IMMUNOREGULATION IN MURINE EXPERIMENTAL AUTOIMMUNE THYROIDITIS

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IMMUNOREGULATION IN MURINE EXPERIMENTAL AUTOIMMUNE THYROIDITIS

by

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Dedicated to the memory of a
professor and friend,

Dr. Garry Ian McTaggart-Cowan
(1940-1997)

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LIST OF ABBREVIATIONS

a.a.	Amino Acid
Ab	Antibody
Ag	Antigen
AITD	Autoimmune Thyroid Disease
APC	Antigen Presenting Cell
APL	Altered Peptide Ligand
BB	BioBreeding
bTg	Bovine Thyroglobulin
BUF	Buffalo
cDNA	Complementary DNA
CFA	Complete Freund's Adjuvant
con A	Concavalin A
CTLL	Cytotoxic T Lymphocyte Line
CNS	Central Nervous System
CPM	Counts per Minute
DEPC	Diethypyrocarbonate
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethylsufoxide
DTH	Delayed Type Hypersensitivity
EAE	Experimental Autoimmune Encephalomyelitis
EAT	Experimental Autoimmune Thyroiditis
ELISA	Enzyme-linked Immunosorbent Assay
FBS	Fetal Bovine Serum
GAD	Glutamic Acid Dehydrogenase
GVHD	Graft Versus Host Disease
HEL	Hen Egg Lysozyme
HT	Hashimoto's Thyroiditis
hTg	Human Thyroglobulin
IDDM	Insulin Dependent Diabetes Mellitus
IFA	Incomplete Freund's Adjuvant
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IL-2R	Interleukin-2 Receptor
i.p.	Intraperitoneal
i.v.	Intravenous
LNC	Lymph Node Cells
LPS	Lipopolysaccaride
mAbs	Monoclonal Antibodies

2-ME	2-Mercaptoethanol
MBP	Myelin Basic Protein
MHC	Major Histocompatibility Complex
MOG	Myelin Oligodendrocyte
MS	Multiple Sclerosis
mTg	Mouse Thyroglobulin
mRNA	Messenger RNA
MW	Molecular Weight
NOD	Non-obese Diabetic
OS	Obese Strain
PBS	Phosphate Buffered Saline
PBS-T	PBS with Tween-20
PLP	Proteolipid Protein
PPD	Purified Protein Derivative
pTg	Porcine Thyroglobulin
RIP	Rat Insulin Promoter
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
s.c.	Subcutaneous
scid	Severe Combined Immunodeficiency
T_3	Triiodothyronine
T_4	Thyroxine
TcR	T Cell Receptor
TFC	Thyroid Follicular Cells
Tg	Thyroglobulin
TgP1	rTg(2495-2551)
TgP2	rTg(2695-2713)
Th1	T helper 1 Lymphocyte
Th2	T helper 2 Lymphocyte
TNF	Tumor Necrosis Factor
TPO	Thyroid Peroxidase

CHAPTER 1

INTRODUCTION

1. EXPERIMENTAL AUTOIMMUNE THYROIDITIS (EAT)

1.1 EAT as a model for Autoimmune Thyroid Disease (AITD)

Experimental autoimmune thyroiditis (EAT) in mice serves as an animal model for the human autoimmune thyroid disease (AITD), Hashimoto's thyroiditis (HT). HT was named for Hakaru Hashimoto (1881-1934), the Japanese surgeon who first described the disease in humans (Klein, 1997, p. 656). In HT, organ-specific T lymphocytes specific for thyroid antigens arise, and due to a disorder in immunoregulation, these autoreactive clones are permitted to proliferate (reviewed in Volpé, 1990, p.78). This defect in immunoregulation may be due to a variety of factors such as: loss of suppressor T lymphocyte function (Iitaka et al., 1988a, Iitaka et al., 1988, Volpé, 1981, Volpé, 1988), defective clonal activation of immature self-reactive T and B lymphocytes (Nossal, 1983), disorder of the anti-idiotypic network (Burmann et al., 1985) or molecular mimicry (Ahmann et al., 1987, Burmann and Baker, 1985, Ingbar et al., 1987, Wolf et al., 1988, Wenzel et al., 1988, Weiss et al., 1983). The autoreactive T cells, specific for thyroid antigens such as thyroglobulin (Tg) and thyroid peroxidase (TPO), proliferate and interact with the antigen on thyroid follicular cells (TFC) or intrathyroidal antigen presenting cells such as dendritic cells (Kabel et al., 1987). The activation of T cells induces first CD4+ and later CD8+ T cells that destroy TFC and eventually the follicular architecture of the thyroid gland. Antibodies

directed against thyroid antigens may also be formed, and may result in the destruction of TFC by antibody-dependent cellular cytotoxicity (ADCC) or by binding complement (Volpé, 1990). The thyroid gland is an endocrine gland that synthesizes hormones such as thyroxine, which are essential for proper growth and metabolism (Weir, 1997, pp. 295). Subsequent to immune destruction, the thyroid gland does not produce hormones (or hormone production is reduced) and metabolic processes are slowed. This results in clinical symptoms in the patient, such as sensitivity to cold, fatigue, and obesity and sometimes, goiter (Klein, 1997, pp. 656).

The histology of chronic EAT is very similar to that of HT. EAT was first described by Rose and Witebsky in 1956, who immunized rabbits with homologous thyroid extract in complete Freund's adjuvant (CFA) (Rose and Witebsky, 1956). This immunization resulted in both 'thyroid autoantibodies and characteristic destruction of the thyroid gland architecture' (Roitt, p. 385, 1994). In EAT both antibodies and T cells directed against Tg develop, resulting in thyroid inflammation (Kuby, 1997, pp 486).

1.2 Animal Models of EAT

Animal models of EAT allow the study of the induction, manipulation and possible immune modification of the pathogenic process. There are two types of EAT models currently under investigation, spontaneous EAT and directly induced EAT. In the spontaneous model of EAT, autoimmune destruction of the thyroid gland occurs without experimental immune intervention. Spontaneous EAT occurs in the obese strain (OS) chicken, the Buffalo (BUF) and Biobreeding (BB) rat strains, the Argonne Laboratory Colony of beagle dogs and in non-obese

diabetic (NOD) mouse strain (Bigazzi, 1993).

1.2.1 Induced EAT

EAT can also be experimentally induced in animals by immunization with thyroid antigens such as Tg in CFA The induction of EAT can be further subdivided into two categories, direct and indirect induction. Direct EAT, which is induced in animals immunized with Tg in CFA, was pioneered by the Rose and Witebsky's experiments with rabbits in 1956 (Rose and Witebsky, 1956). Since then, EAT has been induced using both homologous and heterologous Tg in dogs (Terplan et al., 1960), rats (Jones and Roitt, 1961), guinea pigs (McMaster et al., 1961), monkeys (Kite et al., 1966) and mice (Rose et al., 1971). Heterologous or homologous Tg peptides have also been used to induce EAT in mice and rats (Carayanniotis and Rao, 1997). For direct induction of EAT to be successful, the protein or peptide must be emulsified with CFA. Although the precise mechanism by which CFA enhances the immunogenicity and EAT-inducing capabilities of Tg is unclear, CFA has been described as having both inflammatory and antigen-depot forming properties (Weigle et al., 1969, Yamanaka et al., 1992). In addition to adjuvant, a range of innate and environmental factors must also be considered in EAT induction. These include the genetic susceptibility of the host, the antigen dose, the immunization schedule or the route of antigen (Ag) administration.

In addition to the above methods, EAT can be directly induced via conjugation of normal Tg to mouse class II major histocompatibility complex (MHC)-specific monoclonal antibodies (mAb) in the absence of adjuvant (Balasa and Carayanniotis, 1993a) and the placing of fresh

thyroids under the renal capsule or in the peritoneal cavity followed by an intravenous (i.v.) injection with the polyclonal B cell activator lipopolysaccaride (LPS) (Okayasu and Hatakeyama, 1984).

There are also several methods to indirectly induce EAT. The most common indirect method is the adoptive transfer of lymph node cells (LNC) from mice immunized with Tg or Tg peptides, and further stimulated *in vitro* with the immunizing Ag. These cells are transferred into naive healthy hosts and EAT develops 2-3 weeks later. A variation on this method is the induction of 'granulomatous' EAT using mTg-activated spleen cells from mice primed with LPS. The *in vitro* priming of these cells also occurs in the presence of either IL-2 receptor (IL-2R) or IFN-γ-specific Abs in addition to Tg. This results in the transfer of a more severe and destructive form of EAT to the naive donors (Braley-Mullen et al., 1991). EAT is also induced after neonatal thymectomy of mice, which disturbs the available pool of lymphocytes, or thymectomy of adult mice in conjunction with irradiation, which alters the T cell subsets (Rose, 1998).

1.3 Host T cell response

The murine EAT model has proved very efficient in determining the immunological contribution of T and B cells in EAT. Its strengths include the fact that the murine immune system has been extensively studied, along with its MHC, many inbred strains of mice available, allowing for the study of genetics in EAT. Using a murine model, Vladutiu and Rose (1975) discovered that it was T and not B cells that transferred Tg responsiveness in 'good responder' mice, mice that were shown to be susceptible to EAT induction using Tg, thus inferring an effector role for

T cells. Subsequent studies in other animal models have supported this conclusion. In the OS chicken spontaneous model, neonatal thymectomy along with *in vivo* treatment with T cell-specific antibodies resulted in prevention of EAT (Schauenstein, 1998). In addition, EAT could be transferred to a naive host by T cells derived from infiltrated thyroids of bursectomized (B cell deprived) OS chickens. T cells have been shown to proliferate *in vitro* in response to mTg and adoptively transfer EAT to normal, naive animals (reviewed in Kong and Lewis, 1990). This effect is abrogated by the addition of Thy-1 antibody *in vitro*. T cells expanded *in vitro* by the addition of Tg have also been shown to destroy thyroid monolayers (Creemers et al., 1983, Salamero et al., 1985, Simon et al., 1986). These studies suggest that T cells play a more important role than B cells in the initiation of EAT.

The T cells that proliferate in response to mTg have been shown to be CD4+ (Kong , 1986, Simon et al., 1985) and are H-2A-restricted (Simon et al., 1985). The proliferative response of T cells *in vitro* correlates with EAT susceptibility (Simon et al., 1985, Okayasu et al.,1981). The relative role of CD4+ and CD8+ T cells in EAT has been elucidated by a series of experiments by Yi-Chi Kong and associates (Reviewed in Kong and Lewis, 1990). These experiments indicate that CD4+ cells are required for the initiation of EAT while both CD4+ and CD8+ T cells contribute to the severity of the established disease.

Although there is little evidence as to the cytokine profile of infiltrating CD4+ T cells, indirect evidence indicates that they are of the T helper 1 (Th1) subset. Some of this evidence includes the hyperreactivity of peripheral lymphoid cells, due to the intrinsic production of interleukin-2 (IL-2) in the OS chicken (reviewed in Wick et al., 1998) and the exacerbation of EAT by the Th1-inducing cytokine IL-12 (Braley-Mullen et al., 1998), and the prototypical Th1

cytokine IFN-γ (Alimi et al., 1998). Other indirect evidence includes the finding that antibodies to Th1 cytokines have a protective effect in susceptible strains to EAT, and that the use of T helper 2 (Th2) cytokines such as IL-10 (Mignon-Godefroy et al., 1995) also confer protection from EAT. These studies support the currently held belief that Th1 cells often contribute to the induction of organ-specific autoimmunity.

2. CD4+ T HELPER SUBSETS

2.1 T Helper Subset

2.1.1 Definition of T helper subsets

Early studies of immune responses *in vivo* found that infectious agents had the ability to induce either an antibody response or a cell-mediated form of immunity and that these responses were often associated with distinct cytokine profiles (Constant and Bottomly, 1997). It was Mosmann et al. (1986) who first described two distinct subsets of CD4+ T helper cell populations as Th1 and Th2, based on the cytokine profiles of mouse CD4+ T cell clones (Mosmann et al, 1986). It has since been discovered that both rats (Eastcott et al., 1990) and humans (Scott et al., 1988) also exhibit these two distinct subsets.

The cytokine profile of these two subsets has been well characterized. Th1 cells secrete the cytokines IL-2, IFN-γ, lymphotoxin and TNF-β. They have also been implicated in a myriad of immune responses through activation of macrophages, initiation of delayed-type hypersensitivity

(DTH) reactions and IgG2a and IgG3 antibody production. These include protection against intracellular parasites, organ-specific autoimmunity, allograft rejection, Crohn's disease and unexplained recurrent abortions (Romagnani, 1997). Th2 cells produce the cytokine IL-4, IL-5, IL-10 and IL-13. They are involved in antibody responses, such as helping B cells produce IgM, IgG1, IgA and IgE, attraction of eosinophils and inhibition of macrophages (Druet et al., 1996). Th2 responses are often implicated in protection against metazoan parasites, successful pregnancy, vernal conjunctivitis, atopic and allergic disorders, chronic GVHD (graft versus host disease), systemic sclerosis and progression to AIDS in HIV infection (Romagnani, 1997).

Th1 and Th2 cells are believed to develop from the same mature naive CD4+ T cell, often described as Th0 (Constant and Bottomly, 1997). Initiation of either Th1 or Th2 responses likely depends on the cellular environment at the time of antigen stimulation. The presence of IL-12, produced by activated macrophages and dendritic cells, induces a Th1 response through initiation of the transcription factor Stat-4. Knocking out IL-12 (Magram et al., 1996) or Stat-4 (Thierfelder et al., 1996, Kaplan et al., 1996) results in markedly reduced Th1 responses. IFN-γ also has an inducing effect on Th1 cells, partly by enhancing IL-12 secretion by macrophages (Thinchieri et al, 1995) and partly by maintaining function of IL-12 receptors on CD4+ T cells (Guler et al, 1996). Th2 responses are induced through IL-4, produced by basophils, mast cells and T cells (Thomson, 1994) signaled through the transcription factor Stat-6 (Hou et al., 1994). Similarly to IL-12, knocking out IL-4 (Kuhn et al., 1991, Kopf et al., 1993) and Stat-6 (Kaplan et al., 1996, Takeda et al., 1996, Shimoda et al., 1996) results in deficient Th2 responses.

Previous studies have found that only cytokine secretion and subsequent immune responses could differentiate between Th1 and Th2 cells. Recently, however, several studies have

implicated several chemokine receptors as possible markers for Th1 and Th2 cells. The chemokine receptors CCR8 (Zingoni et al., 1998) and CCR4 (Bonecchi et al., 1998) are differentially expressed on activated Th2 cells, while CCR5 and CXCR3 are differentially expressed on Th1 cells (Bonecchi et al., 1998, Siveke et al., 1998). These studies have opened up a large area of research in this field, since now Th1 and Th2 cells can be characterized at the single cell level based on surface markers, instead of defining a population of cells based the cytokines secreted into culture supernatant.

2.1.2 Th cell cross regulation

One of the most studied aspects of T helper subsets is the fact that Th1 and Th2 cells are cross regulatory. The cytokines produced by the Th1 subset have the ability to down regulate a Th2 response and vice versa (reviewed in Constant and Bottomly, 1997). This finding has had a great impact in studies of immunoregulation and in areas of research where a Th1 or Th2 type of response is unfavorable to the host, such as organ-specific autoimmunity (Th1) or allergy (Th2).

Th1-type cytokines responsible for the down regulation of Th2-type responses include IFN-γ and IL-12. IFN-γ inhibits the proliferation of Th2 clones (Gajewski et al., 1988) while IL-12 can be used to reverse an established Th2 response (Romani et al., 1997). The ability of IL-12 to reverse an established Th2 response was demonstrated in experimental leishmaniasis (Nabors et al., 1995). IL-12 was used in conjunction with a leishmaniacidal drug during late phase infection, and was shown to switch the dominant Th2 response to a Th1 response, resulting in healing and resistance to reinfection with *L. major*.

Cytokines produced by Th2 cells known to inhibit Th1 cells and promote Th2 development include IL-4, IL-10 and IL-13 (Romagnani, 1997). Th1 cells grown in culture without exogenous cytokines, such as cells specific for purified protein derivative (PPD), can become Th2-like in culture by the addition of IL-4 early in bulk culture (Parronchi et al., 1992). T cells from hosts that are infected with *L. major*, polarized to the Th1 population have been modified to produce Th2 cytokines by reculture *in vitro* with IL-4, IL-2 and APC (Mocci and Coffman, 1995). IL-10 has similar effects, suppressing Th1 cells while decreasing IL-2 and IFN-γ production (Viera et al., 1991, Taga et al., 1992).

One of the most interesting debates of the Th1/Th2 paradigm is whether Th1 or Th2 cytokine secreting cells can actually 'switch' to produce the opposing subset cytokines. The level of commitment for cytokine production at the single cell level is unclear (Kong and Lewis, 1990). It is also unclear whether during a 'switch' from Th1 to Th2 or vice versa in bulk cultures, the cytokines present in the environment actually cause a switch in the cytokine profile of individual cells, or allow contaminating cells of the opposite phenotype to proliferate, or act on uncommitted Th0 cells in the culture and allow them to proliferate. Murphy et al. (1996) found that Th1 clones and cells that were cultured under chronic Th1 polarizing conditions resisted a switch to Th2, and a reduction of the IFN-γ producing cells was observed. Also, Th2 cells are often resistant to phenotype reversal, possibly due to a loss of responsiveness to IL-12 (Szabo et al., 1995).

There are several other factors that affect the balance between Th1 and Th2 responses. Antigen dose influences the T cell response and generally, low doses favor Th1 responses, while higher doses favor Th2 (Bretscher et al., 1992, Hosken et al., 1995). Co-stimulatory signals in T cell activation also play a role. In some systems the type of B7 molecule that binds to CD28 has

an effect, with B7-1 inducing Th1 development and B7-2 favoring Th2 (Kuchroo et al., 1995). Other studies indicate that high levels of costimulation in general favor a Th2 response (DiMolfetto et al., 1998). Lower costimulation may result in Th1 response because Th1 cells may be less dependent on the level of T cell activation as they get their stimulatory cytokine IL-12 from APC (Murphy et al.,1994). Some other factors include the genetic background of the animal, as demonstrated by *L. major* studies where susceptibility to *L. major* was dependant on the strain of mouse, and the route of antigen administration.

2.2 Th1/Th2 cells in autoimmunity

2.2.1 Overview of the role of Th1 cells in organ-specific autoimmunity

Many organ-specific autoimmune diseases, such as insulin-dependent diabetes mellitus (IDDM), multiple sclerosis (MS) and HT are mediated by Th1 cells (Liblau et al., 1995). The cross regulation of the T helper subsets has focused on the possibility of deviating the T cell response in these diseases from a 'pathogenic' Th1 response to a 'protective' Th2 response in the animal models for MS and IDDM (Liblau et al., 1995, O'Garra et al., 1997, Druet et al., 1996).

Experimental Autoimmune Encephalomyelitis (EAE):

Experimental autoimmune encephalomyelitis (EAE) is a model of certain aspects of MS in humans. It is induced in rodents by injection of myelin antigens such as myelin basic protein (MBP), proteolipid protein (PLP) and myelin oligodendrocyte protein (MOG) in CFA (reviewed

in O'Garra et al., 1997). The pathogenesis of EAE involves CD4+ T cells but not CD8+ T cells. There are several lines of evidence that indicate that Th1 cells are involved in the induction of EAE (reviewed by Druet et al., 1996, Liblau et al., 1995). Firstly, the inflammatory response in the central nervous system (CNS) resembles a DTH reaction, which is known to be Th1-mediated. There is a strong correlation between DTH responses to myelin antigens and the development of EAE. Immunohistochemical studies have revealed the presence of Th1 cytokines, IL-2, TNF-β and IFN-γ in CNS tissues at the height of diseases, and the absence of Th2 cytokines such as IL-4. EAE can be transferred passively into naive hosts by Th1 cell clones specific for encephalitogenic peptides, derived from encephalitogenic lesions, but not by Th2 cell clones (Baron et al., 1993).

Insulin Dependent Diabetes Mellitus (IDDM):

In the case of IDDM, studies in the BB rat and NOD mouse indicate that IDDM is T-cell mediated (O'Garra et al., 1997). There is evidence that both CD4+ and CD8+ T cells are involved in the pathogenesis of IDDM, through studies using depleting antibodies against either subset (Kikutani et al., 1992). However, there are other studies that show CD4+ T cells alone can induce IDDM (Katz et al., 1995). In the IDDM model the specific pathogenic Ag has not been determined, yet the β-cell protein glutamic acid decarboxylase (GAD) seems a likely candidate (Druet et al., 1996). The idea that IDDM is a Th1-mediated disease is supported by the observation that T cells from NOD mice produce large amounts of IFN-γ in response to GAD (Kaufman et al., 1993, Tisch et al., 1993). Also, the increased expression of IFN-γ under the control of the rat insulin promoter (RIP) resulted in inflammation of islets and diabetes in

transgenic mice (Sarventnick et al., 1990, Stewart et al., 1993). Both IFN-γ (Campbell et al., 1991) and IL-12 (Trembleau et al., 1995) have been shown to promote IDDM in mouse models. In addition, as in the EAE model, T cell clones specific for GAD, that had the ability to accelerate IDDM in NOD mice, produced Th1 type cytokines after restimulation *in vitro* (Bergman et al., 1994).

2.2.2 Th1 to Th2 immune deviation in autoimmunity

Many studies have focused on switching the 'proinflammatory' Th1 response in organ-specific autoimmunity to a 'protective' Th2 response (reviewed in O'Garra et al., 1997). However, the effectiveness of Th2 cells in having a protective and/or therapeutic effect in organ-specific autoimmunity has yet to be definitively shown. In some cases such as in immunocomprimised hosts, Th2 cells have been shown to be pathogenic in their own right (Lafaille et al., 1997, Pakala et al., 1997).

EAE:

In the EAE model, the first attempts to ameliorate the disease were made using monoclonal antibodies to the proinflammatory cytokines IL-12 and IFN-γ. Administration of a antibodies to IL-12 in mice following adoptive transfer of PLP-primed LNC (Leonard et al., 1995) or immunization with PLP in CFA (Gijbels et al., 1997) inhibited the development of EAE. In contrast, the use of IFN-γ specific mAbs had no effect on EAE (Doung et al., 1992) or was shown to enhance its development (Billiau et al., 1988). Other studies in humans indicated that

treatment with IFN-γ exacerbated MS symptoms (Panitch et al., 1987).

The use of Th2 cytokines to skew the inflammatory response has also been studied in this model. In EAE, as in the human condition, there are often stages of relapse and remission, and during the stage of recovery, IFN-γ RNA and protein levels are low while IL-4, IL-10 and TGF-β levels are high, implicating a Th2 response with recovery (Khoury et al., 1992, Kennedy et al., 1992). In the EAE model, administration of IL-4, IL-10 and IL-13 has shown beneficial effects on the progress of the disease (Racke et al., 1994, Rott et al., 1994, Cash et al., 1994). Yet there have been some discrepancies between these findings and the studies of others, partly because of differences in animal models and time of cytokine introduction. For example, early treatment with IL-4 ameliorates EAE in an adoptive transfer model (Racke et al., 1994), yet IL-4 administration at the time of disease induction or onset exacerbated EAE (Gijbels et al., 1997). In the same study, the use of IL-10 had little effect. Encephalitogenic T cell hybridomas transduced with a retroviral gene to express IL-4 delay the onset of EAE when adoptively transferred into mice immunized with MBP (Shaw et al., 1997), and similarly PLP-specific memory cells transduced with IL-10 cDNA were also shown to inhibit EAE (Mathisen et al., 1997). Finally, the use of PLP-derived altered peptide ligands (APL) which induced Th2 cells, conferred protection from EAE induced by the administration of PLP in CFA when adoptively transferred into mice (Nicholson et al., 1995).

In addition to the above studies, Khoruts et al. (1995) found that PLP (139-151)-specific Th2 cells could not suppress EAE induced by Th1 cells. Lafaille et al. (1997) found that in immunodeficient hosts, Th2 cells caused a form of EAE with allergy-like pathology . The above data suggest that the idea that Th1 cells are pathogenic and Th2 cells are protective may be too

simplistic and that there may be some other type of regulatory mechanism coming into play than the Th1/Th2 balance.

IDDM:

In the IDDM model, antibodies to IL-12R and IFN-γ have had beneficial effects on the disease (Liblau et al., 1995). Also, administration of IL-4 prevents diabetes in NOD female mice (Rapoport et al., 1993). Administration of IL-10 ameliorates disease in NOD mice (Pennline et al., 1994) yet mouse β cells with IL-10 expressed by a transgene are destroyed more rapidly (Wogensen et al., 1994). This destruction may be due to a localized high concentration of the cytokine. Recent findings in this model have been similar to data obtained in the EAE model, in that Th2 cells themselves are capable of inducing diabetes in immunocompromised NOD-scid mice (Pakala et al., 1997). These results also suggest that another type of regulatory T cell, such as T regulatory cell 1 (Tr1) or Th3 may be important in the development of autoimmune diseases (Pakala et al., 1997).

Novel approaches to the study of the Th1/Th2 balance in autoimmune states include models such as IL-10 and IL-4 transgenic and deficient mice (Bettelli et al., 1998), TcR antagonists (Anderton et al., 1998), genetic immunization with cytokine genes (Ramshaw et al., 1997) and the use of androgens to affect the Th1/Th2 shift. The results in these experiments support the idea that other factors besides the Th1/Th2 balance, such as regulatory T cells, are involved in organ-specific autoimmunity, and that any potential immunotherapy must take these factors into account.

2.2.3 Th cells in EAT

The role of Th1 and Th2 cells in immunoregulation of EAT has not been as well characterized. EAT induced by immunization of mice with Tg in CFA, is considered to be mediated by CD4+ T cells (Kong et al., 1990) and these cells are believed to be of the Th1 type (Druet et al., 1996). It has also been shown that the CD4+ T cells infiltrating the thyroids of patients with HT are of the Th1 type (Miossec, 1997). This has not been definitively shown in the mouse model, although, as described below, much indirect evidence indicates that they are Th1.

IL-12 has been shown to exacerbate the granulomatous form of EAT, resulting in an extremely severe and destructive form of EAT in recipient mice (Braley-Mullen et al., 1998). Intrathyroidal injection of IFN-γ alone has also been able to induce EAT (Remy et al., 1987). MAbs to IFN-γ during the development of EAT significantly attenuated the disease in mice (Tang et al., 1993). Using mice that were deficient in the IFN-γ receptor gene, Alimi et al. (1998) found that although IFN-γ was not required for the induction of EAT, it was required for progression to full blown disease. Contradicting this finding, injection of mAbs to IFN-γ into mice immunized with Tg has resulted in an exacerbated form of granulomatous EAT in naive hosts, which is induced using mTg-activated spleen cells of the immunized mice cultured in the presence of anti-IL-2-R (Tang et al., 1998). Immunization of IFN-γ– deficient mice with Tg has also resulted in this exacerbated form of granulomatous EAT in the naive hosts (Stull et al., 1992). The above data seem to contradict the assumed role of Th1 cells as initiators of EAT.

The use of Th2 cytokines IL-4 and IL-10 has also yielded interesting results in EAT studies. Mignon-Godefroy et al. (1995) studied the effect of *in vivo* administration of recombinant

human IL-10 (rhIL-10) on the development of EAT following direct immunization with mTg in CFA or adoptive transfer of mTg-specific T cells into naive hosts. They found that administration of rhIL-10 was very efficient in both preventing and treating EAT. They contributed this finding in part to rhIL-10's enhancement of T-cell death. In the 'transfer EAT' model, spleen cells from CBA/J donors previously primed *in vivo* with mTg were cultured with or without rIL-4 or rIL-10. IL-10 was shown to decrease mTg-specific proliferative and cytotoxic T cell responses, while IL-4 had little effect (Mignon-Godefroy et al., 1995). Another group, de la Vega et al. (1998) found that IL-10 mRNA expression in human thyroid can be increased in thyroid autoimmunity but only in cases of severe infiltration. The presence of a Th1- inhibiting cytokine in lesions that contain T cells of the Th1 cytokine profile was not explained. In contrast to this finding, the application of a plasmid containing IL-10 onto the thyroid *in vivo* had a curative effect on EAT (Batteux et al., 1999). The use of the plasmid was shown to induce fast and long lasting expression of IL-10 in mouse TFC. It resulted in a lowering of the mononuclear cell infiltration of thyroid glands and a diminished anti-Tg T cell proliferation. It also induced a trend toward the Th2 response, indicated by a decreased production of IFN-γ and increase in the ratio of IgG1/IgG2a. In the granulomatous EAT model, the addition of rIL-4 *in vitro* to cultures of spleen cells from donor mice immunized with Tg had little to no effect on the development of EAT in the naive recipient mice (Tang et al., 1998).

These results indicate that for EAT, the most powerful Th1-inhibiting factor is IL-10, with IL-4 playing little role in decreasing the severity of EAT. They also show that novel techniques, such as the use of plasmids encoding cytokines to induce cytokine expression by TFC (Batteux et al., 1999), may be successful in both the prevention and treatment of thyroid autoimmunity in the

future.

3. T CELL DETERMINANTS IN EAT

3.1 Thyroglobulin as an antigen

Thyroglobulin is a large, highly conserved glycoprotein with a homodimeric molecular weight of approximately 660 kDa. It is transcribed from a gene found on chromosome 8 in humans and on chromosome 15 in mice. Tg is synthesized in the endoplasmic reticulum of thyrocytes, modified post-translationally in the Golgi, and then secreted into the follicular lumen (Van Herle et al., 1979 and Ekholm and Bjorkman, 1990). It is expressed only in the thyroid gland, where it is the main constituent of the colloid of the thyroid follicles (Caturegli et al., 1997). Its main function is in the synthesis of thyroid hormones, through iodination of its tyrosines (Caturegli et al., 1997) to form T_4 (thyroxine) and T_3 (triiodothyronine), yet it has been recently shown to possess TGF-β activity (Huang et al., 1998).

Tg has been the major autoantigen implicated in the pathogenesis of EAT. In HT, both Tg and TPO are believed to be major autoantigens. In 1956, Roitt et al. showed that patients with HT developed Tg-specific antibodies while Rose and Witebsky (1956) found that immunization of rabbits with Tg in CFA resulted in thyroid infiltration. Since that time, numerous studies have confirmed the pathogenic role of Tg in autoimmune thyroid disease, and Tg-specific T cells in both the induction and effector phases of EAT (reviewed by Kong, 1994).

Tg is also a highly conserved molecule. Three species of Tg have been cloned and fully

sequenced at the cDNA level. These are human Tg (hTg) (Malthiery et al., 1987), bovine Tg (bTg) (Merken et al., 1985) and recently mouse Tg (mTg) (Caturegli et al., 1997). The C-terminus of rat thyroglobulin (rTg) has also been sequenced (DiLauro et al., 1985). Among these species there is a >70% identity in the a.a. sequence with a high degree of conservative substitutions (Carayanniotis and Rao, 1997, Caturegli et al., 1997). This high degree of homology indicates that pathogenic epitopes of Tg may also be highly conserved across species. Tg also has a high degree of internal homology (Malthiery et al., 1987, Merken et al., 1985) where more than 75% of the sequence is composed of 3 types, or domains of repetitive sequences (Carayanniotis and Rao, 1997). These domains are; Domain A with 10 tyrosine- and cysteine-rich repeats of 50 a.a. between positions 29-1196, Domain B which has 3 repeats of 14-17 highly conserved residues between position 1436-1483, Domain C with 5 repeats between position 1583-2109. The last Domain (D) lacks internal homology and is at the C-terminal end of the molecule.

There are at least two features of Tg synthesis that may affect generation of its pathogenic epitopes. Firstly, Tg is not a sequestered antigen, but it is continuously released into the circulation, along with T_3 and T_4 (Carayanniotis and Rao, 1997). It was believed to be a sequestered antigen until the development of sensitive techniques to measure Tg in circulation (Schneider and Ikekubo, 1979). It has not been determined if this released Tg is intact or released in smaller fragments. Processing of Tg fragments by APC *in vivo* may differ from the processing of intact Tg *in vitro*, resulting in the generation of pathogenic epitopes. Secondly, iodination of Tg may influence its pathogenicity. The Tg molecule, consisting of two identical subunits, is more stable when iodinated (Champion et al., 1987). Champion et al. (1987) also found that poorly iodinated Tg failed to elicit EAT in mice, indicating that dominant pathogenic epitopes may be

iodinated. Also, epidemiological evidence suggests that geographical areas of high iodine correlate with the incidence of thyroid autoimmunity (Boukis et al, 1983, Szabolcs et al., 1997).

3.2 Epitope Mapping of the Thyroglobulin molecule

3.2.1 Mapped thyroglobulin epitopes

Identification of pathogenic T cell epitopes of Tg is vital to study of thyroiditis. It is also crucial to the development of potential strategies for immune therapy. Due to the molecular size of Tg, epitope mapping techniques based on overlapping peptides are both impractical and costly. Thus, other means of finding epitopes must be employed, taking into account current knowledge of how peptides interact with T cells. These other methods include algorithms that predict peptides that may bind to MHC molecules, and thus are candidates for T cell activation. Others include studying the homology of Tg with other thyroidal antigens such as TPO, the exploration of peptides containing the hormonogenic sites of Tg, and the use of hybridomas derived from thyroid lesions.

To date, there are five mapped T cell epitopes in Tg. The largest of these is a 40 a.a peptide [Tg(1672-1711)], discovered by Texier et al.(1992). This group used a cytotoxic T cell hybridoma (HTC2), as a mapping tool. HTC2 had the ability to lyse *in vitro*, macrophages pulsed with either intact porcine thyroglobulin (pTg) or a small trypsin fragment of pTg. They also found that the smaller fragment of pTg had the ability to induce EAT in $H-2^k$ strain mice. Pre-immunization of these mice with HTC2 conferred protection from EAT. This indicated that the

peptide fragment was indeed pathogenic. They purified and sequenced the pTg-peptide fragment and found that it had 70% homology with the known hTg cDNA sequence. As the sequence of pTg was unknown, they synthesized a peptide from the human Tg sequence homologous to this region. They immunized mice with hTg(1672-1711) and discovered that EAT was induced. They also found that autoantibodies in the sera of these mice were directed only to the peptide, hTg(1672-1711) and not intact hTg.

The rest of the four mapped T cell epitopes cluster at the C-terminal end of thyroglobulin, a cysteine-poor region homologous to acetylcholinesterase and other esterases (Malthiery and Lissitzky, 1987, Takagi et al., 1991). Roitt and colleagues studied a sequence containing a thyroxine site as a possible pathogenic epitope. In humans, T_3 and T_4 residues may be formed from tyrosines at positions 5, 2553, 2567, and 2746 (Hutchings et al, 1992). They believed that iodination at these positions may affect immunogenicity. Previous studies by this group indicated that two murine Tg autoreactive hybridomas recognized an epitope containing T_4 at position 2553. The peptide epitope Tg(2549-2560) lost its immunogenicity if the T_4 at position 2553 was substituted with any other a.a. The pathogenic potential of this peptide was tested in CBA/J (H-2^k) mice and it was found that this peptide induced EAT by adoptive transfer but not by direct challenge into recipient mice. This peptide also induced T-cell proliferative responses and antibody responses in mice immunized with Tg(2549-2560).

Hoshioka et al. (1993) investigated the two known thyroid antigens, Tg and TPO and they searched for common sequences that may act as T-epitopes. They scanned the human Tg and TPO sequences and found four 5 a.a. residue sequences that were common in both. They then tested the Tg and TPO peptides containing these identical residues for their immunogenicity in

mice. They found that Tg(2730-2743) was antigenic, as was its TPO counterpart, TPO(118-131) (Hoshioka et al., 1993) in that they induced EAT in mice, yet only through an indirect method. They found that when splenocytes from mice immunized with mTg were stimulated *in vitro* with these peptides, as well as with mTg, they transferred thyroiditis into naive recipients. The fact that thyroiditis could not be directly induced in animals challenged with this peptide in CFA was a major drawback of this study.

Carayanniotis and colleagues have mapped two pathogenic T cell epitopes at the C-terminal end of Tg. The first, rTg(2495-2511), designated TgP1, was found using the computer algorithms 'AMPHI' and 'tetramer motif '(Chronopoulou and Carayanniotis, 1992). This 17-mer peptide which induced infiltration in the thryoids of EAT susceptible strains of mice (SJL/J, C3H and B10.BR) but not EAT resistant strains (BALB/c and B10). It was later found that this peptide also induced EAT in rats (Balasa et al., 1993). TgP1 was identified as non-immunodominant, as TgP1-primed T cells did not response to intact mTg or rTg *in vitro* and, conversely, mTg- and rTg- primed T cells failed to respond to TgP1. Serologically, hTg-primed mice showed strong mTg-specific IgG response but no response to TgP1. However, TgP1-primed mice were shown to elicit strong IgG responses to TgP1 that cross react with mTg, rTg, hTg, bTg and pTg, indicating a strongly immunogenic peptide. This group has since delineated a 9-mer peptide of TgP1, (2496-2504) as a minimal pathogenic epitope of Tg (Rao et al., 1994).

Another peptide, rTg(2695-2713) was also found using the same computer algorithms, and was designated as TgP2. This peptide had a unique genetic pattern of induction of EAT. It caused EAT in SJL/J mice ($H-2^s$) mice but not in $H-2^k$ mice (C3H, B10.BR), which are typically 'good responders' to EAT (Carayanniotis et al., 1994). EAT in SJL/J mice was induced both

directly and via adoptive transfer, and the T cells of these mice proliferated *in vitro* to TgP2. All strains tested exhibited TgP2-specific IgG (Carayanniotis et al., 1994).

Of the five T cell epitopes that have been discovered, none has been shown to be immunodominant. This is not particularly surprising considering the size of the Tg molecule. However, recent developments in the field, such as the determination of the sequence for mTg (Caturegli et al., 1997) and the crystal structure of the mouse A^k molecule (Fremont et al., 1998) may lead to the discovery of other T cell epitopes. Due to the high homology across many species of Tg, these new epitopes of mTg will likely be shared across other species of Tg.

3.2.2 The use of algorithms in epitope mapping

Vladutiu and Rose (1971) discovered that mice of $H\text{-}2^k$ or $H\text{-}2^s$ haplotypes are 'good responders', highly susceptible to Tg-induced EAT. Mice of the $H\text{-}2^b$ and $H\text{-}2^d$ haplotype are classified as 'poor responders' with lower T cell responses and little thyroid infiltration (Vladutiu and Rose, 1971). Susceptibility to Tg-induced EAT is controlled by genes in the H-2A subregions, while the H2-D and H-2K subregions have minimal control (Beisel and Rose, 1983). For this reason, algorithms screening the Tg sequence for the existence of peptides binding to H-2A and H-2E in susceptible $H\text{-}2^k$ mice are used to map pathogenic T cell epitopes (Altuvia et al., 1994).

There are other methods used to predict T cell epitopes. Antigen-specific T cell hybridomas derived from inflammatory lesions have been used to predict T cell epitopes of myosin in myocarditis (Donermeyer et al., 1995) and Tg in thyroiditis (Texier et al., 1992). For smaller

antigens, such as myelin proteolipid protein (PLP), an antigen implicated in the pathogenesis of EAE, overlapping peptides covering the whole molecule were tested for their ability to bind to A molecules, and ultimately cause EAE (Greer et al., 1996). This overlapping-peptide technique would be very costly and time consuming if performed on the Tg molecule.

Early algorithms were developed empirically and were based on common features from peptides binding to A or E molecules. A breakthrough in this area came from Allen et al., 1987, who first demonstrated that certain features of a peptide associated with the A^k molecule (in this case HEL(52-61) a dominant epitope from hen egg lyzozyme) were responsible for its immunogenicity. They were able to show through a.a substitution analysis that within a peptide, certain a.a. residues make contact with the A^k molecule and others make contact with the TcR. These discoveries laid the foundation for studies that looked at multiple immunogenic peptides and ascertained common a.a. sequence features shared by all peptides that bound to the A^k. They established that the presence or absence of certain a.a. at given positions within a peptide has a direct impact on its immunogenicity. The study of many peptides that bind to molecules such as A^k and the common features of these peptides have resulted in the formation of algorithms.

Both A^k and E^k molecules have been studied extensively and several algorithms published, estimating peptides that should bind to it (reviewed in Nelson et al., 1996). Altuvia et al. (1994) developed a computerized method for recognition of peptides binding to A^k or E^k using a cohort of peptides that were known to bind to these molecules and elicit a T cell response. As a control, they used another cohort of peptides that failed to bind to A^k or E^k. Motifs were constructed that consisted of both physical-chemical and structural properties of the amino acids that could occupy a particular position in the prospective peptide (Altuvia et al., 1994). These motifs specify a.a at

certain positions as critical for the binding of peptide to MHC. A study by Kelner et al., 1992 supported the idea that A^k and E^k-binding peptides have critical anchor residues. The study demonstrated that a single a.a. substitution in the A^k-restricted peptide Mt cyt (93-104) (mouse testicular cytochrome c) at position 96 from a.a. lysine to alanine changed the restriction of the peptide to E^k. This indicated that this a.a. was somehow critical for the isotype specificity. Several other studies (Nelson et al., 1996 and Itoh et al., 1996) have also demonstrated that certain a.a. are essential for a peptide to bind to the A^k molecule.

An A^k-peptide binding motif has recently been published, stemming from the discovery of the crystal structure of A^k in complex with the dominant epitope HEL(50-62) (Fremont et al., 1998). This discovery gives a visual representation and a structural basis for an accurate A^k peptide-binding motif. The crystal resolution indicated that a peptide binds to the A^k molecule by the 'burial' of 5 a.a. side chains into pockets in the A groove. These pockets are found at position 1, 4, 6, 7 and 9 (Freemont et al., 1998). Of all these pockets, P1 exhibits the most exclusive binding, with the a.a aspartic acid a near perfect fit. Position P6 has the next most exclusive fit, accepting either glutamine or glutamic acid. Position 9 typically accepts small residues such as serine, but will accept larger residues such as threonine. Positions 4 and 7 will accept a larger number of substitutions, yet for P4 medium-size hydrophobic residues are preferred and P7 can accept any amino acids except those with a positive charge. In addition, the crystal structure also implicates several positions as possible TcR contact residues, due to their high exposure to solvent. These occur at positions 3, 5, 8 and 11. This study confirms many of the assumptions of previous motifs and it provides clear rules that dictate peptide-binding to A^k.

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CHAPTER 2

MATERIALS AND METHODS

2.1 ANIMALS

Female SJL/J, C3H, CBA/J, AKR, and male SJL/J mice were purchased from the Jackson Laboratories, Bar Harbor, ME, USA. The female mice were used for immunizations between 4-10 weeks of age. The male SJL/L mice were used for extraction of thyroid, spleen and liver tissue only and were used at 20-25 weeks of age.

2.2 ANTIGENS

Peptides designated TgP1 [rTg(2495-2511)] and TgP2 [rTg(2695-2713)] were synthesized at >95% purity at the Alberta Peptide Institute (Calgary, Alberta, Canada). They were synthesized on an Applied Biosystems (Foster City, CA) 430A synthesizer using a general procedure for solid-phase synthesis outlined by Erikson and Merrifield (1976) with modifications by Hodges et al. (1988). This procedure involved hydrogen fluoride cleavage of peptide resin at -5°C for one hour in hydrogen flouride:anisole:dimethyl sulfoxide (DMSO): p-thiocresol: peptide resin (10ml:1ml:0.5ml:0.2ml:1g). Peptide purity was assessed by HPLC and mass spectroscopic analysis.

Peptides of rTg used to immunize mice were chosen using overlapping A and B motifs from

Altuvia et al. (1994), as outlined in section **2.3**. These were Tg(1827-1837), Tg(2029-2040), Tg(2108-2125), Tg(2424-2437) and Tg(2597-2609). These peptides were synthesized by Synpep (Dublin, CA, USA).

2.3 ALGORITHM-BASED SEARCH FOR A^k-BINDING PEPTIDES IN TG

The paper entitled 'Sequence Features that Correlate with MHC Restriction' by Altuvia et al. (1994) described an algorithm for prediction of peptides that may bind to A^k or E^k molecules. From this paper, we used two motifs to screen for peptides that may bind to the A^k molecule (**Table 2.1**). The pcGENE software was used to scan the 967 a.a. on the C-terminal end of the rTg molecule (DiLauro et al., 1985), and later the complete mTg sequence (Caturegli et al., 1997). We initially used rTg because the complete sequence for mTg had not been published. Using this program, we found sequences within this portion of rTg that fit motif A and motif B, separately. We then chose peptides in which motif A and motif B completely overlapped within the peptide sequence. We then aligned the portion of the rTg sequence with the known mTg molecule (without the presence of the leader sequence) to get the peptide co-ordinates of our candidate peptides.

Table 2.1 Motifs A and B within A^k-binding peptides (Altuvia et al., 1994)

Position #	Characteristic of a.a.	Include/Exclude a.a.
MOTIF A		
1	hydrogen acceptor, non-hydrophobic, not small, aliphatic	**includes**: aspartic acid, glutamic acid, histidine, asparagine, glutamine
2	any a.a. except amides	**excludes**: asparagine, glutamine
3	any a.a.	**includes** all a.a.
4	medium-sized, aliphatic, hydrophobic no charge, cannot be aromatic or amide	**includes**: isoleucine, leucine, threonine, valine
5	any a.a	**includes** all a.a.
6	any a.a. except aromatic	**excludes**: phenylalanine, histidine, tryptophane
7	hydrophobic, no charge and not be An amide	**includes**: alanine, cysteine, phenylalanine, isoleucine, leucine, methionine, proline, threonine, valine, tryptophane, tyrosine
MOTIF B		
1	hydrogen acceptor, not small, polar, non-aliphatic	**includes**: cysteine, aspartic acid, glutamic acid, histidine, asparagine, glutamine
2	any a.a. except negatively charged	**excludes**: aspartic acid, glutamic acid
3	any a.a.	**includes** any a.a.
4	medium-sized, aliphatic, hydrophobic no charge, cannot be aromatic or amide	**includes**: isoleucine, leucine, threonine, valine
5	hydrogen acceptor, non-hydrophobic, not small, aliphatic	**includes**: aspartic acid, glutamic acid, histidine, glutamine, asparagine

2.4 CULTURE MEDIA

All assays were performed in Dulbecco's modified Eagle (DMEM) medium (Gibco, Burlington, Ontario, Canada) supplemented with 10% fetal bovine serum [(FBS)(Bioproducts for Science, Indianapolis, IN, USA], 20mM HEPES buffer, 2 mM L-Glutamine, 100 units/ml pennicillin, 100 µg/ml streptomycin (all from Gibco) and 5 x 10^{-5} M 2-mercaptoethanol (ME)(Sigma Chemicals, St. Louis, MO, USA).

2.5 ANTIGENIC CHALLENGE OF ANIMALS

2.5.1 Induction of antigen specific LNC

Mice were immunized with 50-200 µg of TgP1 or TgP2, or 100 nmol of either of the six peptides [Tg(1827-1837), Tg(2029-2040), Tg(2108-2125), Tg(2424-2437), or Tg(2597-2609)] in a 1:1 emulsion in Complete Freunds Adjuvant (CFA)(with *Mycobacterium butyricum*, Difco Laboratories Inc. Detroit, MI, USA). They were immunized subcutaneously (under ether anaesthesia) at 3 sites along the back. After 9-11 days, some mice were euthanised with ether and their draining inguinal, auxiliary and brachial lymph nodes collected aseptically. A single cell suspension was prepared by passing the lymph node tissue through a sterile stainless steel wire mesh (Sigma, St. Louis, MO, USA), and washed in complete culture media (DMEM + 10% FBS).

LNC were cultured in 96 well plates at a concentration of 4 x 10^5 cells/well for 4 days in the

presence of titrated amounts of the appropriate antigen in 200 μl microcultures. Eighteen hours prior to harvesting, 1 μCi of ^{3}[H]-thymidine was added to each culture in 25 μl of culture medium. The cells were harvested 18 hour later, using a semi-automated cell harvester (Skatron, Sterling, VA) and incorporated thymidine was counted in a liquid scintillation counter (LS3801; Beckman Instruments, Palo Alto, CA, USA). Stimulation index is defined as (CPM in the presence of antigen/CPM in absence of antigen).

To determine if the peptides were thyroiditogenic, some mice were boosted at 3 weeks with 50 nmol of peptide in Incomplete Freund's Adjuvant (IFA). After two weeks, these mice were bled, and the resultant sera used in antibody ELISA (see **2.6.2**). Their thyroid glands were then removed *en bloc* with the trachea and fixed in buffered formalin. The lobes were then dissected from the trachea and embedded in methacrylate. Approximately 40 sections at 3.0 μm intervals, were obtained throughout each gland. They were fixed to glass slides and stained with haematoxylin and eosin. Scoring was performed as follows: 0 = no infiltration; 1=interstitial accumulation of inflammatory cells; 2= one or more foci of inflammatory cells at least the size of one follicle; 3=extensive infiltration, 10-40% of the total area, 4= extensive infiltration, 40-80% of the total area; 5=extensive infiltration, >80% of the total area. The highest infiltration score observed per gland was assigned to each mouse.

2.5.2 Induction of Th1 and Th2 cells

Antigen-primed LNC generated as described in **2.5.1** were cultured *in vitro* in two separate setups. They were placed in cultures containing either 10^6 cells/ml, 10 μg/ml peptide and 30 ng/ml

rIL-4 (Gibco), or a culture with 5 x 10^6 cells/ml, 10 μg/ml peptide and no exogenous cytokines. This followed the protocol outlined in Khoruts et al., 1997. Cells were then cultured for 4 to 10 days. LNC are resuspended from culture and washed in complete media. 1-5 x 10^7 cells are put into 2 ml of media, and 3 ml sterile Ficoll-Hypaque (Pharmacia Biotech Inc., Baie d'Urfé, Quebec, Canada) layered beneath it. The tube was then spun in a centrifuge (Beckman GPR Centrifuge, Beckman, Palo Alto, CA, USA) at 2000 rpm for 15 minutes with no brake. The cells were then removed from the media/Ficoll interface and washed in complete media and counted using 0.02% trypan blue (Gibco, Burlington, Ontario, Canada). Viable LNC were cultured at a concentration of 10^6 cells/ml and restimulated in the presence of 10 μg/ml of peptide and 5 x 10^6 cells/ml of mitomycin C-treated splenocytes. Spleens were collected aseptically from naive healthy SJL/L mice and single cell suspensions were prepared by passing the spleen tissue through sterile stainless steel mesh (Sigma, St. Louis, MO, USA). The resulting cell suspension was pipetted vigorously to obtain an uniform suspension of cells. The cells were washed 3 times in complete medium, adjusted to 5 x 10^7 cells/ml and incubated at 37°C with 100 μg of Mitomycin C (Sigma, St. Louis, MO, USA) for 20 minutes, avoiding exposure to light. The cells are then washed three times in complete media and used as APC. In addition, viable LNC were also used in LNC proliferation assays as outlined in **2.5.1**. After 48 hours in culture, the cell supernatant was collected for determination of cytokine levels via cytokine ELISA.

2.6 ENZYME-LINKED IMMUNOSORBENT ASSAYS

2.6.1 Cytokine ELISA for assessment of cytokines in cell culture supernatant

The enzyme linked immunosorbent assay (ELISA) was used for detection of cytokines as follows. Disposable polyvinyl chloride 96-well ELISA plates (Dynatech Laboratories, Chantilly, VA, USA) were coated with purified anti-cytokine capture monoclonal antibodies at a concentration of 2 µg/ml. These mAbs were: purified rat anti-mouse IL-2 and IL-10 (PharMingen, Mississauga, Ontario, Canada), and the purified mAbs from the supernatant from the cell line MB170 and 11B11 (American Type Culture Collection (ATCC), Rockville, MD, USA), for IFN-γ and IL-4 respectively. Plates were incubated overnight at 4°C and washed with PBS buffer. They were then blocked overnight with 1% BSA in PBS. After washing with PBS containing Tween-20 (PBS-T), standards of each cytokine were added, in addition to the sample supernatants, to generate a standard curve, using recombinant IL-2, IL-10 (both from PharMingen. Mississauga, Ontario, Canada), IL-4 (R&D Systems, Minneapolis, MN, USA), and IFN-γ (Gibco, Burlington, Ontario, Canada). The plates were then incubated overnight at 4°C, and washed again with PBS-T. They were then incubated with biotinylated rat anti-mouse anti-IL-2, IL-10, IL-4 and IFN-γ detecting mAbs (PharMingen, Mississauga, Ontario, Canada) at 0.5 µg/ml. The plates were incubated at room temperature for 45 minutes, washed 3 times with PBS-T, then streptavidin-alkaline phosphatase (Sigma Chemicals, St. Louis, MO, USA) added. After a one-hour incubation and washing, 100 µl of substrate [1 mg/ml p-nitrophenylphosphate (Sigma Chemicals, St. Louis, MO, USA) in 10% diethanolamine (Fisher Scientific, Napean, Ontario,

Canada)] was added. After an hour incubation at room temperature, the plate was read on a microplate reader (Molecular Devices, Sunnydale, CA, USA) at 405 nm.

2.6.2 Antibody ELISA for assessment of antibody in sera

The presence of specific serum IgG antibody was determined by ELISA. Microwells of polyvinyl chloride plate (Dynatech Laboratories, Chantilly, VA, USA) were coated overnight with 10 µg of mTg or the specific peptide (described above) dissolved in carbonate buffer, pH 9.6. The plates were then blocked overnight with PBS + 0.1% BSA. Sera samples or the Tg-specific mAb 55H8 (a kind gift of Dr. P. Lymberi, Hellenic Pasteur Institute, Athens, Greece) as a control, were added to the plates for one hour at room temperature and the plates were then washed three times with PBS-T. Then the second antibody, an alkaline-phosphatase conjugated goat anti-mouse IgG (Sigma, St. Louis, MO, USA) was added to each well. After an hour at room temperature, the plates were again washed three times with PBS-T and p-nitrophenylphosphate substrate solution (Sigma, St. Louis, MO, USA) was added (1 mg/ml p- nitrophenylphosphate in 10% diethanolamine, 100 µl/well) and incubated for 30 minutes. Absorbance of the p-nitrophenylate product was measured at 405 nm using an automated microplate reader (Molecular Devices, Sunnydale, CA, USA).

2.7 ANALYSIS OF ENDOGENOUS THYROID CYTOKINE mRNA LEVELS

2.7.1 RNA extraction

Mice were euthanised using ether, the thyroid gland removed *en bloc* still attached to the trachea, and then the thyroid lobes teased from the trachea. A piece roughly similar in size to the thyroid gland was removed from the liver and spleen of the same animals. The tissue was homogenized, then placed in 0.5 ml of TRIzol (Molecular Research Centre Inc, Cincinnati, OH, USA). The tissue was then separated into two phases using 0.1 ml chloroform and centrifuged at 4°C at 1200 rpm. The RNA remains in the aqueous phase. This phase was placed in a fresh tube and precipitated using 0.25 ml of isopropanol, and again centrifuged. The resultant pellet was then washed with 0.5 ml of 75% ethanol and air dried. The pellet was then resuspended in 8 µl of diethypyrocarbonate (DEPC) treated water. The purified RNA absorbance was then read in a spectrophotometer at the OD of 260 and 280 nm to determine RNA yield and purity.

2.7.2 cDNA preparation

First strand cDNA synthesis was performed using a kit from Pharmacia (Uppsala, Sweden). Briefly, 1-5 µg of RNA (made up to a volume of 20 µl with DEPC-treated water) was placed in an Eppendorf tube and denatured in a Perkin-Elmer™ thermocycler at 65°C for 10 minutes, then chilled on ice. The RNA was then added to a pre-mixed solution of 11 µl Bulk mix, 1 µl dithiothreitol solution, 1 µl Not I-d(T)18, all provided by the First Strand cDNA synthesis kit.

The resultant mixture was then heated in the thermocycler (Perkin Elmer DNA Thermocycler, Cetus, Norwalk, CT, USA) at 37°C for 1 hour, and terminated by heating the tube at 65°C for 10 minutes. The cDNA was stored at -70°C.

2.7.3 Amplification by RT-PCR

PCR amplification was carried out using reagents from the PROMEGA™ PCR kit (Promege Biological Research Products, Madison, WI, USA). Briefly, 1 µl of cDNA was added to 2 µl of each of the forward and reverse primers , purchased from University Core DNA services, University of Calgary (Calgary, Alberta, Canada), 0.4 mM dNTP mix, 4 mM $MgCl_2$, 2.5 units of Taq DNA polymerase. The primers used were for glyceraldehyde-3-phosphate dehydrogenate (GAPDH)[5' CCATCACCATCTTCCAGGAG, 3' TTGAGATGATGCTTTGACA] , Thyroglobulin [5' CGGGATCCACCATGGGCCCTTTCCACTACTGGGG, 3' GGTTGTCGATGTCGTTTACTCCTAGGGC] and the cytokines, IL-2[5' AACAGCGCACCCACTTCAA, 3' TTGAGATGATGCTTTGACA], IL-4 [5' TAGTTGCATCCTGCTCTT, 3' CTACGAGTAATCCATTGC] and IFN-γ [5'AACGCTACACACTGCATCT, 3' TGCTCATTGTAATGCTTGG]. The resultant mixture was then layered with 50 µl of mineral oil and placed in the thermocycler (Perkin Elmer DNA Thermocycler, Cetus, Norwalk, CT, USA). A 30-cycle step program (95°C for 1 minute, 56°C for 1 minute and 72°C for 1 minute) was preceded by a 5 minute denaturation step at 94°C and was followed by a 7 minute extension step at 72°C.

2.7.4 Analysis of PCR Products

The amplified PCR products were subjected to electrophoresis on a 1.5% agarose gel containing 0.5 μg/ml ethidium bromide, in an electrophoresis chamber for approximately 1 hour at 100v. The gel was viewed and photographed using a ChemiImager 4000 computer system from AlphaInnotech Corporation (San Leandro, CA, USA), and saved on disk for later quantitation and analysis.

2.8 MEASUREMENT OF PEPTIDE BINDING TO A^k OR E^k

Determination of peptide binding to A^k of E^k molecules was achieved using a competitive inhibition assay. This assay used the T cell hybridomas, 3.47 (A^k-restricted, Dai et al., 1999) and 8F9 (E^k-restricted, Dai et al., 1999) recognizing the peptides T_4(2553) (a kind gift from Dr. Yi Chi Kong) and TgP1 (Rao et al., 1994), respectively. In a flat-bottom microwell plate, 10^5 hybridoma cells were incubated with a constant amount of their respective ligand (0.05 μg/ml of T_4(2553) and 0.04 μg/ml of TgP1), and serial dilutions of the inhibitor peptide (highest dilution 100 μg/ml) and 10^5 TA3 cells as APC. TA3 express H-2A^d/H-2A^k and H-2E^d/H-2E^k (Allen et al., 1985), which were a kind gift from L.H. Glimcher at Harvard Medical School, and were courteously provided by T.Watts at the University of Toronto. The cells were cultured in a total of 200 μl culture medium per well. After 24 hours, 100 μl of supernatant was collected from each well, transferred to a new plate, and kept frozen at -20°C. The relative IL-2 content of the culture supernatant from the competitive binding assay was determined by thawing the microtitre plates

and adding 10^4 IL-2 dependent CTLL-2 [(Gillis et al., 1977)(ATCC, Rockville, MD, USA)] per well. After 18 hours in culture ^{3}[H]thymidine (1μCi/well) was added. The cells were harvested 6 hours later and the incorporated radioactivity measured as outlined in section 3.4.2. CTLL-2 cells were maintained in culture medium supplemented with 10% supernatant from concavalin-A activated rat splenocytes as a source of IL-2.

CHAPTER 3

ATTEMPTS TO GENERATE THYROIDITOGENIC TH2 CELLS SPECIFIC FOR THYROGLOBULIN PEPTIDES

3.1 ABSTRACT

Cross regulation of Th1 and Th2 cells by the cytokines of the opposing Th subset has been used in immunotherapy of Th1-mediated, organ-specific diseases. The role of Tg-peptide-specific Th2 cells in murine EAT has yet to be assessed *in vivo*. This study attempts to 'switch' Tg-peptide-specific Th1 populations of LNC by culturing them *in vitro* with rIL-4, a prototypical Th2 cytokine. It also examines any endogenous cytokine production by the thyroid gland in normal mice using RT-PCR. Both the original Th1 cell populations and the Th2 cell population produced were to be adoptively transferred into naive mice and their effect on EAT assessed both by histology of, and cytokine mRNA expression in the thyroid glands. The normal murine thyroid gland has no endogenous cytokine mRNA expression. However, Tg-peptide-specific Th2 cells could not be produced using rIL-4 *in vitro*. The property of IL-4 as an autocrine growth factor may have affected its ability to switch the cytokine profile of Th1 cells, and retain their antigen specificity. The inability to generate Tg-peptide-specific Th2 cells by this method prevented us from exploring the biological function of Th2 cells in murine EAT.

3.2 INTRODUCTION

Experimental autoimmune thyroiditis (EAT) is induced in susceptible strains of mice (H-2^k and H-2^s) following challenge with Tg in CFA. The Tg peptides TgP1 [rTg(2495-2510] and TgP2 [rTg(2695-2713)] also have the ability to induce EAT, and act as subdominant epitopes (Chronopoulou and Carayanniotis, 1993, Rao et al., 1994). Many organ-specific autoimmune diseases, such as thyroid disease, multiple sclerosis and insulin-dependent diabetes mellitus are thought to be mediated by CD4+ T cells, and Th1 cells in particular. CD4+ T cells differentiate into two distinct subsets, with Th1 cells producing IL-2 and IFN-γ and involved primarily in cell mediated responses, while Th2 cells produce the cytokines IL-4, IL-10 and IL-13 and are typically involved in humoral responses (O'Garra and Hosken, 1997).

Th1 and Th2 cells are cross-regulatory, meaning that cytokines produced from one subset have the ability to down regulate the response of the opposing subset. The objective of my research was to induce an Tg-peptide-specific Th2 population by the deviation of an established Th1 cell population using the Th2 cytokine IL-4, and to examine the effect of the Th2 subset on the induction and progression of EAT in SJL/J mice. The method I used was based on the work by Khoruts et al. (1995). In their experiments in the EAE animal model for MS, they immunized mice with an encephalitogenic peptide PLP(139-151) in CFA and after 9-11 days *in vitro*, removed the LNC, which were of the Th1 phenotype. They then proceeded to 'switch' these cells from a Th1 to a Th2 pattern of cytokine secretion by the addition of rIL-4 *in vitro*. They then examined the disease-inducing potential of Th1 and Th2 cells in the EAE animal model, by adoptive transfer into syngeneic SJL/J female mice. We attempted to reproduce this protocol in

the EAT model, using the thyroiditogenic peptides TgP1 and TgP2 as antigens.

3.3 RESULTS

3.3.1 There is no detectable mRNA for the cytokines IL-2, IL-4 or IFN-γ in normal thyroid gland.

The measurement of the intrathyroidal cytokine mRNA aimed at establishing a baseline such that in later experiments, the mRNA expression of cytokines in the thyroid glands of mice could be used to indicate a Th1 or Th2 type of response. Ten normal SJL/J mice were tested for the presence of intrathyroidal mRNA for the cytokines IL-2, IL-4 and IFN-γ. The mRNA was extracted from the thyroid gland of 5 male and 5 female mice, along with a similar size piece of liver and spleen. The mRNA was used to produce cDNA which was amplified using RT-PCR, and viewed on an agarose gel. As seen in **Table 3.1**, of the ten SJL/J mice tested, none showed the presence of IL-2, IL-4 or IFN-γ mRNA, yet all showed the presence of the constitutive enzyme GAPDH and thyroglobulin (**Figure 3.1**). As a control, mRNA was extracted from both spleen and liver. Of the mRNA extracted from the spleen, most mice (6 of 9 tested) showed the presence of IL-4, while 2 of 9 showed the presence of IL-2. None exhibited IFN-γ expression. Screening of liver mRNA showed similar results (data not shown), indicating that there is expression of cytokine genes in some tissues such as liver and spleen, but none in normal thyroid tissue. This indicates that the level of cytokine mRNA expression could be used to detect the Th subset present in glands that have been infiltrated after adoptive transfer of Tg-peptide-specific Th1 and

Table 3.1: Comparison of endogenous mRNA levels in SJL/J mouse thyroid and spleen, as assessed by mRNA isolation, cDNA preparation and RT-PCR*

		THYROID mRNA					SPLEEN mRNA				
Mouse	Sex⊕	GADPH	Tg	IL-2	IL-4	IFN-γ	GADPH	Tg	IL-2	IL-4	IFN-γ
1	M	+	n d	-	-	-	n d†	n d	n d	n d	n d
2	M	+	n d	-	-	-	+	-	+	+	-
3	M	+	+	-	-	-	+	-	-	+	-
4	M	+	+	-	-	-	+	-	-	+	-
5	M	+	+	-	-	-	+	-	-	+	-
6	F	+	+	-	-	-	+	-	-	-	-
7	F	+	+	-	-	-	+	-	-	+	-
8	F	+	+	-	-	-	+	-	+	-	-
9	F	+	+	-	-	-	+	-	-	+	-
10	F	+	+	-	-	-	+	-	-	-	-

* Thyroid and spleen tissue and mRNA extracted, cDNA prepared as per Materials and Methods. RNA purity (as assessed by A_{260}/A_{280}) ranged from 1.5-2.7 for thyroid tissue and 1.4-2.8 for spleen tissue.

⊕ Male mice ranged from 20-25 weeks old while female mice ranged from 6-7 weeks old.

† nd = not determined

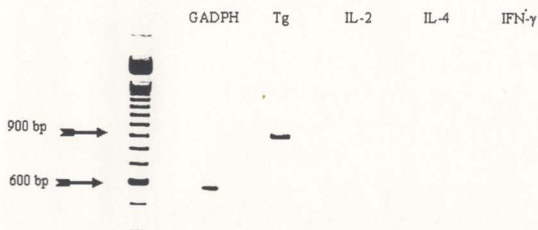


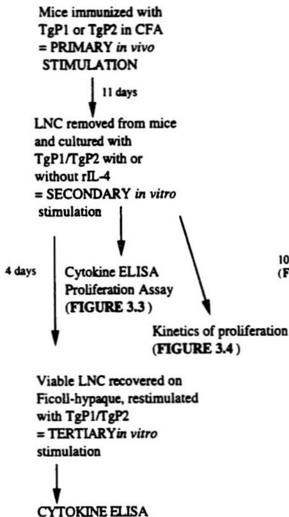
Figure 3.1 Representative gel from RT-PCR of male SJL/J thyroid gland

Th2 cells.

3.3.2 TgP1- and TgP2-specific Th1 cytokine secreting cells are produced *in vitro* without the use of exogenous cytokines.

Following immunization of mice with either of the thyroiditogenic peptides, TgP1 or TgP2, single cell suspensions of LNC were cultured *in vitro* with the corresponding antigen, and a proliferation assay performed (**Figure 3.2**), and supernatants taken at day 1, 2 and 3 for cytokine analysis via cytokine ELISA. The proliferation data (**Figure 3.3**, A and B) indicated that LNC from mice immunized with TgP1 or TgP2 showed significant proliferation in response to the appropriate antigen. The ELISA results (**Figure 3.3**, C and D) showed antigen-specific production of the Th1 cytokines IL-2 and IFN-γ in LNC from mice immunized with either TgP1 or TgP2. However, the amount of cytokines produced in response to TgP1 and TgP2 differed. In TgP1-primed LNC, the amount of IL-2 and IFN-γ produced in response to antigen present in culture was significantly higher than that with TgP2-primed LNC. These data suggest that pathogenic Tg peptides differ in their capacity to activate T cells to cytokine production. Further restimulation of cells *in vitro* with the corresponding peptide allowed the differentiation of peptide-specific cells into the Th1 subset as assessed by cytokine and proliferation data (not shown). The data indicate that immunization of mice with Tg peptides in CFA followed by restimulation of draining LNC *in vitro* with the corresponding peptide leads to the generation of Th1 cells. This is the first direct observation that thyroiditogenic peptides activate Th1 cells.

The proliferation data in the above experiments were not representative of all experiments.



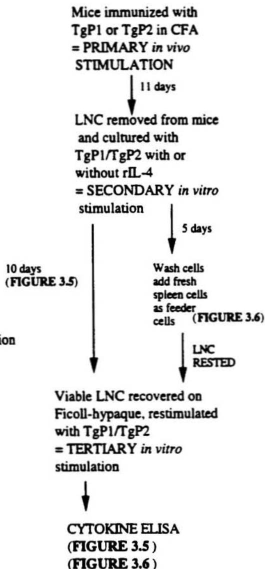
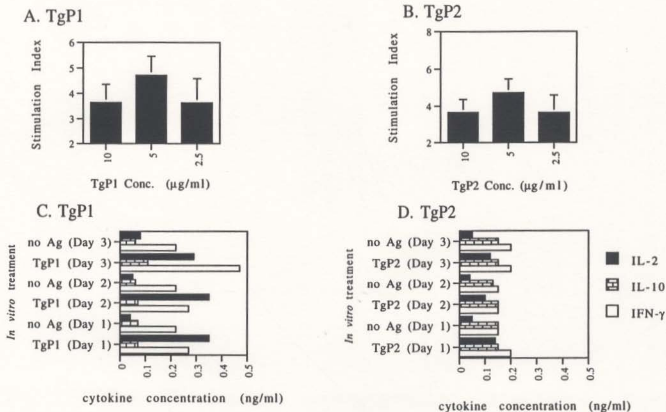


Figure 3.2: Schematic drawing of culture conditions of LNC from mice immunized with TgP1 or TgP2

Figure 3.3 Detection of Th1-type cytokines in the culture supernatant of LNC from mice immunized with TgP1 and TgP2



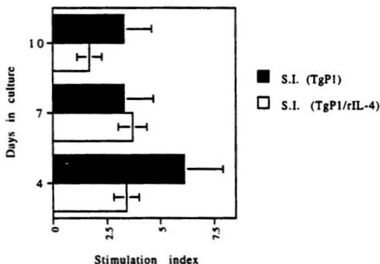
Cytokine ELISA (C,D) and proliferation data, taken on day 4 (A, B), of LNC from mice immunized with the peptide. LNC placed *in vitro* culture without any exogenous peptides at a concentration of 5 x10^6 cells/ml. SD <1%, not seen on graph. Background CPM values are, for TgP1 2883 (A) and for TgP2 is 3238 (B).

In other experiments, the LNC removed from mice immunized with either TgP1 or TgP2 were highly proliferative in the absence of peptide, as indicated by high background CPM in proliferation assays (data not shown). The addition of rIL-4 to culture seemed to accentuate this increased proliferation. For this reason, a study of the kinetics of the proliferation of LNC from mice immunized with TgP1 was performed (**Figure 3.4**). It indicated that the LNC were highly proliferative for up to 10 days of secondary *in vitro* stimulation. Although the response with rIL-4 in culture appears lower, as expressed by stimulation index values, the background counts are actually many times higher than the background counts for cultures containing no exogenous cytokines . On the basis of these data, we decided to culture LNC for 10 days instead of 4 days *in vitro* as Khoruts et al. had done (**See Figure 3.2**), thus modifying our original protocol.

3.3.3 Adding exogenous rIL-4 *in vitro* to LNC from mice immunized with TgP1 or TgP2 does not produce peptide-specific Th2-cytokine secreting cells after 10 days in culture.

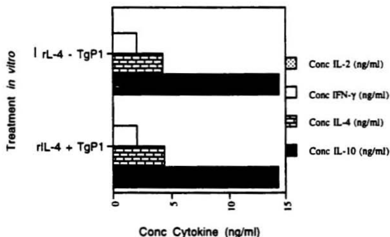
After modifying the original protocol to secondarily stimulate LNC for 10 days, with rIL-4 added only in one 30 ng/ml dose, the preferential production of large amounts of the Th2 cytokines IL-4 and IL-10 was observed (**Figure 3.5**). However, this cytokine production was not antigen-specific because it was present also in cultures that did not contain TgP1. This loss of antigen specificity may be due to the LNC stimulation of rIL-4 in an autocrine fashion. It may also be due to the rIL-4 acting on other cells in the LNC suspension, downregulating the cells of the antigen-specific Th1 subset and stimulating the proliferation of other, non-antigen specific cell types. In subsequent experiments we 'rested' the LNC between primary and secondary

Figure 3.4: Kinetics of proliferation of LNC from mice immunized with TgP1



Mice were immunized with TgP1 in CFA. Nine days later, the draining LNC were cultured *in vitro* with or without exogenous rIL-4 in the absence of antigen. Proliferation assays were performed on day 4, 7 and 10 as indicated on the y axis. Background CPM values were as follows; for TgP1 only: 709 (Day 4), 662 (Day 7), 146 (Day 10); for TgP1 + rIL-4: 3539 (Day 4), 4328 (Day 7), and 3133 (Day 10).

Figure 3.5: Production of cytokines by LNC of mice immunized with TgP1 and cultured *in vitro* in the presence or absence of peptide with rIL-4



Five mice were immunized with 100 µg of TgP1 per mouse. Subsequently, their brachial, inguinal and auxillary LNC underwent a secondary stimulation in culture in the presence of 30 ng/ml of rIL-4 with or without TgP1. The quantitation of the indicated cytokines in the culture supernatant (at day 10) was assayed by sandwich ELISA. S.D. <1%, not visible on the graph. Results are representative of 3 experiments.

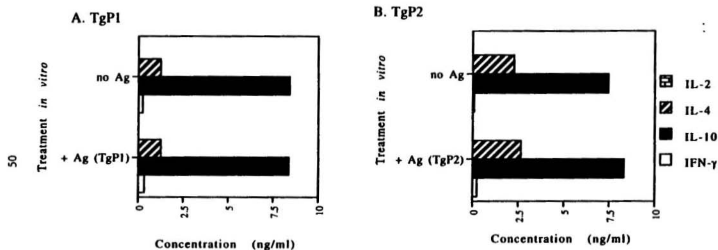
stimulation *in vitro* (see **Figure 3.2**). They were cultured in the absence of all cytokines and antigens for 5 days, after stimulation in culture for 5 days. This again resulted in high production of Th2 cytokines IL-4 and IL-10 (**Figure 3.6**), that was again not antigen- specific. This was seen when mice were immunized with either TgP1 (**A**) or TgP2 (**B**), in relatively equal amounts. This indicated that TgP1 or TgP2 - specific Th2 cells could not be produced using this protocol. As our objective was to use Tg-peptide specific Th1 and Th2 cells in biological experiments, we decided to abandon the production of Tg-peptide specific Th2 cells by this method.

3.4 DISCUSSION

The objective of my research has been two-fold: a) to produce a Tg-peptide-specific Th2 cell population and b) to adoptively transfer Tg-peptide-specific Th1 and Th2 cells into SJL/J mice and to determine their effect on EAT development. An important prerequisite to studying the effect of Th1 and Th2 cells in the thyroid gland during EAT is to determine the background levels of any endogenous cytokines within the normal thyroid gland. Analysis by RT-PCR showed that there is no endogenous production of mRNA for the Th1 cytokines IL-2 or IFN–γ or the Th2 cytokines, IL-4 or IL-10. This would facilitate the detection of intrathyroidal Th1 or Th2 cells, following adoptive transfer of Tg-specific Th1 or Th2 cells into naive hosts.

The present data directly demonstrate that LNC from mice immunized with thyroiditogenic peptides in CFA differentiate by default into Th1 cells upon secondary stimulation in culture with TgP1 and TgP2. Most of the research implicating the role of Th1 cells in EAT has been indirect. It has been shown that injection of Th1-inducing cytokines such as IL-12 (Braley-

Figure 3.6: Production of cytokines by LNC from mice immunized with TgP1 or TgP2, with rIL-4 *in vitro*, with a resting of cells in the absence of stimuli between secondary and tertiary stimulation



Mice were immunized with 100 μg of TgP1 or TgP2 in CFA. Draining LNC were cultured *in vitro* with rIL-4 added to cultures at 30 ng/ml. After 5 days *in vitro*, live cells were isolated, and cultured for a further 5 days in the absence of Ag, with mitomycin-C treated splenocytes (APC) as feeder cells. They were restimulated after 5 days with peptide and fresh APC. S.D. <1%, not visible on graph. Each graph is representative of 5 experiments.

Mullen et al., 1998) and IFN-γ (Remy et al., 1987) promote the development of EAT, while prototypical Th2 cytokines such as IL-10 (Mignon-Godefroy et al., 1995) have a curative effect on EAT. Cell lines and clones that transfer EAT from thyroid lesions from T-cell depleted (C57BL/6xC3H/He)F1 mice also produce Th1 cytokines (Sugihara et al., 1993). However, ours is the first study that indicates that immunization with TgP1 or TgP2 induces LNC that produce Th1 cytokines in response to the pathogenic peptide *in vitro*.

These Th1 cytokine-producing LNC could not be switched to produce Th2-type cytokines in the presence of exogenous rIL-4 *in vitro*. Firstly, LNC removed from mice immunized with TgP1 or TgP2 are highly proliferative for up to 10 days in the absence of either peptide of exogenous cytokines. Addition of rIL-4 *in vitro* accentuated this proliferation yet did not mediate a switch in cytokine secretion of the LNC after 4 days of culture. IL-4 has been shown to inhibit cytokine production by Th1 cells (Fiorentino et al., 1989, Peleman et al., 1989, Vieira et al., 1991). IL-4 also shares features with the prototypical Th1 cytokine IL-2 in that it is an autocrine growth factor for T cells (Kupper et al., 1987). This could allow for LNC that were already highly proliferative *in vitro* and producing high amounts of IL-2 independent of the presence of peptide in culture to have enhanced proliferation following addition of rIL-4. As this increased IL-2 production/proliferation was not demonstrated by Khoruts et al. (1995) who immunized mice with PLP(139-151), one could assume that this finding may be due to a property of the immunizing peptides, TgP1 and TgP2. Both TgP1- (Chronopoulou and Carayanniotis , 1992) and TgP2- (Carayanniotis et al., 1994) specific LNC have shown strong proliferative responses *in vitro*, and the response of TgP1/TgP2 and PLP(139-151) has not been compared. However, prolonged incubation of LNC with rIL-4, or even a resting of cells between first and second

antigen stimulation *in vitro*, did not generate Ag-specific Th2 cells. There was an increase in the IL-4 and IL-10 in culture, along with a corresponding decrease in IL-2 and IFN–γ, but there was no antigen specificity in these responses. It is possible that after long term culture with IL-4, an autocrine growth factor, cells are stimulated to proliferate and secrete cytokines in the absence of antigen.

In some of the experiments exploring the effect of Th2 cells on Th1-mediated autoimmunity, the Th2 cells used were not autoantigen-specific. Das et al. (1997) reported that the generation of Th2 clones specific for PLP(139-151) met with limited success using cytokines in culture. They used two techniques that may have worked in our model. One was to follow immunization with PLP(139-151) with an i.p. injection of the monoclonal antibody anti-B7.1. The second was to use a more common technique for producing Th2 cells, altered peptide ligands (APL). These APL are produced by a single a.a. substitution that produces a partial agonist (Lafaille, 1998). This approach has not been attempted in the EAT model, yet in the EAE model, APL's have been successful in producing Th2 clones. Das et al., 1997 used the APL of PLP(139-151), Q144, that was previously shown to improve disease outcome in SJL female mice immunized with native PLP peptide (W144) in CFA (Nicholson et al., 1995). Other experiments showed that the APL L144/R147 also protected against EAE (Nicholson et al., 1997). Although Th2 cytokine production could not be directly implicated in the protective effects of these APL's, both the cytokine profile of clones produced using APL and a separate study showing that the protective effect of APL's could be abolished by anti-IL-4 mAbs, (Brocke et al., 1996) strongly infer its involvement.

Th2 cells can also be produced by the use of cytokine-specific mAbs that downregulate

Th1 cells during LNC culture (Astrup et al., 1997). For example, adding anti-IL-12 along with the Th2 cytokine IL-4 to a culture of LNC will both promote Th2 development (IL-4) while preventing the ability of any IL-12 in culture to promote a Th1 response (anti-IL-12 Ab)(Austrup et al., 1997). A similar approach may have worked in our model.

IL-4 has been shown to have a protective effect in several models of organ-specific autoimmunity, including EAT (Racke et al., 1994), but only if applied early in the preclinal stages of the disease. In our study, TgP1- or TgP2-specific T cells were probably already committed to the Th1 lineage, and the added IL-4 in culture could not reverse this effect (Racke et al., 1994, Gijbels et al., 1997). In addition, a protective role for IL-4 in EAT has not been conclusively confirmed. On the other hand, the Th2 cytokine IL-10 might have been a much better candidate for such experiments. IL-10 blocks macrophage effector function, and as Th1 induced inflammation is mediated mostly through activated macrophages, IL-10 is a natural candidate as a down regulator of Th1 cells (Lafaille, 1998). Indeed, IL-10 has been demonstrated *in vitro* to downregulate a Th1 response (Lafaille, 1998). In the EAT model, rhIL-10 has been implicated in the enhanced cell death of T lymphocytes in EAT lesions (Mignon-Godefroy et al., 1995) and both *in vivo* administration and plasmid administration of IL-10 had the effect of both preventing and curing established EAT (Mignon-Godefroy et al., 1995, Batteux et al., 1999). For these reasons, in the EAT model immune deviation might be more successful if IL-10 was used instead of IL-4.

Are antigen-specific Th1 cells actually being switched by the addition of rIL-4 to culture? After the addition of rIL-4 to culture, cells proliferate regardless of Ag presence and produce Th2 cytokines. This culture contains a heterogenous mix of cells. Under conditions where no

exogenous cytokines are added, Ag-specific LNC produce cytokines in their specific response to antigen. It is possible that the rIL-4 in culture downregulates production of antigen-specific Th1 cells while it stimulates other cells to proliferate, producing Th2 cytokines. The heterogeneity of even highly polarized Th1 populations has been shown at the single cell level using flow cytometry (Murphy et al., 1996). In the future, Th1 and Th2 cells may also be able to be sorted based on surface markers such as CCR8 (Zingoni et al., 1998), CCR4, CCR5 (Bonnecchi et al., 1998) or CXCR3 (Siveke et al., 1998).

It remains unclear whether adoptive transfer of Th2 cells can be effective in treating Th1-mediated autoimmune disease. Cytokines have been shown to be beneficial in many animal models of autoimmunity, yet this method is impractical for human use because it calls for large quantities of cytokines which have a short half life *in vivo* and often cause serious side effects to the patient (Thomson, 1996). Although the effect of Th2 cells has not been directly assessed in EAT, studies in other organ-specific autoimmune models have not been promising (reviewed in Lafaille, 1998). In many cases, co-injection of Th1 and Th2 cells has had no beneficial effect on disease progression, even when Th2 cells are in 10-fold excess (Katz et al., 1995, Khoruts et al., 1995). Several other experiments (Chen et al., 1994, Kuchroo et al., 1995) have shown Th2 cells to be protective if the autoimmunity was attempted to be induced after the injection of Th2 cells. Thus from these results, it seems that Th2 cells are effective in preventing a Th1 response in naive hosts yet are ineffective in controlling the effector functions of activated Th1 cells. This questions a putative curative role of Th2 cells in organ-specific autoimmunity.

3.5 FUTURE DIRECTIONS

The effect of Tg-peptide specific Th2 cells on the development of EAT remains an open question which cannot be answered unless a reliable method for producing Th2 cells can be found. One possibility is to use a combination of Th2 cytokines such as IL-4 and/or IL-10 and mAbs to Th1-inducing cytokines such as IL-12 and IFN-γ. In addition to assays of cytokines in culture supernatants, assays for Th2 cell markers such as CCR8 should be used to verify the Th2 cell induction. Other methods to produce Th2 cells should also be explored, including the successful method of using APL's of a given pathogenic epitope. As this study aimed to reproduce a successful protocol from another model whereby the production of new peptides was not required, the use of APL's was not attempted.

The role of Th2 cells as a 'cure' for a Th1-mediated disease does not seem, however, promising from results from other autoimmune diseases (O'Garra et al., 1997), and other therapeutic approaches to immune deviation should be investigated. There is the possibility that other types of T cells may be involved in the breakdown in immunoregulation associated with organ-specific autoimmunity. For example, a third type of T helper cell, Th3 which secretes large amounts of TGF-β1 and low amounts of IL-4, IL-10 and IFN-γ may have an effect on autoimmunity as it is believed to have a role in oral tolerance (Chen et al., 1994). There may also be another type of CD4+ T cell type, secreting high IL-10, low IL-4 and TGF-β called T regulatory cell 1 (Tr1) (Groux et al., 1997). The combination of IL-10 and TGF-β seems to have a role in the prevention of colitis in an adoptive transfer model and prevents IDDM in young NOD mice (Han et al., 1996). These cells types should be explored in the EAT model. Exploring

the role of these types of cells may lead to a better understanding of the complex network of T cell signals involved in thyroid autoimmunity and how to manipulate the Th1/Th2 balance to benefit the patient.

CHAPTER 4

AN ALGORITHM-BASED APPROACH FOR MAPPING A^k-BINDING PATHOGENIC THYROGLOBULIN EPITOPES

4.1 ABSTRACT

The mapping of pathogenic epitopes in autoantigens has helped in the understanding of pathogenesis and possible treatment of autoimmune disorders. In earlier studies, Tg peptide-binding to A^k was shown to be a prerequisite to T cell activation and the I-A subregion has been implicated in the genetic control in EAT. Here, we have used an algorithm-based approach to identify five rTg peptides with A^k-binding potential. Three strains of H-2^k mice (CBA/J, AKR, and C3H/HeJ) were challenged with these peptides and the capacity of these antigens to induce T-cell and Ab responses, as well as EAT was examined. One of the peptides, Tg(2597-2609) has emerged as a pathogenic epitope, inducing peptide-specific serum IgG Abs, and mononuclear cell infiltration of thyroids in all three strains of mice. However, T cell activation *in vitro* by cytokine detection in culture supernatant or proliferation in response to the antigen was not detected for reasons that are still unexplained. This study has confirmed that algorithms that predict binding of peptides to A^k are successful in predicting pathogenic T cell epitopes of Tg. Application of this and similar algorithms to the recently published mTg sequence may lead to the discovery of further T cell epitopes in EAT.

4.2 INTRODUCTION

Tg has been implicated as the primary autoantigen in the pathogenesis of experimental autoimmune thyroiditis. As Tg is a large autoantigen (homodimer, MW 660 kDa) it is likely to encompass many pathogenic T cell epitopes. Currently, five T cell epitopes of Tg have been mapped (Carayanniotis and Rao, 1997) but an immunodominant epitope is yet to be discovered. The discovery of new epitopes may help in the understanding of the mechanisms of AITD and how immune tolerance to these self antigens is abrogated, leading to autoimmune disease.

H-2^k mice are susceptible to EAT following immunization with Tg and thus 'good responders' to EAT induction. The H-2 control of EAT has been localized in the I-A subregion (Beisel and Rose, 1983). For this reason, peptides of Tg that bind to A or E molecules of H-2^k mice are possible candidates for pathogenic T cell epitopes.

There are several algorithms that predict peptide binding to the A^k or E^k molecules. The objective of my research was to use one of these algorithms (Altuvia et al., 1994) to scan the Tg molecule for peptides that may bind to A^k. This algorithm contained two motifs, A and B, for screening peptide binding to MHC. From this initial scan, peptides that contained overlapping A and B motifs were identified. These peptides were then tested in three strains of H-2^k mice (AKR, CBA/J and C3H/HeJ) for their capacity to bind to the A^k and E^k molecules and induce T-cell and antibody responses as well as for their ability to induce EAT.

4.3 RESULTS

4.3.1 Algorithm-based prediction of seven Tg peptides that bind to A^k molecules

The last 967 a.a. stretch of rTg was scanned using the motifs A and B outlined in **Table 2.1**, using pcGENE software. This search resulted in 29 peptides that fit motif A and 17 that fit motif B, and we selected peptides that fit both motifs A and B. Seven peptides had completely overlapping motifs (**Table 4.1**) while six peptides were found with partially overlapping motifs (**Table 4.2**). For reasons of economy we focused on peptides with completely overlapping motifs and five were chosen as new candidates for mouse EAT induction. Two were previously documented as existing pathogenic T cell epitopes: peptide (2494-2500) is a portion of TgP1 (2495-2511) (Chronopoulou and Carayanniotis, 1992) and peptide (2547-2553) is part of the peptide designated T_4(2553) (Hutchings et al., 1992). Subsequent to the synthesis of these peptides, the mTg sequence was published (Caturegli et al., 1997). This sequence was also scanned using motifs A and B, and A^k-binding sequences were similarly determined (**Table 4.3** and **Table 4.4**). These sequences included many predicted using the rTg molecule, plus others found at the N-terminal end. The algorithm derived from features of the crystal structure of A^k (Freemont et al., 1997) was also used to scan both the mTg and rTg sequences (**Table 4.5**) and several of these sequences overlapped with sequences from the other algorithm, and are marked in the respective tables. These peptides are also good candidates for T cell epitopes in H-2^k mice.

Table 4.1: Prediction of A^k-binding T cell epitopes within rat Tg using completely overlapping A and B motifs from Altuvia et al., 1994.

Peptide coordinates ☆	rat sequence ★
(1827-1837)	EGADMATELFS
(2029-2040)	GSEDTEVHTYPF
(2108-2125)**	DFCLQECSRHQDCLVTTL
(2424-2437)	NSSVQEVVSVFRQK
(2494-2500)	GSQDDGLINRAKAVK
(2547-2553)	YYSLEHSTDDYASFS
(2597-2609)	YGHGSLELLADVQ

☆ Coordinates corresponding to a.a. positions within the mature hTg polypeptide (does not include leader sequence)
★ Sequences delineated by motif A are represented in bold letters. Sequences denoted by motif B are underlined.
** Indicates motif also found using algorithm from Freemont et al., 1997.

Table 4.2: Prediction of A^k-binding T cell epitopes within rat Tg using partially overlapping A and B motifs from Altuvia et al., 1994.

Peptide coordinates ☆	rat sequence ★
(2029-2045)	GSEDTEVHTYPFGWYQK
(2114-2131)	ECSRHQDCLVTTLQIQQ
(2115-2132)	CSRHQDCLVTTLQIQQG
(2295-2312)	GQLNIDGSILLAVNLIV
(2425-2442)	PNSSVQEVVSCFRQKPA
(2563-2580)	ENATRDYFIICPIVNMA

☆ Coordinates corresponding to a.a. positions within the mature hTg polypeptide (does not include leader sequence)
★ Sequences delineated by motif A are represented in bold letters. Sequences denoted by motif B are underlined.

Table 4.3: mTg sequences predicted using algorithm from Altuvia et al. (1994) that contains completely overlapping A and B motifs.

Mouse sequence position	sequence
107-121	yapv **<u>QCDLQRV</u>** qcwc
224-238	rela **<u>ETGLELL</u>** ldei
228-242	etgl **<u>ELLLDEI</u>** ydti
306-320	yqtv **<u>QCQTEGM</u>** cwcv
824-838	qdvp **<u>QVVLEGA</u>** ttpp
837-851	ppge **<u>NIFLDPY</u>** ifwq
1577-1591**	splv **<u>QCLTDCA</u>** ndea
1823-1837	dfpg **<u>DMATELF</u>** spvd
2025-2039	cgse **<u>DTEVHTY</u>** pfgw
2103-2117**	smaq **<u>DFCLQQC</u>** srhq
2490-2504	gsqd **<u>DGLINRA</u>** kavk
2543-2557	yysl **<u>EHSTDDY</u>** asfs
2595-2609	esyg **<u>HGSLELL</u>** advq

Mouse Tg sequence from Caturegli et al., 1997, containing the leader sequence. Motifs A and B from Altuvia et al., 1994. mTg sequence scanned with motif A and B using pcGENE software. Underlined sequence denotes sequences delineated by motif A while bolded sequence denotes sequences delineated by motif B.
** Indicates that part of this sequence was also predicted using the algorithm found in Freemont et al., 1997.

Table 4.4: mTg sequences predicted using algorithm from Altuvia et al. (1994) with partially overlapping A and B motifs.

Mouse sequence position	sequence
301-318	hrdg HYQTQCQTE gmcw
824-842	qdvp QVVLEGATTPP geni
869-887	mple HFNLRSCWCVD eagq
1020-1038	pvqc HAGTGQCWCVD grge
1049-1067	sqmp QCPTNCELSRA sgli
1385-1403	sgrf QLHLDSKTFSA dttl
1572-1589 **	vpsa DSPLVQCLTD cand
2025-2041	cgse DTEVHTYPF gwyq
2113-2131	csrh QDCLVTTLQIQ pgvv
2114-2131	srhq DCLVTTLQIQ pgvv
2119-2137	clvt TLQIQPGVVRC vfyp
2290-2308	gsgg QLTIDGSILAA vgnf
2420-2438	vgcp TSSIQEVVSCL rqkp
2562-2579	natr DYFIICPMVN masl

mTg sequence from Caturegli et al., 1997, with the inclusion of the leader sequence. Sequence scanned using pcGENE software. Underlined sequences denote sequences delineated using motif A while bolded sequences denote sequences delineated using motif B.
** Indicates sequence predicted using algorithm from Fremont et al., 1997

Table 4.5: Tg sequences predicted using an algorithm for peptide binding to A^k from Freemont et al., 1997.

Sequence position	Sequence
A. MOUSE TG	
1592-1608	vpsa DSPLVQCLT dcan
1601-1617	qclt DCANDEACS fltv
2123-2139	smaq DFCKQQCSR hqdc
B. C-TERMINAL RAT TG*	
2123-2139	slvq DFCLQRSCR hqdc

* mTg sequence aligned with rTg partial sequence using PALIGN software of pcGENE and sequence position taken from this. Sequence in capital letters denotes motif. Sequence position includes the leader signal sequence.

MOTIF FROM FREMONT ET AL., 1997:

D-X-X-[ILVN]-X-[EQ]-{HRK}-X-[STRQAG]

[] mean to include the amino acids contained within them.
{} mean to exclude the amino acids contained within them.
X means any amino acid at that position

4.3.2 The rTg peptides found using the algorithm are highly homologous to the corresponding mTg peptides.

The rTg sequences predicted using the algorithm for A^k-binding peptides were compared to the recently published mTg sequence (Caturegli et al., 1997). As seen in the **Table 4.6**, of the five peptides selected, two [rTg(2029-2040) and rTg (2597-2609)] were completely homologous to the corresponding mTg sequence, while one [rTg(2108-2125)] was highly homologous (94.4%) to mTg. Only one a.a. was different between the two peptides, and the mTg sequence contained both A and B motifs. Two peptides [rTg(1827-1837) and rTg(2424-2437)] had a corresponding mTg peptide which did not contain the motifs from Altuvia et al. (1994) and were not identified by scanning the mTg sequence with the algorithm (**Table 4.4**). In the corresponding mTg sequence for rTg(1827-1837) there was a gap in the mouse sequence inserted when mTg was aligned with bTg or hTg (Caturegli et al., 1997), indicating that the mTg sequence has a deletion of these two a.a.

4.3.3 The peptide Tg(2597-2609) binds strongly to the A^k molecule, while Tg(2424-2437) binds strongly to the E^k molecule.

We first examined the relative binding of each of the peptides to MHC via a competitive inhibition assay. This assay used either an A^k-or an E^k-restricted hybridoma (3.47 and 8F9 respectively) recognizing T_4(2553) and TgP1 respectively, on TA3 cells which express A^k and E^k molecules. Inhibition of T-cell hybridoma activation was then tested in the presence of increasing

Table 4.6 Homology comparison of rTg peptides with putative A^k-binding capacity selected using A^k binding algorithm to corresponding peptides of mTg*

Sequence position	sequence	%homology
rTg(1827-1837)	EGADMATELFS	
mTg(1825-1835)	PG__MATELFS**	72.3
rTg(2029-2040)	GSEDTEVHTYPF	
mTg(2026-2037)	GSEDTEVHTYPF	100
rTg(2108-2125)	DFCLQECSRHQDCLVTTL	
mTg(2107-2124)	DFCLQQCSRHQDCLVTTL	94.4
rTg(2424-2437)	NSSVQEVVSCFRQK	
mTg(2421-2437)	TSSI QEVVSCLRQK	78.6
rTg(2597-2609)	YGHGSLELLADVQ	
mTg(2597-2609)	YGHGSLELLADVQ	100

* Peptides chosen using algorithm outlined in Altuvia et al., 1994. Mtg sequence is from Caturegli et al., 1997.

** within the published sequence for mTg, there are gaps which are designated by an _ in the sequence. These gaps were inserted during the alignment of mTg with other Tg (Caturegli et al., 1997).

Sequences that are not identical in both sequences are in bold in the rTg sequence

amounts of the inhibitor peptide. As shown in **Figure 4.1 and Table 4.7**, the only peptide to show drastic inhibition of the activation of 3.47 was Tg(2597-2609) (shown as an open triangle) with an IC_{50} value of 10 mM. The mouse lysozyme peptide (46-62) (ML46-62) is a known A^k-binder that was used as a positive control, with an IC_{50} value of 8 μM. Slight inhibition was also seen with Tg(2424-2437) and Tg(2029-2040). These results implicate Tg(2597-2609) as a relatively strong binder to the A^k molecule.

All peptides slightly inhibited the activation of 8F9 at high concentrations (**Figure 4.2 and Table 4.7**) yet the peptides can be divided into strong, medium and weak binders. The peptide Tg(2424-2437) strongly inhibited 8F9's activation (IC_{50} values of 20 μM) and therefore is the strongest E^k-binder among the Tg peptides tested. Next are the peptides Tg(2029-2040) and Tg(2597-2609) with IC_{50} values of 35 μM each. The other two peptides Tg(1827-1837) and rTg(2108-2125) may bind very weakly to E^k as they only inhibit 8F9 activation at the highest concentrations.

4.3.4 T cell reactivity in response to the Tg peptides

The immunogenicity of the Tg peptides was determined using proliferation assay and cytokine ELISA. Two mice per strain were immunized with 100 nmol of the appropriate peptide, and 9 days later, the draining inguinal, auxiliary and brachial lymph nodes were collected and single cell suspensions prepared. The LNC were then cultured in the presence of the immunizing peptide and a proliferation assay and cytokine ELISA preformed as outlined in the Materials and Methods. None of the five Tg peptides induced significant proliferation (i.e. Stimulation index

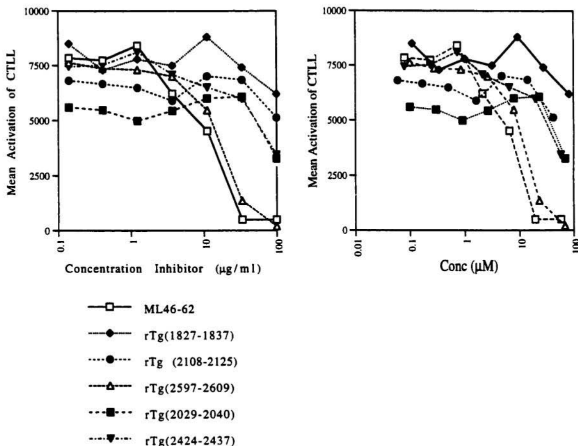
Table 4.7: Results from competive inhibition assays, indicating relative binding of each of the Tg peptides to the A^k or E^k molecules

Peptide	IC_{50} Values (mM)*	
	A^k	E^k
Tg(1827-1837)	>83	>83
Tg(2029-2040)	>70	35
Tg(2108-2125)	>42	>42
Tg(2424-2437)	52	20
Tg(2597-2609)	10	35
ML (46-62)	8	ND**

* Binding to A^k or E^k was determined using a competitive inhibition assay outlined in Materials and Methods. IC_{50} was calculated as the point where activation of CTLL cells was reduced to 50%. Results representative of 3 experiments.

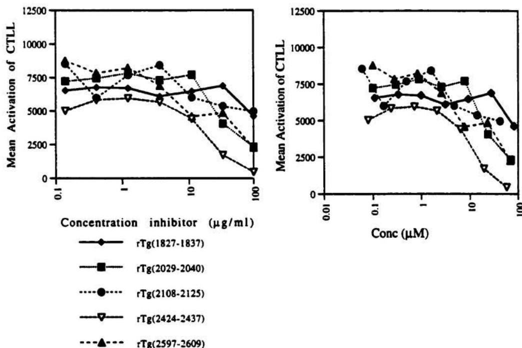
** ND = not determined

Figure 4.1 : Competitive Inhibition Assay indicating A^k binding in peptides chosen using algorithm



Hybridoma cells (3.47, A^k -restricted, T4(2553) specific) were cultured with the APC TA3 in the presence of T4(2553) at 0.05 μg/ml. Increasing concentrations of the inhibitor peptide were added and activation of the hybridoma was measured using CTLL assay. ML46-62, a peptide known to bind to A^k was used as a positive control. The figure is representative of 3 separate experiments.

Figure 4.2: Competitive Inhibition Assay indicating E^k binding in peptides chosen using an algorithm that predicts A^k binding



Hybridoma cells (8F9, E^k -restricted, TgP1-specific) were cultured with the APC TA3 in the presence of TgP1 at 0.04 mg/ml. Increasing concentrations of the inhibitor peptide were added and activation of the hybridoma was measured using CTLL assay. The figure is representative of 3 experiments.

Table 4.8: Immunogenicity of peptides assessed in H-2^k strains of mice

Peptide	Mouse Strain	S.I.*	Cytokine ELISA (ng/ml) IFN-γ	IL-2	IL-10	IL-4
Tg (1827-1837)	C3H	<2	0.04	<0.04	<0.37	<0.04
	AKR	<2	0.1	<0.04	<0.37	0.08
	CBA/J	<2	0.22	<0.04	<0.37	0.14
Tg (2029-2040)	C3H	<2	0.06	<0.04	<0.37	<0.04
	AKR	<2	0.06	<0.04	<0.37	<0.04
	CBA/J	<2	0.04	<0.04	<0.37	<0.04
Tg (2108-2125)	C3H	<2	<0.49	<0.04	ND	<0.04
	AKR	<2	0.45	<0.04	ND	<0.04
	CBA/J	<2	1.16	<0.04	ND	<0.04
Tg (2424-2437)	C3H	<2	0.13	<0.04	ND	<0.04
	AKR	<2	<0.49	<0.04	ND	<0.04
	CBA/J	<2	<0.49	<0.04	ND	<0.04
Tg (2597-2609)	C3H	<2	<0.49	<0.04	ND	<0.04
	AKR	<2	<0.49	<0.04	ND	<0.04
	CBA/J	<2	<0.49	<0.04	ND	<0.04

Mice were immunized with the above peptides and both proliferation assays and cytokine ELISA assays were performed as described in the Materials and Methods. Detection limit for cytokine ELISA was as follows; IFN-γ : 0.49 ng/ml, IL-4: 0.04 ng/ml, IL-2: 0.04 ng/ml, IL-10 0.37 ng/ml. ND= not determined. Cytokine ELISA setups were performed at 30 nmol/ml of the appropriate peptide.

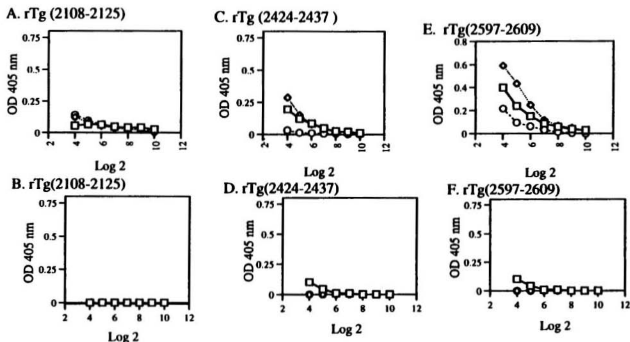
* In the proliferation assays, background CPM did no exceed 1000 in any of the cultures tested. S.I.= Stimulation index.

>2) of the LNC and there was very little cytokine production in any of the LNC cultures (**Table 4.8**). LNC culture of CBA/J mice that were immunized with Tg(2108-2125) produced the highest amount of cytokines, most significantly IFN-γ. The detection limit of the cytokine ELISA was quite low, ranging from 0.04 ng/ml for IL-4 and IL-2 to 0.37 ng/ml for IL-10 and 0.49 ng/ml for IFN-γ, so any change in cytokine levels in that range could have been detected. In addition, the low S.I. values could not be attributed to high background CPM. The conclusion from these data is that none of the Tg peptides significantly stimulate T cells to either proliferate or produce cytokines *in vitro*.

4.3.5. There was significant production of IgG antibody in response to Tg(2424-2437) and Tg(2597-2609) with little cross reactivity with mTg.

The three H-2^k mouse strains (CBA/J, C3H/HeJ and AKR) were immunized with one of the 5 peptides in CFA, and boosted 3 weeks later with the same peptide in IFA. Five weeks from the initial challenge the sera were analyzed for the presence of peptide-specific IgG. There was low but significant amounts of peptide-specific IgG produced in response to Tg(2597-2609) and Tg(2424-2437) (**Figure 4.3**). Both of these peptides fit the motif by Altuvia et al. (1994) as A^k-binding peptides yet only Tg(2597-2609) was shown to be a relatively strong A^k binder (IC_{50} of 10 μM) while Tg(2424-2437) bound more effectively to E^k (IC_{50} of 20 μM) as compared to A^k (IC_{50} of 52 μM). Comparing the three strains of mice, CBA/J produced the highest amount of antibody (as determined by titre in an antibody ELISA assay) followed by AKR mice, with the lowest Ab response exhibited by C3H mice. There was little cross reactivity of the Abs from the

Figure 4.3 IgG antibody from sera of k strain mice immunized with peptides selected using an algorithm reflecting Ak binding of peptides



—□— AKR
·····◇····· CBA/J
····○···· C3H

Three mice from each strain were immunized with the appropriate peptide (100 nmol) in CFA and boosted with the same peptide (50 nmol) in IFA. Mice were bled 3 weeks later. Antibody ELISA was performed as per Materials and Methods. Panel A,C and E were from plates coated with the designated peptide and panels B, D, and F were from plates coated with mouse Tg.

sera of mice immunized with any of the Tg peptides to mTg.

4.3.6 The peptide Tg(2597-2609) causes mononuclear cell infiltration of thyroids in mice.

The three strains of mice were immunized with either Tg(2108-2125), Tg(2424-2437) or Tg(2597-2609) in CFA and boosted with the same peptide in IFA 3 weeks later. Five weeks after the initial challenge, the thyroid glands were removed and histologically examined as outlined in the Materials and Methods. Tg(1827-1837) and Tg(2029-2040) were not used to immunize mice in these experiments because they did not bind to either A^k or E^k (**Figure 4.1** and **Figure 4.2**), did not induce a peptide-specific T-cell response (**Table 4.8**) and did not induce a peptide-specific IgG Ab response (**Figure 4.3**). For these reasons, we decided that the possibility of these peptides inducing mononuclear cell infiltration of thyroid glands was low. Of the peptides tested, Tg(2597-2609) consistently induced thyroiditis in all three strains of mice (**Table 4.9, Figure 4.4**). The degree of mononuclear cell infiltration of the thyroid in each strain correlated with the degree of peptide-specific serum IgG antibody response. For example, CBA/J mice gave the highest antibody response (**Figure 4.3**), and they also showed the highest grade of infiltration in the thyroids, with a mean infiltration index of $3.33 \pm .577$ (**Table 4.9**). This high serum IgG antibody response may indicate more CD4+ T helper cells activated, which interact with B cells to produce antibodies. As CD4+ and CD8+ T cells are involved in the initiation of EAT, this increased antibody response may indicate that more CD4+ T cells are activated by the peptide, and thus are able to participate in the infiltration of thyroid glands. It is also worthwhile to note that this peptide was found to bind relatively strongly to A^k [Tg(2597-2609)] (**Figure 4.1**).

Table 4.9. Comparison of histology results from different $H\text{-}2^k$ mouse strains immunized with Tg peptides

Peptide	Strain	IC_{50} (μM)		Infiltration Index						# mice with EAT	Mean I.I.*
		A^k	E^k	0	1	2	3	4	5		
Tg	C3H			3	0	0	0	0	0	0/3	0
(2108-2125)	AKR	>42	>42	3	0	0	0	0	0	0/3	0
	CBA/J			3	0	0	0	0	0	0/3	0
Tg	C3H			3	0	0	0	0	0	0/3	0
(2424-2437)	AKR	52	20	2	1	0	0	0	0	1/3	0.33 ± .577
	CBA/J			1	2	0	0	0	0	2/3	0.66 ± .577
Tg	C3H			0	0	1	2	0	0	3/3	2.67 ± .577
(2597-2609)	AKR	10	35	1	0	1	1	0	0	2/3	1.67 ± 1.53
	CBA/J			0	0	0	2	1	0	3/3	3.33 ± .577

Mice were immunized and thyroid glands removed for histology as outlined in Materials and Methods. IC_{50} was calculated using competitive inhibition assay as shown in Table 4.3. Three mice per strain per peptide were immunized and thyroid glands removed for histology. * infiltration index

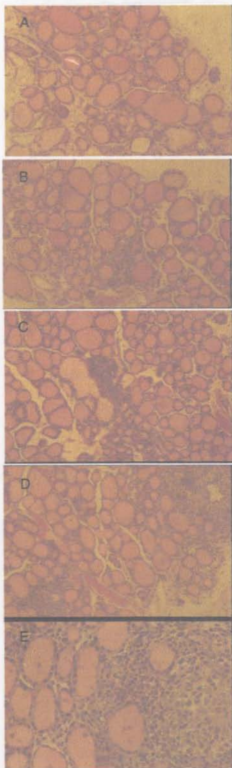


Figure 4.4 Mononuclear cell infiltration of thyroid glands from AKR mice immunized with Tg(2597-2609)

Figure 4.4

Mice were immunized with 100 nmol Tg(2597-2609) in CFA and boosted 21 days later with 50 nmol peptide in IFA. Fourteen days after boosting, thyroid glands were collected and sectioned, stained and scored as outlined in Materials and Methods. Section A is normal thyroid gland, infiltration index (I.I.) of O; B = I.I. of 1; C = I.I of 2, D= I.I. of 3, all viewed at 100 x magnification. E = I.I. of 3, a 250 x magnification of section D.

4.4 DISCUSSION

Among the different methods employed to map T-cell epitopes in autoantigens, the use of algorithms is quite common and has been used to delineate pathogenic Tg epitopes (reviewed in Carayanniotis and Rao, 1997). T cells recognize antigenic determinants of autoantigens as peptides associated with self-MHC. Binding of immunogenic peptides to MHC is a prerequisite for T cell activation (Babbitt et al., 1985) and T cell activation against Tg is known to lead to EAT induction. For this reason, algorithms that screen for peptide-binding to MHC molecules may be helpful in identifying pathogenic peptides within large antigens.

H-2^k mice have been shown to be 'good responders' to EAT and the genetic control of EAT has been mapped to the I-A subregion (Beisel and Rose, 1983). Thus, Tg peptides that bind to A^k are good candidates for pathogenic T-cell epitopes. The algorithm used in this study was created by compiling information on many peptides and their ability to bind either A^k or E^k molecules (Altuvia et al., 1994) This algorithm used a database of A^k- or E^k-binding peptides and searched for motifs that were common to these binding peptides. These motifs then related to the sequence features of the a.a. in each position, such as polarity, charge, size and hydrophobicity. The computerized scan of A^k-binding peptides resulted in 3 separate motifs, designated A, B and C. In our study, two of these motifs (A and B) were used to scan the C-terminal end of rTg, as the sequence of mTg was unknown at this time. The scan of the last 967 a.a. of rTg identified 29 peptides that contained motif A and 17 that contained motif B. Of these peptides, seven peptides had completely overlapping A and B motifs while 6 had partially overlapping motifs (see **Table**

4.1). Of the seven peptides with completely overlapping motifs, two peptides had been previously studied and determined to be pathogenic. The fact that these algorithms identified two peptides that were already known to be pathogenic (Hutchings et al., 1992, Chronopoulou and Carayanniotis, 1992) highlights its value in predicting new pathogenic epitopes.

A subsequent scan of the complete mTg identified seven more peptides that had completely overlapping A and B motifs. These peptides are good T-cell epitope candidates in future EAT studies. Using the rTg fragment, five of the seven corresponding mTg peptides contained both motifs. In one of the mTg peptides that didn't fit, [rTg(2424-2437)], a substitution within the mTg molecule resulted in an a.a. that no longer corresponded with the motif. The published complete sequence of mTg (Caturegli et al., 1997) will allow a more systematic search of pathogenic Tg epitopes. Up to now, T-cell epitopes in murine EAT have been mapped using heterologous Tgs and it is possible that a single a.a. difference in a T-cell epitope site may abolish its pathogenic properties and, therefore, its detection by an algorithmic approach.

Recently, the crystal structure of A^k has been revealed, and from this structure, a motif for peptide binding has been presented (Freemont et al., 1998) based on the ability of individual a.a. to fit within pockets in the A molecules groove. This motif spans 9 a.a. positions and has an exclusive requirement for aspartic acid at position 1 and either glutamine or glutamic acid at position 6 (Freemont et al., 1998), making it more restrictive than previous motifs. When this motif was used to scan the a.a. sequence both mTg and the rTg fragment, it yielded 3 and 1 peptides respectively. These peptides also corresponded with peptides found containing motif A and B in the present study. However, only one of the peptides synthesized in our study, rTg(2108-2125) actually fit the crystal structure motif, yet this peptide exhibited no detectable

binding to A^k in the competitive binding assay (see **Table 4.7**). This is probably due to low peptide purity (75%) or degradation during storage. It may also indicate a failure of this particular motif.

Although the peptides, Tg(2424-2437) and Tg(2597-2609) showed detectable binding to both A^k and E^k molecules in the competitive binding assay (see **Figure 4.1, Figure 4.2,** and **Table 4.7**), and induced specific IgG responses (**Figure 4.2**), neither peptide stimulated an *in vitro* proliferative response of LNC from mice immunized with that peptide. EAT induction with Tg(2597-2609) indicates that it must activate effector cells as an antibody response indicates the presence of T-cell help. Thus it is unclear why these peptides did not induce a proliferative LNC response.

Peptide degradation during storage may play a role. For example, preliminary studies by G.Carayanniotis found a strong antibody response to rTg(2108-2125) in C3H mice immunized with that peptide, but this was not detected in subsequent studies (**Figure 4.3**).

The present data clearly demonstrate that the peptide Tg(2597-2609) causes mouse EAT. Although *in vitro* T cell proliferation could not be demonstrated, it is implied by the other results. The presence of a strong IgG response to the peptide implies T helper activation and the infiltration of thyroid glands by mononuclear cells also indicates the involvement of specifically activated effector T cells. The ability of this peptide to bind to A^k molecules is compatible with its immunogenic role. The role of anti-Tg antibodies in the pathogenesis of EAT is controversial (reviewed in Tomer, 1997). In some experiments, EAT was passively transferred into naive mice by anti-Tg Abs (Vladutiu et al., 1971, Nakamura et al., 1969, Clagett et al., 1974, Tomazic et al., 1971, Polley et al., 1981). This supports a role of anti-Tg antibodies, and possibly anti-Tg peptide

Abs in the pathogenesis of EAT. Yet, while EAT can be reliably shown to be transferred using Tg-specific T cells, the ability of Tg-specific IgG to transfer EAT has not been consistently shown (Tomer, 1997). For this reason, Ab data indicating Tg-peptide specific IgG in the sera of mice immunized with that peptide, cannot indicate its pathogenesis. However, when this Ab data is merged with data indicating infiltration of the thyroid gland by mononuclear cells in mice immunized with the same peptide, it indicates that this peptide is pathogenic. The fact that antibodies are produced indicates help from T cells, as does infiltration. In this study, antibody response did correlate with pathogenesis. Significant production of anti-peptide Abs served as an indicator of infiltration of thyroid glands by mononuclear cells in mice immunized with the same peptide. However, the antibody response to the peptide was not cross reactive with mTg, indicating that the pathogenic peptide [Tg(2597-2609)] is likely a serologically non-dominant epitope.

This algorithm has been successful in identifying peptides that may bind to A^k molecules, yet not all Tg peptides predicted were shown to bind to A^k (see **Table 4.7**). However, the Tg peptide that was shown to bind strongly A^k was also shown to be pathogenic when injected into mice. In this manner, this algorithm is a successful method to predict T cell epitopes in Tg.

4.5 FUTURE DIRECTIONS

The published sequence of mTg is an asset to the mapping of pathogenic T-cell epitopes in

murine EAT. The algorithm used here and the motif from the crystal structure has been used in mTg to predict Tg peptides that may bind to A^k. These peptides should be tested for their ability to bind to A^k and if they are shown to bind, they should be tested in H-2^k mice for their pathogenicity in EAT. The E^k motifs should also be used to predict pathogenic peptides of Tg. These approaches would allow the prediction of epitopes in the N-terminal region of the Tg molecule, as to date, most epitopes are clustered at the C-terminal end (Carayanniotis and Rao, 1997). These algorithms may also allow for a dominant epitope of Tg to be mapped.

The pathogenicity of Tg(2597-2609) should be confirmed by using Tg(2597-2609)-specific LNC in adoptive transfer experiments. CBA/J mice may be the best prospect for these experiments, as they were shown to give the best response to this peptide. Also, >95% pure preparations of both the pathogenic Tg(2597-2609) and Tg(2108-2125) should be made and the immune response of mice to these peptides retested . With respect to Tg(2108-2125), earlier work indicated that a high titre of peptide-specific IgG were produced in response to mouse immunization with this peptide, but subsequent experiments failed to reproduce this. This peptide was also predicted as an A^k-binder by the crystal structure motif, yet competitive binding assays failed to indicate binding to A^k. As the peptide preparation may not be pure enough, or it may have degraded over time, fresh peptide should be used to immunize mice before concluding that it is not a pathogenic epitope.

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