

Dana Jean Fraser. **THE IMMUNOMODULATION OF MYELIN BASIC PROTEIN-SPECIFIC T HELPER CELLS BY INTERLEUKIN-2.** (Under the direction of Dr. Thomas J. McConnell) Department of Biology, July 1995.

ABSTRACT

The purpose of this thesis is to examine the regulation of interleukin-2 (IL-2) by myelin basic protein (MBP)-specific rat T cells, and to look at the effects of IL-2 on those same T cell lines. MBP-specific T cells have been implicated in Experimental Autoimmune Encephalomyelitis (EAE), an autoimmune disease which is inducible in many animal species including Lewis rats. EAE is studied as an animal model for multiple sclerosis because they are similar in etiology. This research focuses on IL-2 because it may play an important role in autoimmunity.

Previously, the available source of rat IL-2 was the supernatant of Concanavalin A-activated rat splenocytes, which contains not only IL-2 but a number of other rat proteins including cytokines. To pursue this research, a source of rat IL-2 was needed which contained no other rat cytokines. The rat IL-2 gene was cloned, sequenced, and used to express the IL-2 protein *in vitro* in a baculovirus system. Through CTLL-2 bioassays and proliferative assays it was determined that the IL-2 protein was fully functional.

Several aspects of the role of IL-2 in T cell regulation were examined using T cell proliferative assays and CTLL-2 bioassays. When MBP-specific T cells are reactivated within 4 days, they are refractory to IL-2 production. This refractory period may play a role in limiting the activation of autoreactive T cells. W3/25 is an anti-CD4 monoclonal

antibody (mAb) that is inhibitory to T cell proliferation and IL-2 production. Even though W3/25 inhibited IL-2 production, proliferation, and rendered MBP-specific T cells anergic, IL-2 did not reverse the W3/25-mediated inhibition of proliferation. These results indicate that its effects are not just restricted to inhibition of IL-2 production, but may also involve inhibition of IL-2 receptor expression. When T cells were cultured in the absence of IL-2, they lost antigenic reactivity. T cells cultured in media supplemented with recombinant rat IL-2 were six-fold more responsive to antigen specific activation than T cells cultured in unsupplemented media. This loss of antigenic responsiveness in the absence of IL-2 may indicate yet another role for IL-2 in the maintenance of self-tolerance. Together these data contribute to the understanding of T cell regulation by IL-2. Increased knowledge of T cell regulation may provide insight to the origins of autoimmunity.

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T HELPER CELLS BY INTERLEUKIN-2

A Thesis
Presented to
the Faculty of the Department of Biology
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In Partial Fulfillment
of the Requirements for the Degree
Master of Science in Molecular Biology and Biotechnology

by
Dana Jean Fraser
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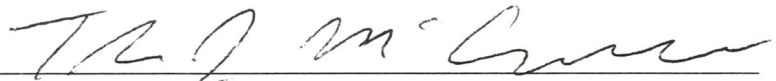
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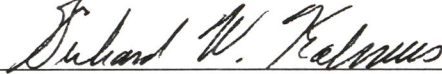
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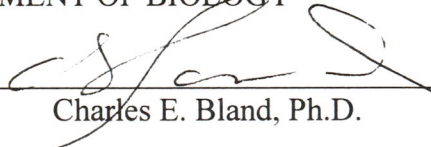
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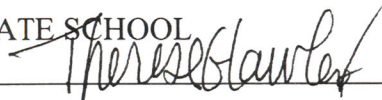
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DEDICATION

To my husband James for all of his love, patience, and support while I pursued this degree.

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INTRODUCTION

REGULATION OF THE IMMUNE RESPONSE

One function of the immune system is to protect the body from invading microbes, and it consists of many different components which act through both general and specific mechanisms to eliminate foreign antigen. The immune system is self-regulating in that it functions to remove the antigen responsible for triggering an immune response, and the removal of the stimulus results in the termination of the immune response. While carrying out its function of protection, the immune system must somehow distinguish foreign protein from self protein. There is a complex network of cells and cytokines which govern the regulation of the immune response and function in the maintenance of tolerance to self protein.

T Lymphocytes. Lymphocytes are the major cells which are responsible for the recognition of antigen. One subset of lymphocytes are the T lymphocytes which are responsible for cell-mediated immunity which occurs through the destruction of microbes or the lysis of infected cells. T lymphocytes may be further subdivided into two categories, the helper T (T_H) cells and the cytotoxic T cells. Both subsets have membrane-bound receptors, referred to as T cell receptors (TcR), that recognize specific peptide antigens only when non-covalently bound to major histocompatibility complex (MHC) proteins on the surface of antigen presenting cells (APC). The cytotoxic T cells

are identified by the expression of the cell surface marker CD8, and are known to lyse cells that are presenting foreign antigen bound to class I MHC molecules. A T_H cell can be distinguished from a cytotoxic T cell by the expression of a CD4 cell surface molecule instead of CD8. When a T_H cell is activated by the presentation of specific peptide antigen bound to class II MHC molecules, it will begin to secrete proteins called cytokines which function to promote proliferation and differentiation of T cells as well as other immune system cells. T_H cells can be further subdivided based on their pattern of cytokine secretion. T_H1 lymphocytes produce IL-2, γ -interferon, and tumor necrosis factor β , while T_H2 cells produce interleukin-4, interleukin-5, interleukin-6, interleukin-10, and interleukin-13 (Paul and Seder, 1994).

The T lymphocytes arise in the bone marrow and migrate to the thymus, which is the major site of maturation for both subsets. Initially thymocytes do not express a TcR, CD4, or CD8 and are incapable of effector functions. In the cortex of the thymus, where the most immature thymocytes are found, a high rate of mitosis occurs. However, less than five percent of the immature thymocytes produced survive the migration to the medulla where they will exit the thymus as mature T lymphocytes, entering the periphery. During this migration through the thymus the cells will begin to express TcR and cell surface markers and undergo positive and negative selection, the processes which select for cells recognizing foreign antigen or eliminate autoreactive T cells, respectively.

Thymocytes begin to express TcR, which are generated by a random rearrangement of germline gene sequences, early in their transit through the thymus. The

most common TcR consists of an α and β chain containing variable, joining, and constant regions, with the β chain also containing diversity regions. It is the rearrangement of these genes that creates the enormous diversity of the T cell repertoire. The TcR is noncovalently associated with the CD3 complex which consists of at least five noncovalently associated membrane proteins and is responsible for some of the signals associated with cellular activation. On T_H cells the TcR is also closely associated with the CD4 cell surface marker which is thought to stabilize the TcR and antigen presenting cell interaction (Doyle and Strominger, 1987). CD4 is physically associated with the tyrosine protein kinase $p56^{lck}$ and may transduce positive signals, which augment the ability of the cell to become activated, or negative signals, resulting in cellular inhibition (Janeway *et al.*, 1988; Veillette *et al.*, 1988).

T Cell Activation. When a T_H cell is activated through presentation of specific antigen by an APC, the two main responses of the cell are secretion of cytokines and proliferation, resulting in the clonal expansion of the antigen specific T cell. The clonal expansion of cells with the same antigenic specificity through mitotic division augments the immune response against a particular antigen. These T cell responses to activation result from signal transduction events leading to transcriptional activation of a variety of genes, including those of cytokines and new cell surface molecules such as cytokine receptors. Immediately following activation the plasma membrane protein phosphatidylinositol 4,5 biphosphate is hydrolyzed by phospholipase C, resulting in

increased cytoplasmic levels of diacylglycerol and inositol 1,4,5-triphosphate (Rudd *et al.*, 1989). This enzymatic reaction is followed by an increase in cytoplasmic calcium levels and the subsequent activation of protein kinase C. Protein kinase C and other protein kinases are responsible for the phosphorylation of membrane proteins such as the CD3 δ and γ chains, and CD4. In addition, these protein kinases may activate cytoplasmic proteins such as DNA binding proteins through phosphorylation, thereby regulating transcriptional activity of genes essential for T cell activation.

The interaction between the TcR and antigen bound to an MHC molecule is not the only contact necessary between a T_H and an APC to result in activation. In addition to CD4, other accessory molecules such as CD2, LFA-1, CD28, and CD45 might be required to interact with their respective ligands on an APC for activation to occur. The precise function of all accessory molecules is not known, but they are believed to be involved in various roles such as cell-adhesion and signal transduction. While some of these accessory molecules are necessary for the activation of a T cell, others affect the level of activation. For example, the costimulatory signal provided by an anti-CD28 monoclonal antibody (mAb) can prevent the induction of anergy in T cells (Harding *et al.*, 1992). The binding of CD28 by its ligand during activation of a T cell has been shown to induce the formation of a protein complex which binds to the interleukin-2 (IL-2) gene resulting in increased levels of IL-2 transcripts (Fraser *et al.*, 1991; Linsley *et al.*, 1991).

Discrimination of self from non-self. The immune system must be capable of discriminating self proteins from foreign protein so as not to mount an immune response against the body. Self-tolerance is an acquired process and may occur through several mechanisms. During maturation in the thymus, the lymphocytes undergo the processes of positive and negative selection. Positive selection of lymphocytes is a mechanism which helps to avoid the accumulation of useless lymphocytes, such as those with no receptor or a useless receptor (von Boehmer, 1994). Positive selection also serves to enhance the population of T cells that potentially recognize foreign peptides which are presented by MHC molecules. Those T cells bearing a TcR capable of recognizing self peptide are subject to negative selection and will undergo clonal deletion (Nossal, 1994).

The mechanisms of positive and negative selection in the thymus can not entirely explain peripheral tolerance of lymphocytes to self antigen. There are self proteins which may not be present in the thymus, because they are anatomically sequestered or are not yet expressed. T lymphocytes with that particular antigenic specificity will escape clonal deletion. In the case of an anatomically sequestered antigen, the lack of antigen availability for presentation may aid in the maintenance of peripheral tolerance (Miller and Heath, 1993). If there is a T cell in the periphery that recognizes self antigen, which is available for presentation, there must be mechanisms in place to render those cells nonresponsive. The functional inactivation, or anergy, of autoreactive T lymphocytes may serve this purpose. When a T cell is activated in the periphery without the proper costimulus, the outcome is clonal anergy (Kronenberg, 1991; Sloan-Lancaster *et al.*,

1993; Johnson and Jenkins, 1994). Johnson and Jenkins (1993) suggested that presentation of antigen without the appropriate costimulatory signal will result in the delivery of a negative signal resulting in anergy. Defects in the maintenance of peripheral tolerance may result in autoimmune disease.

Clonal Anergy. Clonal anergy is the functional inactivation of T cells, which renders them nonresponsive to their respective antigen without compromising cellular viability. When a T cell recognizes specific antigen in the absence of the necessary costimulatory signal, the result is clonal anergy (Miller *et al.*, 1991; Johnson and Jenkins, 1993; Larche' *et al.*, 1994). In addition, anergy can be induced when the proper costimulatory signals are present but the cell is not allowed to proliferate. For example, the activation of cells with normal costimulatory APC in the presence of anti-IL-2 and anti-IL-2 receptor mAbs will result in the induction of anergy (Johnson and Jenkins, 1993). Anergy induction appears to involve a change in T cell phenotype as cell surface molecules such as TcR, CD28, and CD4 are downregulated (Cobbold *et al.*, 1992; Larche' *et al.*, 1994). The main effect of anergy on T cells appears to be a reduced level of IL-2 transcription. Defects in the binding of transcription factors to the AP-1 and NF-AT sites of the IL-2 promoter have been observed (Lasalle and Hafler, 1994). The existence of "anergy proteins" has been proposed to explain the IL-2 mRNA deficit of anergic T cells (Johnson and Jenkins, 1993). Protein synthesis is necessary for the induction of anergy as it can be stopped by the addition of protein synthesis inhibitors such as cyclohexamide (Johnson

and Jenkins, 1993; Larche' *et al.*, 1994). Other defects in antigen stimulated anergic cells are a decreased level of tyrosine kinase activity, a decreased rise in intracellular Ca^{++} , and the expression of constitutively reduced amounts of p56^{lck} (Gilbert, 1994; Lasalle and Hafler, 1994; Kronenberg, 1991).

Experimental Autoimmune Encephalomyelitis.

Experimental Autoimmune Encephalomyelitis (EAE) is a T cell mediated autoimmune disease directed against Myelin Basic Protein (MBP), which is a major component of the myelin sheath surrounding the axons of the central nervous system (Heber-Katz and Acha-Orbea, 1989). Because the symptoms and pathology of EAE are similar to that of multiple sclerosis, it is often studied as a model of this disease. EAE has been induced in a number of animal species such as the Lewis rat, which is highly susceptible to the disease. The disease may be induced by immunization with MBP in an emulsion of complete Freund's adjuvant, by adoptive transfer with lymphocytes from immunized donors, and by immunization with synthetic encephalitogenic peptides (Mannie *et al.*, 1985; Paterson and Swanborg, 1988; Miller *et al.*, 1991).

Anti-CD4 mAb administered at the time of immunization with encephalitogenic MBP or synthetic peptides have been shown to prevent the induction of EAE (Shizuru *et al.*, 1992). Anti-CD4 mAb have been used *in vivo* to deplete CD4^{+} T cells and to inhibit T cell function and have been shown to block *in vitro* responses to specific antigen through inhibition of cytokine production and proliferation (Shizuru *et al.*, 1992; Mannie

et al., 1993). As a result of their ability to induce tolerance, anti-CD4 mAb have been investigated for use in immunotherapy of autoimmune disorders. Two possible mechanisms by which anti-CD4 mAb may affect CD4⁺ T cells are by physically blocking the interaction of CD4 with a MHC molecule, thereby inhibiting the TcR recognition of foreign antigen and interfering with normal signal transduction, or by causing the transduction of a negative signal through CD4 (Shizuru *et al.*, 1992).

W3/25 is an anti-CD4 mAb which has been studied to elucidate its mechanisms for inhibition of T cells and its ability to induce anergy (Mannie *et al.*, 1993, 1994). W3/25 inhibits MBP-stimulated proliferation of sensitized lymph node cells and partially inhibits the response of cloned and uncloned lines of MBP-specific T cell lines (Mannie *et al.*, 1993). W3/25 also blocks antigen specific production of the autocrine growth factor IL-2 (Mannie *et al.*, 1993, 1994). In addition, the inclusion of W3/25 in an antigen specific activation has been shown to induce anergy in MBP-specific T cell lines (Marinakakis, 1995; Mannie, personal communication).

Interleukin-2. Cytokines are soluble factors involved in the immune response which have a wide range of effects on their target tissues and cells. Many cytokines function redundantly; that is, several different cytokines may act on the same cell type in a similar manner. In addition, one cytokine may have a variety of effects on different cell types (Kishimoto *et al.*, 1994). IL-2 is a cytokine produced by T_H1 cells upon activation whose many functions include inducing the proliferation of T cells, and the reversal of anergy

(Umlauf *et al.*, 1993). IL-2 may also regulate other cells involved in the immune response such as B cells and monocytes (Cohen, 1990). The human and murine IL-2 genes contain four exons and code for 153 amino acids, of which the first 20 are a signal polypeptide (Fuse *et al.*, 1984, Holbrook *et al.*, 1984). The molecular weight of IL-2 is 15.5 kD in the absence of post-translational modifications (McKnight and Classon, 1992), and its structure contains anti-parallel α -helices (Waldmann, 1993).

The Interleukin-2 Receptor. Like other cytokines, IL-2 exerts its biological effects through a specific cell surface receptor (Kishimoto *et al.*, 1994). The high affinity IL-2 receptor is a heterotrimer consisting of the α , β , and γ chains which associate in a noncovalent manner (Waldmann, 1993). The α chain is a densely glycosylated 55kD subunit which binds IL-2 with low affinity (Waldmann, 1993; Kaempfer, 1994). The β chain is 70 - 75 kD, and with the 64 kD γ chain forms an intermediate affinity receptor. The γ chain is required for receptor mediated internalization of IL-2 and alters the β chain so that it may bind IL-2 (Minami *et al.*, 1993). The β subunit of the high affinity receptor is necessary for signal transduction, and the α subunit serves to enhance the affinity of the complex for the ligand (Kishimoto *et al.*, 1994). The α and β chains of the high affinity IL-2 receptor complex are inducible by mitogenic or antigenic stimulation of T cells (Kaempfer, 1994). The γ chain is constitutively expressed in lymphoid cells (Taniguchi and Minami, 1993). The α chain of the high affinity receptor is very unstable with a half life of two to three hours. This short half life provides for rapid regulation of

the high affinity form of the receptor. When transcription of the α chain ceases, responsiveness to IL-2 halts quickly (Kaempfer, 1994).

Signaling begins by the ligand-induced dimerization of the receptor components, which causes a signaling cascade (Kishimoto, 1994). While there are no enzymatic motifs in the IL-2 structure, there is evidence for two distinct signaling pathways resulting from the interaction of the IL-2 high affinity receptor with its ligand. The β chain has two functional domains, one membrane-proximal serine-rich region found to be important in IL-2 induced proliferative responses as well as a membrane-distal acidic region which is physically associated with tyrosine kinases. When the latter region is missing, proliferative signals are still delivered through the IL-2 receptor. Both regions seem to mediate the induction of the transcription factors c-myc and c-fos.

Interleukin-2 and Peripheral Tolerance. IL-2 has been implicated as an important cytokine in the maintenance of peripheral tolerance. It has been shown that anergy can be reversed by culturing the cells in the presence of IL-2 and allowing the cells to undergo several rounds of cell division (Johnson and Jenkins, 1993). This observation supports the idea that anergy proteins exist that affect the transcription of IL-2. Proliferation may increase the turnover rate of proteins or effectively dilute out the concentration of the inhibitory proteins. The cell surface molecule CD28 provides more insight into the significance of IL-2 in peripheral tolerance. CD28 provides signaling which enhances the production of IL-2, and anti-CD28 mAb can prevent the induction of anergy (Fraser *et*

al., 1991; Linsley *et al.*, 1991). CD28 has been shown to be downregulated in anergic cells which might explain some of the loss of IL-2 production in anergic T cells (Larche' *et al.*, 1994).

The T cells found in autoimmune lesions have been shown to produce IL-2 and express IL-2 receptors, and IL-2 is known to enhance the activation of many cells of the immune system (Sinha *et al.*, 1990). Clonal anergy can be reversed *in vivo* by infection suggesting that T cell clones become autoreactive under inflammatory conditions (Lasalle and Hafler, 1994). An infection would cause IL-2 production by T cells and would result in the recruitment of cells to the area of infection. If anergic, autoreactive T cells *in vitro* are exposed to IL-2, the condition is reversed and the same could be true of autoreactive T cells *in vivo*. This "bystander activation" caused by IL-2 production could result in a breakdown of peripheral tolerance.

IL-2 has been demonstrated to be an important cytokine in the regulation of MBP-specific Lewis rat T cell lines (Mannie *et al.*, 1993, 1994, 1995). Antigen specific activation of the cell lines results in a seven to ten day period of refractoriness to IL-2 production, and IL-2 production can be inhibited by activation in the presence of the mAb W3/25. To resolve questions about the ability of IL-2 to reverse W3/25-mediated inhibition and how a lack of IL-2 could affect T cells during their refractory period, a source of rat IL-2 was needed which did not contain other rat proteins. A recombinant rat IL-2 was produced using a baculovirus expression system. It was then used to further examine the regulation of IL-2 by MBP-specific T cells and to study the effects of IL-2

on the cell lines this research utilized. The study of the *in vitro* regulation of IL-2 and MBP-specific T cells may provide insight into the maintenance of peripheral tolerance or the activity of autoreactive T cells.

***IN VITRO* BACULOVIRUS PROTEIN EXPRESSION**

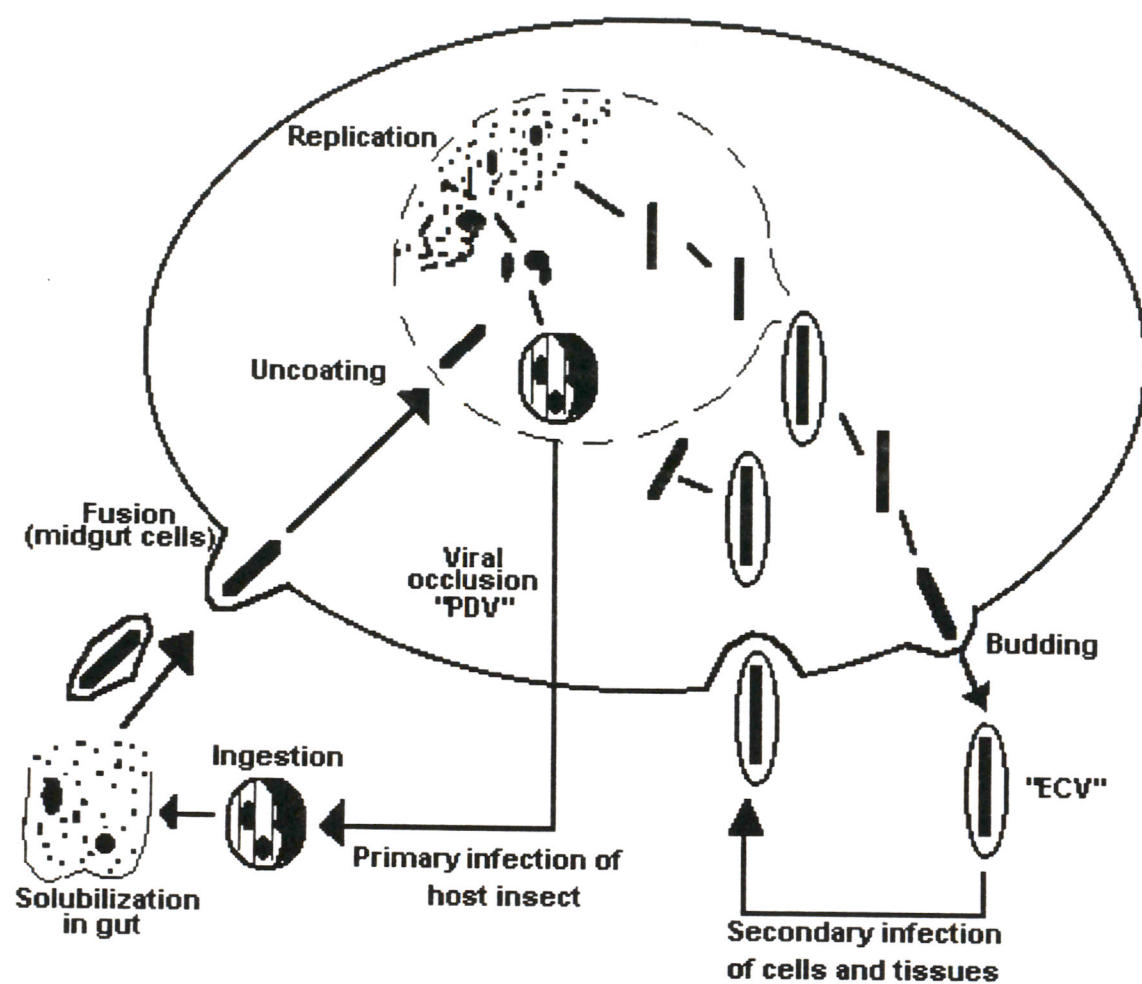
A number of systems, both prokaryotic and eukaryotic, have been employed for *in vitro* protein expression. While many genes can be successfully expressed in a prokaryotic system, some eukaryotic gene products are not biologically active. The failed biological activity is generally related to improper tertiary structure or post-translational modification (Miller, 1989). Many eukaryotic systems have been developed using hosts such as yeast, mammalian, insect, and plant cells, which provide a more suitable environment for eukaryotic gene expression. Insect cells have become a popular eukaryotic system because of a high level of protein expression, the ability to insert large fragments of foreign DNA, and the simplicity in handling insect cell cultures and the viruses (Fraser, 1992).

Protein expression in insect cells is accomplished by the infection of the insect cells with recombinant baculoviruses which contain the gene of interest. The baculoviruses include nuclear polyhedrosis viruses (NPVs), granulosis viruses (GVs), and non-occluded baculoviruses (NOBVs). The baculovirus morphology is a rod-shaped nucleocapsid surrounded by a lipoprotein envelope. Each envelope can contain one or

multiple (3 to 10) nucleocapsids. The viruses contain a covalently closed circular DNA genome containing approximately 80 genes (Kelly, 1982).

The baculovirus *Autographa californica* is a nuclear polyhedrosis virus (AcMNPV) which infects cells from the moth *Spodoptera frugiperda*. Using recombinant AcMNPV and a *Spodoptera frugiperda* ovarian cell line (Sf9), protein may be expressed *in vitro*. The life cycle of the virus includes two forms of infectious virus particles which are genetically identical but have different phenotypes (Figure 1) (Fraser, 1992). The extracellular virus (ECV) particles are responsible for the spread of infection within a host insect, and the occlusion bodies (OB) are responsible for establishment of the primary infection in the gut of insects. Infection begins when insect larvae ingest occluded virus particles. The major component of OB is polyhedrin protein which is alkali labile (Kelly, 1982). The basic nature of the larval gut aids in the degradation of the polyhedrin protein and therefore of the OB (Fraser, 1992). Each OB may contain as many as 100 virus particles (Kelly, 1982). In the first phase of infection (0 to 20 hours post infection) nucleocapsids are assembled in the nucleus, move through the cytoplasm, and then bud through the plasma membrane as ECVs, where they are released into the insect hemolymph. This process allows the virus access to the rest of the insect tissue for further infection (Kelly, 1982). In the second phase of infection (20 to 70 hours post infection) nucleocapsids are encapsulated in OB (Fraser, 1992). By the time of death, OB account for 10 - 20% of an insect's dry weight (Miller, 1989). The OB protect the virus

Figure 1. The baculovirus life cycle. Baculovirus infection begins when insect larvae ingest occluded virus particles. The polyhedrin protein is degraded and the infectious virus particles are released into the gut. The virus particles move to the nucleus of the gut cells where viral DNA replication occurs within 6 hours. Early in infection (0 to 20 hours post-infection), extracellular virus particles (ECV) are assembled, bud through the membrane and infect neighboring cells and tissues. Late in infection (after 18 hours post-infection), the polyhedrin protein assembles and virus particles become imbedded, forming occluded virus particles. The viral occlusions are released to the environment when the insect dies, and the polyhedra derived virus (PDV) are responsible for the infection of other insects upon ingestion. Figure adapted from MaxBac[®] Baculovirus Expression Manual (Invitrogen, San Diego, CA).



from harsh conditions such as extreme temperatures and dessication until they are ingested by insect larvae.

The polyhedrin protein which makes up the OB is a 29 kD protein (Miller, 1989) that can account for up to 60% of the total cellular protein in Sf9 cells late in infection (Kang, 1988). Polyhedrin is one of few viral proteins which is produced throughout the entire second phase of infection, and is therefore allowed to accumulate to higher levels than other viral proteins. The polyhedrin gene is nonessential for infection of Sf9 cells by the baculovirus or for the replication of the virus (Kang, 1988). The combination of a strong promoter and the nonessential nature of the gene makes it a good candidate for replacement with a foreign gene which is to be expressed. When the polyhedrin gene is replaced with a foreign gene, the level of protein expression can be as high as 1 mg/ml of culture supernatant (Miller, 1989). Sf9 cells infected with the recombinant virus are OB negative, and therefore can be morphologically distinguished from cells infected with nonrecombinant virus (OB positive).

To generate a recombinant virus, the wild-type AcMNPV virus and a plasmid containing the foreign gene to be expressed are cotransfected into insect cells. The plasmid typically contains the following features: (1) vector sequences allowing replication and selection in *E. coli*, (2) restriction sites to enable insertion of the foreign gene, and (3) recombination sequences flanking the area into which the foreign gene will be inserted (Miller, 1989). A plasmid may also contain a *lacZ* gene which will become part of the recombinant virus and will allow color screening of the plaques. In this

research a plasmid was used which contains all of these features, pBlueBac III (Figure 2) (Invitrogen, San Diego, CA). When the plasmid and the viral DNA are cotransfected into the insect cells, the genetic recombination system present in the host cell will combine the DNAs by homologous recombination (Figure 3). The *lacZ* gene, the polyhedrin promoter, and the foreign gene within the plasmid will be inserted into the viral DNA genome (Miller, 1989). Isolation of recombinant virus is achieved by screening for infected Sf9 cells which are OB negative or by use of blue/white color screening.

There are many advantages of the baculovirus system in addition to the ones previously mentioned. Many of the enzymes and pathways for post-translational modification in mammals have similar counterparts in insect cells (Miller, 1989). Phosphorylation, N-linked and O-linked glycosylation, and signal sequence cleavage all occur in the baculovirus system (Miller, 1989; Fraser, 1992). Proper cellular compartmentalization such as secretion or localization to the nucleus, cytoplasm or membrane also occur (Fraser, 1992). Numerous proteins have been successfully expressed in the baculovirus system including cytokines such as human beta interferon (Smith *et al.*, 1983), human IL-2 (Smith *et al.*, 1985), murine interleukin-4, and murine interleukin-5 (Rodewald *et al.*, 1990).

Figure 2. *pBlueBac III plasmid map.* The pBlueBac III plasmid has the following features to aid in the cloning and sequencing of genes, and their expression in the baculovirus system: (1) vector sequences allowing selection and replication in *E. coli*, (2) a multiple cloning site allowing insertion of the foreign gene, (3) recombination sequences flanking the multiple cloning site, and (4) the *lacZ* gene which allows a blue/white color screening of plaques. Figure adapted from MaxBac[®] Baculovirus Expression Manual (Invitrogen, San Diego, CA).

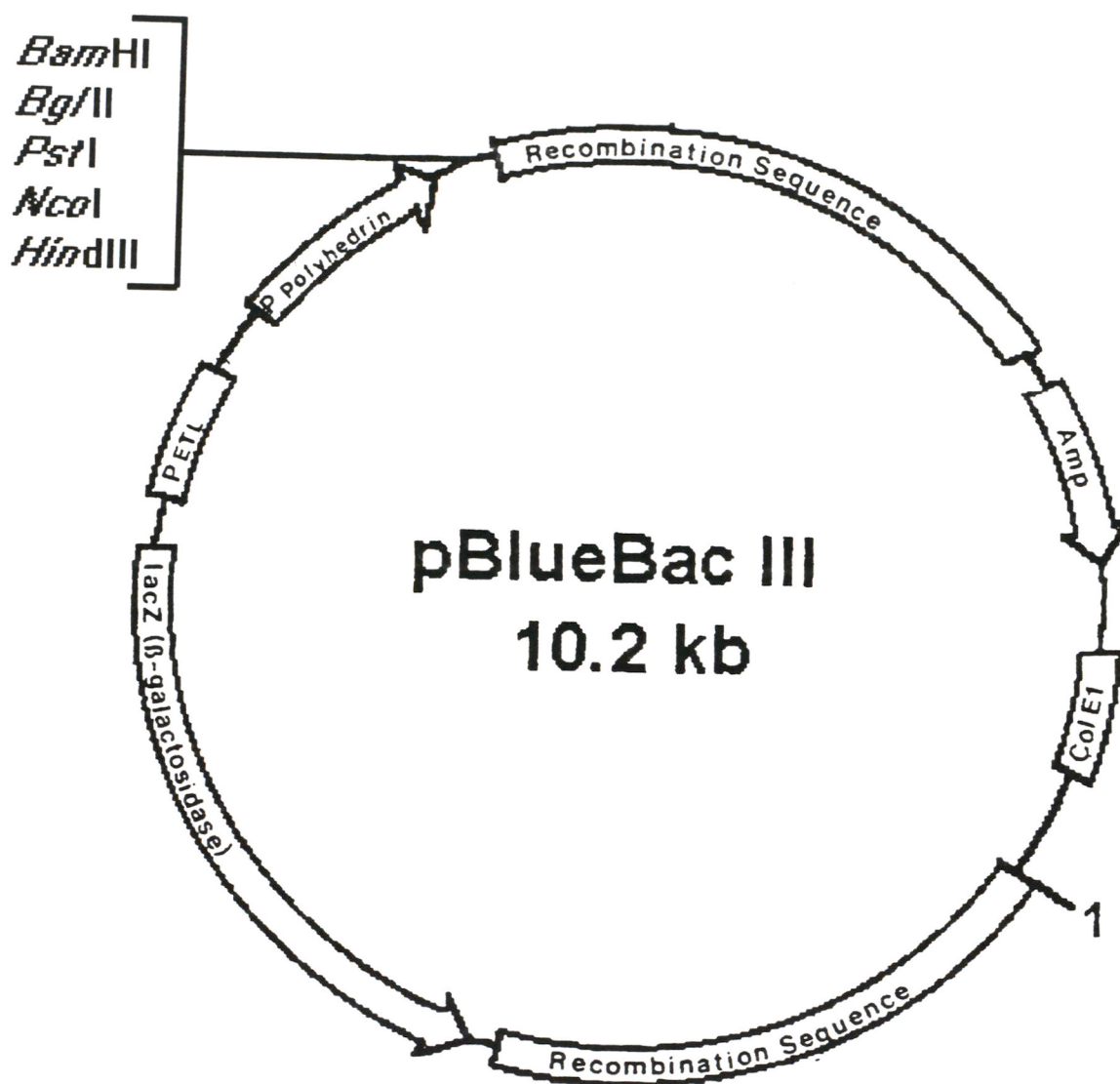
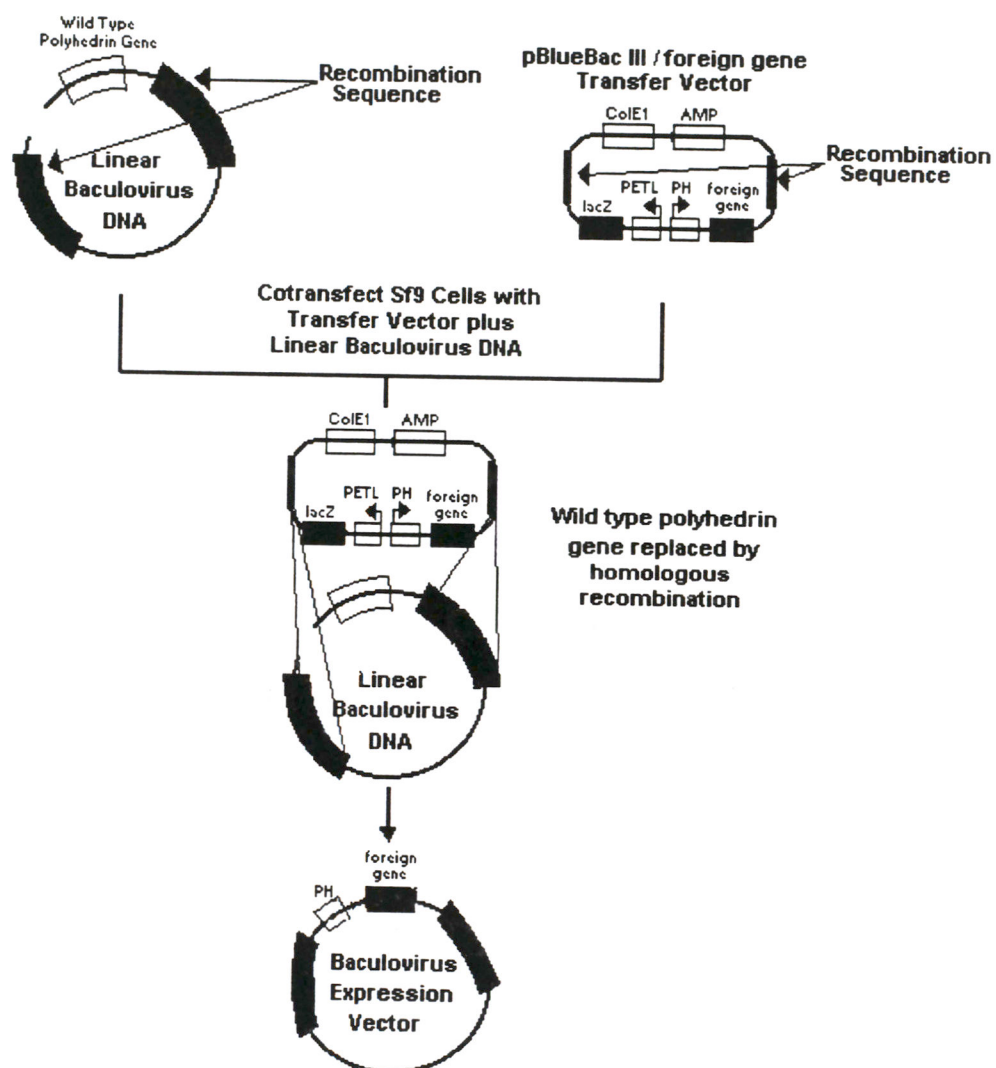


Figure 3. Recombination of the Baculovirus. A foreign gene is cloned into the pBlueBac III plasmid and then cotransfected with linear baculovirus DNA into Sf9 insect cells. In some of the cotransfected cells, the polyhedrin gene of the virus will be replaced with the foreign gene through homologous recombination. These recombinant baculoviruses can be plaque-purified and used to infect Sf9 insect cells for the purpose of *in vitro* protein expression.



MATERIALS AND METHODS

ANIMALS.

Lewis rats (Harlan-Sprague-Dawley, Indianapolis, IN) were bred in the animal facility at the East Carolina University School of Medicine. Rats received a standard rat chow and water *ad libitum*.

T CELL CULTURE.

Production of T Cell Growth Factor (TCGF). Spleens were aseptically obtained from nine Lewis rats and were processed into a single-cell suspension using a wire mesh screen. The splenocytes (SPL) were then washed three times with Hanks Balanced Salts Solution (HBSS) and resuspended at a concentration of 20×10^6 cells per ml of complete RPMI (cRPMI). cRPMI is RPMI 1640 medium (GIBCO BRL Life Technologies, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Hyclone Laboratories, Logan, UT), 2 mM glutamine, 100 $\mu\text{g/ml}$ streptomycin, 100 U/ml penicillin (Whittaker Bioproducts, Walkersville, MD), and 50 μM 2-mercaptoethanol (Fisher Scientific). Ten μM Indomethacin (Sigma) and 2.5 $\mu\text{g/ml}$ of Concanavalin A (ICN Biomedicals, Costa Mesa, CA) were added. Cells were incubated at 37°C for 3 days. At the end of each 24 hours, the supernatants were collected and the cells were resuspended under the same conditions and returned to the incubator. The supernatants were incubated for 20 minutes with α -methyl-mannoside at a concentration of 4.8 mg/ml of

supernatant and were stored at -20°C. Supernatants were serially diluted and tested for IL-2 using a CTLL-2 bioassay. TCGF was added to cRPMI which was used to propagate cell lines and was used as a positive control for CTLL-2 bioassays.

Preparation of Accessory Cells. Spleens were aseptically dissected from untreated Lewis rats. The spleens were processed into a single-cell suspension using a wire mesh screen. The SPL were washed three times with HBSS, placed on ice, and irradiated by exposure to 3000 rads of γ -irradiation from a Gammator-50 source of ^{137}Cs . The irradiated splenocytes (IRRSPL) were centrifuged at 4°C, and resuspended in cRPMI for use in activation cultures.

***In vitro* Activation of T Cell Lines.** T cells were washed in HBSS and were activated with various concentrations of Guinea Pig MBP (GPMBP) in the presence of IRRSPL. MBP was purified from guinea pig spinal cords (Pel-Freez, Rogers, AR) (Bellini, 1986). Cells were activated at a ratio of 2.5×10^5 T cells/ 5×10^5 IRRSPL/0.02 ml of cRPMI unless otherwise stated. Cultures were incubated at 37°C at 5% CO_2 . After specified times, cells and supernatants were harvested. Cells used for RNA isolation were frozen in liquid nitrogen for later use. Cells for proliferative assays were used immediately. Supernatants were placed at -20°C until they were needed.

Measurement of T Cell Proliferation. T cells were activated in 96 well plates with GPMBP in the presence of IRRSPL (2.5×10^4 T cells / 5×10^5 IRRSPL / 0.02 ml cRPMI), with or without various other reagents. The cultures were placed at 37°C at 5% CO₂ for 46-48 hours. After 24 hours, the cells were pulsed with 0.5 µCi [³H]-thymidine and were then placed back in the incubator. At the end of the incubation, 120 µl per well of supernatant were transferred to a replicate plate for assay of IL-2 activity. The T cells were harvested onto fiberglass filters with a Titertek harvester (Flow Laboratories, Rockville, MD), and the filters were placed into vials with scintillation fluid (Ecoscint, National Diagnostics, Mansville, NJ). The incorporation of [³H]-thymidine was measured using a Beckman LS7000 counter. Assays were set up in triplicate or quadruplet and counts per minute (CPM) represent an average of those groups.

CTLL-2 Bioassay of Interleukin-2. IL-2 activity was measured using the murine IL-2 dependent CTLL-2 cell line. Assays were set up in a 96 well plate format with each well containing 120 µl of supernatant from a proliferative assay, or 100 µl serial dilutions of supernatant from a T cell activation or recombinant baculovirus infections. TCGF positive controls and cRPMI negative controls were also assayed. CTLLs ($2.5 - 5.0 \times 10^4$) were added per well and the plates were placed at 37°C with 5% CO₂ for 20 - 24 hours until the cells in the negative control wells began to die. At this time, the wells were pulsed with 10 µl MTS (2.9 mg/ml) /PMS (0.1 mg/ml) and were placed in the incubator for another 20 - 24 hours. The plates were read at 492 nm on an Anthos Elisa Reader

(model 2001, ACCSales, Chapel Hill, NC). The level of IL-2 was calculated by subtracting the optical densities of the negative control wells from the experimental wells.

***Spodoptera frugiperda* CELL CULTURE.**

Maintenance of *Spodoptera frugiperda* (Sf9) Cell Line. Cells were cultured at 27°C in SF900II serum-free media (GIBCO BRL Life Technologies, Grand Island, NY), or complete TNM-FH (cTNM-FH) which is Grace's medium (GIBCO BRL Life Technologies, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (Hyclone Laboratories, Logan, UT), 10 µg/ml gentamycin (Sigma), 20 ml 50X Lactalbumin Hydrosylate (GIBCO BRL Life Technologies, Grand Island, NY), and 20 ml 50X Yeastolate Solution (GIBCO BRL Life Technologies, Grand Island, NY) per liter of media. Cells were maintained in a monolayer and were allowed to grow to confluency. When the cells were confluent, they were diluted ten-fold with fresh cTNM-FH or SF900II media.

MOLECULAR BIOLOGY TECHNIQUES

RNA Isolation. GP2E5.30 MBP-specific T cells (5×10^6) were activated with 2 µM GPMBP in the presence of 50×10^6 IRRSPL. After 17 hours the cells were removed from activation and the RNA was isolated using RNazol™ B (Tel-Test, Inc, Friendswood, TX) following the manufacturer's suggested protocol. The RNA was

resuspended in sterile water and was quantified by reading the optical density at 260 nm on a spectrophotometer.

Reverse Transcriptase-Polymerase Chain Reaction Amplification of Interleukin-2

mRNA. An oligonucleotide specific for the 3' region of the IL-2 mRNA (5' GAGAAGCTTTTACTGAGTCATTGTTGAGAT 3') was used to prime the synthesis of the rat IL-2 cDNA. One μ g of total RNA and 400 ng of this primer were added to DEPC water in a 0.7 μ l thin-walled microfuge tube for a final volume of 20 μ l. The samples were placed in a thermal cycler (MJ Research, Inc., Watertown, MA) and heated to 70°C for 10 minutes and were then held at 50°C. The samples were centrifuged briefly and were returned to the thermal cycler at 50°C. A mixture containing 4 μ l of 10x *Taq* DNA Polymerase buffer (Promega, Madison, WI), 3.2 μ l of 50 mM MgCl₂ (GIBCO BRL Life Technologies, Grand Island, NY), 1 μ l 10 mM dNTP (GIBCO BRL Life Technologies, Grand Island, NY), 1 μ l 0.1 M DTT, 100 U of Superscript II™ Reverse Transcriptase (GIBCO BRL Life Technologies, Grand Island, NY), and 10.3 μ l of DEPC water was added to each tube. The reactions were heated for 50 minutes at 50°C, and then cooled to 4°C. The tubes were briefly centrifuged to collect the condensate and 400 ng of oligonucleotide primer specific for the 5' end of the IL-2 gene and containing a 5' *Bam*HI restriction endonuclease site (5' GAGGGATCCATGTACAGCATGCAGCTCGCA 3') was added to each tube. The reactions were overlaid with 100 μ l of mineral oil (Sigma) and placed back in the thermal cycler, where they were heated to 99°C for 5 minutes.

The reactions were cooled to 80°C and a 9 µl mixture, containing 6.7 µl DEPC water, 1 µl 10x Taq Polymerase buffer, 0.8 µl of 50 mM MgCl₂, and 2.5 U of *Taq* Polymerase (GIBCO BRL Life Technologies, Grand Island, NY), was added to each tube. The cDNA was then PCR amplified for 30 cycles of 94°C for 1 minute, 62°C for 2 minutes, and 72°C for 2 minutes. Following the 30 cycles, the reactions were incubated at 72°C for 10 minutes and were then held at room temperature. A reaction was included which did not contain RNA template as a control for contaminants in the RT-PCR reagents.

Agarose Gel Electrophoresis. RT-PCR products were visualized on an ethidium bromide stained 1.5% agarose gel. A 10 µl aliquot of each reaction was electrophoresed along with a 100 basepair (bp) DNA molecular weight standard, or a 1 KB ladder molecular weight standard (GIBCO BRL Life Technologies, Grand Island, NY) to determine the size of RT-PCR products. The electrophoresis gel [0.375 gm agarose (GIBCO BRL Life Technologies, Grand Island, NY) in 25 ml of 1x TBE] was melted by boiling in a microwave. Ethidium bromide was added to the gel at a final concentration of 2 ng/µl, and to 1X TBE, for use as electrophoresis buffer, at a final concentration of 70 ng/ml. To 10 µl of each RT-PCR reaction 1 µl of 10x DNA loading dye [25% (w/v) Ficoll (type 400), 0.25% (w/v) bromophenol blue in sterile distilled water] was added. Agarose gels were run at 35-40 milliamps for 2 hours. Gels were visualized on a UV-transilluminator (International Biotechnologies, Inc., New Haven, CT) and photographs

were taken with a Polaroid Quick Shooter Model QSP camera (International Biotechnologies, Inc., New Haven, CT).

Cloning of Interleukin-2 RT-PCR Product. The RT-PCR sample which contained a fragment of the appropriate size was prepared to be cloned into a plasmid vector. The sample was removed from under the mineral oil overlay and was transferred to a sterile 1.5 ml microfuge tube. An equal volume of PCI (25:24:1 phenol, chloroform, and isoamyl alcohol respectively) was added to the sample, the tube was inverted twice, and the sample was centrifuged at 14,000 rpm for 5 minutes at room temperature. The upper, aqueous phase was transferred to a new microfuge tube and a second PCI extraction was performed. After the sample was transferred to a new microfuge tube, 2.5 volumes of 100% ethanol and one-tenth volume of 3.0 M Sodium Acetate (pH 5.2) were added. After an overnight incubation at -70°C, the sample was centrifuged at 14,000 rpm for 15 minutes at 4°C to collect the RT-PCR product. The supernatant was discarded and the pellet was washed with 70% ethanol, dried at room temperature, and resuspended in 20 µl of autoclaved Millipore water.

One µl of the RT-PCR product was placed in a 0.7 ml microfuge tube along with 6 µl of sterile water, 1 µl T4 DNA ligase (GIBCO BRL, Grand Island, NY), 1 µl 5x T4 DNA ligation buffer (GIBCO BRL, Grand Island, NY), and 50 ng of pCR II plasmid vector (Invitrogen, San Diego, CA). The reaction was placed in a 12°C water bath overnight. Transformation of the ligation product into XL-1 Blue competent cells

(Stratagene, La Jolla, CA) was done following the TA cloning vector protocol provided by Invitrogen (San Diego, CA). Transformants were plated on Luria agar plates containing ampicillin. Seventy-five μ l of 20 mg/ml of Blueo-gal (GIBCO BRL Life Technologies, Grand Island, NY) had been spread on the surface of each plate to allow blue/white color screening of colonies. The plates were incubated at 37°C overnight, at which time they were removed and transformants which might contain the IL-2 insert were identified.

Plasmid DNA Isolation. Fifty ml centrifuge tubes containing 12 ml of Luria Broth (1 ml 1 M NaOH, 10 g Bacto-tryptone [GIBCO BRL Life Technologies, Grand Island, NY], 5 g yeast extract [GIBCO BRL Life Technologies, Grand Island, NY], and 5 g NaCl per liter of medium), supplemented with 50 μ g/ml ampicillin (Sigma), were aseptically inoculated with colonies suspected to contain the IL-2 insert. The caps were placed loosely on the tubes, and the tubes were placed in a 37°C shaking incubator overnight. One ml was removed from each culture and placed in a 1.5 ml microfuge tube for later use. The remaining culture was centrifuged at 2000 rpm for 10 minutes, and the supernatants were discarded. The bacterial pellet was resuspended in 90 μ l of solution I (50 mM glucose, 10 mM EDTA, 25 mM Tris pH 8.0), and was transferred to a 1.5 ml microfuge tube. Next, 10 μ l of Lysozyme (10 mg/ml in solution I) was added to the microfuge tube, and the lysate was incubated for 5 minutes. A fresh alkali solution (200 μ l of solution II: 0.2 M NaOH, 1% SDS) was added, and the tubes were gently inverted.

Next, 150 μ l of solution III (1 M KOAc, 11.5 % glacial acetic acid) was added, the tubes were again inverted, and then placed on ice for 15 minutes. The tubes were centrifuged at 14,000 rpm for 15 minutes at 4°C, the supernatants were transferred to a new microfuge tube, and 2 PCI extractions were performed. Twice the volume of ethanol was added and the samples were incubated for 5 minutes at room temperature before being centrifuged at 14,000 rpm for 5 minutes. The pellets were dried, and the samples were resuspended in 100 μ l of T₁₀E₁ (10 mM Tris, 1 mM EDTA). RNase was added at a final concentration of 0.25 μ g/ μ l, and the samples were incubated for 10 minutes at room temperature. Sterile water (260 μ l) and 40 μ l of 3.0 M NaOAc were added to each tube, and then one PCI extraction was followed by a chloroform extraction. Two volumes of 100% ethanol were added, and the tubes were placed at -70°C for an hour. Samples were centrifuged at 14,000 rpm at 4°C for 15 minutes to pellet the plasmid DNA, the pellets were washed with 70% ethanol, and then they were dried at room temperature. Samples were resuspended in 23 μ l of T₁₀E₁.

Restriction Endonuclease Digests. A 3 μ l aliquot of each plasmid DNA sample was digested with *Bam*HI restriction endonuclease. A *Bam*HI restriction site was present in the upstream IL-2 primer and a second *Bam*HI site was present in the pCR II plasmid cloning vector 39 bp downstream from the cloning site. Three μ l of each plasmid sample were placed in a 0.7 ml centrifuge tube along with 20 U of *Bam*HI (New England Biolabs, Beverly, MA), 1 μ l 10x *Bam*HI digest buffer (New England Biolabs, Beverly,

MA), 1 μ l 10x BSA (New England Biolabs, Beverly, MA), and 4 μ l sterile water for a final volume of 10 μ l. The digests were placed in a 37°C water bath overnight. A 5 μ l aliquot of each digest was electrophoresed on a 1.5% agarose gel and the gel was visualized on a UV-transilluminator.

Preparation of Insert for Ligation Into pGEM and pBlueBac III Plasmids. Four 10 ml overnight cultures were grown of the bacteria transformed with the pCR II plasmid suspected to contain the IL-2 insert. Plasmid minipreps were performed as previously described. The plasmid DNA from each miniprep was resuspended in 20 μ l of sterile water. The four plasmid preparations were combined and a *Bam*HI digest was performed on the DNA. The 80 μ l volume was digested with 180 U *Bam*HI, 10 μ l 10x *Bam*HI digest buffer, and 1 μ l 100x BSA in a 100 μ l total volume. The reaction was incubated overnight at 37°C. The digest was electrophoresed on an ethidium bromide stained, 1.5% agarose prep gel. The fragment digested out of the plasmid was cut out of the gel, and was placed in dialysis tubing with 100 μ l of 1x TBE. After clamping both ends of the tubing, it was placed in an electrophoresis buffer chamber with 1x TBE. Current was applied, and the movement of the DNA within the gel slice was monitored using a hand-held UV light. After the DNA migrated out of the gel slice, the power source was turned off, and the dialysis tubing was removed from the electrophoresis chamber. The TBE containing the DNA was transferred to a 1.5 ml microfuge tube. The dialysis tubing was rinsed with fresh TBE, which was also transferred to the microfuge tube. Two PCI

extractions were done and the aqueous layers were transferred to sterile 1.5 ml microfuge tube. An equal volume of 100% ethanol and one-tenth the volume of 3 M sodium acetate (pH 5.2) were added, and the sample was placed at -70°C for 1 hour to precipitate the DNA. The sample was removed from the freezer and was centrifuged 15 minutes at 14,000 RPM at 4°C. The pellet was washed with 70% ethanol and was dried under vacuum. The plasmid DNA was resuspended in 20 µl of sterile water and was used for ligation into pGEM and pBlueBac III (Invitrogen, San Diego, CA) plasmids.

Ligation of IL-2 Insert Into pGEM and pBlueBac III Plasmids. A 6 µl aliquot of DNA insert was placed into each of two 0.7 ml microfuge tubes. The pGEM and pBlueBac III plasmids used for the ligations were prepared using a Qiagen tip 100 following the protocol provided by Qiagen (Chatsworth, CA). To one tube, 2 µl of *Bam*HI digested pGEM plasmid was added and 2 µl of *Bam*HI digested pBlueBac III was placed in the other tube. One µl of T4 DNA Ligase and 1 µl of T4 DNA Ligation buffer were added to each of the reactions. The tubes were incubated overnight in a 12°C water bath. The ligation mix was transformed into XL-1 Blue competent cells following the manufacturer's protocol (Stratagene, La Jolla, CA). Twenty µl and 200 µl of each transformation mix were plated on Luria agar plates containing 75 µl of 20 mg/ml IPTG, in addition to 75 µl of 20 mg/ml of Bluo-gal. Transformants were screened for inserts by restriction digests as previously described. When transformants were shown to have an insert, the remaining miniprep plasmid DNA was used for DNA sequencing.

Automated Sequencing of Interleukin-2 cDNA. Approximately 1 μg of miniprep plasmid DNA was used for each sequencing reaction. Sequencing primers used include M13 forward and reverse, pBlueBac III forward and reverse and the PCR primers used to generate the IL-2 cDNA. The reactions performed were fluorescence-based dideoxy sequencing following the PRISM™ Ready Reaction DyeDeoxy™ Terminator Sequencing Kit protocol (Applied Biosystems, Inc., Foster City, IA). The sequencing reactions were analyzed on the Applied Biosystems model 373A DNA Sequencing System following the manufacturer's recommended protocols.

BACULOVIRUS PROTEIN EXPRESSION

Transfection of Sf9 Insect Cells. A 30 mm tissue culture plate (Corning) was seeded with 1×10^6 log phase Sf9 cells which were allowed to attach for 30 minutes at 27°C. A transfection mix containing 1 ml of unsupplemented Grace's media, 1 μg linear *Autographica californica* mononuclear polyhedrosis viral DNA, 3 μg of plasmid DNA, and 20 μl Cationic Liposome solution (Invitrogen, San Diego, CA) was vortexed, and was incubated at room temperature for 15 minutes. The pBlueBac III plasmid containing the IL-2 gene was prepared using the plasmid miniprep DNA isolation protocol previously described. The media was removed from the 30 mm tissue culture dish, and 2 ml of unsupplemented Grace's media were added. The plates were incubated for an additional 10 minutes, and again the media was removed. The transfection mixture was added dropwise to the plate, and was then placed on a side/side rocking platform (Bellco

#774020020) at a setting of 2.5 for 4 hours. One ml of complete TNM-FH was added to the plate, and it was placed in a 27°C incubator with high humidity for 48 hours. After 48 hours, the media (the viral stock containing the recombinant baculovirus) was harvested and placed at 4°C.

Plaque Purification of Recombinant Virus. Ten-fold dilutions of the recombinant baculovirus stock were performed using unsupplemented Grace's media. The 10^{-3} and 10^{-4} dilutions were plated following the Invitrogen (San Diego, CA) baculovirus protocol. Each plate contained 750 µg of Bluo-gal to allow a blue/white color screen for recombinant (blue) plaques. Plates were incubated at 27°C with high humidity for 6-8 days. Plates were screened for plaques by examination under a light microscope. Each plaque was removed from the plates using a micropipettor and was placed in one well of a 24 well tissue culture plate which was seeded with 2.5×10^5 Sf9 cells. One well of the plate was left uninfected to serve as a control. The plate was incubated at 27°C for 3 days at which time 750 µl of media were removed from each well to be analyzed for recombinant virus by PCR. The remaining culture was placed back in the incubator until lysis of the cells was observed. The supernatant was harvested, and another round of plaque purification was performed.

PCR Analysis of Viral Supernatant. Viral DNA was isolated from the viral stocks following Invitrogen's (San Diego, CA) protocol. The supernatants of uninfected Sf9

cells were treated with the same viral DNA isolation procedure, and PCR reactions were performed using the resulting DNA as a control. The DNA was resuspended in 10 μ l of sterile water, half of which was used in the PCR reaction. Invitrogen's suggested PCR protocol was also used with the following modifications: a 94°C incubation for 8 minutes was added before the PCR cycles, 40 cycles of amplification were performed, and PCR primers used were at a concentration of 200 ng/ μ l. Ten μ l of each PCR reaction were analyzed on a 1.5% Agarose gel. The expected size of a PCR band for the recombinant IL-2 virus was 630 bp plus the size of the insert. Wild-type virus will yield a 630 bp band and the uninfected cells control should have no band. PCR reactions lacking template were included as negative controls.

Preparation of Baculovirus Supernatants. Sf9 cells cultured in SF900 II serum-free media or cTNM-FH were seeded at 1×10^6 cells per well in a 24 well plate. Seven wells were inoculated with 20 μ l viral supernatants per clone. Control wells were infected with wild-type virus or no virus. Supernatants were harvested each day for 7 days, and were stored at 4°C until needed.

Sample Preparation for SDS-Polyacrylamide Gel Electrophoresis. Supernatants which were generated in serum-free media were run on an SDS-PAGE gel. The samples were concentrated approximately 10 times by centrifugation under vacuum without heat for 16 hours. Ten μ l of each sample were added to 40 μ l of sample buffer (4.8 ml water,

1.2 ml 0.5 M Tris-Cl (pH 6.8), 2.0 ml 10% SDS, 1.0 ml glycerol, 0.5 ml 0.5% bromphenol blue (w/v), and 50 μ l 2-mercaptoethanol per 0.95 ml). After the SDS-PAGE apparatus was assembled, the samples were denatured for 4 minutes at 95°C, and were immediately loaded onto the gel. A 2.5 μ l aliquot of protein molecular weight standard, with a range of 2,850 to 43,000 daltons (Gibco BRL Life Technologies, Grand Island, NY), was added to 10 μ l of sample buffer, was denatured, and electrophoresed along with the other samples.

SDS-Polyacrylamide Gel Electrophoresis. A 15% SDS-PAGE gel was used to analyze the baculovirus supernatants suspected to contain IL-2. A separating gel was mixed which contained 11.5 ml water, 25 ml 30% acrylamide, 12.5 ml 1.5 M Tris (pH 8.8), and 0.5 ml 10% SDS. Immediately prior to pouring the gel, 500 μ l of fresh ammonium persulfate (10% w/v) and 20 μ l TEMED were added. The separating gel was poured to 1 cm below the bottom of the comb. The gel was overlaid with 2-butanol and allowed to polymerize. After polymerization, the 2-butanol was poured off, the top of the gel was rinsed with water, and the excess water was removed. The 5% stacking gel was then prepared, containing 6.1 ml water, 2.5 ml 0.5 M Tris (pH 6.8), 1.3 ml of 30% acrylamide stock solution, and 0.1 ml 10% SDS. The comb was set into place, 50 μ l of ammonium persulfate (10% w/v) and 10 μ l TEMED were added, and the stacking gel was poured immediately. After polymerization of the stacking gel, the bottom spacer and the comb were removed. The gel was then clamped into the SDS-PAGE apparatus. Electrode

buffer (0.3 g Tris base, 1.4 g glycine, 1 ml 10% SDS per 100 ml buffer) was poured into the buffer chambers, and air bubbles were removed from between the glass plates at the bottom of the gel. The samples were loaded and the power source was set at 35 milliamps until the samples reached the interface between the stacking and separating gels. At that time, the power source was increased to 45 milliamps. The gel was electrophoresed until the dye in the sample buffer reached the bottom of the gel.

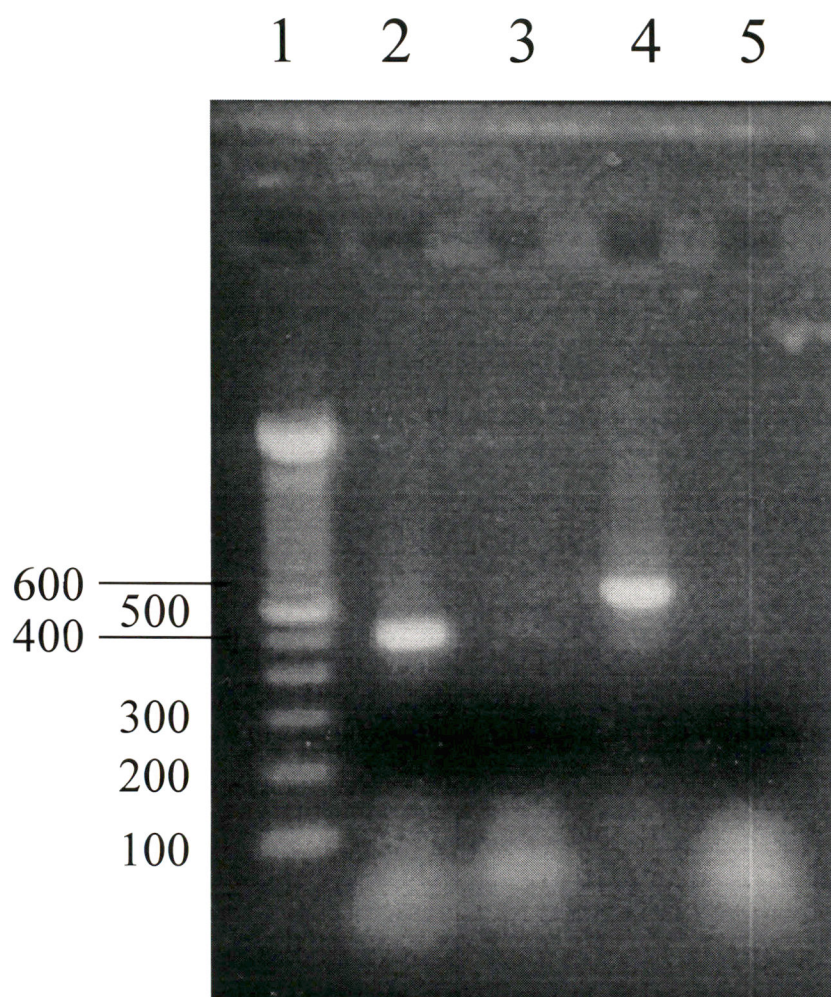
Silver Staining. The gel was placed in 200 ml of fixing solution (30 parts ethanol: 10 parts glacial acetic acid: 60 parts water) on a shaking platform for 4 hours. Next, it was placed in 200 ml of 30% ethanol for 30 minutes. The rinse was replaced and the gel was returned to the shaking platform for another 30 minutes. Three washes were performed on the shaking platform with 400 ml of water each. Then the gel was placed in 200 ml of 0.1% silver nitrate and allowed to shake for 30 minutes. Each side of the gel was rinsed with water for 20 seconds, and the gel was placed in 200 ml of developer (Bio-rad, Richmond, CA) on the shaking platform until the desired contrast was reached. Then the gel was washed in 1% acetic acid for 3 minutes and placed in water until it was photographed.

RESULTS

The goals of this research were to examine the regulation of rat IL-2 by MBP-specific T cell lines as well as the effect of IL-2 on those same cell lines. To achieve this goal a source of rat IL-2 was needed. Therefore, the rat IL-2 cDNA was cloned, sequenced, and the protein was expressed in a recombinant baculovirus system.

Cloning and sequencing of rat Interleukin-2. Oligonucleotide primers were chosen based on published rat IL-2 sequences (McKnight *et al.*, 1989). The 5' oligonucleotide contained a *Bam*HI restriction endonuclease site at its 5' end, and its region of homology with the IL-2 sequence began at the start codon. The 3' oligonucleotide contained a *Hind*III restriction endonuclease site at its 5' end, and its region of homology extended through the IL-2 stop codon. When RT-PCR was performed using these primers and activated GP2E5.30 T cell RNA as a template, a single cDNA product was generated. The fragment was 486 bp in length as predicted (Figure 4). The fragment was cloned by ligation into the pCR II plasmid vector (Invitrogen, San Diego, CA). The insert was removed from the plasmid by *Bam*HI restriction endonuclease digest using *Bam*HI restriction sites which were present in the 5' oligonucleotide primer and in the pCR II vector 40 bp downstream from the insert. The resulting digestion fragment was 525 bp in length. This fragment was ligated into the plasmid vector pBlueBac III (Figure 2); its orientation with respect to the polyhedrin promoter was confirmed by restriction

Figure 4. RT-PCR amplification of rat IL-2 mRNA from activated GP2E5.30 MBP-specific T cells. 5×10^6 T cells were activated with 2 μ M GPMBP and IRRSPL. After 17 hours of activation, the cells were harvested and total RNA was isolated. RT-PCR was performed and the products were electrophoresed on a 1.5% agarose gel. A 100 bp ladder was included as a molecular weight standard. The samples in each lane are as follows: 1) 100 bp molecular weight standard, 2) rat IL-2, 3) rat IL-4, 4) β -actin, and 5) negative control (no RNA template included in RT-PCR reaction).



digest, and it was then sequenced using a fluorescence-based chain termination methodology.

The DNA sequence obtained was compared to the published sequence (McKnight *et al.*, 1989), and was confirmed to be rat IL-2. The sequence was identical to the published sequence with one exception, bp number 31 was found to be adenine while in the published sequence it was guanine (Figure 5). This base is the first of a codon and changed the deduced amino acid from alanine to threonine. The rat IL-2 cDNA was amplified in two independent RT-PCR reactions and both were sequenced. In both cases base number 31 was found to be adenine. The murine IL-2 amino acid sequence also contains a threonine at position 11 (Yokota *et al.*, 1985) so it is possible that the rat sequence does as well (Figure 6).

Identification of recombinant IL-2 baculovirus. After confirming the DNA sequence, cotransfection of the pBlueBac III/rat IL-2 vector construct and the linear baculovirus DNA into Sf9 insect cells was performed. The recombinant baculovirus was plaque purified, the viral DNA isolated, and PCR was performed to identify recombinant IL-2 baculovirus clones using primers specific for regions flanking the recombination site. Both wild-type virus and recombinant virus were observed (Figure 7). PCR amplification of the wild-type and the recombinant viral DNA produced 630 and 1,155 bp fragments, respectively. Three recombinant clones were identified, and were subjected to a second round of plaque purification to ensure clonality. After PCR amplification of the second

Figure 5. Lewis rat IL-2 gene and deduced amino acid sequences. Comparison of the rat IL-2 deduced amino acid sequence (first) and DNA sequence (second) from this study and the published rat IL-2 DNA sequence (third) (McKnight *et al.*, 1989) and deduced amino acid sequence (fourth) (Yokota *et al.*, 1985).

1	M	Y	S	M	Q	L	A	S	C	V	T	L	T	L	V	L
1	ATGTACAGCATGCAGCTCGCATCCTGTGTTACACTGACGCTTGTCCTC															
	G															
	A															
17	L	V	N	S	A	P	T	S	S	P	A	K	E	T	Q	Q
49	CTTGTCAACAGCGCACCCACTTCAAGCCCTGCAAAGGAAACACAGCAG															
33	H	L	E	Q	L	L	L	D	L	Q	V	L	L	R	G	I
97	CACCTGGAGCAGCTGTTGCTGGACTTACAGGTGCTCCTGAGAGGGATC															
49	D	N	Y	K	N	L	K	L	P	M	M	L	T	F	K	F
145	GATAATTACAAGAATCTGAAACTCCCCATGATGCTCACGTTTAAATTT															
65	Y	L	P	K	Q	A	T	E	L	K	H	L	Q	C	L	E
193	TACTTGCCCAAGCAGGCCACAGAATTGAAACATCTTCAGTGCCTGGAA															
81	N	E	L	G	A	L	Q	R	V	L	D	L	T	Q	S	K
241	AATGAACTCGGAGCTCTGCAGCGTGTGTTGGATTTGACTCAAAGCAAA															
97	S	F	H	L	E	D	A	G	N	F	I	S	N	I	R	V
289	AGCTTTCACTTGGAAGACGCTGGAAATTTTCATCAGCAATATCAGAGTA															
113	T	V	V	K	L	K	G	S	E	N	K	F	E	C	Q	F
337	ACTGTTGTAAAACTAAAGGGCTCTGAAAACAAATTTGAGTGCCAATTC															
129	D	D	E	B	A	T	V	V	E	F	L	R	R	W	I	A
388	GATGATGAGCCAGCAACTGTGGTGGAATTTCTGAGGAGATGGATAGCC															
145	I	C	Q	S	I	I	S	T	M	T	Q					
433	ATCTGTCAAAGCATCATCTCAACAATGACTCAG															

Figure 6. Lewis rat and murine IL-2 deduced amino acid sequences. The deduced amino acid sequences of rat IL-2 (upper) and murine IL-2 (lower) (Yokota *et al.*, 1985). A dot (.) indicates homology between sequences while a dash (–) indicates a break in sequence. Note the threonine at position 11 in both sequences which is marked with an asterisk (*).

*

1 M Y S M Q L A S C V T L T L V L L V N S A P

23 T S S P A K E T - - - - -
 . . . S T S S S T A E A Q Q Q Q Q Q Q Q Q Q

45 Q Q H L E Q L L L D L Q V L L R G I D N Y K
 M . . . E . . S R M E . . R

67 N L K L P M M L T F K F Y L P K Q A T E L K
 R

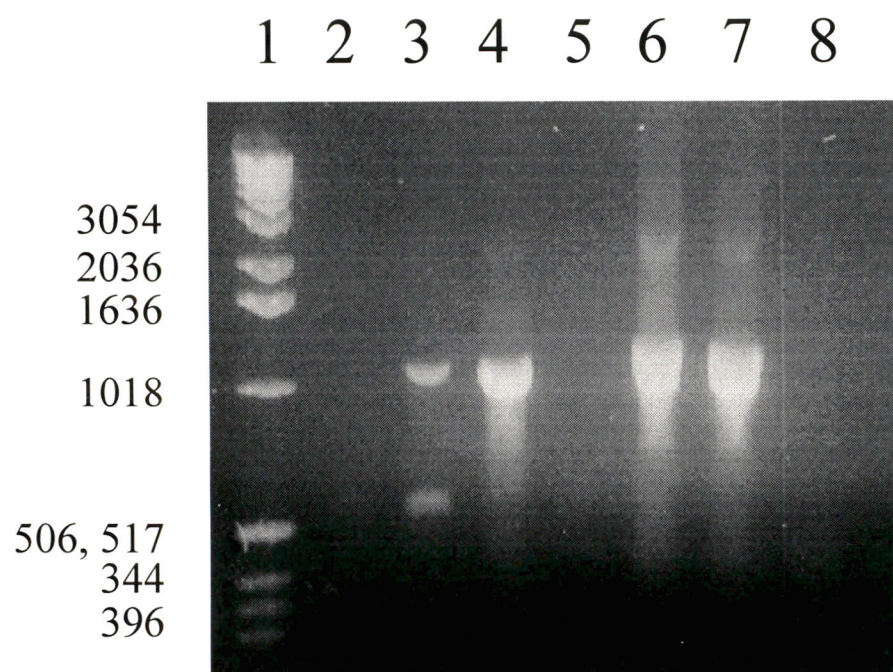
89 H L Q C L E N E L G A L Q R V L D L T Q S K
 D D . . . P . R H

111 S F H L E D A G N F I S N I R V T V V K L K
 . . Q E

132 G S E N K F E C Q F D D E B A T V V E F L R
 . . D S D . . .

154 R W I A I C Q S I I S T M T Q
 F S P .

Figure 7. PCR analysis of baculovirus supernatants. Viral DNA was isolated from the cells and supernatants of expanded baculovirus clones. PCR reactions were performed on the viral DNA using primers specific for the polyhedrin locus. The fragments were generated from 40 cycles of PCR. The PCR products were electrophoresed on a 1.5% agarose gel stained with ethidium bromide. A 1 kilobase ladder was included as a molecular weight standard. Wild-type baculovirus clones and recombinant IL-2 baculovirus clones yield 630 and 1,155 bp bands, respectively. The sample in each lane is as follows: 1) molecular weight markers, 2) recombinant IL-4 baculovirus, 3) recombinant IL-4 baculovirus and wild-type baculovirus, 4) recombinant IL-4 baculovirus, 5) uninfected cells, 6) recombinant IL-2 baculovirus, 7) recombinant IL-2 baculovirus, and 8) negative control containing no DNA template.

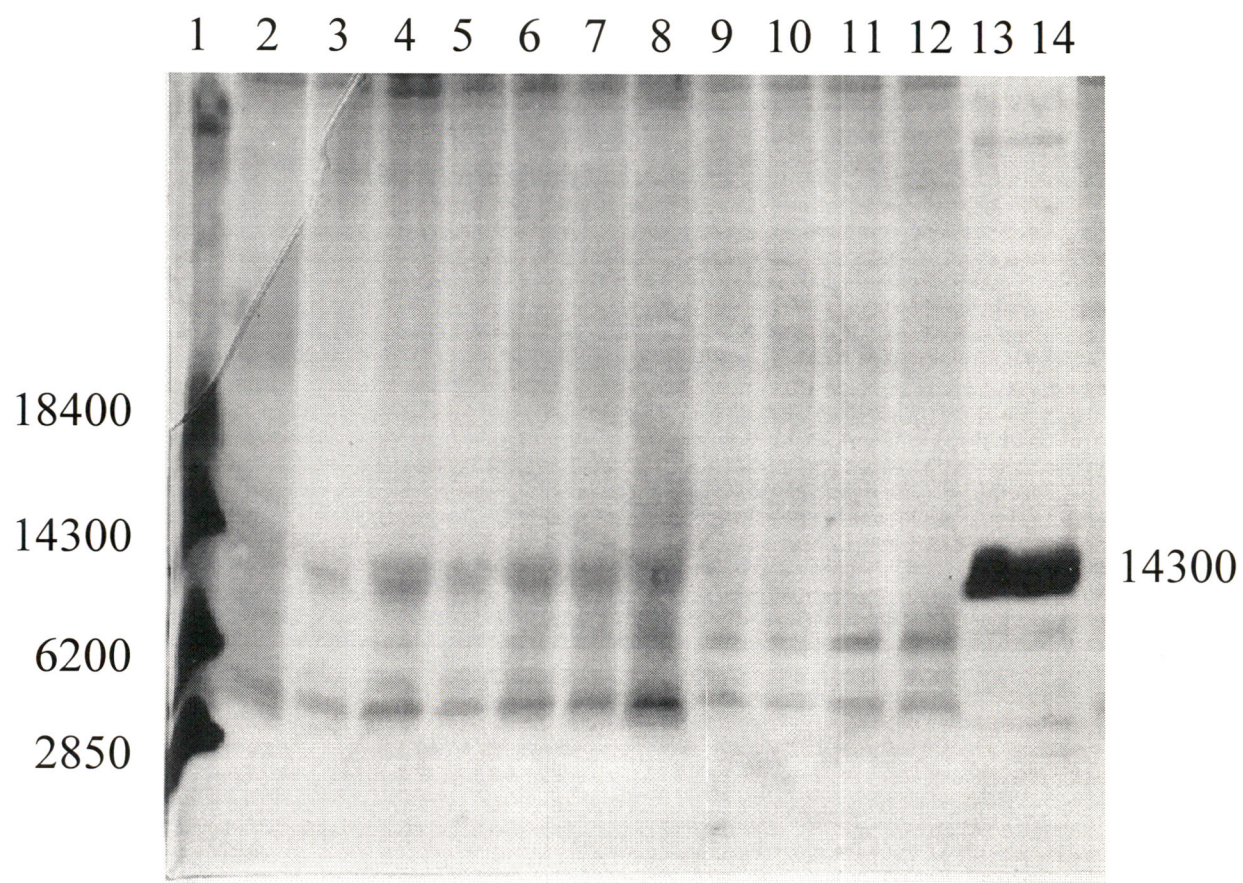


round purification viral DNA, three recombinant IL-2 baculovirus clones (clones 1A, 2A, and 4A) were identified and analyzed.

SDS-PAGE analysis of recombinant IL-2 baculovirus supernatants. Supernatant samples were generated by infecting Sf9 insect cells cultured in serum-free media with recombinant or wild-type baculovirus. The supernatant samples were concentrated and were analyzed by SDS-PAGE (Figure 8). The major protein band present in the recombinant IL-2 baculovirus (clone 4A) infected supernatants, which was not present in the wild-type baculovirus infected supernatants, was approximately 15 kilodaltons, which corresponds with the known molecular weight of rat IL-2. The band was present in the day one supernatant and increased slightly through day seven.

Baculovirus supernatants contain functional recombinant rat IL-2. After confirming the presence of a protein in the recombinant baculovirus supernatants, the next step was to assay for bioactivity with the CTLL-2 bioassay. Both serum-free supernatants and supernatants generated in Grace's media supplemented with 10% FBS were tested. For each media type, supernatant samples were generated using clones 1A, 2A, and 4A. As Figure 9 shows, the CTLL-2 cell line responds to recombinant baculovirus supernatants indicating the presence of functional recombinant IL-2. The majority of the protein was expressed in the first 48 hours with a slight increase in the following days, and the IL-2 protein remained stable when incubated at 27°C for up to 7 days (Figure 8). The

Figure 8. SDS-PAGE analysis of recombinant baculovirus supernatants. Sf9 cells cultured in SF900II serum-free media (Gibco BRL Life Technologies, Grand Island, NY) were infected with a recombinant baculovirus containing the rat IL-2 gene. The supernatants were harvested every 24 hours and stored at -20°C until they were needed. The supernatants were concentrated approximately 10-fold and to 10 µl of each concentrate 40 µl of sample buffer was added. The samples were heated for 5 minutes at 95°C and were loaded onto the gel. The gel consisted of a 5% stacking gel and a 15% separating gel. The gel was silver stained as described in the Materials and Methods section. The sample in each lane is as follows: 1) molecular weight standards, 2-8) supernatants from Sf9 cells infected with recombinant IL-2 baculovirus clone 4A from day 1 to day 7 post-infection, 9-12) supernatants from Sf9 cells infected with wild-type baculovirus from day 1 to day 4 post-infection, and 13 and 14) lysozyme.



response was significantly greater to supernatants where the infected Sf9 cells were cultured in Grace's media supplemented with 10% FBS as opposed to SF900 II serum-free media (Gibco BRL Life Technologies, Grand Island, NY). In the CTLL-2 assay the wells containing serum-free supernatants were supplemented with FBS to maintain a concentration of 10% so that the viability of the CTLL-2 cell line was not affected by a lower level of serum. TCGF was also assayed with a CTLL-2 bioassay; the response was less than that of the serum-supplemented supernatants, and was similar to that of the serum-free supernatants. The bioassay data in Figure 9 is for recombinant IL-2 expressed from clone 4A. The results from supernatants generated with recombinant IL-2 clones 1A and 2A were similar. CTLL-2 assays were also performed on cRPMI, SF900 II serum-free media, serum-supplemented Grace's media, and on supa, and on supernatants from cells infected with wild-type virus (mock-infected). All of the absorbance readings for these control samples were at or below background.

MBP-specific T cell lines proliferate in response to recombinant rat IL-2. After establishing that the supernatants contained functional recombinant IL-2 in supernatants, the GP2E5/R1 MBP-specific T cell line was assayed for a proliferative response to the expressed protein. The proliferative response to the serum-supplemented supernatants was greater than to the serum-free supernatants, indicating higher levels of recombinant IL-2 (Figure 10). The high level of proliferative response confirmed that the recombinant

Figure 9. Quantitation of IL-2 bioactivity in T Cell Growth Factor, serum-supplemented baculovirus supernatants, and serum-free baculovirus supernatants.

The supernatants assayed were generated by infecting Sf9 cells in SF900 II serum-free media or Grace's media supplemented with 10% FBS with clone 4A. The data represents supernatants which were collected on day 3 of the infection. Supernatants were serially diluted with cRPMI and 2.5×10^4 CTLLs were added to each well. The plates were placed at 37°C for 24 hours and then were pulsed with MTS. Twenty-four hours later absorbance readings were obtained. The bioactivity of IL-2 is plotted as the concentration of supernatant versus the absorbance reading. Each data point is the mean of quadruplet wells. Error bars are less than two standard deviations.

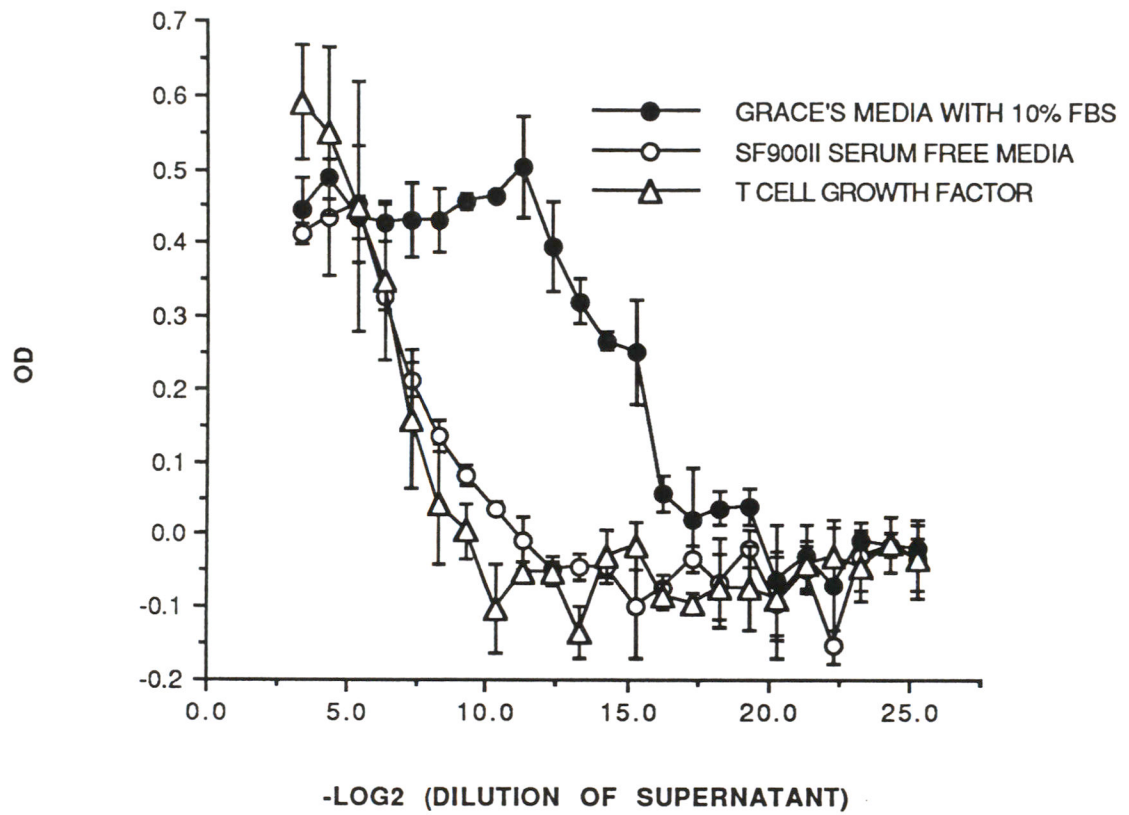
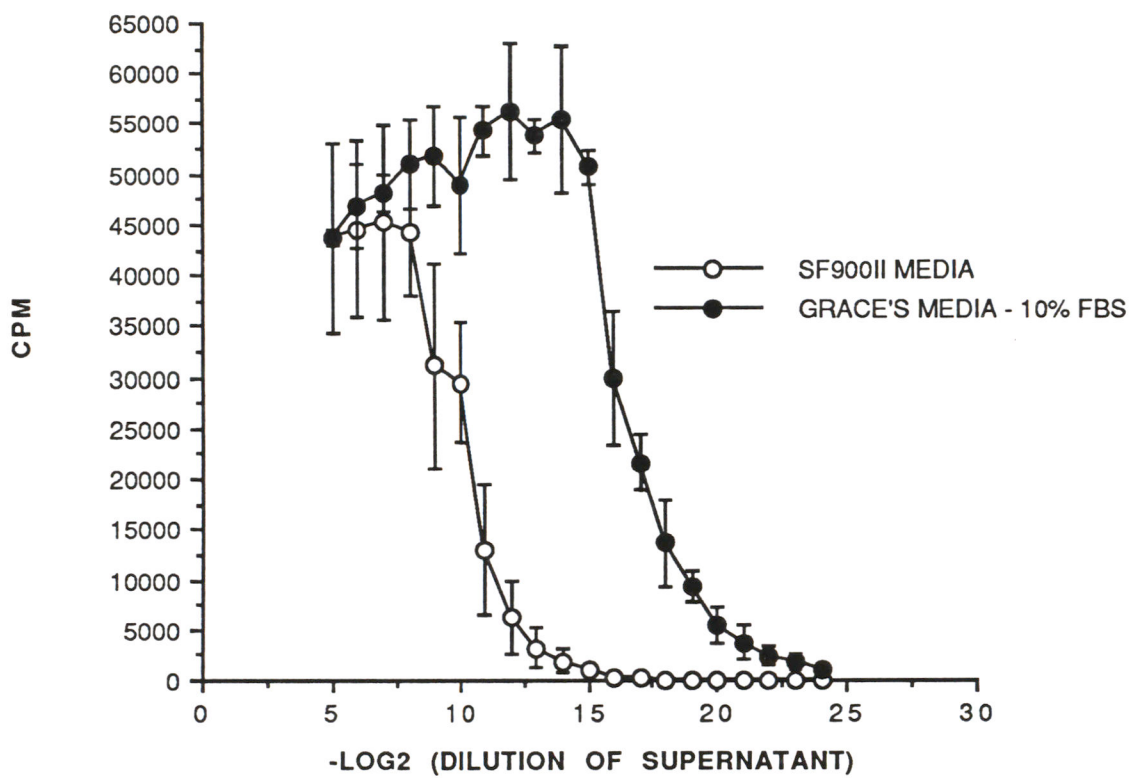


Figure 10. Myelin Basic Protein-specific T cells proliferate in response to recombinant rat IL-2 produced from a baculovirus system. Baculovirus supernatants were serially diluted with cRPMI in a 96 well plate, and 2.5×10^4 GP2E5/R1 T cells were added to each well. After 24 hours of culture, the wells were pulsed with [3 H]-thymidine and returned to the 37°C incubator for another 24 hours. At that time, the cells were harvested and [3 H]-thymidine incorporation was measured. The proliferative response is plotted as the concentration of supernatant versus counts per minute (CPM). Each data point represents the mean of day 4 supernatants produced by recombinant IL-2 baculovirus clones 1A, 2A, and 4A. Error bars are less than two standard deviations.

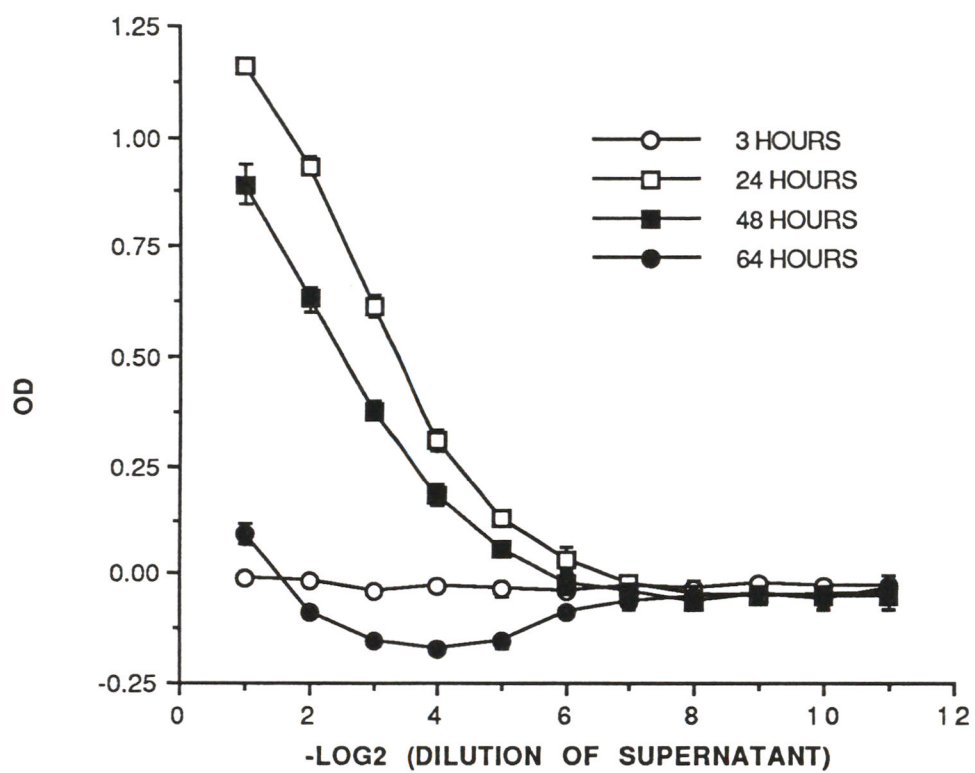


IL-2 would be suitable for supplementing cRPMI for use in T cell culture. Once a functional source of rat IL-2 was available, this research began to focus on IL-2 and T cell regulation.

Time course of IL-2 production. IL-2 is inducible in T cells by antigen specific or mitogenic activation. Figure 11 demonstrates the time course of IL-2 production by the GP2E5.30 MBP-specific rat T cell line upon activation with GPMBP and IRRSPL. At 3 hours post-activation IL-2 is not detected by the CTLL-2 bioassay. As with all bioassays the CTLL-2 bioassay has limited sensitivity so that low levels of IL-2 may go undetected. In addition, the nature of the assay does not allow the detection of IL-2 which has been consumed by the cells, only that IL-2 which remains in the supernatant at the time of collection will be detected. By 24 hours post-activation the levels of IL-2 found in the supernatant are highest. At 48 hours a slight drop is seen and by 64 hours the levels of IL-2 are below background. A previous study has shown that IL-2 mRNA levels begin to drop between 12 and 24 hours post-activation so it is likely that the decrease in IL-2 in the supernatant at 48 hours is due to the decrease in IL-2 production in addition to the rate of consumption (Marinakis, 1995).

MBP-specific T cells are refractory to IL-2 production upon reactivation. When reactivated within 7-10 days of a prior activation, MBP-specific T cell lines are refractory to the production of IL-2 (Mannie *et al.*, 1994). During this refractory period there is a

Figure 11. Time course of IL-2 production in activated T cells. For each time point assayed, 1×10^6 GP2E5.30 T cells were activated with 2 μ M GPMBP in the presence of 20×10^6 IRRSPL. The cultures were placed at 37°C, and the supernatants were harvested at the given timepoints. Supernatants were serially diluted with cRPMI and assayed for IL-2 activity using the CTLL-2 assay. The bioactivity of IL-2 is plotted as the concentration of supernatant versus the absorbance reading. Each data point is the mean of triplicate wells. Error bars are less than two standard deviations.

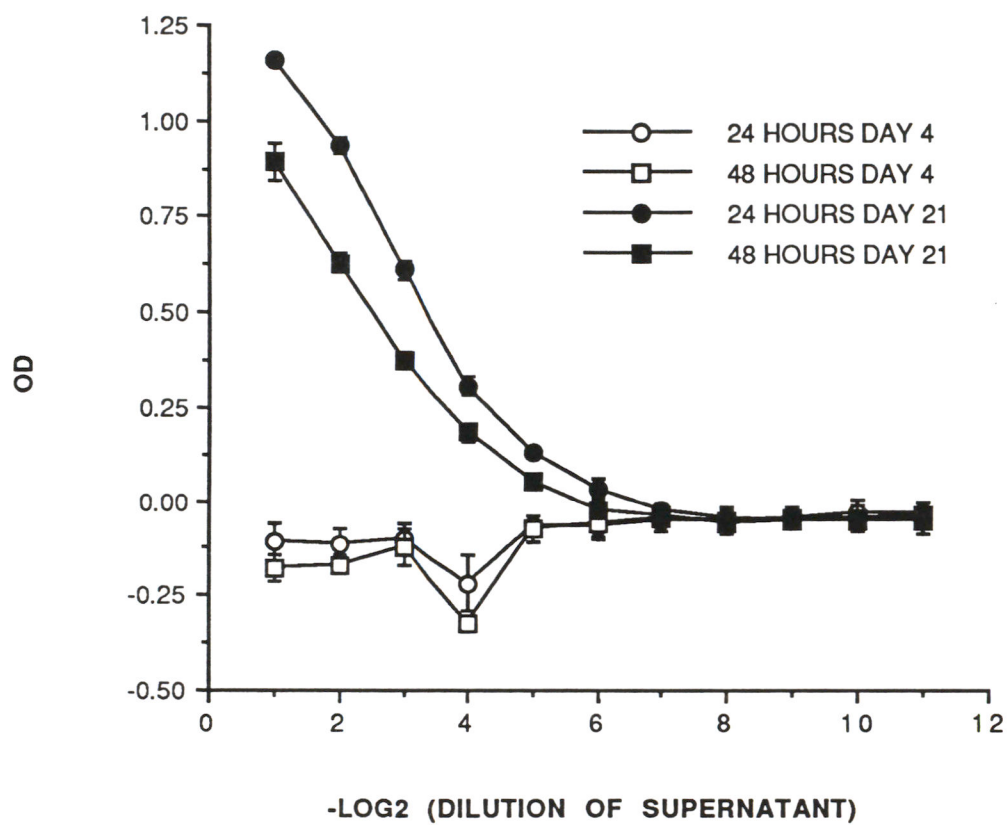


reduction in IL-2 mRNA production as measured by RT-PCR (Mannie, personal communication). In this study, the GP2.E5.30 cell line was activated with GPMBP and IRRSPL for three days at which time they were returned to IL-2-conditioned media. The cells were rested in the conditioned media for 4 or 21 days and were then reactivated under the same conditions. The supernatants were collected, and IL-2 production was measured by the CTLL-2 bioassay. As illustrated by Figure 12, the cell line that was rested for only 4 days produced no measurable IL-2 while the cells that rested for 21 days produced normal levels of IL-2. Despite the lack of IL-2 production the refractory cell line proliferated in response to activation.

Both antigen specific and mitogenic activation will result in refractoriness to IL-2 production in a cell line that has been reactivated within a 7-10 day period, indicating that this refractory phase is a result of cellular activation. Based on this observation, it was suggested that the refractory phase represents a form of T cell self-regulation (Mannie *et al.*, 1994).

The addition of exogenous IL-2 to an activation does not prevent W3/25-mediated inhibition. The anti-CD4 mAb W3/25 has been shown to inhibit MBP-induced T cell activation as measured by the expression of EAE transfer activity (Swanborg, 1983). Antigen specific activation of T cell lines is inhibited, but not mitogenic activation (Mannie *et al.*, 1993). The activation of the GP2.E5/R1 T cell line with GPMBP and IRRSPL in the presence of W3/25 induced anergy as measured by proliferative response

Figure 12. MBP-specific T cells are refractory to IL-2 production when reactivated after four days of rest. 1×10^6 GP2E5.30 T cells were activated for 1 or 2 days as designated with 2 μ M GPMBP in the presence of IRRSPL (20×10^6). Cells were resting either 4 or 21 days as designated. Supernatants were harvested and assayed for IL-2 activity using the CTLL-2 bioassay. The bioactivity of IL-2 is plotted as the concentration of supernatant versus the absorbance reading. Each data point is the mean of triplicate wells. Error bars are less than two standard deviations.

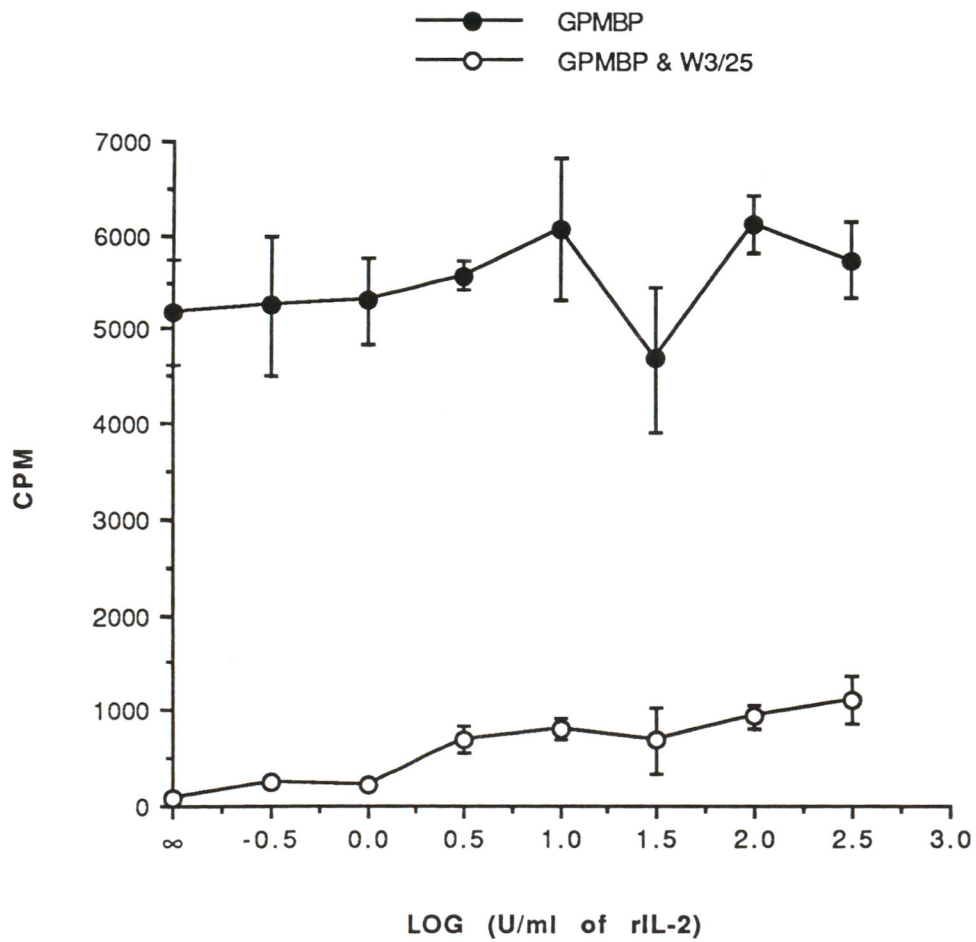
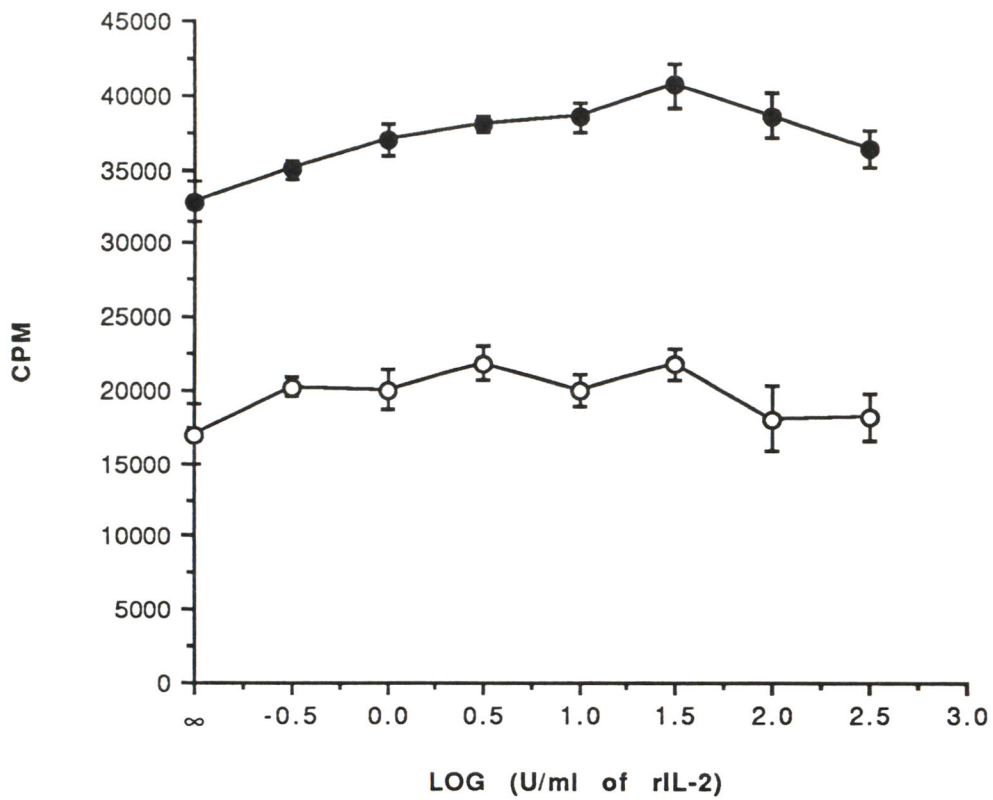


in subsequent activations (Marinakis, 1995). Cell division in the presence of IL-2 has been shown to reverse anergy (Johnson and Jenkins, 1993).

In an attempt to answer whether the presence of exogenous recombinant IL-2 would reverse the inhibitory effects of W3/25, the GP2.E5/R1 and GP2.E5/R1.33 MBP-specific T cell lines were activated with various concentrations of exogenous recombinant IL-2 (0 U/ml to 320 U/ml) in the presence of W3/25. The proliferative response was measured by [³H]-thymidine incorporation. Activation of both cell lines in the presence of W3/25 resulted in almost complete inhibition of IL-2 production. The lack of IL-2 production in the GP2.E5/R1 cell line was accompanied by a partial inhibition of proliferation, and in the GP2.E5/R1.33 cell line there was a nearly complete inhibition of proliferation (Figure 13). The addition of recombinant IL-2 to an activation culture containing W3/25 had no effect on the proliferative response indicating that the lack of proliferation was not solely due to the inhibition of IL-2 production.

IL-2 augments antigenic responsiveness. The R2 T cell line is a MBP-specific, IL-2 dependent cell line. When these cells are cultured in a resting state, the media must be supplemented with IL-2. When the cell line is removed from IL-2-conditioned media and is cultured in cRPMI, it will lose antigenic responsiveness. In this study, the R2 cell line was cultured in cRPMI for 4 days prior to activation with GPMBP and IRRSPL. The proliferative response of the cells was markedly reduced when assayed by activation with GPMBP. When the same cell line is cultured in media supplemented with 1%

Figure 13. Addition of exogenous recombinant IL-2 does not prevent W3/25-mediated inhibition. A proliferative assay was set up in a 96 well plate format. 2.5×10^4 GP2E5/R1 (top panel) or GP2E5/R1.33 (bottom panel) T cells were activated with $2\mu\text{M}$ GPMBP in the presence of 5×10^5 IRRSPL. Activations were set up with or without W3/25 as indicated. Recombinant IL-2 was added to each well in concentrations ranging from 0 to 320 U/ml. The cultures were incubated at 37°C for 24 hours and were then pulsed with [^3H]-thymidine. After another 24 hour incubation, the cells were harvested and [^3H]-thymidine incorporation was measured. The proliferative response is plotted as the concentration of recombinant IL-2 versus CPM. Each data point represents the mean CPM of triplicate wells. Error bars are less than two standard deviations.



recombinant IL-2 containing supernatant and is assayed by activation with GPMBP, the proliferative response is substantially increased (Figure 14). A third assay was performed on the same cell line which had been cultured in cRPMI supplemented with IL-1. The proliferative response of those cells was comparable to the cells cultured in unsupplemented cRPMI. Despite the difference in proliferative response between the cell lines, the level of IL-2 production was similar indicating that the loss of response is not the result of lack of IL-2 production (Figure 15). In fact, when 1% rat recombinant IL-2 was added to the activation cultures the proliferative response was not affected.

Figure 14. *IL-2 augments antigenic responsiveness.* R2 T cells (2×10^6) were cultured in 40 ml of cRPMI or cRPMI supplemented with 1% recombinant IL-2 for four days. T cells (2.5×10^4) of each line were activated with GPMBP at concentrations ranging from 20 nM to 2 μ M in the presence of 5×10^5 IRRSPL. After 24 hours the cultures were pulsed with [3 H]-thymidine and were returned to 37°. After a second 24 hour incubation the cells were harvested and assayed for [3 H]-thymidine incorporation. The proliferative response is plotted as the concentration of GPMBP versus the CPM. Each data point represents the mean CPM of triplicate wells. Error bars are less than two standard deviations.

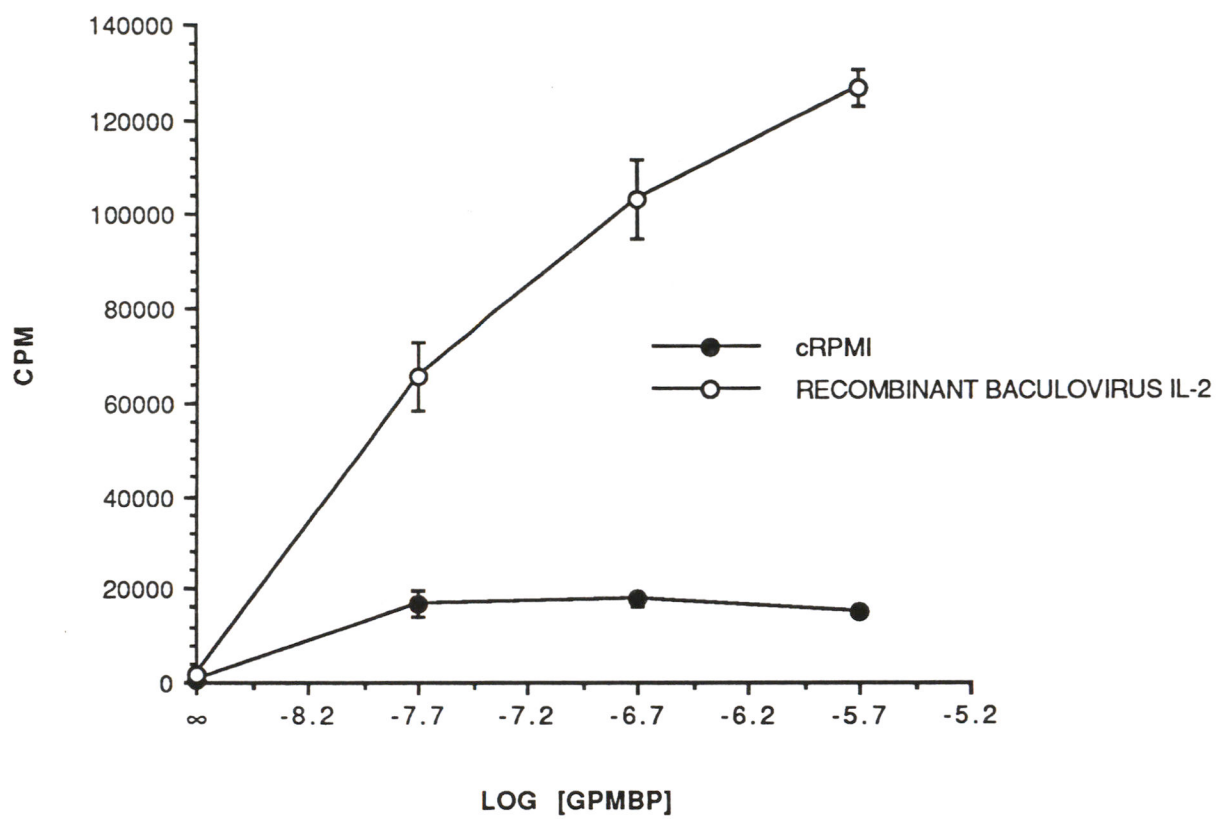
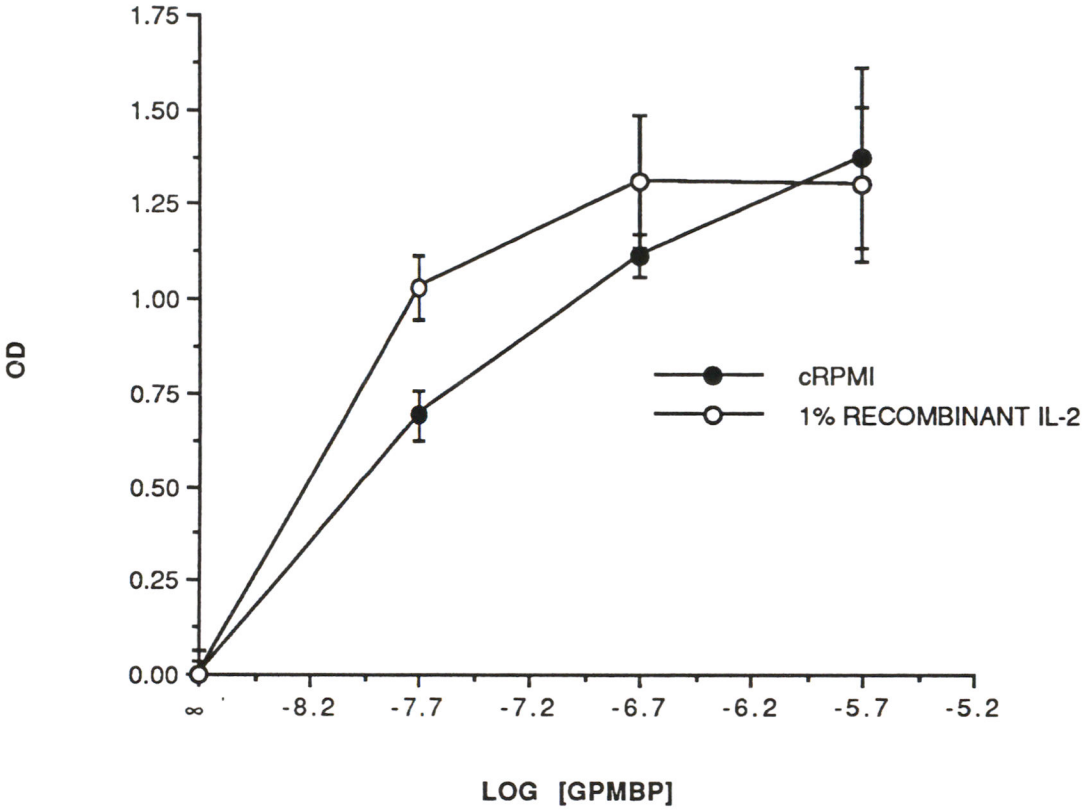


Figure 15. When the R2 T cell line is cultured with or without IL-2 it retains the ability to produce IL-2 upon antigen specific activation. R2 T cells (2×10^6) were cultured in 40 ml of cRPMI or cRPMI supplemented with 1% recombinant IL-2 supernatant for four days. T cells (2.5×10^4) of each line were activated with GPMBP at concentrations ranging from 20 nM to 2 μ M in the presence of 5×10^5 IRRSPL. After 24 hours 120 μ l of supernatant were removed from each activation culture and assayed for IL-2 activity with the CTLL-2 assay. The bioactivity of IL-2 is plotted as the concentration of GPMBP versus the absorbance reading. Each data point represents the mean absorbance readings of triplicate wells. Error bars are less than two standard deviations.



DISCUSSION

The experiments in this study were designed to analyze the regulation of IL-2 by MBP-specific T cell lines and to look at the effects of IL-2 on those same cell lines. This thesis offers some insight as to how autoreactive T helper cells may self-regulate as a way of limiting an autoreactive immune response. In addition, this thesis describes a recombinant rat IL-2 which will prove useful in the study of T cell regulation.

Baculovirus recombinant rat IL-2 provides a source of the T cell growth factor which is free from other rat proteins. When T cells recognize specific antigen on the surface of APCs, they become activated. Two of the results of antigen specific activation are the production of the autocrine growth factor IL-2 and proliferation of the T cells. The supernatant from mitogen activated rat splenocytes, hereafter referred to as T Cell Growth Factor (TCGF), can be harvested and used as an IL-2 supplement in media and a source of IL-2 for experimentation. One complication that arises with the use of TCGF is that it not only contains rat IL-2, it also contains the numerous cytokines and other proteins produced by activated splenocytes. Included in the TCGF could be other growth factors or inhibitory cytokines that would introduce unwanted variables into an experiment. The generation of recombinant rat IL-2 using a baculovirus system has provided a source of the growth factor independent of other rat cytokines which is valuable in studying the effects of rat IL-2 on MBP-specific T cell lines. By nature, TCGF contains a great variety of rat proteins in addition to IL-2. When generated in

SF900II serum-free media there is little other protein (Figure 8) as compared to the protein found in Grace's media from the FBS supplement. In addition, the baculovirus system produced levels of IL-2 higher than those found in TCGF (Figure 9) and to which T cell lines were responsive (Figure 10).

W3/25 inhibits MBP-specific T cell lines through mechanisms affecting proliferative signals in addition to an inhibition of IL-2 production. The anti-CD4 mAb W3/25 inhibits the IL-2 production and proliferation of MBP-specific T cell lines (Figure 13). W3/25 selectively affects the levels of IL-2 mRNA production; however, it is not a general inhibitor as it has been shown that IL-4 and β -actin mRNA are not affected (Marinakis, 1995). W3/25 specifically inhibits antigen specific activation while mitogenic activation is left intact (Mannie et al., 1993). Possible mechanisms for the inhibition are that W3/25 may disrupt the interaction between CD4 and MHC class II molecules resulting in the destabilization of the TCR/MHC/antigen complex or a negative signaling via CD4 pathways (Marinakis, 1995). Another explanation may be that W3/25 affects the interaction between the CD4:p56^{lck} and TCR/CD3 complexes. It has been proposed that these complexes may physically associate during antigen presentation, and form an aggregate receptor that enhances the generation of intracellular signals leading to proliferation (Burgess *et al.*, 1991). Any of these hypotheses are consistent with the results shown in Figure 13 in that they could affect many aspects of T cell activation and proliferation.

The addition of various concentrations of recombinant rat IL-2 to T cells activated with GPMBP, IRRSPL, and W3/25 does not restore proliferative responses. In addition to inhibiting IL-2 production, other proliferative signals may be affected by W3/25. One explanation could be the downregulation of IL-2R or an effect on the IL-2R signaling. For example, Carrera and co-workers (1987) showed that the anti-CD4 mAb HP2/6 almost completely inhibited the expression of IL-2R.

W3/25 only partially inhibits the proliferative response of GP2.E5/R1 MBP-specific T cells (Figure 13) and other MBP-specific T cell lines (Mannie *et al.*, 1993, 1994). There is a resistant component which allows proliferation, possibly via another growth factor such as IL-4 (Mannie *et al.*, 1993; Marinakis, 1995). This indicates that the lack of proliferative response to IL-2 is not attributable to the inhibition of a general mechanism of proliferation but to a specific aspect of IL-2 driven proliferation.

Post-activation refractoriness contributes to the maintenance of peripheral tolerance by promoting antigenic non-responsiveness. Some T cells will become refractory to IL-2 production if reactivated with 7-10 days of a prior activation (Mannie *et al.*, 1994). When the GP2.E5.30 cell line is allowed only 4 days of rest, it does not produce IL-2 when activated with specific antigen (Figure 12). During this refractory period, the T cells are more sensitive to inhibitors such as W3/25 and prostaglandin E₂ and are more susceptible to the induction of anergy (Mannie, personal communication). *In vitro* this refractory period could be a mechanism that exists to limit the immune response. In the

case of infection, the invading organism would rapidly multiply and would consist of a diverse array of foreign proteins. More T cells of diverse specificities would be recruited to the area, and they would be presented specific antigen resulting in their activation. The activation would result in IL-2 production by the newly recruited T cells which could be utilized by the refractory cells to perpetuate the immune response. Under circumstances where an autoreactive T cell was activated by self antigen, it would not elicit an immune response of the magnitude that a foreign organism would evoke. The self tissue would not provide the diversity and quantity of antigenic determinants provided by a foreign organism. As a result, fewer IL-2 producing T cells would be recruited to the site. While the original activated T cells would produce high levels of IL-2 in the first 48 hours, the level of IL-2 production would rapidly decline (Figure 11) and the cells would then require a paracrine source of IL-2 to stimulate proliferation. If no other T cells were recruited, the cells would become more susceptible to the induction of anergy. This increased susceptibility to anergy would occur at a time when necessary costimulus is not available due to low numbers of activated T cells. Thus, when presented specific antigen in the absence of costimulus, the autoreactive cells will become tolerant. The level of antigenic complexity within the microenvironment of the immune response would determine whether the outcome is anergy or an immune response (Mannie, personal communication).

Supporting this hypothesis is the fact that when a large amount of aqueous antigen is administered without an adjuvant, the result is an inhibition of the immune response

(Abbas *et al.*, 1991). The presence of an adjuvant would create an inflammatory response and lead to the recruitment of lymphoid cells to provide the necessary costimulatory signals and IL-2 production necessary to support the continuation of an active immune response. In the absence of adjuvant, and therefore the absence of newly recruited T cells, the autoreactive cells would be presented antigen in the absence of costimulus and become anergic. In addition, the levels of IL-2 would drop and a loss of antigenic reactivity would occur (Figure 14).

The implication of bacterial and viral infections in the etiology of autoimmune disease (Sinha *et al.*, 1990) may also lend support to a significant role for IL-2 in autoimmunity. An infection may trigger autoimmune responses in two ways: (1) The inflammatory response to an infectious agent may provide the cytokines necessary to activate nonresponsive autoreactive T cells, or (2) A pathogen may contain antigenic determinants which cross-react with an autoreactive T cell, referred to as molecular mimicry (Sinha *et al.*, 1990). In the event of molecular mimicry, not only would an autoreactive T cell be activated, and undergo clonal expansion, but a number of other T cells would be activated in response to the diverse antigenic determinants of the infectious agent. It is the activation of these additional T cells which would support the immune response through the production of IL-2. It has been shown that MBP does share sequence homology with a number of viruses (Martin *et al.*, 1992). In both cases, enough IL-2 would be produced to overcome the refractory period of the initial activated T cells and to recruit other lymphoid cells to support an autoimmune response.

The length of the refractory period is highest when T cells are activated at lower densities (Mannie, personal communication). When cells were activated at high densities ($2.5\text{-}5.0 \times 10^5$ cells/ ml activation culture) IL-2 production pathways were recovered in full by 13 days post-activation while low density activations ($1.0\text{-}2.0 \times 10^3$ cells/ ml activation culture) recovered only part of their IL-2 production pathways after 41 days and were less responsive to antigen. A high density activation results in more IL-2 production to support an immune response. A low density activation may not provide sufficient support in the form of paracrine IL-2 and result in the loss of some antigenic reactivity.

The idea that a paracrine source of IL-2 is necessary to prevent the induction of anergy in T cells requires that autoreactive T cells exist in low numbers in the periphery. If autoreactive T cells existed in high numbers, upon T cell activation, enough cells may be recruited to the area to support the previously activated T cells. While autoreactive T cells do not undergo negative selection it is likely that they do not undergo positive selection due to the absence of their respective antigen in the thymus (von Boehmer, 1994). T cells in the periphery which recognize foreign antigen will have undergone positive selection resulting in clonal expansion, and will therefore be present in larger numbers than autoreactive cells in the periphery.

T cells require interleukin-2 to maintain antigenic responsiveness. It has been shown in two models of graft tolerance that IL-2 is down regulated in the grafts of tolerant

animals while levels of other cytokines are similar to those seen during graft rejection (Dallman *et al.*, 1993). In one model, the tolerant mice displayed a decrease in IL-2 mRNA and in another model, tolerant rats were unable to produce biologically active IL-2. Thus it seems that IL-2 is a cytokine which plays a role in limiting reactivity of T cells in the periphery and therefore may participate in the maintenance of nonresponsiveness in autoreactive T cells. When the R2 MBP-specific T cell line is cultured in the absence of an IL-2 source it develops a diminished response to specific antigen and IRRSPL. When the same cell line is cultured in cRPMI supplemented with 1% recombinant rat IL-2 there is a six-fold increase in proliferative response when activated under the same conditions (Figure 14). While in many models of anergy IL-2 transcription is primarily affected, the absence of IL-2 seems to make T cells more susceptible to anergy (Mannie, personal communication) and affects the antigenic reactivity of T cells (Figure 14).

While cells cultured in the absence of IL-2 lost antigenic reactivity, it was not due to a lack of IL-2 production. When the cell line was cultured with and without IL-2, the subsequent antigen specific activations resulted in high levels of IL-2 production, yet only the former retain full antigenic reactivity (Figure 15). When a T_H1 cell is fully activated with specific antigen and APCs, it can be rendered nonresponsive by simply washing away the IL-2 produced by the cell (Johnson and Jenkins, 1994). The cells require continuous exposure to IL-2 to maintain antigenic responsiveness. There is a lasting effect on cells cultured in the absence of IL-2 that affects the mechanisms of proliferation. The TcR and IL-2R are coupled to different signaling mechanisms so one

function may be lost without the impairment of the other (Gilbert, 1994). When IL-2 is withheld from a T cell culture, the TcR function and signaling appear to remain intact as IL-2 production by the cells during subsequent activations is normal. However, the response to IL-2 is affected as measured by a proliferative deficit (Figure 14).

Anti-CD3 mAbs have been shown to block entry of T_H1 clones into the cell cycle completely inhibiting their proliferative response to endogenously produced and exogenous IL-2 (Abbas *et al.*, 1991). Perhaps the absence of IL-2 may affect the function of a cell surface molecule such as CD3, resulting in inhibition of proliferation. Anti-CD3 mAbs have also been implicated in the induction of anergy, suggesting that the molecule plays a role in the maintenance of peripheral tolerance. While T cells which are chronically stimulated with anti-CD3 produce IL-2, they fail to elicit a proliferative response (Abbas *et al.*, 1991; Johnson and Jenkins, 1994).

Another possible explanation for the diminished responsiveness could be the presence of a repressor protein which prevents transcription of a gene necessary for IL-2 driven proliferation of T cells. A similar idea has been suggested for a protein which would repress transcription of IL-2 (Johnson and Jenkins, 1993, 1994). If a cell was stimulated and not allowed to proliferate, the protein would build up to a level where it could inhibit IL-2 transcription. Perhaps the absence of IL-2 in a T cell culture allows the accumulation of a protein which inhibits the proliferation but not the IL-2 production of a cell. If the cells are placed back in IL-2 supplemented media, they will eventually regain the lost antigenic reactivity (Mannie, personal communication). The proliferation in

culture might create a diluting out effect for a repressor protein reducing it to ineffective levels.

While an autoreactive T cell may receive IL-2 as a bystander of an immune response and become responsive for a short period of time, as the antigen is cleared, the self-regulatory nature of the immune system will cause the response to subside. As the immune response declines, so will the levels of IL-2, and in the absence of IL-2 any autoreactive cells that may have been responsive will become susceptible to anergy induction and again lose antigenic reactivity. This mechanism of loss of antigen reactivity in the absence of IL-2 may ensure that peripheral tolerance is maintained in autoreactive cells.

IL-2 affords a T cell with a method of self-regulation which seems to be an integral part of maintenance of peripheral tolerance. The understanding of this self regulation may provide insight to the delicate balance between T cell tolerance and autoimmunity.

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