated syngeneic tumor cells. The transformation of Auto D1.4 and PE-9 was evidenced by the autonomous growth and increased rate of proliferation even in the absence of external stimuli such as rIL-2 or through the TCR.

Thus, the first objective of this study was to determine whether T cell autocrine growth factors, such as IL-2 and IL-4, were responsible for the induction of spontaneous transformation of such T cell lines. In order to address this, the transformed clone (Auto D1.4T) was grown in the presence of

autocrine growth factor antagonists. Such antagonists include: 1) mAbs against IL-2, IL-2R, a combination of both and mAbs against IL-4. 2) immunosuppressive drug, CsA, which is known to down-regulate the expression of several cytokines including IL-2 and IL-4. 3) antisense oligonucleotides directed against the growth factor mRNA. Incubating the Auto D1.4T cells with the mAbs against IL-2 and IL-2R significantly inhibited the *ex vivo* proliferation of the transformed cells. However, the anti-IL-4 mAbs did not block the growth of this transformed cell line. CsA as well as the anti-IL-2 mRNA antisense oligonucleotides dramatically inhibited the *ex vivo* proliferation of the Auto D1.4T transformed cells. Furthermore, the Auto D1.4T transformed cells constitutively expressed the IL-2 gene as shown by RT-PCR. These results indicated that the Auto D1.4T transformed clone was dependent on the continuous production of and responsiveness to IL-2. To examine whether the transformed cells were tumorigenic *in vivo,* we injected the transformed cells into normal syngeneic and immunodeficient mice. Interestingly, the *ex vivo* transformed cells grew in nude mice but they failed to grow in a normal syngeneic host. Next, the *in vivo* growth was checked for IL-2 dependence. This was achieved by injecting nude mice with transformed cells and growth factor antagonists simultaneously. Groups of mice which received anti-IL-2, anti-IL-2R, a combination of both or CsA had significant reduction in tumor size as compared to mice injected with similar concentrations of rat IgG. Furthermore, injection of high concentrations of Abs

against IL-2 completely inhibited the tumor growth. These results demonstrated for the first time that *ex vivo* spontaneous transformation can occur exclusively due to IL-2 autocrine stimulation.

Next, we tested whether *in vivo* originated T cell lymphomas also employed similar mechanisms for tumorigenic transformation. To this effect, the role of IL-2 and IL-4 as autocrine growth factors responsible for autonomous growth of four murine tumor cell lines was investigated. The tumor cell lines included, LSA, a Rad LV- induced T cell lymphoma; EL-4, a chemically triggered T cell lymphoma; PE-3T, a T cell line which underwent spontaneous transformation *ex vivo* and P815, a mastocytoma. All tumor cell lines screened constitutively expressed IL-2R and IL-4R genes. However, only LSA and PE-3T cells expressed IL-2 and IL-4 genes constitutively while EL-4 and P815 tumor cells expressed only IL-4 but not IL-2. Monoclonal antibodies against IL-2, IL-4 or a combination of these, as well as mAbs against IL-2R significantly inhibited the proliferation of LSA but not that of other tumor cell lines *ex vivo*. To exclude the possibility that in other tumor cell lines, the autocrine growth factor may interact with its receptor within the cell, the ability of antisense phosphorothioate oligonucleotides (APO) to inhibit the growth of the tumor cells was tested. The APO specific for IL-2, IL-4, IL-2R β, or IL-2R γ chains, added in culture, could markedly inhibit the growth of LSA but not other tumor cell lines screened.

Inasmuch as, IL-2R β and γ subunits also serve as a component of the receptors for IL-4, IL-7, IL-9 and IL-15, the above data suggested that such cytokine redundancy was not responsible for autonomous growth of the other tumor cell lines. However, addition of CsA, *ex vivo*, could inhibit the proliferation of all four tumor cell lines screened. Interestingly, the LSA tumor growth in nude mice, was significantly inhibited following treatment of these mice with a combination of mAbs against IL-2 and IL-4. Together our studies demonstrated for the first time that IL-2 and IL-4 may serve as autocrine growth factors in the autonomous proliferation of tumor cells, particularly those that are retrovirally induced. Secondly, some tumor cell lines despite expressing certain cytokines and their receptors constitutively, may not depend exclusively on such factors for autocrine growth. However, the ability of CsA to inhibit the growth of such tumor cells suggests that other cytokines may participate in the autocrine loop, regulating the tumor growth.

Because, both *ex vivo* originated T cell line Auto D1.4T and the *in vivo* originated LSA cell line were found to be dependent on autocrine growth factor stimulation for their tumorigenic transformation, we wanted to investigate why the *ex vivo* originated tumor cells failed to grow *in vivo* in a normal host while the other could. To address that question, we investigated the involvement of several factors that affected the rejection or the survival of a tumor in an

immunocompetent host. To that effect, we screened the Auto D1.4T, PE-3T, LSA, EL-4 and P815 tumor cell lines for the expression of MHC molecules and whether they were able to induce CTL or NK activity in normal host. All tested cell lines expressed the MHC molecules except the Auto D1.4T cell line. Also, all cell lines induced CTL activity whereas Auto D1.4T triggered strong NK activity. Thus, these results explained why the *ex vivo* transformed cell T lines would not grow in an immunocompetent host. However, such results did not explain why *in vivo* originated tumors though immunogenic, could grow in the normal host. We next screened the tumor cell lines for the expression of immunosuppressive cytokines such as IL-10 and TGF-β. These results demonstrated that LSA was the only cell line to express both cytokines constitutively. This finding lead us to test the effect of IL-10 neutralizing mAbs on the survival of LSA-bearing mice. Although such a treatment led to an increase in the mean survival time, the host still died of tumor growth. This could have resulted from increased tumor load. Alternatively, IL-10 was not playing an important role . Thus we investigated additional factors which may facilitate the growth of T cell lymphomas.

One such mechanism was the interactions between Fas ligand and Fas expressed on tumor cells and activated T cells respectively. It was hypothesized, that tumor cells of T cell origin expressed Fas-ligand and mediated apoptosis in activated T lymphocytes that came in contact with them.

The data suggested that both the *in vivo* originated T cell lymphomas, LSA and EL-4, constitutively expressed Fas-ligand. Moreover, we tested whether these cell lines would be able to trigger apoptosis in activated T cells. Interestingly, both cell lines induced significant apoptosis of Fas^+ but not Fas^- syngeneic T cells. These results, demonstrated for the first time a novel mechanism used by tumor cells to evade the action of the immune system by triggering apoptosis of immune T cells.

Together these studies, have demonstrated for the first time that dysregulation in autocrine growth factor production and responsiveness, can be responsible for the autonomous growth *ex vivo* and tumorigenicity *in vivo,* of certain T cell lymphomas. Furthermore, such tumor cell lines are highly immunogenic due to constitutive production of T cell growth factors. However, in order to survive in the host, such T cell lymphomas may use different approaches to avoid the action of the immune system. One such important mechanism involves the constitutive expression of Fas-ligand by the tumor cell which may kill any T cell of the host that comes in contact with the tumor cell. This novel mechanism may help in the survival of the tumor cell in an otherwise hostile immunocompetent environment.

Table 5.1. Summary of the expression of various genes and functional properties exhibited by the tumor cell lines disscused in the current study.

Tumor Cell Lines	Auto D1.4T	PE-3T	LSA	EL-4	P815
IL-2 mRNA	+	+	+	-	-
IL-4 mRNA	+	+	+	+	+
IL-2R mRNA	+	+	+	+	+
IL-4R mRNA	+	+	+	+	+
IL-10 mRNA	+/-	+/-	+	-	-
TGF-β mRNA	+	+	+	+	+
Fas-Ligand mRNA	-	-*	+	+	-
Perforin mRNA	+	-*	-	-	+
IL-2R	+*	+	+	+	+
H-2	-	+	+	+	+
Fas	-	-	-	-	-
Fas-Ligand	-	-	+	+	-
CTL	+	+	+*	+*	+*
NK	+	ND	ND	ND	ND
In Vivo tumors in normal host	-	-	+	+*	+*
In Vivo tumors in nude mice	+	+	+	+*	+*

* Data not shown, ND Not Detrmined.

Conclusion and Significance of the Current Study:

The current study has demonstrated that autocrine growth factors play an important role in T cell transformation and tumorigenesis *in vivo*. Thus, growth factor antagonists can be employed to treat such cancers. We also reported that long term culture of normal T cells can lead to transformation of such cells and ability to induce tumors in an immunodeficient host. Inasmuch as, cultured T cells are being used to treat cancers and viral infections, extreme caution should be employed to ensure that such an adoptive transfer does not include any transformed cells. Otherwise, in an immunodeficient host, such cells may indeed induce additional tumors. Also, we have characterized a number of mechanisms that the tumor cells may use to escape from the potential damage caused by the immune T cells of the host. One such novel mechanism involves the use of Fas-ligand by tumor cells, which is normally used by T cells to kill tumor cells. Thus, the tumor cells that express Fas may escape from the potential damage from the host's antitumor defense. It is also possible that soluble Fas-ligand may be secreted by such tumor cells which may cause major toxicity in the cancer-bearing host. Thus, it should be possible to treat such

cancers as well as reduce toxicity using soluble Fas or mAbs against Fas ligand. Together, the current study has provided novel and useful information on the origin and control of lymphomas or leukemias of T cell origin.

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Research

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- Matched by the Biology Department, $400, Spring, 1992.
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- Awarded first place in the student paper competition, Virginia Academy of Science (Medical Sciences section), $50, May, 1994.
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Professional Experience:

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Publications:

1. Hammond, D., Nagarkatti, P., Gote, L., Seth, A., Hassuneh, M. and Nagarkatti, M. 1993. Double-negative T cells from MRL-Lpr/Lpr mice

mediate cytotoxic activity when triggered through adhesion molecules and constitutively express perforin gene. *J. Exp. Med.* **178**:2225.

2. Nagarkatti, M., Hassuneh, M., Seth, A. and Nagarkatti, P. S. 1993. Inhibition of Tumorigenicity of an *ex vivo* transformed T cell clone using antibodies against interleukin-2 and interleukin-2 receptors. Recent Adv. Chemotherapy p:998-999.

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4. Hassuneh, M., Nagarkatti, P.S. and Nagarkatti, M. Dysregulation of Cytokine Gene Expression as a cause of T cell Transformation and *In vivo* Tumorigenicity. In Molecular Biology of Hematopoiesis, Ed. N. G. Abraham, Plehum Press, New York. (1995, In press).

5. Hassuneh, M., Nagarkatti, P. S. and Nagarkatti, M. Evidence for the participation of interleukin-2 and interleukin-4 in the regulation of autonomous growht and tumorigenesis of transformed cells of lymphoid origin. (submitted, 1996).

6. Hassuneh, M., Nagarkatti, P. S. and Nagarkatti, M. "Reverse Apoptosis": Killing of host T cells by tumor cells using Fas-FasL interactions. (manuscript in preperation).

7. Hassuneh, M., Nagarkatti, P. S. and Nagarkatti, M. Nature of anti-tumor immunity against autocrine growth factor-induced tumors. (manuscript in preperation).

Abstracts:

1. Hassuneh, M.,and Nagarkatti, M. Role of IL-2 in the transformation of T cells and induction of cancer *in vivo.* Published in the ninth Annual Graduate Research Symposium booklet. March, 1993.

2. Hassuneh, M. and Nagarkatti, M. Role of IL-2 as an autocrine growth factor in T cell transformation and tumorigenesis. Published in the American Cancer Society, 13th Annual Cancer Researchers in Virginia Symposium booklet. April, 24th, 1993.

3. Hassuneh, M. and Nagarkatti, M. Role of Autocrine Growth Factors in Tumorigenic Transformation of T cells. Virginia Journal of Science Vol.44,No (2):169; 1993.

4. Gote, L., Hassuneh, M. and Nagarkatti, M. Activation of T cells via homing receptors includes interleukin-2 gene expression and cell proliferation. Virginia Journal of Science, Vol.44,No (2):168; 1993.

5. Hassuneh, M. and Ngarkatti, M. Role of IL-2 as an autocrine growth factor in the transformation and maintaenance of T cell lymphomas. 14th Annual Seminar of Cancer Researchers in Virginia. March,26th, 1994.

6. Hassuneh, M. and Nagarkatti, M. The transformation and maintenance of T cell lymphomas as a result of IL-2 autocrine stimulation. Virginia Journal of Science, Vol.45, No (2):97, 1994.

7. Hassuneh, M. Nagarkatti, P.S. and Nagarkatti, M. Dysregulation of the cytokine gene expression as a cause of T cell transformation and *in vivo* tumorigenesity. National Am. Assoc. Immunol. Meeting, April, 1995. Atlanta, GA.

8. Nagarkatti, M. and Hassuneh, M. Dysregulation of cytokine gene expression as a cause of T cell transformation and *in vivo* tumorigenecity. Acta Haematologica 9th symposium of molecular biology of hematopoiesis. June 23-27, 1995.

Presentations at Meetings:

Hassuneh, M.,and Nagarkatti, M. Role of IL-2 in the transformationof T cells and induction of cancer *in vivo*. Poster presented at the ninth Annual Graduate Research Symposium. March, 1993. VPI & SU, Blacksburg, VA.

Hassuneh, M. and Nagarkatti, M. Role of IL-2 as an autocrine growth factor in T cell transformation and tumorigenesis. Presented at the American Cancer Society, 13th Annual Cancer Researchers in Virginia Symposium. April, 24th, 1993. Richmond, VA.

Hassuneh, M. and Nagarkatti, M. Role of autocrine growth factors in tumorigenic transformation of T cells. Presented at the 71st meeting of the Virginia Academy of Science at old Dominion University. May, 20-21 1993. Norfolk, VA.

Gote, L., Hassuneh, M. and Nagarkatti, M. Activation of T cells via homing receptors includes interleukin-2 gene expression and cell proliferation. Presented at the 71st meeting of the Virginia Academy of Science at old Dominion University. May, 20-21 1993. Norfolk, VA.

Nagarkatti, M., Hassuneh, M. and Seth, A. Inhibition of autocrine growth and tumorigenicity induced by a T cell clone *in vivo* using monoclonal antibodies against cytokines and cytokines receptors. Presented at the 18th International Congress of Chemotherapy. July,1993. Stockholm Sweden.

Hassuneh, M. and Nagarkatti, M. Perturbation in Autocrine Growth Factor Production as a Cause of T cell Transformation and its Regulation using Growth Factor-Specific Antibodies or Antisense Oligonucleotides. Presented at the Annual Meeting of the Virginia Branch of American Society for Microbiology. November, 1993. Lexinton,VA.

Hassuneh, M. and Ngarkatti, M. Role of IL-2 as an autocrine growth factor in the transformation and maintaenance of T cell lymphomas. 14th Annual Seminar of Cancer Researchers in Virginia. March, 26th, 1994. Blacksburg, VA.

Hassuneh, M. and Nagarkatti, M. The transformation and maintenance of T cell lymphomas as a result of IL-2 autocrine stimulation. Presented at the 72nd annual meeting of the Virginia Academy of Science at James Madison University. May, 21st, 1994.

Nagarkatti, M. and Hassuneh, M. Dysregulation of the IL-2 gene expression as a cause of T cell transformation and its potential hazard in immunotherapy or immunorehabilitation. Presented at the International Congress of Immunorehabilitation. July, 1994. Russia.

Hassuneh, M. Nagarkatti, P.S. and Nagarkatti, M. Dysregulation of the cytokine gene expression as a cause of T cell transformation and *in vivo* tumorigenesity. Presented at the National Am. Assoc. Immunol. Meeting, April, 1995. Atlanta, GA.

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Nagarkatti, M. and Hassuneh, M. Dysregulation of cytokine gene expression as a cause of T cell transformation and *in vivo* tumorigenecity. Presented at the 9th symposium of the Molecular Biology of Hematopoiesis. June 23-27, 1995. Genoa, Italy.