INFLUENCES OF IODINE OR ENZYMES ON THE FORMATION OF PATHOGENIC THYROGLOBULIN PEPTIDES

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INFLUENCES OF IODINE OR ENZYMES ON THE FORMATION OF PATHOGENIC THYROGLOBULIN PEPTIDES

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LIST OF ABBREVIATIONS

a.a.	Amino Acid
Ab	Antibody
AEP	Asparagine Endopeptidase
Ag	Antigen
AITD	Autoimmune Thyroid Disease
APC	Antigen Presenting Cell
BB-DP	BioBreeding Diabetes-Prone
bTg	Bovine Thyroglobulin
cDNA	Complementary DNA
CFA	Complete Freund's Adjuvant
CTLA-4	Cytotoxic T Lymphocyte Antigen-4
CTLL	Cytotoxic T Lymphocyte Line
cpm	Counts per Minute
CNBr	Cyanogen Bromide
DEPC	Dimethypyrocarbonate
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethylsulfoxide
EAT	Experimental Autoimmune Thyroiditis
ER	Endoplasmic Reticulum
FBS	Fetal Bovine Serum

HAT-containing	Hypoxanthine Aminopterin Thymidine-containing
HEL	Hen Egg Lysozyme
HLA	Human Leukocyte Antigen
HT	Hashimoto's Thyroiditis
HT-containing	Hypoxanthine Aminopterin Thymidine-containing
hTg	Human Thyroglobulin
IDDM	Insulin Dependent Diabetes Mellitus
IFN-γ	Interferon Gamma
Ig	Immunoglobulin
IL-2	Interleukin-2
LPS	Lipopolysaccharide
LNC	Lymph Node Cells
MAbs	Monoclonal Antibodies
2-ME	2-Mercaptoethanol
MBP	Myelin Basic Protein
MHC	Major Histocompatibility Complex
mTg	Mouse Thyroglobulin
mRNA	Messenger RNA
MW	Molecular Weight
NOD	Non-obese Diabetic
O.D.	Optical Density
OS	Obese Strain

RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
SAT	Spontaneous Autoimmune Thyroiditis
s.c.	Subcutaneous
T3	Triiodothyronine
T4	Thyroxine
TCR	T-cell Receptor
TFC	Thyroid Follicular Cells
TPO	Thyroid Peroxidase

CHAPTER 1

INTRODUCTION

1.1 Autoimmune Thyroiditis

1.1.1 Hashimoto's thyroiditis (HT)

Hashimoto's thyroiditis (HT) is one of the common autoimmune thyroid diseases (AITD) in humans. HT, also named autoimmune or chronic lymphocytic thyroiditis, was first described by the Japanese surgeon, Hakaru Hashimoto in 1912 (Hashimoto 1912). This disease occurs as a result of the failure of immunologic self-tolerance. Autoreactive T-cells, both CD4+ T-cells and CD8+ T-cells, escaping from intrathymic deletion and anergy, are activated in local lymph nodes under certain conditions, then accumulate and attack the thyroid gland where organ-specific self antigens, such as thyroglobulin (Tg) and thyroid peroxidase (TPO) are expressed (Ai et al. 2003, Weetman and McGregor 1994). Anti-thyroglobulin antibody (Tg-Ab) (Campbell et al. 1956) and anti-thyroid peroxidase antibody (TPO-Ab) (Kotani et al. 1986) also arise in patients with HT. These two autoantibodies work as indicators for HT diagnosis and predictors of future autoimmune hypothyroidism (Weetman 2004). However, the presence of Tg-Ab and TPO-Ab alone does not indicate the occurrence of HT because Tg-Abs and TPO-Abs are also detected in healthy people, with variable prevalence according to gender and age

(Weetman 2004). Also, about 20% of patients with thyroid carcinoma are Tg-Ab positive (Weetman 2004). Due to above immune responses, the thyroid gland is destroyed gradually and shows pathological features such as: goiter, mononuclear cell infiltration, fibrosis, and thyroid cell hyperplasia. With the destruction of the thyroid gland, the secretion of thyroidal hormones, triidothyronine (T3) and thyroxine (T4) is reduced; hypothyroidism and clinical symptoms such as a diffuse goiter, fatigue, depression, sensitivity to cold, weight gain, muscle weakness, coarsening of the skin, dry or brittle hair, constipation, increased menstrual flow appear in patients. The natural course of HT shows a chronic progression with a long subclinical phase in which the patients with HT have no distinct clinical symptoms (Ai et al. 2003).

Besides the immunological factors, genetic and environmental influences all contribute to the initiation and development of HT. The human leukocyte antigen (HLA) region was thought to contain one of the most important HT susceptibility genes because many immune response genes are harbored in this polymorphic region. Epidemiological studies in Caucasians showed that HLA-DR3 is associated with HT (Tandon et al. 1991). The association of goitrous HT with HLA-DR5 has been reported in other studies, with a relative risk (R.R.) of 3.1 (Farid et al. 1981). Other immune related genes have also been considered as susceptibility genes, such as the cytotoxic T lymphocyte antigen-4 (CTLA-4) (Ban and Tomer 2003). However, CTLA-4 does not confer specific susceptibility to HT (Ban and Tomer 2003). In fact, it is associated and linked with many autoimmune diseases including AITD (Nithiyananthan et al. 2002, Kotsa et al. 1997). The role of

iodine, as the most important environmental factor in HT induction, has been confirmed in clinical (Bournaud and Orgiazzi 2003) and animal models (Ruwhof and Drexhage 2001). Both sudden changes in iodine intake (Boukis et al. 1983) and prolonged exposure to a high but constant iodine intake (Harach et al. 1985) facilitate the incidence of thyroiditis and the development of thyroid autoantibodies.

1.1.2 Experimental autoimmune thyroiditis (EAT) as an animal model of HT

Experimental autoimmune thyroiditis (EAT), an animal model of HT, provides unique insights into the etiology and pathogenesis of autoimmune thyroiditis. Studies related to the two types of EAT, induced and spontaneous, will be discussed in the next two sections.

1.1.2.1 Induced EAT

1.1.2.1.1 Induced EAT animal models

The first EAT model was described by Witebsky and Rose in 1956. They reported that rabbits immunized with homologous thyroid tissue emulsified in complete Freund's adjuvant (CFA) produced thyroid autoantibodies and showed lymphocytic infiltration in the thyroid gland (Rose and Witebsky 1956). Since then, by using similar methods, EAT has been successfully induced in various animal species: guinea pigs, dogs (Terplan et al.

1960), rats (Jones and Roitt 1961), monkeys (Rose et al. 1966) and mice (Rose et al. 1971). Although both heterologous and homologous Tg are used in EAT induction, homologous Tg usually elicits more severe EAT than heterologous Tg. This may be due to thyroiditogenic epitopes unique to self Tg, in addition to sharing conserved thyroiditogenic epitopes . Other protocols have also been proven to be efficient in inducing EAT. In 1977, Esquivel and his colleagues found that the use of lipopolysaccharide (LPS) as an adjuvant was equivalent to CFA at the same mouse Tg (mTg) dose (Esquivel et al. 1977). Later, mTg conjugated to a monoclonal antibody (MAb) specific for MHC-II molecule (anti-I-A^k), instead of an adjuvant, was found to successfully induce EAT in CBA mice (Balasa and Carayanniotis 1993). Moreover, EAT can be induced by repeated high doses of syngeneic mTg in the absence of adjuvant (ElRehewy et al. 1981).

1.1.2.1.2 Association of EAT susceptibility with MHC gene expression

During the establishment of the mouse EAT model, it was shown that some mice respond well to mTg immunization, while others respond poorly or not at all under the same conditions. These observations led to the discovery that susceptibility of mouse strains to EAT is linked to H-2 genes (Vladutiu and Rose 1971). Mice with H-2 haplotypes *k* and *s* are susceptible, while mice with H-2^b, H-2^d, H-2^f-haplotypes are resistant and mice with H-2^q are intermediate (Vladutiu and Rose 1971). In addition, the evidence for human leukocyte antigen (HLA) association with HT was explored by using HLA transgenic

mouse models (Kong et al. 2003, Kong et al. 1996). It was found that EAT-resistant B10.M mice became susceptible after introduction of the HLA-DR3 transgene (Kong et al. 1996). In contrast, HLA-DR2 and HLA-DR4 transgenic mice did not develop EAT after challenge with mTg or human Tg (hTg) (Kong et al. 2003).

1.1.2.1.3 EAT as T-cell mediated disease

The central role of T-cells in the induction of EAT has also been demonstrated in animal models. By adoptive transfer of mTg-primed and activated spleen cells to normal syngeneic recipients, Braley-Mullen *et al.* observed that similar incidence and severity of EAT was induced in these syngeneic recipients and in mice directly immunized with mTg (Braley-Mullen et al. 1985). They confirmed that EAT was transferred by T-cells since removal of B-cells from the culture did not abolish the ability of cells to transfer EAT. Subsequent studies illustrated that both CD4+ and CD8+ T-cells were required to initiate thyroiditis (Flynn et al. 1989, Conaway et al. 1989, Conaway et al. 1990). CD4+ T-cells were the primary population responsible for the initial transfer and development of EAT (Flynn et al. 1989). Kinetic analysis of T-cell subsets infiltrating the thyroid clearly showed CD4+ T-cells accumulating with higher percentages than CD8+ T-cells in the early phase of transferred EAT with a relative increase of CD8+ T-cells later. These CD8+ T-cells were probably involved in EAT pathogenesis by cytotoxic effects (Conaway et al. 1989, Conaway et al. 1990).

1.1.2.2 Spontaneous autoimmune thyroiditis (SAT)

Spontaneous autoimmune thyroiditis (SAT), a type of disease similar to HT, also occurs in animals (Weetman and McGregor 1994). Several animal models of SAT have been described (Wick et al. 1989, Sternthal et al. 1981, Many et al. 1995, Braley-Mullen et al. 1999). One of the well established models is the obese strain (OS) chicken, which is characterized as hypothyroidism after autoimmune thyroidal destruction (Wick et al. 1989). The biobreeding diabetes-prone (BB-DP) rat, a model for insulin-dependent diabetes mellitus (IDDM), has also shown high incidence of SAT (Sternthal et al. 1981). Usually, there is no significant hypothyroidism following the development of lymphocytic thyroiditis in BB-DP rats (Sternthal et al. 1981). Another animal model of autoimmune diabetes, the nonobese diabetes (NOD) mouse, also suffers from autoimmune thyroiditis (Many et al. 1995). Interestingly, the NOD MHC-congenic strain, NOD.H-2^{h4} develops only SAT, not diabetes (Braley-Mullen et al. 1999). Studies of SAT models indicate that a combination of genetic, immunological and environmental factors results in the susceptibility to autoimmune thyroiditis.

1.2 Mapping T cell epitopes in EAT

1.2.1 Thyroglobulin (Tg) as an autoantigen in EAT

The important role of Tg as an autoantigen in autoimmune thyroiditis was elucidated based on two major findings: in 1956, Campbell *et al.* (Campbell et al. 1956) reported that Tg-specific Abs were detected in sera of patients with HT; in the same year, by the efforts of Rose and Witebsky (Rose and Witebsky 1956), EAT was successfully induced in rabbits by immunization with homologous thyroid extract in adjuvant. These animals showed similar pathological features as HT in humans and developed autoantibodies recognizing Tg. Since then, numerous studies have confirmed the immunopathogenicity of Tg in EAT and pointed out the pivotal role of Tg-specific T-cells in the induction and effector phase of EAT (Weetman and McGregor 1994).

Tg represents up to 75% of the total protein in the thyroid gland (Malthiery et al. 1989). This large, highly conserved glycoprotein consists of two monomeric polypeptide chains making up a molecular weight of approximate 660 kDa. The two constituent chains are thought to be identical when synthesized, but heterogeneous after post-translational modifications such as glycosylation and iodination (Dunn and Dunn 2000). Tg is synthesized in the endoplasmic reticulum (ER) of thyrocytes, then transported to the Golgi body for sulfation and glycosylation, and eventually secreted to the apical cell

surface where iodination occurs (Van Herle et al. 1979, Ekholm 1990). Metabolically, Tg is working as a prothyroid hormone. It provides the matrix which incorporates and stores iodine in iodotyrosine residues to form the thyroid hormones: triiodothyronine (T3) and thyroxine (T4) (Taurog 2000).

Tg is also highly conserved among different species (Vali et al. 2000). So far, the complete primary sequences of human (h) (Malthiery and Lissitzky 1987), bovine (b) (Mercken et al. 1985) and mouse (m) (Caturegli et al. 1997) have been deduced from their mRNA and comprise 2767, 2769 and 2768 amino acid residues respectively. High identity in the amino acid sequence is revealed among these species: hTg and bTg (77.3%), bTg and mTg (73.5%), and mTg and hTg (71.8%) (Vali et al. 2000). Moreover, Tg shows significant internal homology (Vali et al. 2000). The N-terminal end of this molecule has three types of repetitive cysteine-rich domains. The type I domain contains 10 repeats of about 60 amino acid residues between position 1-1200. The type II domain has 3 repeats of 14-17 residues between position1436-1483 and there are 5 repeats between position1583-2170 in the type III domain (Vali et al. 2000). The functional role of these repetitive structures has been hypothesized by Molina *et al.* (Molina et al. 1996). The type I repeats may function as binders and reversible inhibitors of the proteases that are involved in proteolytic processing of Tg. In contrast, the C-terminal end lacks internal homology, but this cysteine-poor region is homologous to acetylcholinesterase (Ach) and other carboxylesterases (Takagi et al. 1991).

There are two biosynthetic features, which influence the immunological effects of Tg (Carayanniotis 2003). First, Tg is not a sequestered antigen since it is continuously released into the circulation by transcytosis (Herzog 1983). It is not clear whether Tg is released in the circulation in an intact form or as smaller immunoreactive fragments. Processing of smaller Tg fragments by antigen-presenting cells (APC) in vivo to generate thyroiditogenic epitopes may be different from the processing of intact Tg in vitro (Carayanniotis and Rao 1997). Second, iodination of Tg may influence the immunopathogenicity of this molecule (Carayanniotis 2003). Poorly iodinated Tg dissociates easily, while increasing iodination stabilizes this dimer (Dunn and Dunn 2000). Mice develop more severe EAT when immunized with highly iodinated Tg (Dai et al. 2002), suggesting that iodination of Tg enhances its pathogenicity. Epidemiological data have also shown an increasing prevalence of thyroiditis which parallels dietary supplementation with iodine (Bournaud and Orgiazzi 2003).

1.2.2 T-cell epitope mapping in Tg

The findings that T-cells play a critical role in eliciting thyroiditis encouraged the identification of pathogenic T-cell epitopes within Tg. Given the huge molecular size of Tg, the traditional method of epitope mapping, such as the use of overlapping peptides to

screen and test pathogenic determinants in autoantigen, is not suitable for Tg. Despite the technical challenges, efforts from different research groups have so far mapped 23 thyroiditogenic Tg peptides by diverse approaches (Carayanniotis 2003, Flynn et al. 2004, Li and Carayanniotis 2006). Fifteen known EAT-causing peptides have been identified in mTg, six in hTg and two have been localized around hormonogenic sites identical between hTg and mTg (Carayanniotis 2003, Flynn et al. 2004, Li and Carayanniotis 2006).

mTg-derived pathogenic peptides

Most of mTg-derived pathogenic peptides have been uncovered by the use of computerized algorithms that scan the primary a.a. sequence for potential MHC-binding peptides (Chronopoulou and Carayanniotis 1992, Carayanniotis et al. 1994, Rao et al. 1994, Rao and Carayanniotis 1997, Verginis et al. 2002, Li and Carayanniotis 2006). This method is based on the observation that the susceptibility of EAT is linked to H-2 genes. H-2^k and H-2^s strains are good responders, while H-$2^{b,d}$ strains are low responders (Vladutiu and Rose 1971). Thus, pathogenic determinants were found presented in the context of E^k, A^k or A^s molecules and different MHC class II haplotypes were found to influence the pathogenicity of Tg peptides. For example, the 13 mer peptide (2596-2608) elicits EAT in H-2^k, but not H-2^s mice (Verginis and Carayanniotis 2004) and vice versa, the peptide (2694-2711) induces thyroiditis in H-2^s but not H-2^k mice (Carayanniotis et al. 1994), indicating that EAT may be induced via recognition of distinct Tg determinants among H-2-differnet susceptible strains. Interestingly, the minimal 9 mer (2496-2504) Tg

epitope, causes EAT in *k* and *s* strains and it turns out that this peptide binds to both E^k and A^s molecules (Rao et al. 1994). Thus, there are also shared Tg determinants that are pathogenic in both H-2^k and H-2^s mice.

hTg-derived pathogenic peptides

Utilizing a cytotoxic T-cell hybridoma, Texier *et al.* discovered the first pathogenic hTg peptide (1671-1710) in a trypsinized hTg fragment. This 40mer peptide caused EAT in CBA mice by direct challenge (Texier et al. 1992). Computerized algorithm methods have also been used in mapping immunopathogenic T-cell epitopes in hTg, taking advantage of the knowledge that the HLA-DR3 allele is associated with HT in humans (Ban and Tomer 2003). HLA-DR3-transgenic mice provided a wonderful tool to test the pathogenicity of hTg peptides (Kong et al. 1996). By searching for HLA-DR3 binding sequences, Flynn *et al.* (Flynn et al. 2004) identified four immunogenic peptides (hTg 181, hTg418, hTg1518, hTg2079) in hTg, while only two of them (hTg181, hTg2079) showed thyroiditogenicity. All four peptides elicited a significant proliferative response to hTg-primed cells from DR3+ transgenic mice, suggesting that these peptides are naturally generated by the processing of hTg in vivo. Considering that HLA-DR is homologous to the mouse I-E molecule, these two peptides may also induce EAT in H-2^k mice. In fact, one 16mer mTg peptide (179-194), overlapping the analogue sequence of 15mer hTg peptide (181-195), showed immunopathogenicity in EAT when administered to CBA/J mice (H-2^k) (Li and Carayanniotis 2006). In addition, an E^k-restricted hTg peptide (2340-2359), which was initially found encompassing B-cell epitope(s)

recognized by autoantibodies from Graves' disease sera (Thrasyvoulides et al. 2001), elicited autoimmune thyroiditis in AKR/J (H-2^k) mice (Karras et al. 2003). Recently, its pathogenicity has been also described in HLA-DR3 transgenic mice (Karras et al. 2005).

Pathogenic Tg peptides containing hormonogenic sites

Based on the observation that iodine-deficient Tg fails to induce autoimmune thyroiditis in mice (Champion et al. 1987), it has been hypothesized that immunodominant epitopes might be iodinated peptides. Initial attempts to identify pathogenic iodinated determinants focused on the hormonogenic sites (Champion et al. 1991, Hutchings et al. 1992, Kong et al. 1995, Wan et al. 1997). The 12mer peptide (2549-2560), having T4 at residue 2553, was first found to cause EAT in H-2^k mice and was presented in the context of A^k (Champion et al. 1991, Hutchings et al. 1992, Kong et al. 1995, Wan et al. 1997). This peptide elicited EAT in recipient mice only by adoptive transfer of peptide-activated splenocytes, but not by direct challenge (Hutchings et al. 1992). It is noteworthy that p2549 was the first peptide found to be presented by the processing of intact Tg in vivo, since it was recognized by Tg-primed LNC (Kong et al. 1995). Another EAT-causing peptide (a.a.1-12) with T4 at a.a. residue 5 has also been identified (Kong et al. 1995). Like p2549, this peptide also elicited a cytotoxic T cell response (Wan et al. 1998). However, studies with peptides encompassing the other two hormonogenic sites showed that the peptides (2559-2570 and 2737-2748) with T4 at residues 2567 and 2746, respectively, lacked immunogenicity (Wan et al. 1997). These data illustrated that the bulky two-phenyl-ring side chain of T4 per se is not sufficient to impart immunogenicity

to any given Tg peptide, and as expected, the amino acid backbone played a pivotal role (Wan et al. 1997).

Another six pathogenic Tg peptides containing iodotyrosyl residues were recently identified by Li *et al.* (Li and Carayanniotis 2006). These six pathogenic peptides were localized outside of hormonogenic sites of Tg and induced EAT in CBA/J mice (Li and Carayanniotis 2006). These results will be described in more detail in section 1.3.3.

1.2.3 Use of computerized algorithms in mapping potential dominant epitopes in Tg

To date, none of the 23 known pathogenic Tg peptides have been shown to encompass dominant determinant(s). The conventional definition of dominant epitopes might not apply to determinants derived from Tg antigen due to its large size, but nevertheless, mapping potential dominant peptides within Tg is still possible. Further understanding of the processing of Tg by APC and parameters determining this process will be helpful in identifying dominant pathogenic Tg peptides.

The theoretic basis of computerized algorithms

Computerized algorithms stem from the findings that peptide binding to MHC molecules follows stringent rules according to different MHC isotypes (Allen et al. 1987, Sette et al. 1987, Ogasawara et al. 1990, Kurata and Berzofsky 1990, Boehncke et al. 1993). Allen *et*

al. were the pioneers in studying the interaction between a peptide and a MHC molecule, and the interaction between TCR and a peptide-MHC complex (Allen et al. 1987). Using the hen egg lyzozyme (HEL) peptide 52-61, they demonstrated that certain a.a. residues served in the interaction between a MHC molecule, in this case A^k, and HEL52-61, while other amino acid residues were responsible for TCR recognition. Numerous studies have confirmed that specific a.a. substitutions alter TCR contact or affect MHC binding affinity (Sette et al. 1987, Ogasawara et al. 1990, Kurata and Berzofsky 1990, Boehncke et al. 1993). Peptide sequences presented by the same MHC molecule, E^k or A^k, share binding motifs (Leighton et al. 1991, Altuvia et al. 1994).

Compilation of A^k and E^k-binding motifs

The computerized algorithm described by Altuvia *et al.* was used in identification of Tg peptides (Altuvia et al. 1994, Verginis et al. 2002, Li and Carayanniotis 2006). This algorithm was derived from a database of peptides, known to bind to A^k or E^k molecules and elicit T-cell responses. It also characterized the common features within these groups of peptides. The motifs of peptides took into account the physic-chemical and structural properties of the amino acids such as hydrophobicity, charge, size and polarity that were essential for MHC binding. Three A^k and five E^k-binding motifs were described in this study.

Early studies on searching dominant Tg epitopes by computerized algorithms

So far, a total of 19 of 23 Tg peptides have been identified using computerized algorithm approach (Chronopoulou and Carayanniotis 1992, Rao et al. 1994, Carayanniotis et al. 1994, Rao and Carayanniotis 1997, Verginis et al. 2002, Flynn et al. 2004, Li and Carayanniotis 2006). In 2002, Verginis *et al.* systematically scanned the complete mTg sequence to detect dominant pathogenic T-cell epitopes by using A^k- binding motifs (Verginis et al. 2002). This approach was justified by previous observations: 1) the autoimmune response to Tg and thyroiditis were under the genetic control of the I-A^k subregion (Vladutiu and Zaleski 1981, Beisel et al. 1982); 2) administration of anti-I-A^k Abs prevented EAT (Vladutiu and Steinman 1987). The above observations indicated that dominant epitope(s) might be presented in the context of A^k in the susceptible H-2^k strain. In this study, they identified five pathogenic peptides but none of them showed dominant characteristics. Verginis *et al.* found 99 additional A^k-binding sites in mTg and it remains, therefore, quite likely that dominant epitopes may be found in this subset and only a few of these A^k-binding peptides may become immunodominant epitopes after the processing of intact Tg in APC in vivo and in vitro.

Intracellular proteolysis of Tg

The proteolysis of Tg is thought to occur in lysosomes following its uptake by macropinocytosis (Dunn and Dunn 2000). The investigation of the enzymes involved in degrading Tg showed that thyroid proteases were commonly found in lysosomes of most organs (Dunn and Dunn 2000). The following proteases may play an important role in

Tg degradation: cathepsin D (aspartic endopeptidase) (Dunn and Dunn 1982), cathepsin B, H, L, and S (cysteine endopeptidases) (Dunn et al. 1991, Nakagawa and Ohtaki 1984, Petanceska and Devi 1992) and two exopeptidases, dipeptidase I and dipeptidyl peptidase II (Dunn et al. 1996). In 1991, Dunn *et al.* (Dunn et al. 1991) investigated the cleavage sites of Tg by human cathepsins B, D, L. Three cleavage sites for cathepsin B and four for cathepsin D were found scattered within the Tg molecule, while four cleavage sites for cathepsin L were clustered at the C-terminal of Tg. Although the exact processing of Tg has not been well defined yet, the combined action of endopeptidases and exopeptidases is likely to generate some T-cell determinants but, at the same time, to destroy others. The limited Tg fragments produced via proteolysis are delivered to the assembly of MHC-II/peptide complexes at the end of endocytic pathway (Watts 1997).

A clarifying insight is that T-cell determinants in a degraded antigen fragment are not randomly or equally captured by class II MHC molecules (Sercarz et al. 1993). Accumulated evidence has shown that not all antigenic determinants are efficiently presented for T-cell recognition (Maizels et al. 1980, Bixler et al. 1985, Shimonkevitz et al. 1984). Generally, only a few or one determinant(s) - the immunodominant ones, derived from the processing of a given antigen, elicit an antigen-specific T-cell response, while the rest of determinants are invisible to T-cells and are referred to as cryptic epitopes (Sercarz et al. 1993). 'A gradient of presentation effectiveness' comprises the stringent hierarchy of the epitopes within self-antigens, which determines which epitope(s) will be dominant or cryptic (Sercarz et al. 1993, Moudgil and Sercarz 2005).

The determinants undergoing appropriate processing will compete each other for binding effectively to MHC molecules (Sercarz 2002). In this competition, the first available determinant, close to a cleavage site, has been thought to have the priority (Gammon et al. 1987, Moudgil and Sercarz 2005). This hypothesis, termed 'determinant capture', has been supported by several studies (Sercarz et al. 1993, Schneider et al. 2000). The processing of HEL provides examples of 'determinant capture' (Sercarz et al. 1993). Both NOD and BALB/c mice are good antigen-responders for HEL. Only the dominant HEL peptide (11-25) and subdominant HEL peptide (91-105) are known to elicit peptide-specific T-cell responses in NOD mice. In addition, the E^d-restricted HEL peptide (106-116) elicits a dominant T-cell response in BALB/c mice. Therefore, in (NOD x BALB/c) F1 mice, codominant responses to peptide (11-25) and peptide (106-116) were observed as expected. However, the subdominant response to peptide (91-105) was totally abrogated. Strikingly, when these mice were challenged with cyanogens bromide (CNBr)-treated HEL, which was cut at a.a. position 12 and 105, a significant response to p(91-105), equivalent to other dominant peptides, was unmasked. They concluded that the dominance of peptide (91-105) revealed here, was due to the precise scission that exposed the determinant (91-105) as the first accessible binding motif (Sercarz et al. 1993). Furthermore, by introduction of an endopeptidase cleavage site in HEL, Schneider *et al.* demonstrated enhancement of presentation of a determinant adjacent to the cleavage site (Schneider et al. 2000). The above studies highlight that the dominant determinants for a given antigen, may occur adjacent to the endopeptidase cleavage sites.

Based on the above findings, we hypothesize that the dominant determinants of mTg may be A^k-restricted and may be found in the regions close to the endopeptidase cleavage sites. In chapter 4, we describe a theoretical prediction of dominant epitopes in mTg by using computerized algorithm to search for A^k-binding site adjacent to cathepsin cleavage sites in mTg.

1.3 The effect of iodine on autoimmune thyroiditis

1.3.1 Iodine metabolism

Iodine is an indispensable component of thyroid hormones, T3 and T4, constituting 59% and 65% of their molecular weight, respectively (Dunn and Dunn 2001). Therefore, iodine metabolism is of great relevance to hormone formation. Basically, the formation of T3 and T4 contains following steps: 1) active uptake of iodide; 2) formation of iodotyrosyl residues of Tg; 3) coupling of iodotyrosyl residues to form T3 and T4; 4) proteolysis of Tg to release hormones; 5) deiodination of iodotyrosines and recirculation of iodide (Taurog 2000).

To utilize iodide, thyroid follicular cells first actively transport iodine against an electrochemical gradient from plasma to the cytoplasm (Wolff 1964). This process of crossing the basolateral plasma membrane of the follicular cells is mediated by the Na^+/I^- symporter (NIS) and consumes cellular ATP (O'Neill et al. 1987). The thyroid

stimulating hormone (TSH) has been considered as one of the most important regulators for I^- transport since TSH upregulates NIS protein expression (Levy et al. 1997, Uyttersprot et al. 1997). The other well known regulator is iodide itself (Wolff and Chaikoff 1948). Either high doses or low doses of iodide inhibit the accumulation of iodide in the thyroid (Braverman and Ingbar 1963, Eng et al. 1999). Intracellular iodide passively passes through a putative I^- channel to the apical membrane where it is oxidized for further utilization (Dunn and Dunn 2001).

Once iodide is translocated into the follicular lumen, the second step, iodination of Tg, occurs, mediated by thyroid peroxidase (TPO) (Taurog 1964). Except TPO, H_2O_2 and O_2 as the oxidizing agents are also necessary for iodination (Taurog 1964). TPO has two substrate sites, one favoring iodine and the other favoring tyrosine. The reaction of Tg iodination starts with the oxidation of TPO by H_2O_2, then oxidation of iodine and tyrosine. The corresponding free radicals, the iodinium ion (I^+) and hypoiodite (OI^-) yielded from the above oxidation reactions attach to each other to form monoiodotyrosine (MIT) or diiodotyrosine (DIT). Within the Tg molecule, further oxidation occurs, resulting in the formation of T4 and T3 by coupling two DIT residues or one DIT and one MIT (Taurog 2000). The tyrosyl residues in Tg are not equally susceptible to iodination (Dunn and Dunn 1999). Generally, the favored sites for hormone formation, also named as hormonogenic sites, are residues 5, 2553, 2567 and 2746. (Dunn and Dunn 1999). However, many other tyrosyl residues are also capable of incorporating of iodine (Marriq et al. 1989, Ohmiya et al. 1990).

Further release of T_4 and T_3 requires iodinated Tg reentry into the thyroid cells (Dunn and Dunn 2001). Tg is internalized by macropinocytosis initially fuses with early endosomes, then with lysosomes, where the proteolytic degradation of Tg and hormone liberation take place (Dunn and Dunn 2001). Finally, the released hormones T4 and T3 are secreted to the circulation. The iodotyrosine residues removed from Tg undergo deiodination by an iodotyrosine-specific deiodinase in follicular cells and their released iodide is reused for hormone synthesis (Rosenberg and Goswami 1979).

1.3.2 Iodine excess with AT

It has been widely accepted that high iodine intake is associated with the onset and development of AT (Bournaud and Orgiazzi 2003). This conclusion is based on the accumulated evidence both from epidemiological and experimental studies (Bournaud and Orgiazzi 2003). Epidemiological studies show an increasing incidence of HT during the implementation of iodine prophylaxis, that is food supplementation with iodine to prevent endemic goiter in iodine-deficient areas (Bournaud and Orgiazzi 2003). A comparative epidemiological study was examined in two populations, in Jutland where iodine intake was relatively lower (40-80 μg/day) than the recommended daily iodine requirement of 150 μg and in Iceland where iodine intake was much higher with a the median urinary iodine excretion rate of 300 μg/day (Laurberg et al. 2000). High incidence of hypothyroidism was found among elderly subjects in Iceland, 3.9% in females and

0.6% in males. However, hypothyroidism was uncommon in the same age groups in Jutland. Also, the investigations in the high iodine intake areas such as Japan, Whickham and Worcester suggested the increase of HT in parallel with the increase of iodine in the diet (Robuschi et al. 1987, Vanderpump et al. 1995). In addition to a moderate increase in iodine level by a persistently long period of exposure, a short-term exposure to large doses of iodine contained in certain drugs such as amiodarone also leads to iodine excess (Roti and Vagenakis 2000).

Studies on animal models have provided a more clear profile of the effect of iodine on induction and development of AT. Bagchi *et al.* demonstrated that excess dietary iodine enhanced the incidence of AT in the Cornell strain (CS) chicken (Bagchi et al. 1985). They fed the CS chickens with different concentrations of KI-containing water during the first 10 weeks of life. Then, AT induction was accessed by examining lymphocytic infiltration of thyroid glands and the presence of anti-Tg, T3 and T4 antibodies. About 7.3% of the chickens treated with excess iodine developed AT. However, in the normal iodine intake group, only 1.28% of the chickens had AT. Moreover, the OS chickens, raised under conditions of iodine-deficiency, had a dramatically decreased incidence of thyroid autoantibodies. Similar results were observed in other animal models such as the BB-DP rat (Mooij et al. 1993). Mooij *et al.* administered an enriched iodine diet (EID 100 μg iodine /day) or a normal iodine diet (NID 7 μg iodine /day) to young BB rats (< 3 wk old) for 18 weeks (Mooij et al. 1993). About 50% of BB rats fed with 100 μg iodine

per day developed the focal lymphoid cell infiltrates in the thyroids, suggesting high iodine intake accelerates the development of AT.

While the mechanisms by which iodine excess influences thyroid autoimmune reactivity are not clear, three possible mechanisms have been proposed (Ruwhof and Drexhage 2001). 1) Iodine excess may have toxic effect on thyrocytes, resulting in thyrocyte damage directly (Li and Boyages 1994, Many et al. 1995). 2) Iodine could stimulate immune cells such as dendritic cells, macrophages and T cells to facilitate the development of autoimmune response (Toussaint-Demylle et al. 1990, Li et al. 1993). 3) The incorporation of iodine increases the immunogenicity of Tg (Carayanniotis 2003). In the next section, we will focus on studies related to the last hypothesis.

1.3.3 Iodination increases Tg immunogenicity

It has been suggested that iodination of Tg enhances its immunogenicity (Ruwhof and Drexhage 2001, Carayanniotis 2003). In 1992, Ebner *et al.* examined the Ab responses against Tg with different iodine content in BB/W rats immunized with normally iodinated Tg (NTg) or low iodine Tg (LTg) (Ebner et al. 1992). The NTg or LTg was obtained from thyroid glands of rats that were fed with a normal diet or an addition of 0.5% methimazole which inhibits the conversion of iodide to iodine by binding to TPO. They found that 31% of the BB/W rats immunized with NTg showed significant thyroidal lymphocytic infiltration, while none of the BB/W rats in the LTg-immunized group

developed lymphocytic thyroiditis. In addition, higher levels of anti-Tg antibodies were detected in the NTg-challenged rats compared to the LTg-challenged rats, indicating that iodination increases the immunogenicity of Tg. Similar results had been observed in CS chickens and mice (Sundick et al. 1987, Dai et al. 2002). Using CS chickens, Sundick *et al.* demonstrated that highly iodinated Tg (about 60 iodine atoms /Tg) elicits a stronger anti-Tg response than low iodinated Tg (Sundick et al. 1987). Later, Dai *et al.* (Dai et al. 2002) investigated the immunogenicity of Tg with 15-20 iodine atoms or 55-70 iodine atoms in SJL strain mice. More severe EAT was induced in mice immunized with high-iodinated Tg (55-77 iodine atoms) than mice immunized with normal Tg (15-20 iodine atoms). Moreover, the LNC from high iodinated Tg-primed mice proliferated vigorously in the presence of high-iodinated Tg, but mildly in the presence of normal Tg.

In regard to the underlying mechanisms of the enhanced immunogenicity of Tg induced by iodination, it is likely that iodination modification of Tg results in the formation of neo-epitopes containing iodothyronines / iodotyrosines and generation of non iodinated pathogenic epitopes that are normally cryptic to the immune system (Carayanniotis 2003). These epitopes may break the established T-cell tolerance, leading to autoimmune thyroiditis.

The first iodinated T-cell epitope (2551-2559) containing T4 at position 2553 was identified by Champion *et al.* (Champion et al. 1991). Using two clonal T-cell hybridomas, they demonstrated that recognition of this epitope required the presence of the T4 residue, since substitution of T4 iodothyonine at position 2553 with any other amino acids abrogated the ability of the peptide to stimulate the T-cell hybridoma clones. Studies on the pair of analogue peptides (2549-2560) carrying T0 or T4 provided further understandings on the role of iodine in the formation of T-cell determinants (Kong et al. 1995). The peptide T0 (2553), lacking four iodine atoms at position 2553 was immunogenic in CBA mice and induced EAT by adoptive transfer of T0-primed LNC in recipients, suggesting that the immunogenicity of T0 (2553) depends on the amino acid backbone not iodine atom modification. In this study, they also found that T4 (2553)-primed LNC could be stimulated by T4 (2553) but could not be cross-stimulated by T0 (2553). This finding indicated iodine modification was sufficient to recruit a distinct subset of T-cells which only recognizes only iodine-modified epitope.

Another possible mechanism of the enhanced immunogenicity of the iodinated Tg is that iodine modification changes the processing of Tg in APC, thus rendering the generation of normally cryptic epitopes (Dai et al. 2002, Carayanniotis 2003). Tg peptide (2494-2510) has shown crypticity features in SJL mice (Chronopoulou and Carayanniotis 1992). However, LNC from highly iodinated Tg (HI-Tg) containing 55-70 iodine atoms per monomeric subunit proliferated well in the presence of p2494. Additionally, p2494-

specific T-cell clonal hybridomas were significantly activated by processing of HI-Tg in macrophages or dendritic cells in vitro. Therefore, the iodination of Tg converted the cryptic p2494 into a dominant determinant.

In a recently published study, Li. *et al.* identified six pathogenic Tg peptides with iodotyrosyl residues out of hormonogenic sites (Li and Carayanniotis 2006). Strikingly, three Tg peptides, peptide I-p117 (a.a.117-132), peptide I-p304 (a.a 304-318) and peptide I-p1931 (a.a. 1931-1945) were immunopathogenic only in their iodotyrosyl-containing form in CBA mice. The iodotyrosine residue facilitated binding of I-p304 and I-p117 to A^k, while the iodotyrosine residue of I-p1931 may constitute a central contact residue for TCR. These results illustrate that neo-antigenic determinants formed via iodine modification of Tg peptides cause thyroiditis. However, further studies with additional Tg peptides indicated that iodine modification does not always enhance the immunogenicity of Tg peptides. By comparing iodinated peptides with their non-iodinated analogs, we found that iodination increased the immunogenicity of p179 (a.a. 179-194), decreased the immunogenicity of p2540 (a.a. 2540-2554) and did not alter the immunogenicity of p2529 (a.a. 2529-2545) (unpublished data). These findings provided, for the first time, evidence for differential influences of iodination on immunogenicity of Tg T-cell determinants. Iodination might regulate the immune response to Tg by enhancing the immunogenicity of some Tg peptides, while simultaneously decreasing the immunogenicity of other Tg peptides. This process is perhaps mediated by the influence

of iodine atoms on peptide MHC binding or T-cell recognition. However, these observations do not contradict earlier studies that iodination increases Tg immunogenicity (Dai et al. 2002, Sundick et al. 1987). This effect can be explained as a consequence of the total influences of iodotyrosyl formation on Tg determinants.

CHAPTER 2

MATERIALS AND METHODS

2.1 Animals

Female CBA/J (H-2^k) mice purchased from Jackson Laboratories (Bar Harbor, ME) were used in experiments at 6-8 weeks of age.

2.2 Antigens

2.2.1 Purification of mTg

mTg was extracted from thyroids of outbred ICR mice (Bioproducts for Science, Indianapolis, IN) as previously described (Chronopoulou and Carayanniotis 1992). Briefly, frozen glands (Bioproducts for Science, Inc., Indianapolis, IN) were homogenized in phosphate buffer (pH 7.0) with protease inhibitors: Leupeptin (10^{-6} mol/L), Pepstatin A (10^{-5} mol/L) PMSF (10^{-3} mol/L) and the supernatant was centrifuged three times at 14,000 rpm. mTg was obtained from the supernatant by passing through a Sepharose CL-4B column (Pharmacia, Baie d'Urfe, Quebec, Canada). The fractions of peak II (**Figure 2.1**) were pooled, dialyzed in double distilled water (Fisher Chemicals) three times, then concentrated to 3-5 mg/ml by ultra-filtration cells (Amicon, Danvers, MA), filter-sterilized, lyophilized and stored at -20°C. Tg concentration was assessed

spectrophotometrically ($O.D._{280}$) and calculated as the molarity of the monomeric form (330 kDa, 100 μg/ml = 0.303 μM).

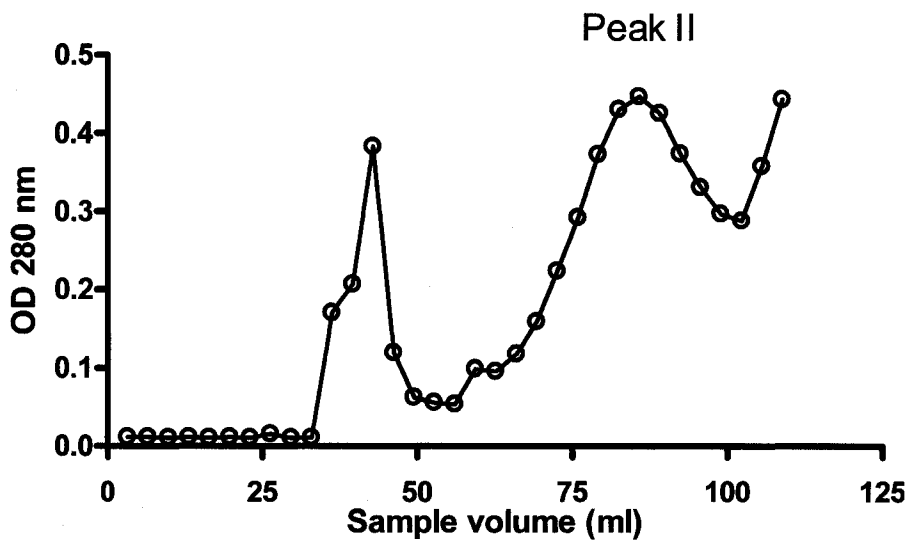

Figure 2.1 Purification of mTg.

One hundred frozen thyroid glands from outbred ICR mice were homogenized in 2.5 ml ice-cold PBS containing protease inhibitors (Leupeptin, Pepstatin A, PMSF). The supernatant was loaded on the Sepharose column following centrifugation (14,000 rpm, 10 min). Tg was collected in tubes (3.3 ml/tube) and its concentration was measured spectrophotometrically at 280 nm. The fractions of peak II from tubes 19 to 28 (62.7 ml to 92.4 ml in the figure) were pooled together, dialyzed three times in double distilled water, concentrated, filtered and lyophilized. The Tg powder was stored at -20°C.

2.2.2 Peptides

The Tg peptide (179-194) NTTDMMIFDLIHNYNR (p179), its iodinated form I-p179 (NTTDMMIFDLIHNY(I)NR) and I-p304 (a.a 304-318) were synthesized at >80% purity at the Dalton Chemical Laboratories Inc. (Toronto, Ontario, Canada). All truncated peptides (>90% purity) were synthesized at Biosynthesis, Inc. (Lewisville, TX). mTg peptide (a.a. 2496-2504), p2496 (Rao et al. 1994) used in the studies was previously synthesized at the Alberta Peptide Institute (Edmonton, Alberta, Canada) and peptide (a.a. 1826-1835), p1826 (Verginis et al. 2002) was synthesized by Sigma-Genosys (The Woodlands, TX). All peptides were blocked with an acetyl group at the N-terminal and with an amide group at the C-terminal end.

2.3 Proliferation assay of antigen-specific LNCs

Mice were immunized s.c with 100 nmol Tg peptide p179 or 100 μg mTg in CFA (with *Mycobacterium butyricum*; Difco Laboratories). Nine days later, the inguinal, brachial, and axillary lymph node cells were cultured (5×10^5 cells /well) for 4 days with or without Ag in 200 μl microculture. Eighteen hours before harvesting, 1 μCi [^{3}H] thymidine (PerkinElmer, Life and Analytical Sciences, Boston, MA) was added to each well. The cells were harvested 18 h later by a Harvester 96® Mach III M (Tomtec, Hamden, CT, USA) and radioactivity incorporation was counted in the Topcount

NXTTM microplate counter (Canberra Packard Canada, Mississauga, ON, Canada). Stimulation index is defined as (cpm in the presence of Ag)/(cpm in the absence of Ag).

2.4 Cell culture

2.4.1 Cell lines and culture media

Cell culture was performed in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen Life Technologies) supplemented with 10 % fetal bovine serum (FBS) (Cansera, Ontario, Canada), 20 mM HEPES buffer, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin (all from Invitrogen Life Technologies), and 5×10^{-5} M 2-ME (Sigma-Aldrich, St. Louis, MO) (complete medium). The antigen presenting cell line LK35.2 (H-$2A^{k/d}$, H-$2E^{k/d}$) (Kappler et al. 1982), the IL-2 dependent cell line CTLL (Gillis and Smith 1977) and hybridomas specific for A^k (10-3.6.2, IgG2a) (Oi et al. 1978), E^k (14-4-4s, IgG2a) (Ozato et al. 1980) and influenza A nucleoprotein (H16-L10-4R5, IgG2a) (Yewdell et al. 1981) were purchased from the American Type Culture Collection (Manassas, VA). The respective monoclonal antibodies were purified from culture supernatants by affinity chromatography on a protein G-Sepharose 4 Fast Flow Column.

2.4.2 Generation of peptide-specific T-cell hybridoma clones

The IL-2-secreting T cell hybridomas 1E7, 4A11, 2D5, 1B3 and 2H2 were generated following a modified method described by Perkins *et al.* (Perkins et al. 1991). Briefly, CBA mice were challenged s. c. with 100 nmol p179 or I-p179 in 100 µl of PBS/CFA emulsion and nine days later, draining LNCs were collected and 4 x 10^6 cells/ml were cultured in the presence of 20 µM of the respective peptides. After three days, the stimulated cells were fused at a 1:2 ratio with BW5147$\alpha^-\beta^-$ lymphoma cells (White et al. 1989) by using polyethylene glycol (Boehringer Mannheim, Indianapolis, IN). Hybridomas appeared after 7-10 days of culture initially with HAT-containing medium (Sigma), then HT-containing medium (Sigma), and then finally normal complete medium. The specificity and sensitivity of the hybridomas were screened by an activation assay as described in the following paragraph. Peptide-specific hybridomas were cloned by limiting dilution at 0.3 cell/well in complete medium containing 20% FBS with 1% syngeneic red blood cells (RBC) used as feeder cells.

2.4.3 Activation assay of T-cell hybridomas

An activation assay of clonal T-cell hybridomas was performed by incubating hybridoma cells (10^5 cells/well) and LK35.2 cells (10^5 cells/well) in the presence of relevant peptides. After 24 hours of incubation, 100 µl of supernatant was transferred from each well into new plates and stored at -70°C for at least 2 hours. The release of IL-2 in the culture

supernatant was assessed by measuring the proliferation of the CTLL cells. In brief, 10^4 CTLL cells per well were cultured in the above supernatant. After 18 h, 1 μCi of [^{3}H]-thymidine was added to each well. The cells were harvested 6 h later as described in 2.3.

2.5 MHC-restriction analysis of T-cell hybridomas or T-cells from peptide-primed LNCs

2.5.1 Blocking assay of T-cell hybridomas or T-cells from peptide-primed LNCs

The blocking assay was used to determine the MHC-restriction of T-cell hybridoma clones or T-cells from peptide-primed LNC. Hybridoma cells (10^5 cells/well) were incubated with a constant amount of their respective ligands, LK35.2 cells (10^5 cells/well) and serial dilutions of the blocking Abs in a 200 μl final volume. After 24 hours, 100 μl of supernatant was transferred to a new plate from each well, then frozen at -70°C for at least 2 hours. The IL-2 secretion was determined by CTLL assay as described in 2.3.3. To perform blocking assay of peptide-primed LNCs, CBA/J mice were immunized with 100 nmol of p179 or I-p179 emulsified in CFA and nine days later, draining LNCs proliferative responses to priming peptides were assessed in the presence of anti-I-A^k or anti-I-E^k Abs, as described in section 2.3.

2.5.2 Competitive inhibition assay of T-cell hybridoma activation

T-cell clones 10C1.8 (I-A^k-restricted, I-p304-specific) (Li and Carayanniotis 2006) and 8F9.27 (I-E^k-restricted, p2496-specific) (Rao et al. 1994) were used in the competitive inhibition assay to determine the MHC-restriction of p179 and its iodinated analogue. In a flat-bottom microwell plate, 10^5 of 10C1.8 or 8F9.27 cells were cultured with a constant amount of relevant ligand, serial dilutions of p179 and I-p179 as competitive inhibitors and 10^5 LK35.2 as APC. The supernatant (100 µl/well) was collected in a new plate after 24 hours of incubation, then frozen at -70°C for at least 2 hours. The IL-2 secretion was determined by CTLL assay as described in 2.3.3.

2.6 Analysis of T-cell receptor (TCR) V-β family utilization of peptide-specific T-cell hybridomas

2.6.1 RNA extraction

Total RNA was extracted from 5 x 10^6 of T cell hybridomas using TRIZOL reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instruction. Briefly, cells were lysed in 1 ml of TRIZOL reagent, and then 200 µl of chloroform (Sigma Chemicals) was added for the phase separation. The solution was separated into an upper aqueous phase containing RNA and an organic phase by centrifugation. The aqueous phase (about 600 µl) was transferred in a new

diethylpyrocarbonate-treated tube and RNA was recovered by precipitation with isopropylalcohol (500 µl). The RNA pellet was washed with 1 ml of 70% ethanol, air-dried and dissolved in 20 µl of RNase-free water (Sigma Chemicals). The RNA yield and its purity were measured using a Beckman Du®64 spectrophotometer (Fullerton, CA, USA) at the optical density (O.D.) of 260 and 280 nm. The RNA with purity from 1.6 to 1.8 (260 nm/ 280 nm) was chosen for further RT-PCR analysis.

2.6.2 cDNA synthesis

First-strand cDNA synthesis was performed using a cNDA synthesis kit (Amersham Bioscience, Buckinghamshire, UK). Briefly, 5 µg of total RNA was heated at 65°C for 10 min to remove RNA secondary structure, and then chilled on ice. The denatured RNA was added to a mixture containing 11 µl first-strand bulk mix, 1 µl dithiothreitol (DTT) solution and 0.2 µg NotI-d$(T)_{18}$ primer (1 µl). FPLC$_{pure}$™ murine reverse transcriptase is already contained in the first-strand bulk mix. The total 33 µl reaction solution was then incubated at 37°C for 1 hour and terminated by heating at 75°C for 10 min. The cDNA was stored at -20°C.

2.6.3 PCR amplification

First strand cDNA was amplified by PCR in a reaction mixture containing: 1 x PCR buffer, 1.5 mM $MgCl_2$ (Invitrogen, Carlsbad, CA, USA), 0.2 mM of each dNTP (Gibco-BRL), 2 μl cDNA template, 20 pmoles of each primer (forward and reverse, Operon Technologies, Inc. Alameda, CA, USA) (**Table 2.1**), 2.5 U Taq polymerase (Invitrogen) and RNase-free water (Sigma) to 100 μl final volume. Forward primers specific for Vβ families and a reverse primer specific for Cβ were chosen as described (Casanova et al. 1991). Mineral oil was added to the top of the PCR solution to avoid evaporation. A 35-cycle step program (1 min denaturation at 95°C, 1 min annealing at 55°C, and 1 min extension at 72°C) was performed on the Perkin Elmer DNA thermocycler (Cetus, Norwalk, CT, USA) with a preceding 5 min denaturation step at 94°C followed by 10 min extension step at 72°C. The PCR products were visualized by 1.5% agarose gel electrophoresis containing 0.5 μg/ml ethidium bromide.

2.7 Statistical Analysis

Data were analysed by t test using the GraphPad Prism software.

Table 2.1 PCR primers used in this study

primer	Sequence (5'→3')	PCR product (bp) (Vβ +Cβ)
Cβ	CCAGAAGGTAGCAGAGACCC	
Vβ1	CCCAGTCGTTTTATACCTGAATGC	350
Vβ2	TCACTGATACGGAGCTGAGGC	322
Vβ3	CCTTGCAGCCTAGAAATTCAGTCC	317
Vβ4	GCCTCAAGTCGCTTCCAACCTC	364
Vβ5.1	GTCCAACAGTTTGATGACTATCAC	334
Vβ6	CTCTCACTGTGACATCTGCC	306
Vβ7	TACAGGGTCTCACGGAAGAAGC	346
Vβ8.1	CATTCTGGAGTTGGCTTCCC	305
Vβ8.2	CCTCATTCTGGAGTTGGCTACCC	303
Vβ8.3	ACGCAAGAAGACTTCTTCCTCCTGC	335
Vβ9	TCTCTCTACATTGGCTCTGCAGGC	308
Vβ10	ATCAAGTCTGTAGAGCCGGAGGAC	300
Vβ11	GCACTCAACTCTGAAGATCCAGAGC	315
Vβ12	GAAGATGGTGGGGCTTTCAAGGATC	366
Vβ13	AGGCCTAAAGGAACTAACTCCAC	386*
Vβ14	ACGACCAATTCATCCTAAGCAC	317
Vβ15	CCCATCAGTCATCCCAACTTATCC	343
Vβ16	CACTCTGAAAATCCAACCCAC	312
Vβ17	GAGTAACCCAGACTCCACGA	522
Vβ18	CAGCCGGCCAAACCTAACATTCTC	333
GAPDH (F)	CCATCACCATCTTCCAGGAG	577
GAPDH (R)	CCTGCTTCACCACCTTCTTG	

*PCR product of V/D2/J2.1/C sequence

The above PCR primers were described by Casanova *et al.* (Casanova et al. 1991).

CHAPTER 3

EFFECTS OF IODOTYROSYL FORMATION ON T-CELL RECOGNITION OF A SINGLE THYROGLOBULIN PEPTIDE

3.1 Abstract

We have previously shown that iodotyrosyl formation within certain thyroglobulin (Tg) peptides confers to them immunopathogenic properties. In this study, we focused on the subdominant 16mer Tg peptide p179 (aa.179-194). Iodotyrosyl formation at the Y192 residue enhances the immunogenicity but not the pathogenicity of p179 but the mechanisms underlying this observation are not understood. This peptide has both A^k- and E^k-binding sites, and iodination was found not to alter this MHC-restriction profile. We developed a panel of p179-specific T-cell hybridoma clones, with the view to examining the effects of a single iodine atom on T-cell recognition. Two E^k-restricted clones, 2H2 and 1B3, responded well to both p179 and I-p179 analogs, indicating that the bulky iodine atom did not sterically hinder their TcR engagement. Two other clones, 1E7 (E^k-restricted) and 2D5 (A^k-restricted) recognized only p179, indicating a negative influence of the iodine atom on TcR recognition. Lastly, the E^k-restricted clone 4A11 was reactive only to I-p179, suggesting that the iodine atom is an integral part of its TcR ligand. Truncation analysis localized the determinant seen by all clones within the 11mer peptide p184 (aa. 184-194) suggesting that the cross-reactive clones were not activated

by a minimal epitope lacking Y192, and that the negative influence of iodine on 1E7 and 2D5 was not due to effects from flanking residues. Taken together, these results demonstrate, at the clonal level, differential effects of a single iodine atom on the recognition of a single Tg peptide. Even when a net combined effect is discernible polyclonally this may not be associated with thyroidal damage, if various p179-specific clones do not contribute equally to pathogenicity.

3.2 Introduction

Excessive iodine intake has been associated with the incidence of autoimmune thyroiditis in clinical and experimental studies (Braverman et al. 1971, Bagchi et al. 1985, Boyages et al. 1989, Robuschi et al. 1987, Tajiri et al. 1986, Mooij et al. 1993, Kahaly et al. 1998). The mechanisms underlying this association remain unknown but they may be, at least in part, related to the immunogenicity of thyroglobulin (Tg), the only molecule that incorporates iodine in vivo to facilitate the synthesis of the thyroid hormones triiodothyronine (T3) and thyroxine (T4) (Taurog 1964). Tg represents up to 75% of the total protein in the thyroid gland (Malthiery et al. 1989) and it is the major thyroid autoantigen causing experimental autoimmune thyroiditis (EAT), a T-cell mediated disease (Braley-Mullen et al. 1985, Flynn et al. 1989, Conaway et al. 1989), considered to be a model for Hashimoto's thyroiditis (HT) in humans (Rose and Witebsky 1956, Rose et al. 1971).

Several studies have supported the view that the iodine content in Tg, which varies according to dietary intake, can influence its immunogenicity (Ebner et al. 1992, Champion et al. 1987, Sundick et al. 1987). For example, iodine-deficient Tg fails to induce EAT in mice (Champion et al. 1987) and BB/W rats (Ebner et al. 1992) and conversely, highly iodinated Tg is more immunogenic than normal Tg and elicits severe EAT (Dai et al. 2002, Sundick et al. 1987). Iodination has been shown to enhance Tg

immunogenicity by two ways: 1) by formation of neoantigenic determinants encompassing either hormonogenic or iodotyrosyl-containing sites (Kong et al. 1995, Li and Carayanniotis 2006); and 2) by alteration of Tg processing resulting in generation of non iodinated but pathogenic cryptic epitopes (Dai et al. 2002, Carayanniotis 2003). We have previously reported that iodotyrosyl formation can render certain innocuous Tg peptides (p304, p117 and p1931) immunopathogenic (Li and Carayanniotis 2006). Additional results, however, have suggested that iodotyrosyl formation can have variable effects on the immune recognition of other Tg peptides i.e. it can increase, decrease or not alter their established immunogenicity (Li and Carayanniotis 2006). In this study, we have used as a model antigen the mouse Tg peptide p179 (aa 179-194) because of two interesting features: a) an overlapping peptide analog (aa 181-195) has been reported to result from the natural processing of human Tg in HLA-DR3-transgenic mice (Flynn et al. 2004); and b) the iodinated analog I-p179, carrying an iodotyrosyl at position Y192, elicits stronger proliferative T cell responses than p179, but remains, unexpectedly, mildly pathogenic as p179. (Li et al. submitted for publication). Therefore, we aimed to examine if p179 could be similarly generated following the in vivo processing of mTg and, in addition, investigate at the clonal T-cell level, effects of a single iodine atom on T-cell recognition of p179 that may account for the immunopathogenic behavior of the iodinated analog.

3.3 Results

3.3.1 p179 contains subdominant epitope(s)

We first tested whether the Tg peptides p179 and I-p179 contain dominant T-cell epitope(s). CBA/J mice were immunized with 100 μg of mTg emulsified in CFA and nine days later, draining LNCs were cultured in the presence of mTg or free peptides. Tg-primed LNCs responded strongly against mTg (S.I. = 2.9 - 8.2 in the 0.01- 1 μM range), weakly to higher concentrations of p179 (S.I .= 3.2 +/- 0.3 at 27 μM), but failed to respond to equimolar amounts of I-p179 (**Figure 3.1A**). Conversely, in vivo priming with p179 (100 nmol) elicited strong recall responses to the priming peptide (S.I. = 3.6 - 14.7 in the 0.6 - 5 μM range) and a weak but significant response (S.I. = 4.3-5.5) to equimolar amounts of mTg in vitro (**Figure 3.1B).** In all assays, proliferative LNC responses were not detected against the control Tg peptide p2496 (data not shown). These data indicate that p179 encompasses subdominant T-cell determinant(s).

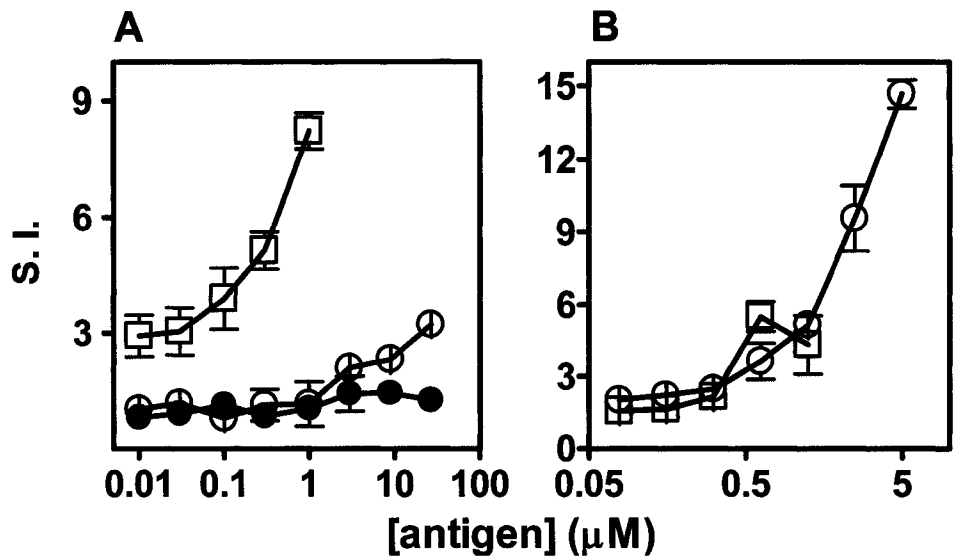

Figure 3.1 p179 contains subdominant epitope(s). CBA/J mice (three mice per group) were challenged with either Tg (A) or p179 (B). Antigen-specific proliferation of LNCs, in the presence of Tg (□), p179 (○) or I-p179 (•) was examined 9 days later. Data show the mean ± SD of S.I. values of triplicate wells and are representative of 2-3 experiments. Background cpm varied from 3499 to 5653.

3.3.2 p179 and I-p179 can be presented in the context of E^k and A^k

Tg peptide 179 (a.a. 179-194) was identified by scanning the mTg sequence (Kim et al. 1998) for A^k binding motifs with tyrosine as the flanking residue as seen in the previous study (Li and Carayanniotis 2006). Coincidentally, we found that this peptide also has an E^k-binding motif (Altuvia et al. 1994) (**Figure 3.2A**). To test whether p179 and I-p179 could be presented in the context of E^k and A^k, we performed blocking assay using peptide-primed LNC. CBA/J mice were immunized with 100 nmol of p179 or I-p179 emulsified in CFA and nine days later, draining LNCs proliferative responses to priming peptides were assessed in the presence of anti-I-A^k or anti-I-E^k Abs. The proliferation of p179-primed LNC to 10 μM of p179 was significantly inhibited (% inhibition = 76.96 +/- 14.67, $p<0.05$) in the presence of 1.25 μg/ml anti-I-E^k Ab (**Figure 3.2B**). The same concentration of anti-I-A^k Ab suppressed by 42.13 +/- 8.58 % ($p<0.05$) the proliferative response of p179-primed LNC to p179 (10 μM) (**Figure 3.2B**). On the other hand, the proliferation of I-p179-primed LNC to 10 μM I-p179 was significantly inhibited (% inhibition = 86.02 +/- 3.86, $p<0.05$) in the presence of 1.25 μg/ml anti-I-E^k Ab (**Figure 3.2C**). The same concentration of anti-I-A^k Ab suppressed by 36.71 +/- 7.79 % ($p<0.05$) the proliferative response of p179-primed LNC to p179 (10 μM) (**Figure 3.2C**). These data indicate that p179 and I-p179 can be presented in the context of A^k and E^k, and the majority of T-cells recognize this pair of peptides in the context of E^k.

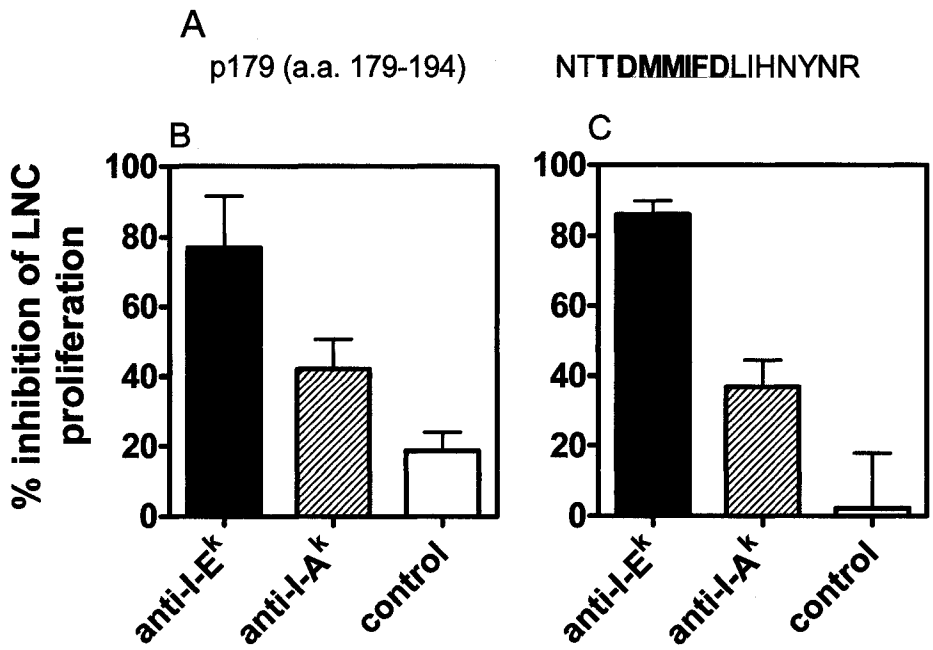

Figure 3.2 p179 & I-p179 can be presented in the context of E^k and A^k.

A, amino acid sequence of p179. A^k binding motif (underlined) and E^k binding motif (bold) were identified following the algorithms described by Altuvia et al. (Altuvia et al. 1994), A^k-binding motif A and E^k-binding motif C, respectively (Altuvia et al. 1994). B p179-primed LNC proliferative response to 10 μM p179 in the presence of anti-I-E^k or anti-I-A^k blocking Abs (1.25 μg/ml). C. I-p179-primed LNC proliferative response 10 μM I-p179 in the presence of anti-I-E^k or anti-I-A^k blocking Abs (1.25 μg/ml). Anti-influenza A nucleoprotein Ab was used as an isotype control for anti-I-E^k or anti-I-A^k blocking Abs. % inhibition = [1- proliferation with blocking Ab (cpm)/proliferation without blocking Ab (cpm)] x 100%

3.3.3 Iodination of p179 does not influence MHC binding

To determine whether iodination of p179 can influence its binding to MHC, we used two hybridoma clones: the E^k-restricted 8F9 clone, specific for the Tg peptide p2496 (Rao et al. 1994) and the A^k-restricted 10C1 clone, specific for the Tg peptide I-p304 (Li and Carayanniotis 2006) in order to examine their activation by their cognate ligands in the presence of p179 or its iodinated analogue in a competitive inhibition assay. Both p179 and I-p179 inhibited 8F9 activation equally well suggesting equivalent binding affinities to E^k (**Figure 3.3A**). Similarly, both analogues strongly inhibited 10C1 activation but this effect was noticeable at high competitor peptide concentrations (125 μM) with the I-p179 showing a slightly higher, although not significantly different ($p<0.05$), inhibition (**Figure 3.3B**). The inhibitory effect was MHC specific because equimolar concentrations of control peptides did not cause any inhibition (**Figure 3.3A&B**). These results indicate that the addition of an iodine atom to the Y192 residue does not significantly alter the MHC binding properties of p179.

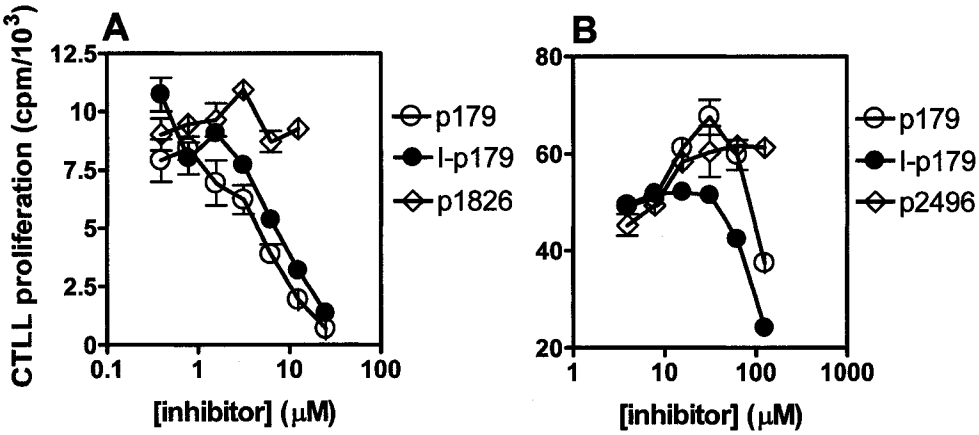

Figure 3.3 Iodination of p179 does not influence its MHC binding. A, Activation of the E^k-restricted clone 8F9 by its p2496 ligand (25 nM) in the presence of increasing concentrations of the inhibitor peptides p179 and I-p179. IL-2 secretion by the activated T cell hybridomas was assessed by CTLL proliferation. p1826 (A^k-restricted) was used as a negative control. B, Activation of the A^k-restricted clone 10C1 to its I-p304 ligand (0.15 μM), in the presence of increasing concentrations of the inhibitor peptides p179 and I-p179. IL-2 secretion was assessed as above. E^k-restricted p2496 was used as a negative control.

3.3.4 Generation of T-cell hybridomas from p179- and I-p179-primed LNCs

To determine whether iodine modification influences T-cell recognition of p179 peptide, we generated T-cell hybridomas from LNCs separately primed with p179 or I-p179. Briefly, CBA/J mice were immunized s. c. with 100 nmol of p179 or I-p179 in 100 μl of PBS/CFA emulsion (*Mycobacterium butyricum*; Difco, Detroit, MI) and nine days later, draining LNC were cultured with 20 μM of the corresponding priming peptide. After three days, the stimulated cells were fused with BW5147$\alpha^-\beta^-$ lymphoma cells (White et al. 1989). The specificity and sensitivity of the hybridomas to p179 and I-p179 were tested by an activation assay based on IL-2 release. In cultures of hybridomas derived from p179-primed LNC, 128 out of 576 wells showed hybridoma growth, and of those, 53 hybridomas were randomly tested. Fifteen hybridomas recognized only p179 and seventeen hybridomas cross-reacted with p179 and its iodinated analog. The remaining 21 hybridomas were non-specific (**Table 3.1**). In cultures of hybridomas derived from I-p179-primed LNCs, 99 out of 576 wells showed hybridomas growth, and of those, 22 hybridomas were randomly tested. Ten hybridomas recognized only I-p179 and seven hybridomas cross-reacted with I-p179 and its non-iodinated analog (**Table 3.2**). The above data demonstrated that I-p179 and p179 elicited cross reactive clones; they also elicited monospecific T-cells, which reacted either with I-p179 only, or p179 only. Thus, TcR may exist that show a predilection for iodine-modified MHC-peptide complexes,

whereas other TcR may not tolerate the presence of iodine within the p179-MHC complex.

Table 3.1 Screening the specificity of T-cell hybridomas generated from p179-primed LNC

Reactivity of hybridomas generated from p179-primed LNC against p179 & I-p179							
hybridoma		p179 (1.25μM)		I-p179 (1.25μM)		No peptide	
specificity	name	Mean(cpm) *	S.D	Mean(cpm) *	S.D	Mean(cpm) *	S.D
p179	HJ 5G6	9422	1946	3350	310	2896	976
	HJ 5G2	16042	2184	243	33	278	244
	HJ 5C1	8929	810	1836	71	506	78
	HJ5E10	9720	386	1981	302	66	1
	HJ 5H8	6890	455	69	25	39	11
	HJ3G11	24608	1488	5109	1499	242	75
	HJ1F6	27517	2975	201	17	4607	6183
	HJ4C7	34739	1941	227	49	208	29
	HJ2D10	30460	1921	228	14	239	55
	HJ 3D12	14560	105	691	34	680	45
	HJ2D5	26021	3982	290	37	167	35
	HJ1F10	27926	2022	382	95	267	30
	HJ4B5	26414	88	263	133	152	40
	HJ5H11	38031	5745	392	34	224	59
	HJ1C1	27074	2784	428	131	242	0
cross-reactive to p179 & I-p179	HJ 5D11	14711	508	15181	597	43	22
	HJ 4F5	9908	692	11747	805	47	6
	HJ 5A4	10427	1180	12542	129	192	199
	HJ 5A12	11878	412	11207	1024	99	21
	HJ 4A10	5811	751	3454	482	101	37
	HJ 5B7	10850	1253	12471	926	117	101
	HJ5F2	26143	1896	21839	144	110	6
	HJ 3F12	8548	537	10467	907	309	18
	HJ1G1	23001	2708	27990	1189	176	21
	HJ2D8	31959	573	29439	998	280	98
	HJ5D12	8816	912	10509	798	117	122
	HJ3G5	19443	986	30049	1008	205	66
	HJ1D7	13006	3072	18392	6153	368	54
	HJ 2F11	17826	131	14572	1961	4318	5546
	HJ 2H2	16732	545	16081	180	147	40
	HJ 3D4	14890	626	15802	1953	283	21
	HJ3G1	34560	4804	39003	2230	336	33
non specific	HJ4A1	23024	752	26753	935	12574	12060
	HJ5D2	18754	12514	26750	4150	24451	2567
	HJ3F9	19281	675	18431	1606	9233	12420
	HJ4C5	32715	1329	36970	1356	29155	5259
	HJ1C4	216	52	2044	291	231	127
	HJ 5A11	10446	831	10923	147	7409	2990
	HJ3H5	23071	156	24424	1245	7860	8248
	HJ2D2	18798	1720	28018	655	29691	1703
	HJ5B11	18388	103	26210	647	25651	1932
	HJ4E4	667	4	797	25	412	155
	HJ 4G6	15583	18	15069	191	18017	129
	HJ 4F12	558	136	238	36	577	285
	HJ 3F6	16195	1122	19291	3465	14230	181
	HJ 3C1	4805	386	2837	259	3800	88
	HJ 3B11	16545	4337	17078	1752	233	146
	HJ 4G4	123	23	257	14	173	55
	HJ 4H12	17310	1635	7879	687	258	143
	HJ 4H6	9292	3174	7397	1049	5284	704
	HJ5E5	1788	220	535	148	194	173
	HJ 5C2	1458	187	188	42	83	19
	HJ5E3	32805	821	29736	637	10788	14489

Table 3.2 Screening the specificity of T-cell hybridomas generated from I-p179-primed LNC

Reactivity of hybridomas generated from I-p179 primed LNC against p179 & I-p179							
hybridomas		I-p179 (5μM)		p179 (5μM)		No peptide	
specificity	name	Mean(cpm) *	S.D	Mean(cpm) *	S.D	Mean(cpm) *	S.D
I-p179	HJ 4A11	37892	8597	1151	54	1079	575
	HJ 6B12	32539	4072	1483	387	1811	242
	HJ 3F4	17151	965	1115	30	1381	374
	HJ 1C7	35808	49	1636	17	460	19
	HJ 1D8	35591	7529	3485	270	5091	803
	HJ 2D12	17310	959	1242	0	565	62
	HJ 2G11	45665	1063	698	106	569	120
	HJ 3G11	42020	54	654	7	599	22
	HJ 4D11	16074	780	2804	2140	1220	12
	HJ 5E4	48018	1060	1009	141	897	127
cross-reactive to p179 & I-p179	HJ 4G8	42349	4606	44186	1678	927	237
	HJ 3A6	40810	2739	35502	3118	4857	3831
	HJ 4F12	27480	2749	16300	70	2186	560
	HJ 1A7	50780	977	49302	2470	1004	222
	HJ 3B7	40973	251	40249	3302	752	57
	HJ 5F12	28806	2001	48431	8126	2871	850
	HJ 1B3	32100	342	37174	70	2343	378
non specific	HJ 2F5	43944	7660	37117	3763	38557	5270
	HJ 6E9	1046	272	744	252	780	90
	HJ 3A12	1938	459	1622	93	1722	180
	HJ 2D1	1516	208	1876	1136	2117	128
	HJ 3C12	52099	7930	43452	2643	46566	1179

* The mean (cpm) of values of duplicate wells.

3.3.5 Variable effects of iodine on T-cell recognition at the clonal level

To examine possible effects of the iodinated Y192 on T cell recognition, we generated a panel of T-cell hybridoma clones: 1E7, 2D5 and 2H2 clones were obtained from p179-primed LNC, whereas 4A11 and 1B3 clones were generated from I-p179-primed LNC. Within the first set, it was found that 1E7 and 2D5 were activated only by p179 and that the incorporation of one iodine atom to Y192 abrogated their receptor engagement (**Figure 3.4A & B**). In contrast, clone 2H2 was activated by both p179 and I-p179 suggesting that the presence of iodine had a neutral effect on its TCR engagement (**Figure 3.4C**). Within the second set, clone 4A11 was activated only by the I-p179 analogue within the 1-125 nM range, demonstrating that the iodine atom was critical for TCR recognition (**Figure 3.4D**). On the other hand, clone 1B3 showed a preference for the iodinated analog but remained reactive to p179 indicating that the iodine atom was not an integral part of its ligand (**Figure 3.4E**). MAb-mediated blocking assays confirmed that all the above clones were E^k-restricted with the exception of 2D5 which is A^k-restricted (**Figure 3.5**). Furthermore, RT-PCR analysis showed that 1E7 utilized Vβ10, 4A11 utilized Vβ4, while 2D5, 1B3 and 2H2 utilized Vβ2 gene families. These results confirmed the clonal nature of these hybridomas and further suggested that there may be no strict segregation of Vβ family use according to the fine TCR specificity (**Figure 3.6**).

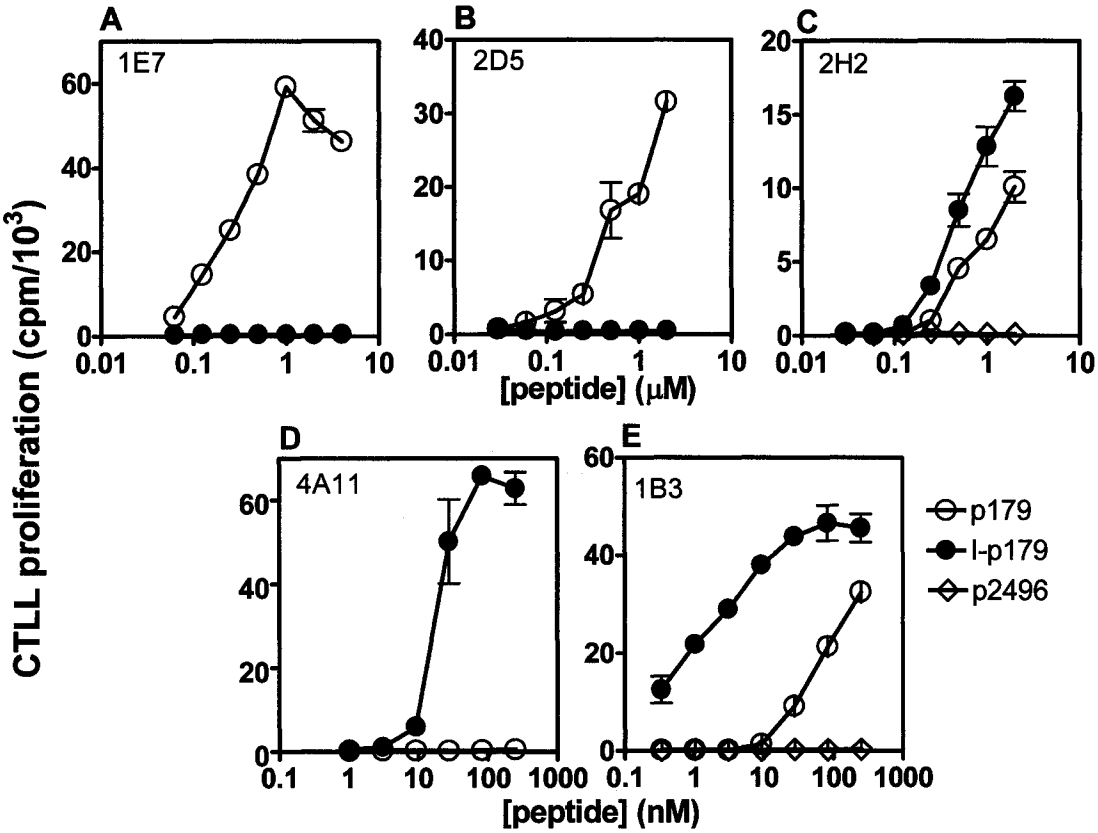

Figure 3.4 Variable effects of iodine on T-cell recognition at the clonal level. Hybridoma clones 1E7 (A), 2D5 (B) and 2H2 (C) were generated from p179-primed LNCs (1E7 and 2D5 were obtained from two independent immunizations.) And clones 4A11 (D) and 1B3 (E) were generated from I-p179-primed LNCs. LK35.2 cell line was used as APC. Data show mean ± SD of cpm values of triplicate wells and are representative of data from three independent assays.

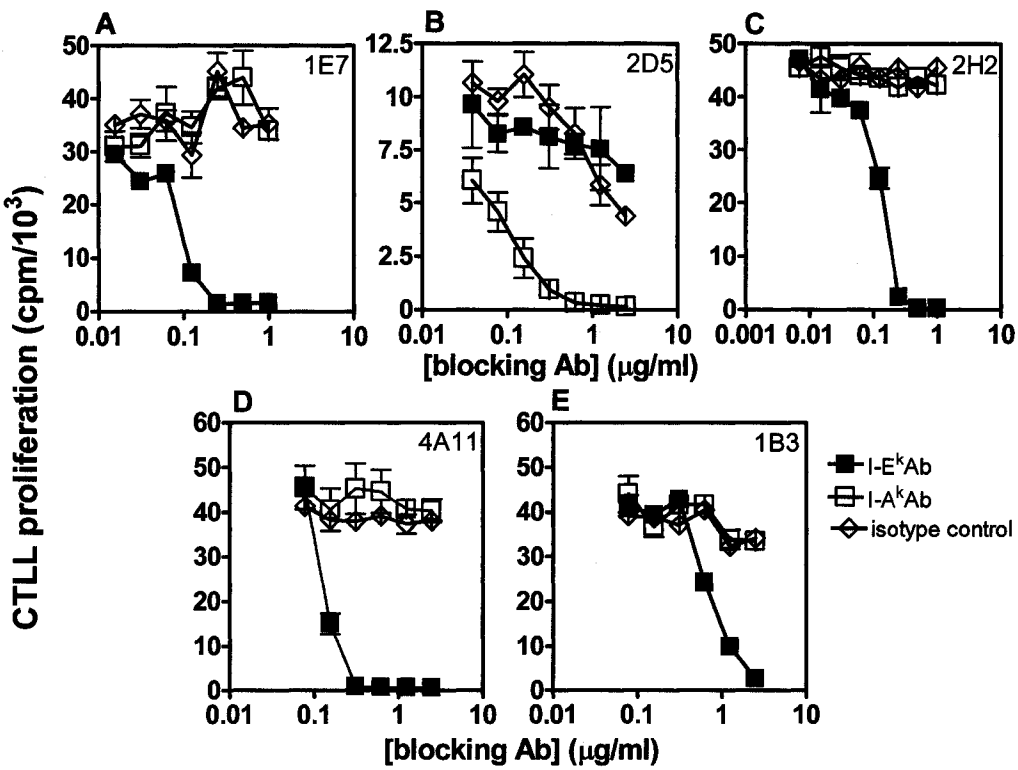

Figure 3.5 MHC-restriction of T-cell clones of 1E7 (A), 2D5 (B), 2H2 (C), 4A11 (D) and 1B3 (E). Reactions of T-cell clones to their ligands were measured in the presence of increasing concentration of blocking Abs, I-E^k Ab or I-A^k Ab. A mAb specific for influenza nucleoprotein was used as an isotype control. LK35.2 cell line was used as APC. Data showed mean ± SD of cpm values of triplicate wells and were representative of three experiments.

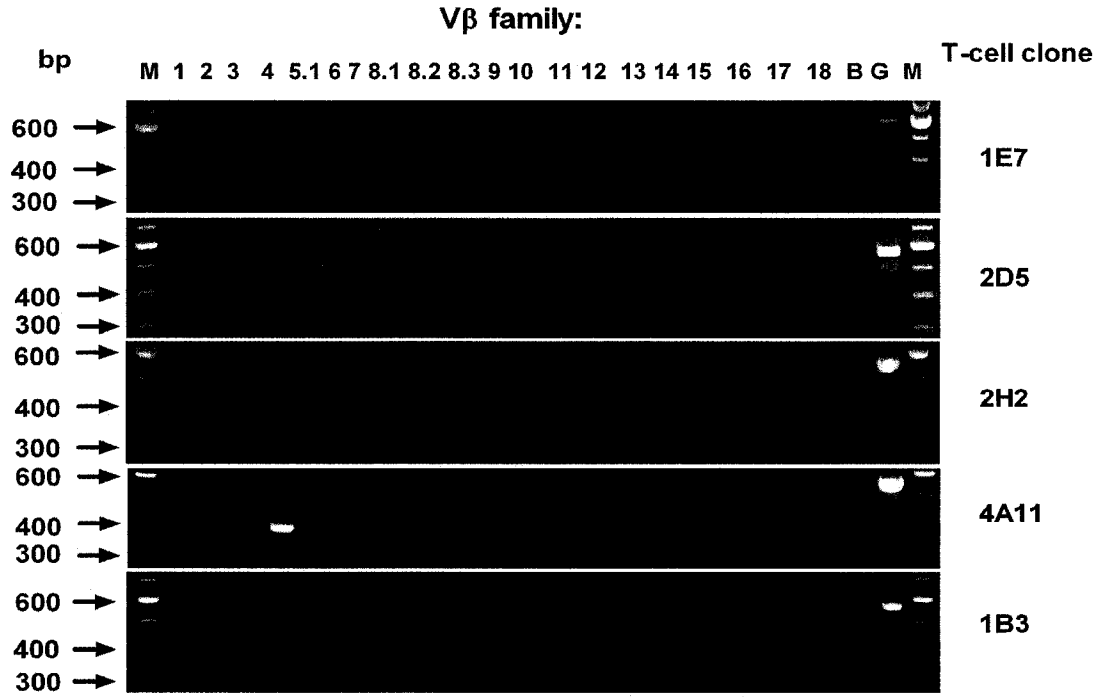

Figure 3.6 Vβ family utilization of T-cell clones. TCR Vβ utilization was examined by RT-PCR using Vβ-specific primers (Ju et al. 1995). M: Marker. B: Blank control. G: GAPDH.

3.3.6 Examination of fine specificity of hybridoma clones by truncation analysis

To investigate whether the fine specificity of the above clones was linked to recognition of distinct determinants within the a.a. 179-194 sequence, we performed truncation analysis using the peptide panel shown in **Figure 3.7A**. The clones 1E7 and 2D5 which reacted only against the p179 analog (pep.7) were found to respond to the 11mer Tg peptide p184 (pep. 5, aa.184-194) but remained unresponsive to the iodinated analogue I-p184 (pep.2) (**Figure 3.7B & C**). In contrast, the clone 2H2 responded to both 11mer analogs (pep. 2 and 5) (**Figure 3.7D**). Conversely, the clone 4A11 recognized only I-p184 (pep.2) not p184 (pep. 5) (**Figure 3.7E**), whereas clone 1B3 responded to both p184 and I-p184 analogs (**Figure 3.7F**). No clone was activated by either pep.3 or pep.6 suggesting that R194 is a critical residue. These results established: a) that the R194 residue is the C-terminal residue of the minimal epitope recognized by all clones and b) that the x-reactive clones 2H2 and 1B3 recognize an epitope that contains the Y192, i.e. they can tolerate the presence of the bulky iodine atom in this position.

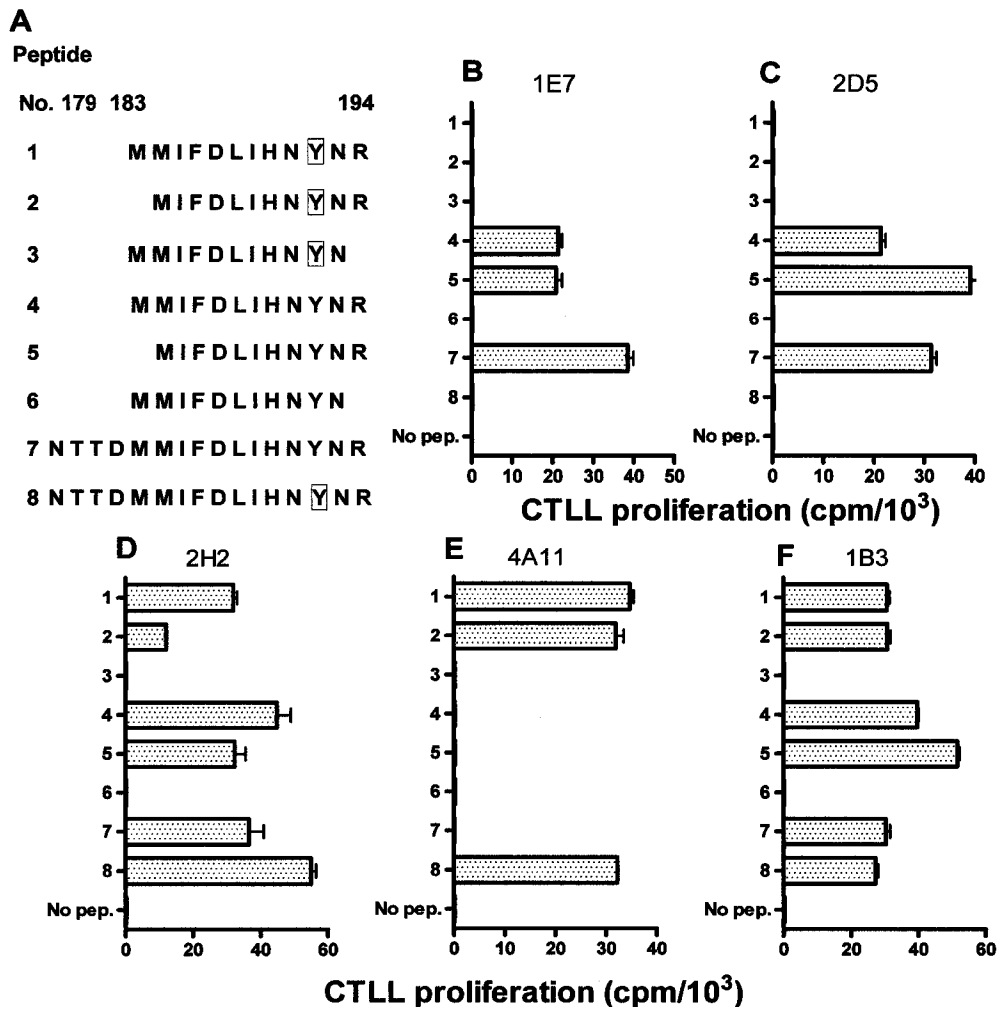

Figure 3.7 Truncation analysis of p179 and I-p179 by T-cell clones. A, Truncated peptide sequence. Peptide 7 and 8 containing full sequence as p179 and I-p179, respectively, worked as peptide quality control. Activation of hybrodomas 1E7 (B), 2D5 (C), 2H2 (D), 4A11(E) and 1B3 (F) was measured in the presence of 10 μM of indicated peptides. LK35.2 cell line was used as APC.

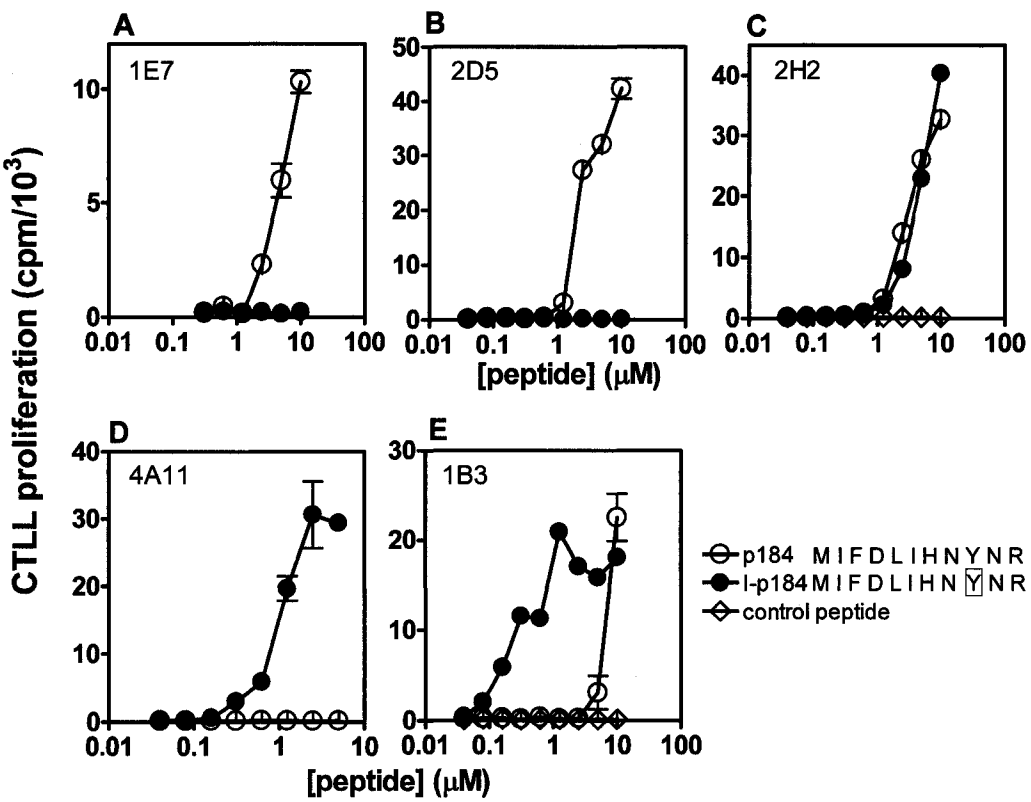

Figure 3.8 T-cell clone recognition of p184 and its iodinated analog. Hybridoma clones 1E7 (A), 2D5 (B) and 2H2 (C) were generated from p179-primed LNCs and clones 4A11 (D) and 1B3 (E) were generated from I-p179-primed LNCs. LK35.2 cell line was used as APC. Data show mean ± SD of cpm values of triplicate wells and are representative of data obtained in three assays.

3.4 Discussion

Tg is the only self antigen that stores iodide and organifies it in the form of iodotyrosyl or iodothyronine residues. This post-translational modification varies according to the environmental supply of iodine and has immunological consequences due to formation of neoantigenic determinants. For example, thyroid-infiltrating T cells are elicited by Tg peptides containing hormonogenic sites (Champion et al. 1991, Kong et al. 1995) or iodotyrosyls (Li and Carayanniotis 2006). Iodine can also have variable influences on peptides of known immunogenicity as it can increase, decrease or not alter their antigenic and immunogenic potency (Li and Carayanniotis 2006). In this context, we have described in a parallel study that iodine enhances the immunogenicity of the p179 peptide with no apparent effects on its pathogenicity (Li et al. submitted for publication). These observations and the prior finding that processing of hTg in HLA-DR3 transgenic mice generates the overlapping peptide (181-195) (Flynn et al. 2004) prompted us to examine how iodine influences recognition of p179 at the clonal T cell level.

Our results demonstrate that processing of mTg in vivo or in vitro seems to weakly prime p179-specific T cells, confirming its subdominant nature. However, mTg-primed LNC do not react against I-p179 suggesting that mTg does not harbour the iodinated analog under steady-state conditions. So far, subdominant Tg T-cell epitopes have been localized within seven Tg peptides: (179-194), (418-432), (1518-1532), (2079-2093), (2340-2359) (2549-2560) and (2695-2713) (Flynn et al. 2004, Carayanniotis et al. 1994, Karras et al.

2005, Hutchings et al. 1992). Identification of a dominant Tg epitope which can activate mTg-primed LNC at concentrations equimolar to those of intact Tg remains problematic, and it is uncertain to what extent a 330 kDa Tg monomer can be quantitatively processed in vitro to yield any given peptide with an approximately 150-fold smaller molecular mass (Hutchings et al. 1992, Carayanniotis et al. 1994).

The I-p179 analog binds to A^k or E^k molecules almost as well as p179, yet the presence of one iodine atom at Y192 can exert extremely variable influences at the clonal T cell level. Clones 1E7 and 2D5 must represent subsets of T cells whose fine specificity is negatively impacted by iodine, presumably through a steric hindrance effect. Conversely, the absence of iodine completely abrogates the reactivity of clone 4A11 which likely represents T cell subsets monospecific for the neoantigenic determinant. These cells will be elicited following animal challenge with I-p179, and they may even occur at a frequency higher than p179-specific T cells to account for the higher immunogenicity of I-p179. However, they are not expected to contribute to EAT if I-p179 is not constitutively expressed in normal Tg. Their putative effector function would be demonstrable only when increased ingestion of iodine might promote iodination of the Y192 residue. Lastly, the cross-reactive receptors on clones 2H2 and 1B3 may be expressed on effector T cells in EAT elicited by either analog, and may account, to a large extent, for the mild thyroiditogenicity of p-179 and I-p179.

The truncation analysis data mapped the minimal epitope recognized by all clones within the 11mer p184 (184-194) peptide. The R194 residue comprises the C-terminal aa residue of this epitope because its removal abrogates the reactivity of all clones. Although the N-terminal end of the minimal epitope has not been formally assigned, this sequence differs only at two aa positions (I185- V185 and N191- S191) from the homologous thyroiditogenic sequence in human Tg (Flynn et al. 2004). The data also demonstrate that the cross-reactivity of clones 2H2 and 1B3 cannot be explained by recognition of an identical minimal epitope lacking Y192. Instead, these findings argue for the presence, within the normal receptor repertoire, of TcR capable of tolerating the bulky iodine atom regardless of whether the initiating stimulus is p179 or I-p179.

In conclusion, the current data support the view that iodination of Tg may have pleiotropic effects on Tg-reactive T cell clones. Any given iodinated neoantigenic determinant may turn on some autoreactive T cells but simultaneously suppress others specific for the non iodinated analog. Thyroiditogenicity may be determined by the combined net effect of this modification at the polyclonal level. Overall, highly iodinated Tg may become more immunopathogenic than normal Tg because of: a) creation of iodinated neoantigenic peptides which preferentially activate thyroiditogenic T cells; and b) generation of non iodinated but cryptic pathogenic peptides generated by altered processing of Tg in APC (Dai et al. 2002). The potential role of each category of determinant in the development of clinical disease remains to be elucidated.

3.5 Future directions

Since p179 is generated following processing of intact Tg, it is plausible that I-p179 may be generated under conditions favoring iodotyrosyl formation at Y192. Future studies can examine whether I-p179 can be generated by processing of highly iodinated Tg (I-Tg), since normal Tg-primed LNC did not respond to I-p179, suggesting that I-p179 cannot be generated naturally following processing of normal Tg (unpublished data). To answer this question, CBA/J mice will be immunized with I-Tg and, then, the I-p179-specific proliferative response of I-Tg-primed LNC will be tested. If a significant proliferative response to I-p179 is detected, it will indicate that I-p179 can be generated by processing of Tg with increased iodine content. In a parallel study, we will examine whether the neoantigenic determinant generated by iodination of p179 is expressed in the thyroid. CBA/J mice will be given excess dietary iodine, such as 0.05 % NaI-containing water to enhance the iodine incorporation of Tg in thyroid for several weeks. Then, primary thyrocytes will be collected from these mice treated with excess iodine and cultured with INF-γ to up-regulate MHC-II molecules (Kimura et al. 2005). These IFN-γ-treated thyrocytes will be used as APC with pathogenic Tg determinants presented on their surface for activation assay of I-p179-specific T-cell clone 4A11. If a significant response is detected, it will indicate that the iodotyrosyl determinant within I-p179 is formed in the thyroid and it recruits a new subset of T-cells which home to the thyroid and are involved in the pathological damage.

The hybridoma clones generated in this study will provide very useful tools to address the following questions: First, p179 is pathogenic in CBA/J mice, as shown in previous studies (unpublished data), suggesting that p179 can be presented on thyroid. The present data show that there are two subsets of T-cells activated following p179 immunization. It is not clear which subset of T-cells leads to the thyroid lesion. We can use clones 1E7/2D5 and 1B3, representing these two subsets of T-cells, to examine which subset can be activated by thyrocytes which process and present Tg in thyroid. In the activation assay, primary thyrocytes from CBA/J mice can be used as APC. Second, we had observed that p179 contained subdominant epitope(s) since Tg-primed LNCs reacted to free peptide 179 at high concentrations. This might be due to the generation of p179 by processing of intact Tg in vivo being not efficient enough to stimulate a large number of peptide-specific T-cells. The subdominance of p179 in intact Tg can be tested by using p179-specific T-cell clones (1E7 & 2D5). Third, the parameters that influence the processing of Tg remain an open question. The pleiotropic cytokine interleukin (IL)-6 has been shown to alter antigen processing and presentation in dendritic cells (DCs) by changing the pH of peripheral, early endosomal compartments (Drakesmith et al. 1998). We can use these T-cell clones to further examine whether treatment of DCs with IL-6 could alter the hierarchy of the subdominant determinant(s) within p179 following processing of intact Tg.

CHAPTER 4

SEARCHING FOR DOMINANT A^k-BINDING PEPTIDES IN MOUSE TG

4.1 Abstract

So far, no dominant peptide has been identified in Tg due to its large size (dimeric MW 660 kD). Studies about antigen processing have shown that the dominant determinant(s) might be presented adjacent to endopeptidase cleavage sites. In this study, we searched A^k-binding motifs close to cleavage sites in mouse Tg (mTg) sequence by computerized algorithms to predict potential dominant determinants. According to the identified cleavage sites in human Tg (hTg), we have identified 11 corresponding cleavage sites in mTg, four cleavage sites for cathepsin L (P'_1 2388, P'_1 2451, P'_1 2490 and P'_1 2657), three cleavage sites for cathepsin B (P'_1 531, P'_1 795 and P'_1 2486) and four cleavage sites for cathepsin D (P'_1 550, P'_1 1833, P'_1 2467 and P'_1 2642). Subsequently, 20 amino acid sequences adjacent to these endopeptidase cleavage sites were scanned for A^k-binding motifs. A total of eight dominant Tg peptide candidates were unveiled in this study. Since these Tg peptides are the closest to cleavage sites in Tg sequence, they might have priority to bind to the A^k molecule, and be presented them on the surface of APCs to auto-reactive T-cells to elicit a dominant response to Tg. The potential dominance of above Tg peptides should be empirically examined in a future study.

4.2 Introduction

Twenty-three pathogenic Tg peptides have so far, been identified in Tg (Carayanniotis 2003, Flynn et al. 2004, Li and Carayanniotis 2006), but none of them comprises an immunodominant determinant (Carayanniotis and Rao 1997, Carayanniotis and Kong 2000). The observations that EAT is under control of the I-A^k subregion (Vladutiu and Zaleski 1981, Beisel et al. 1982) and administration of anti-A^k Abs protects mice from EAT induction (Vladutiu and Steinman 1987) strongly suggest that Tg carries dominant A^k-binding determinant(s). Following these clues, Verginis *et al.* searched for the complete mTg sequence to detect dominant pathogenic T-cell epitopes containing A^k-binding motifs (Verginis et al. 2002). Due to the large size of Tg, a total of 104 A^k-binding sites were found. Therefore, it became apparent that it is unlikely to identify dominant epitope(s) simply depending on this approach.

Antigen processing studies have indicated that determinants close to endopeptidase cleavage sites become more easily available to bind to MHC molecules (Sercarz et al. 1993, Schneider et al. 2000). This renders it likely that dominant determinant(s) are localized adjacent to endopeptidase cleavage points. Data analysis following proteolysis of rabbit Tg by human cathepsins L, B and D allowed Dunn *et al.* to identify the cathepsin cleavage points in the human Tg sequence (Dunn et al. 1991). Based on the above observations, we proceeded to identify the cathepsin cleavage points in mTg and

search for putative dominant A^k-binding peptide(s) localized close to these cleavage points.

4.3 Results

4.3.1 Algorithm based putative dominant A^k-binding peptides in mTg

Extrapolating from the known cathepsin cleavage sites in the hTg sequence (Dunn et al. 1991), we identified homologous sites within mTg sequence (**Table 4.1**). The four cathepsin L cleavage sites showed P'_1 residue at a.a. positions 2388, 2451, 2490 and 2657; the three cathepsin B cleavage sites showed P'_1 residue at a.a. positions 531, 795 and 2486; the four cathepsin D cleavage sites showed P'_1 residue at a.a. position 550, 1833, 2467 and 2642 (**Table 4.1**). We next searched for A^k-binding motifs (Altuvia et al. 1994) within 20 a.a. residue segments adjacent to the above endopeptidase cleavage sites. Eight peptides met this rule in the mTg sequence (**Table 4.2**). We arbitrarily defined the boundaries of these peptides by adding five a.a. residues at the N- and C-termini of the identified A^k-binding motifs. The final eight sequences varied from13-17 a.a., which is a favorite length for MHC class II-binding peptides. Three of eight Tg peptides (p2369, p2438 and p2490) were located closely to cathepsin L cleavage points (P'_1 2388, P'_1 2451 and P'_1 2490); four (p557, p1822, p1838 and p2467) were found adjacent to cathepsin D cleavage points (P'_1 550, P'_1 1833 and P'_1 2467), and two of them, p1822 and p1838 were at both sides of cleavage site P'_1 1833. Only one peptide (p771) was found close to the

cathepsin B cleavage site P'_1 795. Coincidentally, one of the above Tg peptides, p2369 also has three E^k-binding motifs (Altuvia et al. 1994), indicating that this peptide could be presented in the context of both A^k and E^k molecules.

Table 4.1 The possible cleavage sites in mTg by cathepsin L, B and D

Endopeptidase	P_4-P_1 sequence[a]	P'_1-P'_{n+1} sequence[b]	P'_1 residue no.[c]
Cathepsin L	QLFR	KALLMGGSALS	2388
	KLLA	VSGPFHYWGPVVDGQ	2451
	LIGG	SQDDGLINRA	2490
	FSRK	AAEFATPWP	2657
Cathepsin B	RVVG	NFGFKVNLQENQDAL	531
	REVA	SRNFSLFLQ	795
	KVDL	LIGGSQDDGLINRA	2486
Cathepsin D	FLVS	SLLELPEFLVFLQR	550
	ATEL	FSPVDITQVI	1833
	DGQY	LRELPSRRLKRPL	2467
	FSNF	IRSGNPNYPHEFS	2642

[a] P sequences are based on the cDNA-derived sequence of mTg (Kim et al. 1998).

[b] Homologous rabbit Tg residues identified by Dunn *et al.* (Dunn et al. 1991) are underlined.

[c] P' residue no. is based on the cDNA-derived sequence of mTg (Kim et al. 1998).

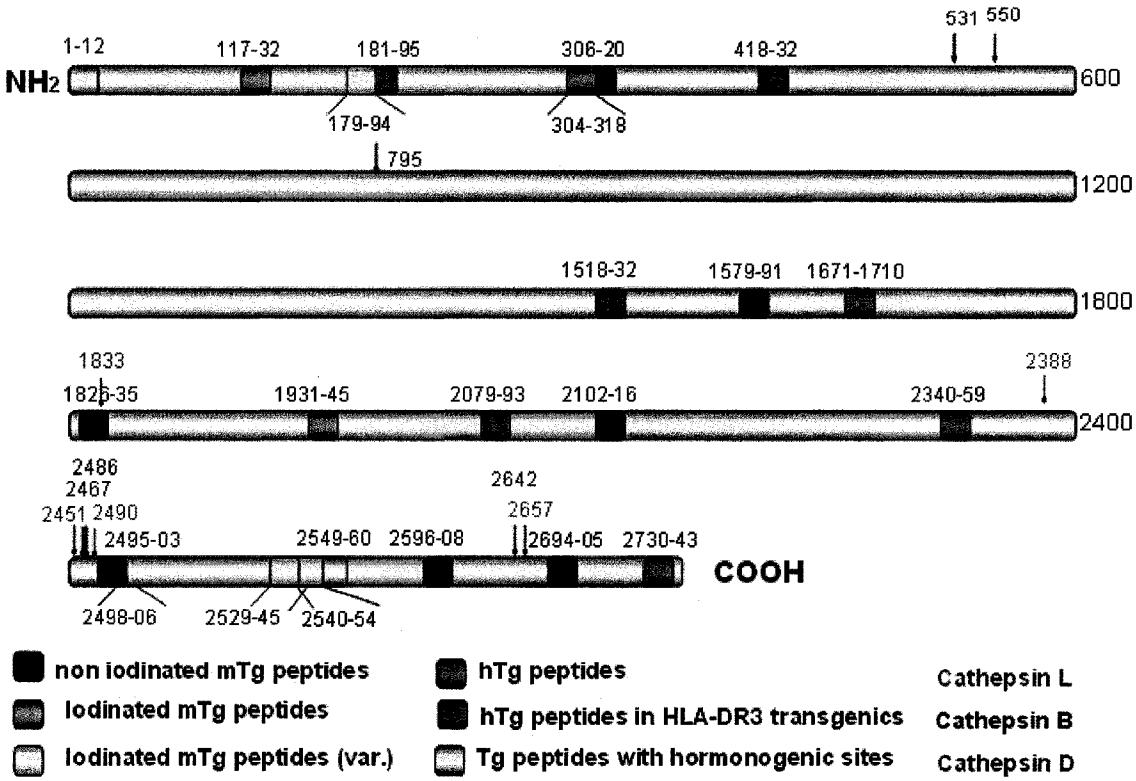

Figure 4.1 Cathepsin cleavage sites within mTg sequence are indicated by arrows. Numbers inducate the a.a. coordinates of Tg peptides with known pathogenicity and/or T-cell reactivity.

Table 4.2 Putative A^k-binding peptides adjacent to possible cleavage sites within mTg

Amino acid coordinates[a]	Motif-containing sequence[b]	Cleavage site P'	Peptide denotation
557-573	FLVFLQRAVSVPEDIAR	550	p557
771-787	QNGDGQELTPAALLMKI	795	p771
1822-1832	SDFPG**DMATE**L	1833	p1822
1838-1850	ITQVIVNTSHSLP	1833	p1838
2369-2387	DVASIHLLISRPTRLQLFR[c]	2388	p2369
2438-2450	ANILNDAQTKLLA	2451	p2438
2467-2478	LRELPSRRLKRP	2467	p2467
2490-2504	SQD**DGL**INRAKAVKQ	2490	p2490

[a] Amino acid coordinates are based on cDNA-derived sequence of mTg (Kim et al. 1998).

[b] The underlined sequences represent motif A: [DEHNQ]-{NQ}-X- [ILTV]-X-{FHWY}-[ACFILMPTVWY]; the bold sequences represent motif B: [CDEHNQY]-{DE}-X-[ILTV]-[DEHQN]; the squared sequences represent motif C: [CILNPTV]-[CHNSTY]-[CKNRST]; [] represents inclusion and {} represents exclusion of indicated amino acids at this position, X denotes any amino acid at this position (Altuvia et al. 1994)

[c] This peptide also contains an E^k-binding motif A: [ACFILMPTVWY]-X-[ACFILMPTVWY]-{DE}-{DE}-{HKR}-{FHWY}-[KR] (Altuvia et al. 1994) (shown in p2369 as ASIHLLISR); two E^k-binding motifs C: [AILSTV]-{NQ}-[ACFILMPTVWY]-{DE}-[ACFILMPTVWY]-{NQ}-[DEKNQR] (Altuvia et al. 1994) (shown in p2369 as IHLLISR and TRLQLFR).

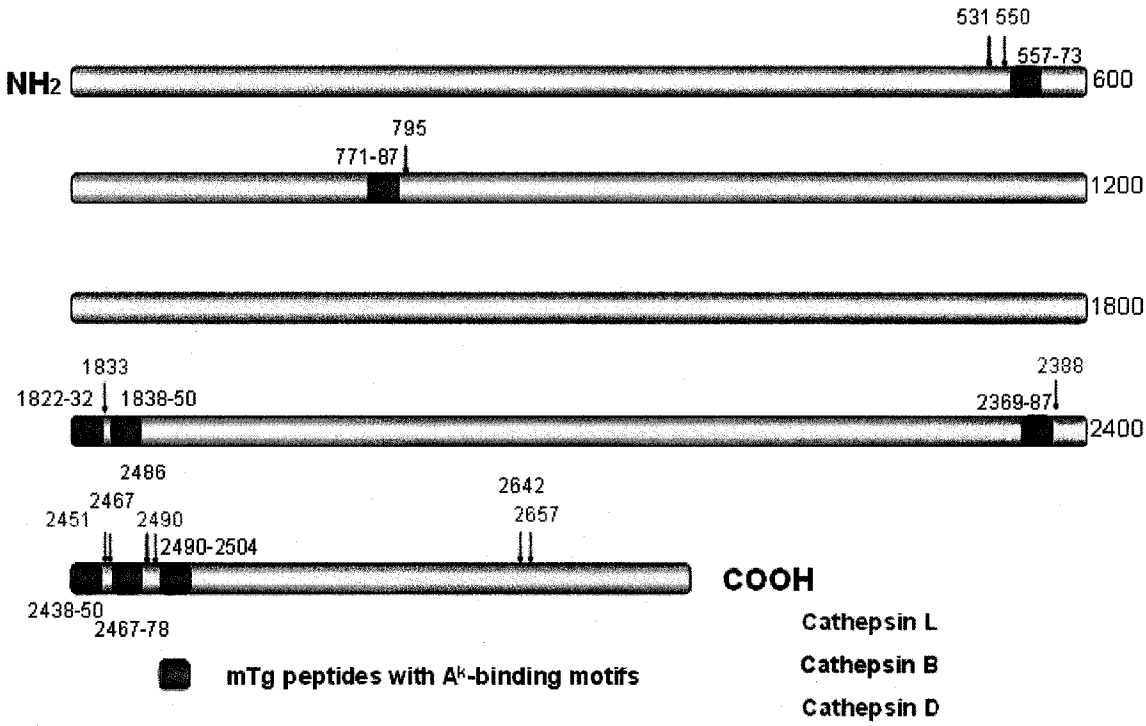

Figure 4.2 Mapping putative dominant mTg peptides with A^k-binding motifs adjacent to cathepsin cleavage sites. Numbers indicate the a.a. coordinates of these peptides.

4.4 Discussion

Despite the fact that 23 pathogenic epitopes have so far been mapped in Tg, none of them meets the conventional criteria for immunodominance, i.e. is not recognized by Tg-primed LNC in vitro and conversely do not prime LNC so that they can be stimulated equally well by equimolar amounts of free peptide or intact Tg in vitro. There has been some skepticism as to whether the large Tg molecule can be efficiently processed in vitro within the 1-10 μM (330-3300 μg/ml) concentration range to yield any given peptide (Carayanniotis 2003, Flynn et al. 2004, Li and Carayanniotis 2006, Carayanniotis and Rao 1997, Carayanniotis and Kong 2000). However, dominant epitopes could still exist but remain undiscovered because of the large size of Tg and the application of the particular methodologies used.

In this study, we explored, on a theoretical basis, the number as well as the localization of putative dominant Tg epitopes based on a rationale different from those applied before in the field. We took into account the results of an earlier study which identified cleavage sites of rabbit Tg by human cathepsins B, D, L (Dunn et al. 1991). The P'_1-P'_{n+1} a.a. were sequenced in proteolytic fragments and the homologous points were localized in hTg since the primary sequence of rabbit Tg is not available. We then extrapolated these sites into the known homologous sequence of mTg. We further took into account a view, expounded mainly by E. Sercarz, that immunodominant peptides are usually localized

close to endopeptidase cleavage sites (Sercarz et al. 1993, Sercarz 2002). According to this view, crypticity may result from either excessive or limited proteolysis of an antigen, facilitating the destruction or the poor generation of a given determinant, respectively. This concept was supported by studies with the myelin basic protein (MBP) peptide (89-101) (Anderton et al. 2002, Manoury et al. 2002). There are three overlapping determinants in this peptide, (89-94), (92-98), and (95-101), but T-cell responses were detected only against the two flanking determinants, when intact MBP was used as an immunogen (Anderton et al. 2002). It was subsequently found that there is an asparagine endopeptidese (AEP) cleavage site at Asn^{94} which probably ruins MBP (92-98) during processing, leading to the crypticity of this peptide (Manoury et al. 2002).

This concept is also eloquently supported by the map of the 23 known pathogenic peptides in Tg. Twenty one of these determinants are found far from endopeptidase cleavage points (**Figure 4.1**). Enzymatic processing of Tg may not adequately expose these peptides, rendering them as cryptic. On the other hand, the A^k-restricted mTg peptide (1826-1835) (Verginis et al. 2002), which has been shown to be thyroiditogenic in CBA/J mice, contains a cathepsin D cleavage site (P'1833) and therefore, p1826 may be destroyed during Tg processing of intact Tg by cathepsin D. An interesting case can be made for the pathogenic but cryptic Tg peptide (2495-2503), presented in the context of E^k and A^s (Rao et al. 1994), which maps closely to the cathepsin L cleavage site (P'2490). According to the view presented above this peptide should have been likely to behave as dominant. However, we have found an A^k-binding motif (a.a. 2493-2497) right after the

P'_1 2490 cleavage point, overlapping two a.a. with p2495. Thus, there may be an additional A^k-restricted epitope at this site which, as the first available epitope, may have priority over p2495 to bind to MHC, rendering p2495 as cryptic.

Among the eight new putative A^k-binding peptides (**Figure 4.2**) adjacent to possible endopeptidase cleavage sites, two, p1822 (a.a. 1822-1832) and p2490 (a.a. 2490-2504) overlap with the previously identified pathogenic peptides p1826 and p2495 (Verginis et al. 2002, Rao et al. 1994). p1826 contains a cathepsin D cleavage point (P'1833) and p2490, although identified earlier as a putative A^k-binder (Verginis et al. 2002), it was never synthesized and tested because of its overlap with p2495. The N-terminus of p557 and p1838 was ≥ 6 a.a. away from the cathepsin cleavage sites and the C-terminus of p771 was 9 a.a. away from the cathepsin B cleavage site (P'_1 795) (**Figure 4.2**). These peptides, if generated, may require further a.a trimming by exopeptidase before or after MHC binding in order to form T-cell activating determinants.

The analysis described herein has several caveats that need to be addressed. First, it is not known whether mouse cathepsins can cleave mTg at the indicated sites extrapolated from the hTg sequence. Second, enzymatic activity may be influenced by cytokines resulting in alternate modes of processing within APC. For example, IL-6 has been shown to alter the hierarchy of T-cell determinants by influencing the pH of early endosomes in DCs (Drakesmith et al. 1998). Third, and most importantly, the source of APC may play a pivotal role in what enzymes participate for the generation of the given determinants. It

should be emphasized that, despite their crypticity, all known Tg epitopes are pathogenic, i.e. they must be generated intrathyroidally and presented in the context of MHC class II molecules to be seen as targets of adoptively transferred $CD4^+$ effector T cells. Currently, it is not clear what cells carry out this APC function in the thyroid, but they must comprise either DC or thyrocytes with upregulated class II MHC expression (Kimura et al. 2005). The present theoretical study aims to identify peptides with immunodominant characteristics in APC of peripheral lymphoid tissues, as outlined by Sercarz (Sercarz et al. 1993, Sercarz 2002).

4.5 Future Directions

We have identified 8 peptide sequences that express the combined features of being mapped close to cathepsin cleavage sites and carrying A^k-binding motifs. The working hypothesis is that at least some of them may contain immunodominant Tg determinants. These peptides can be synthesized at high purity (>90%), emulsified in CFA and delivered into several strains of mice of the H-2^k haplotype. The capacity of these peptides to elicit T-cell and/or B-cell responses, as well as EAT will then be examined. Most importantly, peptide-primed LNC from draining lymph nodes can be assayed for their reactivity to intact Tg in vitro; and conversely, Tg-primed LNC can be tested for their proliferation to each peptide. Positive response in both of these tests will support the discovery of immunodominant determinant(s).

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