EXPERIMENTAL AUTOIMMUNE THYROIDITIS INDUCED IN MICE BY THYROGLOBULIN T-CELL DETERMINANTS

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by

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Dedicated to my parents

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&

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ABSTRACT

Experimental autoimmune thyroiditis (EAT) induced in mice following challenge with thyroglobulin (Tg) in adjuvant, serves as a model for Hashimoto's thyroiditis (HT). Earlier work in our laboratory established that EAT can be induced in mice following challenge with a 17mer MTg(2495-11) or an 18mer MTg(2695-13) peptide, in complete Freunds adjuvant: (2495-11) peptide induced thyroiditis in H-$2^{k, s}$ haplotypes while (2695-13) peptide induced disease only in H-2^{s} haplotype. In the present study, truncation analysis using a panel of peptide-specific TCR $\alpha\beta^{+}$ $CD4^{+}$ $CD8^{-}$ T-cell hybridomas from EAT-susceptible C3H mice, identified two overlapping 9mer minimal determinants (2496-04) and (2499-07) within MTg(2495-11). The E^{k}-restricted (2496-04) determinant was immunogenic and pathogenic not only in C3H but also in SJL mice. Further, the determinants within MTg(2495-11) recognized by A^{s} -restricted SJL T cells were mapped identical to those restricted by the E^{k} and A^{k} molecules. MTg(2496-04)-primed lymph node cells (LNC) secreted IL-2, IFN-γ but not IL-4 upon specific activation *in vitro*, suggesting that induction of Th1 cells follows priming with the minimal Tg peptide in SJL mice. In addition, TCR-Vβ 2, 4 and 17 genes were utilized by a panel of ten MTg(2496-04)-specific IL-2-secreting hybrid T-cell clones generated from this Th1 subset, thus providing the first evidence of multiple TCR-Vβ gene usage in EAT induced with a minimal Tg epitope. In parallel, work involving the 18mer MTg(2695-13) peptide established a panel of TCR $\alpha\beta^{+}$ $CD4^{+}$ $CD8^{-}$ IL-2 secreting T-cell hybridomas from SJL mice. Using two A^{s}-restricted (2695-13)-specific T-cell hybrids and three overlapping 12mer peptides spanning the core, N- and C- terminal regions of MTg(2695-13), two

distinct T-cell determinants were localized within the 18mer peptide. The N-terminal 12mer (2695-06) was highly immunogenic and induced severe EAT following adoptive transfer of peptide-specific Th1 cells. A human homologue of MTg(2695-06) carrying two Ser substitutions of GLn2703 and Thr2704, on the other hand, showed contrasting immunopathogenic properties: it failed to activate Th1 cells; it did not cross-react with MTg(2695-06)-specific T cells and induced only mild thyroiditis. These findings highlight caution against extrapolating the epitope mapping data across heterologous Tgs despite their high homology. Finally, a computer search of SWISS-PROT data bank to identify sequences highly homologous with pathogenic Tg determinants, has led to identification of a 14mer adenoviral E1B(368-81) peptide (AVP). The viral peptide cross-reacted significantly with MTg(2696-04) at B- and T- cell level. AVP, however, failed to induce specific B- or T- cell responses. Despite its poor immunogenicity, AVP, when used as an Ag *in vitro*, conferred the EAT-inducing ability on MTg(2696-04)- primed LNC in adoptive transfer experiments. These findings suggest that viral peptides, such as AVP, when generated during infection, can amplify autoreactive T cells (via molecular mimicry) present in the host and thus, precipitate autoimmune thyroid disease.

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a. a.	amino acid
Ab	antibody
ADCC	antibody-dependent cell mediated cytotoxicity
Ag	antigen
AITD	autoimmune thyroid disease
APC	antigen presenting cell
ATCC	American Type Culture Collection
AVP	adenovirus peptide
BB/W	Bio-breeding/Worcester
BSA	bovine serum albumin
BSS	balanced salt solution
BTg	bovine thyroglobulin
BUF	Buffalo
cAMP	cyclic AMP
cDNA	complementary DNA
CIA	collagen-induced arthritis
CFA	complete Freund's adjuvant
con A	concanavalin A
CS	Cornell strain
cpm	counts per minute
^{51}Cr	^{51}Chromium
CTL	cytotoxic T lymphocyte
CTLL	cytotoxic T lymphocyte line
DIT	diiodotyrosine
DMEM	Dulbecco's modified Eagle's medium
DTH	delayed-type hypersensitivity
EAE	experimental autoimmune encephalomyelitis
EAT	experimental autoimmune thyroiditis
EAU	experimental autoimmune uveitis
EC	endothelial cell
ELISA	enzyme linked immunosorbent assay
FACS	fluorescence activated cell sorter

FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
GD	Graves' disease
HA	haemagglutination
HAT	hypoxanthine/aminopterin/ thymidine
HBSS	Hanks' balanced salt solution
HEL	hen egg lysozyme
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethane-sulphonic acid
HLA	human leukocyte antigen
HPLC	high-performance liquid chromatography
HT	Hashimoto's thyroiditis
HTg	human thyroglobulin
hTPO	human thyroid peroxidase
IDDM	insulin dependent diabetes mellitus
IFA	incomplete Freund's adjuvant
IFN-γ	interferon-γ
Ig	immunoglobulin
IL-2	interleukin-2
IL-4	interleukin-4
IL-5	interleukin-5
IL-10	interleukin-10
i. p.	intraperitoneal
ITL	intrathyroidal lymphocytes
i. v.	intravenous
kDa	kilo dalton
LNC	lymph node cells
LPS	lipopolysaccharide
m.wt.	molecular weight
mAb	monoclonal antibody
MBP	myelin basic protein
2-ME	2-mercaptoethanol
MHC	major histocompatibility complex
MIT	monoiodotyrosine
MLR	mixed lymphocyte reaction

MNC	mononuclear cells
mRNA	messenger RNA
MS	multiple sclerosis
MTg	mouse thyroglobulin
NOD	non obese diabetic
NP	influenza A nucleoprotein
O. D.	optical density
OVA	ovalbumin
PAB	PBS with 0.1% sodium azide plus 0.5% BSA
PBMC	peripheral blood monuclear cells
PBS	phosphate buffered saline
PBST	PBS with 0.1%Tween-20
PE	phycoerythrin
PPD	purified protein derivative
PTg	porcine thyroglobulin
pTPO	porcine thyroid peroxide
RA	rheumatoid arthritis
RFLP	restriction fragment length polymorphism
RTg	rat thyroglobulin
SAT	spontaeneous autoimmune thyroiditis
s. c.	subcutaeneous
SCID	severe combined immunodeficiency
S. I.	stimulation index
ST	subacute thyroiditis
T_4	thyroxine
T_3	triiodo thyronine
TCR	T-cell receptor
TEC	thyroid epithelial cell
Tg	thyroglobulin
TGF-α	transforming growth factor-α
TGF-β	transforming growth factor-β
Th	helper T lymphocyte
TPO	thyroid peroxidase

TRH	thyrotrophin-releasing hormone
TSH	thyroid stimulating hormone
TSH-R	thyroid stimulating hormone-receptor

CHAPTER 1

INTRODUCTION

1. Thyroid gland

1. 1 Structure and Physiology

The thyroid gland is specialized to synthesize, store and release thyroid hormones (T_3 and T_4). In humans, it consists of two pear-shaped lobes that lie on either side of the trachea. Both lobes are joined by an isthmus that lies over the second to fourth tracheal rings. The thyroid follicle (200-300 μm in diameter), a spheroidal structure lined by parenchymal epithelium, encloses the protein thyroglobulin (Tg) and is the functional unit of the thyroid gland. The shape of the follicular epithelial cells changes from normal cuboidal to columnar under excessive stimulation with thyroid stimulating hormone (TSH) (McDougall, 1992). Iodide (I^-) is selectively concentrated within thyrocytes and transported transepithelially into the follicular lumen by means of specific mechanisms (Nilsson 1995). In an extracellular process catalyzed by thyroid peroxidase (TPO), I- is oxidized and coupled to tyrosine residues on the Tg resulting in the formation of monoiodotyrosine (MIT) and diiodotyrosine (DIT). Although large amounts of MIT and DIT are generated, coupling of only some iodinated tyrosines occurs at fixed positions on the Tg molecule. Synthesis and release of thyroid hormones is basically under the control of TSH secreted by the anterior pituitary. TSH not only serves to maintain active iodide transport and iodide peroxidase system but also it stimulates hormonogenesis through a cAMP-responsive system and a phosphatidyl-inositol system (DeGroot et al., 1996). In

addition to the Tg, which comprises 70-80% of the protein, the thyroid gland also contains other iodoproteins (2-13%) such as the thyroalbumin, an iodinated albumin-like protein containing MIT, DIT and iodothyronines that are believed to enter the thyroid gland from the serum (DeGroot et al., 1996).

1. 2 Thyroidal antigens

1. 2. 1 Thyroglobulin

Thyroglobulin (Tg), a huge and highly conserved glycoprotein of 660 kD, encoded by a gene on chromosome 8 in humans, is synthesized in the endoplasmic reticulum of thyroid follicular cells (thyrocytes). After synthesis, Tg is post translationally modified in the Golgi complex, and eventually secreted into the follicular lumen (Van Herle et al., 1979; Ekholm and Bjorkman, 1990). Two monomers of human Tg (HTg) (17 S), each composed of 2748 residues (19 S if iodinated), are linked by disulfide bonds. Iodinated Tg tends to form aggregates while iodine deficiency causes Tg to dissociate more easily into subunits (Rossi et al., 1973) which are degraded by thyroid acid proteases (Lamas and Ingbar, 1978). Tg molecule has 140 tyrosyl residues, of which, about 40 can be iodinated and 8-10 are hormonogenic sites. However, Tg normally contains one T3 and two or three T4 molecules. Immunologically, Tg is not a sequestered antigen as it is continuously released along with T_3 and T_4 into the circulation (Carayanniotis and Rao, 1997). Based on internal homologies, the Tg sequence has been subdivided into 4 domains: Domain A has ten Tyr- and Cys- rich repeats of 50 a. a. each between positions 29 and 1196; Domain B has three repeats of 14-17 highly conserved residues between positions 1436-1483; Domain C has five repeats between positions 1583 and 2109; and Domain D lacks internal

homology (Medeiros-Neto et al., 1993). Amino acid (a. a.) sequence comparison of Tgs from human, bovine Tg (BTg) and rat Tg (RTg) (known portion) revealed: 73-77% identity; 6-7% conservative substitutions and 16-19% dissimilarity (Carayanniotis and Rao, 1997). Due to this high homology between them, Tgs from various species cross-react at the B- or T- cell level.

1. 2. 2 Thyroid peroxidase and thyroid stimulating hormone receptor

Thyroid peroxidase (TPO), also now known as microsomal antigen, is an important enzyme that oxidizes iodide in the presence of H_2O_2 and is situated on the luminal surface of the microvilli of the thyrocytes. In 1957, Trotter and co-workers described reactivity in sera from patients with autoimmune thyroid disease (AITD) to the 'thyroid microsomal antigen'. Several studies done in the mid 1980s had confirmed the identity between the microsomal antigen and the TPO (Czarnocka et al., 1985; Portmann et al., 1985 and 1988). TPO is a transmembrane glycoprotein, of 107 kD in size and 933 a. a. in length, encoded by a gene on chromosome 2p13. It shares 44 % a. a. sequence similarity with myeloperoxidase and has domains similar to acetylcholinesterase and receptors of low density lipoprotein and insulin-like growth factors. Expression of TPO mRNA can be rapidly induced by TSH or cAMP through transcriptional activation (DeGroot et al., 1996). The two important steps in the biosynthesis of thyroid hormones that are controlled by TPO are: first, the iodination of tyrosine residues on Tg and second, the coupling of iodinated tyrosines to form T_3 and T_4. The presence of anti-TPO antibodies in the majority of patients with thyroid dysfunction seems to emphasize the possible role of this thyroidal antigen in AITD.

TSH receptor (TSH-R) is a 120 kD protein expressed on the thyrocytes, that binds to the TSH, the major trophic hormone for the thyroid. Encoded by a gene on chromosome 14, TSH-R is 764 a. a. in length and consists of seven hydrophobic transmembrane regions spanning 346 a. a. and an extracellular portion of 398 a. a. to which TSH binds. The extracellular N-terminal half of TSH-R of human, dog and rat share 85-90% homology (Nagayama and Rapoport, 1992). A 10mer (441-450) sequence of the cytoplasmic C-terminal of TSH-R was shown to be important in generating cAMP response (Chazenbalk et al., 1990) while N-terminal a. a. 38-45 sequence of the receptor had been shown to be crucial for TSH binding (Wadsworth et al., 1990). The extracellular portion of the TSH-R has been shown to be highly immunogenic confirming its importance in GD (Murakami and Mori, 1990).

2. Thyroid autoimmunity

2. 1 Autoimmune thyroid disorders: an overview

AITD is considered an example of an organ-specific autoimmune phenomenon, although patients with autoimmune thyroid disease often exhibit certain associated syndromes including vitiligo, myasthenia gravis, pernicious anemia, rheumatoid arthritis and Sjogren's syndrome. The etiology of these disorders remains complex and their relationship to thyroid autoimmunity is unclear at present (DeGroot et al., 1996). An attempt has been made here to describe AITD from an immunological standpoint.

Depending upon the presence or absence of specific peripheral blood lymphocytes sensitized to thyroidal Ag, human thyroid disorders have been classified as primary or secondary. The primary AITDs include: Graves' disease

(GD), Hashimoto's thyroiditis (HT), lymphocytic thyroiditis of childhood and adolescence, post-partum thyroiditis, chronic fibrous thyroiditis, idiopathic myxoedema and atrophic asymptomatic thyroiditis (Volpé, 1990). Among the various forms of autoimmune thyroid disorders, HT is the most common, affecting people of all ages and both sexes, although it is shown to occur more frequently in women of age 40-60 years (9-25 times higher) than in men of the same age (McDougall, 1992). GD has been recorded in people of all ages, however, it occurs seven times more commonly in women than men of 30-40 years age (Dwyer, 1994). Despite the fact that GD and HT represent the opposite ends of the spectrum of thyroid function, co-occurrence of both the diseases in families and, clinical transition of one disorder to the other within the same individual over time have led some to believe they are pathogenetically related (DeGroot et al., 1996). However, others believe they are distinct entities (Volpé, 1990, Dwyer, 1994). When large populations are compared, differences in HLA associations with GD or HT are apparent; e.g. HLA-DR3 was found to be associated with GD while HLA-DR4 or 5 was found in higher frequency in HT cases (Volpé, 1990).

2. 2 Hashimoto's thyroiditis

Since its first description as "struma lymphomatosa" in 1912 by Hashimoto to characterize the lymphocytic infiltration, eosinophilia of thyroid epithelium and interstitial fibrosis of thyroid glands of four middle aged women, the term Hashimoto's thyroiditis is now used to embrace other variants of thyroid disease including lymphocytic thyroiditis, postpartum thyroiditis, chronic fibrous thyroiditis, idiopathic myxedema etc. (Volpé, 1990).

Over forty years ago, Roitt et al., (1956) detected antibodies against Tg in sera of patients with HT, while Rose and Witebsky (1956) demonstrated induction of thyroiditis in rabbits following immunization with thyroid extract. The etiology of HT still remains elusive (Volpé, 1991) despite much progress in the characterization of autoimmune responses in general.

Epidemiological studies indicated that HT prevalence is increasing. HT affects 0.5 -1.2% of the general population and it is believed that there exist many subclinical cases with focal thyroiditis that can only be diagnosed by autopsy (Bigazzi, 1993). It is estimated that in most populations, 10% of individuals have positive Tg and TPO antibody .

On clinical examination, most patients present with a thyroid gland that may be firm, nodular or diffusely enlarged but rarely painful and tender. There are usually no systemic symptoms unless the patient is severely hypothyroid (reviewed in McDougall 1992). Some may switch from a hypothyroid to hyperthyroid condition perhaps a process reflective of whether inhibiting or stimulating antibodies are produced (Sung and McDougall, 1978). Most patients with autoimmune thyroiditis have antibodies that can react *in vitro* with Tg, microsomal Ag or other cell surface autoantigens. The prevalence of anti-Tg Ab in HT patients seems to vary with their age. Juveniles affected with chronic lymphocytic thyroiditis show Ab to Tg and microsomal Ag (Burek et al., 1982). Although a total correlation exists between the incidence of chronic lymphocytic thyroiditis and the presence of Ab to Tg or microsomal Ag, the latter does not necessarily indicate disease occurrence (Burek and Bresler, 1990). On the other hand, Ab against Tg are rare in children without AITD. In the case of adults with HT, autoantibodies could be detected in 76- 100% patients (Bigazzi, 1993). Using

a radioimmunoassay, circulating antibodies were demonstrated in 86-100% of patients with autoimmune thyroiditis, 87-89% of patients with untreated GD and 69-94% of patients with primary myxedema (Bigazzi and Rose, 1985). Although a large body of literature exists showing correlation between the presence of anti-Tg Ab and thyroiditis in patients, it remains still unclear whether these responses occur secondarily, following thyroid damage or are involved in etiology of the disease. The observation that healthy subjects (8.7%) also have anti-Tg Ab (Bigazzi, 1993) and some patients with histological lesions of HT (Yoshida et al., 1978) and others with thyroid autoimmunity (Tanner et al., 1982) have anti-TPO but not anti-Tg Ab further complicates this issue.

The nature of Tg-epitopes recognized by Ab in patients' sera remains unclear. Nye et al., (1980) suggested that autoantibodies from different human sera recognized the same epitopes on human Tg. On the other hand, through the use of mouse mAbs eight different autoepitopes on HTg were determined by Rose and coworkers (reviewed in Bigazzi and Rose, 1985). In addition, six distinct clusters of reactivity were described by Ruf and co-workers (1983) using a panel of mouse mAb to HTg. Based on the observation that pooled serum from 48 GD patients reacted only with one of the six clusters, they suggested that certain selected regions of the Tg molecule bear pathogenic epitopes. Studies by Bouanani et al., (1989) revealed that sera from healthy subjects contained anti-Tg Ab that bound to a domain distinct from that recognized by serum from subjects with AITD. Another study by Henry et al., (1992), identified an immuno-dominant domain (a. a. 1149-1250) in the central part of HTg recognized by sera from patients with AITD. In addition, patients with AITD were shown to have Abs that react with TPO (Doble et al., 1988; Kaufman et al., 1989), "second colloid

antigen" (van Trotsenburg et al., 1989), thyroid cell surface antigens (Bigazzi, 1993) and TSH receptor (Zakarija and McKenzie, 1990).

Histopathology of thyroid glands, in general, is characterized by the presence of inflammatory cells (lymphocytes, plasma cells and macrophages), distorted follicles and fibrosis (focal or extensive). Electron microscopic studies showed dilatation of capillaries and damage to the basement membranes of the thyroid follicles. Hyperplasia and a tall columnar nature of the follicular cells is observed in the goitrous form of thyroiditis. In some cases, thyroid cells become packed with mitochondria and assume an acidophile staining and are known as Hurthle or Askanazy cells (reviewed in DeGroot, 1996).

Among the intrathyroidal lymphocytes (ITL), $CD4^+$ and cytolytic $CD8^+$ cells were found; activated B cells were also shown to be increased in some patients (reviewed in Volpé 1991). In some studies immune complexes formed between Tg and its Ab were demonstrated around the basement membrane of the follicular cells. It is also believed that formation of such complexes and their subsequent removal by the thyroid might be responsible for low levels of anti-Tg Ab in patient sera (reviewed in DeGroot, 1996). Finally, the intrathyroidal cytokine expression profile in HT revealed expression of a mixed Th1 and Th2 response (Paschke et al., 1994; Ajjan et al., 1996).

3. Experimental autoimmune thyroiditis

3. 1 Animal models

Animal models for thyroid disease have been developed to facilitate a better understanding induction, manipulation and modification of the pathogenic

process. Although it remains unclear as to what extent these animal models mimic the human disease, they provide an opportunity to easily intervene in the development and/or progression of EAT.

A brief outline of four categories of animal models that are currently being used to study AITD is presented. In the spontaneous models including the pure-bred obese strain (OS) of chickens, BUF and BB rats and non-obese diabetes (NOD) mice, thyroiditis develops without the need for manipulation of the immune system or immunization (Bigazzi, 1993). In the second category involving mice, experimental autoimmune thyroiditis (EAT) results following manipulation of T-cell sub-sets: spontaneous thyroiditis was observed in mice following injection of syngeneic $CD4^{+}$ $CD5^{low}$ T cells subsequent to thymectomy, irradiation (Tx-X) and depletion of their own T cells (Sugihara et al., 1988). The third model involves SCID mice. Grafting of human thyroid tissue and infusion of peripheral blood mononuclear cells (PBMC) from patients with thyroid disease allowed further destruction of the grafted thyroid tissue in these SCID mice (Akasu et al., 1993). In the last model principally involving mice and rats, thyroiditis is induced after immunization with thyroid Ag in adjuvant or by adoptive transfer of thyroid Ag-specific lymphocytes.

3. 1. 1 Spontaneous EAT

Rats: BUF and BB rats show development of spontaneous thyroiditis. The disease incidence is low in young females but rises with age. The disease in females (as high as 48% incidence in ex-breeders) is characterized by the formation of germinal centers, presence of plasma cells and macrophages in their thyroid gland. Animals are highly susceptible to the development of severe

thyroiditis following s. c. injection of trypan blue, methylcholanthrene and neonatal thymectomy (Bigazzi and Rose, 1975; Silverman and Rose, 1974).

BB rats develop spontaneous diabetes in addition to thyroiditis. Destruction of pancreatic beta cells occurs in about 30% of the rats of 60-120 days age. In older rats, however, the incidence is much higher up to 59% in the 8-10 month old group (Sternthal et al., 1981). In contrast to diabetes, the incidence of lymphocytic thyroiditis varies in different sublines of this strain: subline NB showed 100% incidence while only 4.9% animals developed thyroiditis in subline BE (Rajatanavin et al., 1991). Treatments such as feeding of iodine-reduced diets and the administration of methimazole or T_4 to suppress TSH, decrease the incidence of thyroiditis but not of diabetes in the BB rats. It was therefore, suggested that autoimmune processes underlying these diseases might be different.

OS Chicken: Originally established by Cole in 1966 at a breeding center where the disease incidence was only 1% in the female birds, OS chickens were selectively bred over the years. Spontaneous autoimmune thyroiditis that develops in OS chicken resembles Hashimoto's thyroiditis in clinical, endocrinological, histopathological and immunological aspects (Wick et al., 1982): (1) germinal centers develop in chicken of age 6-8 weeks and replace the glandular tissue leading to fibrosis of the thyroids, and (2) the presence of immune complex deposits formed by autologous C3, auto-Ab and Tg in the lamina propria of thyroid gland, have particularly enhanced the relevance of this model to study the human disease. Although the etiology of the spontaneous thyroiditis in OS chicken remains far from clear, three different groups of genes have been considered to exert control on the disease process. They include an

essential but yet unidentified autosomal recessive "disease susceptibility" gene, the modulating genes responsible for T-cell and macrophage hyperactivity, and altered iodine metabolism in the thyroid gland (Wick et al., 1989). Molecular mimicry by an endogenous virus present in the thyroid glands was hypothesized as a possible mechanism leading to thyroid autoimmunity in OS chickens (Wick et al., 1985), however, further evidence is lacking.

NOD mice: Auto-antibodies to thyroid membrane antigens and thyroiditis are observed in these mice (Bernard et al., 1992). The incidence of thyroiditis is age but not sex-dependent and is prevalent in both diabetic as well as non diabetic animals.

SCID mice: In an effort to study the behavior of HT or GD and PBMC, Volpé and co-workers developed a new model using SCID mice (Akasu et al., 1993). SCID mice injected i. p. with PBMC and xenografted into their groins with thyroid tissue from HT patients were monitored for up to 6 weeks. The presence of both HTg- and TPO-reactive Ab and histologically worsening of the xenografts was noted in these mice suggesting that activation of the autoreactive lymphocytes continued to occur in these mice.

3. 1. 2 Induced EAT

In 1956, pioneering studies by Witebsky and Rose on experimental induction of autoimmune thyroiditis provided a great impetus to advance the field in this area. Their studies not only showed the involvement of thyroidal Ag in thyroid autoimmunity but also documented a methodology for induction of thyroiditis in rabbits by immunizing them with homogenate from syngeneic glands and CFA (Witebsky and Rose, 1956; Rose and Witebsky, 1956). Later,

thyroiditis was induced in various animal species: dogs (Terplan et al., 1960), rats (Jones and Roitt, 1961), guinea pigs (McMaster et al., 1961), monkeys (Kite et al., 1966) and mice (Rose et al., 1971).

Over the last four decades, Tg-induced EAT provided a great deal of knowledge about the genetics of susceptibility to, and immunoregulation of the thyroid disease, and the nature of the host immune cells participating in the thyroid autoimmunity. Recently, 9mer Tg-peptides in CFA have been found to induce EAT allowing a reductionist approach in further studies of the disease (Carayanniotis and Rao, 1997). It is noteworthy, that EAT resembles closely the autoimmune characteristics of HT but differs histologically in a few respects. For example, germinal centers, a hallmark of HT, are absent and no evidence exists for Hurthle (or Askanazy) cells or signs of fibrosis in the thyroid glands of animals undergoing EAT. In addition, it is unclear whether hypothyroidism follows thyroid infiltration in the animal studies and if so, whether or not it can lead to hyperthyroidism since HT patients in some instances develop GD eventually (Goolden et al., 1971; reviewed in Volpé, 1990). Since the present study focused on mouse EAT, an attempt has been made here to introduce to the reader some of the studies in this area that involved different approaches of EAT induction.

Direct approaches of EAT induction: As mentioned earlier, investigations by Witebsky and Rose in 1956 opened the way to study human thyroiditis experimentally. Initially the thyroiditis induction protocol involved challenge of animals (rabbits) with a emulsified mixture of crude thyroid gland extract or purified fraction containing Tg and CFA (Witebsky and Rose, 1956; Rose and Witebsky, 1956). EAT was confirmed in these animals by the presence of auto-

(MNC). Subsequent studies showed induction of thyroiditis in rabbits following immunization with heterologous or chemically altered homologous Tg (Weigle, 1965). The immunopathogenic effects exerted by Tg were severe when a highly purified preparation was used to challenge rats (Roitt et al., 1965). These studies clearly established the basis for the subsequent era of characterizing Tg-induced EAT.

The precise mechanism(s) whereby CFA mediates its adjuvanticity remains unknown, but its highly inflammatory nature and its Ag depot-forming effects have been considered to be crucial for its efficacy (Weigle et al., 1969; Yamanaka et al., 1992). Besides CFA, various other adjuvants including IFA (Rose et al., 1965), LPS (Esquivel et al., 1977), muramyl dipeptide, a polyclonal B-cell activator (Rose et al., 1981), polyadenylic-polyuridylic acid complex (polyA:U) (Esquivel et al., 1978), and latex particles (Esquivel et al., 1977) have also been used.

In general, both homologous and heterologous Tgs induce mouse EAT either directly or by adoptive transfer (Romball and Weigle, 1984; Charreire, 1989; reviewed in Kong and Lewis, 1990; Carayanniotis and Rao, 1997). EAT induction by direct challenge of animals with Tg or Tg-peptides requires, besides having a genetically susceptible host, the optimization of various parameters such as Ag dose, route of Ag administration, immunization regimen, and the time window of thyroiditis assessment.

Early epidemiological data suggested that excessive iodine intake precipitates autoimmune thyroid disease (reviewed in Burek and Bresler, 1990) and it was further supported by studies on OS chicken (Bagchi et al., 1985) and BB/W rats (Allen et al., 1986). The iodine content of Tg was noted to alter its

immunogenicity leading to loss of self-tolerance (Sundick et al., 1987) but the precise mechanism(s) of this phenomenon remains unclear. Roitt and co-workers have noted that poorly iodinated Tg is not a good inducer of EAT (Champion et al., 1987). Further, they have shown that the HTg-peptide (2549-2570), one of the four conserved sites of T_4 synthesis on HTg, is recognized by mouse T cells that adoptively transfer EAT to syngeneic recipients (Champion et al., 1991). This supports the notion that iodinated regions of Tg constitute sites recognized by pathogenic T cells in EAT. However, recent studies (Kong et al., 1995) have not supported this notion. It was demonstrated that peptide (2549-2570) containing thyronine (T_0) that lacks iodine or T_4 at 2553 are equally pathogenic. The peptides (1-12) and (2559-2570) encompassing other sites of T_4 synthesis on Tg also do not induce EAT. The interpretation of these observations was that the immunogenicity of a hormonogenic epitope is influenced by its primary amino acid sequence and that iodinated Tg-determinants *per se* are not immunopathogenic (reviewed in Carayanniotis and Rao, 1997). The mechanism(s) by which (excessive) iodination of Tg may alter its immunogenicity leading to loss of self tolerance and thyroid pathology remains to be explored.

Another approach followed in our laboratory to induce EAT has been to immunize mice with conjugates of Tg and mouse class II MHC-specific mAb (Balasa and Carayanniotis, 1993a). While the adjuvant-free priming of mice with mAb-Tg conjugates has been successful in abrogating the self-tolerance to Tg, as evidenced by the host IgG-responses against Tg, this regimen failed to induce thyroiditis, thereby suggesting lack of induction and/activation of subset(s) of T cells with pathogenic effector function in EAT.

Finally, Okayasu and Hatakeyama (1984) were able to induce thyroid lesions in mice by implanting fresh syngeneic thyroids under the kidney capsule or in the peritoneal cavity of host mice followed by i. v. injection of LPS, a potent polyclonal B-cell activator.

In most of the studies described above, EAT was induced by Tg (or peptides thereof), the most abundant antigen of thyroid gland. It is noteworthy, however, that trypsinized porcine TPO or its immunodominant peptide (a. a. 774-788) is shown to be capable of inducing thyroid pathology in mice (Kotani et al., 1990; Kotani et al., 1992). Since TPO represents an important target molecule for thyroid autoimmune responses in humans, further work is needed to characterize other relevant epitopes on TPO capable of mediating EAT.

Indirect approaches of EAT induction: These include: 1) adoptive transfer of Ag-specific/sensitized lymphoid cells into naive normal healthy hosts and 2) perturbation of the available pool of host lymphoid cells by neonatal thymectomy, depletion of certain T-cell subset(s) by irradiation.

In the mouse model several groups of workers documented thyroiditis induction following transfer, into syngeneic recipients, of lymph node cells (LNC) primed *in vivo* and boosted *in vitro* with Tg (Braley-Mullen, 1985; Simon et al., 1986); *in vivo* Tg-sensitized T cells further activated *in vitro* by con A, were also shown to transfer EAT (Okayasu, 1985). To date, the only study where adoptive transfer of clonal populations of T cells was observed to induce thyroid pathology was carried out by Romball and Weigle (1987). It is clear from these studies that Tg-specific proliferating cells predominantly of $CD4^+$ phenotype participate in the induction of EAT. Using a synthetic peptide approach, work from other laboratories (Champion et al., 1991; Kong et al., 1995) as well as ours

(Carayanniotis et al., 1994; present study see chapters 5 and 6) have shown that LNC specific for defined Tg T-cell determinants can mediate severe thyroiditis.

When IL-2R or anti IFN-γ-specific Abs were added to MTg-sensitized murine splenocytes cultured in the presence of MTg (i.e., *in vitro* boosting with Ag) and the splenocytes were subsequently transferred to syngeneic hosts, the recipients developed a severe form of thyroiditis called 'granulomatous' EAT (Braley-Mullen et al., 1991; Stull et al., 1992) which, like the lymphocytic form, required the Tg-specific $CD4^+$ T cells.

Another method of EAT induction was developed via the manipulation of the T-cell subsets in Wistar rats. Following thymectomy and sublethal total body irradiation (200 rads x 5 times at 2 week intervals) (Tx-X) the animals developed thyroiditis and Tg-reactive Ab (Penhale et al., 1973). The interpretation given to these data was that self-reactive and pathogenic T cells were neither deleted nor rendered anergic in these Tx-X rats but pathology ensued after the loss of regulatory T cells as a result of the above treatment. In support of this concept, a subsequent study showed that lymphocytes from normal syngeneic rats when transferred into the Tx-X hosts suppressed EAT development (Penhale et al., 1976).

Following a somewhat different approach, Sugihara and co-workers have observed development of thyroiditis after adoptive transfer of $CD5^{dull}$ $CD4^+$ T cells in T-cell-depleted mice (Sugihara et al., 1988). Immunohistochemically, both $CD4^+$ and $CD4^-$ T cells were shown to be present in the thyroid lesions (Sugihara et al., 1989). In addition, co-injection of fractionated $CD4^+$ cells containing $CD4^+$ $CD5^{low}$ cells prevented thyroiditis in the recipients. These findings led to the conclusion that thyroid autoreactivity results from a subset of $CD4^+$ T cells with

low expression of CD5. This subset is kept in check normally by the $CD4^+$ T cells expressing higher levels of CD5. Autoimmune thyroiditis may result when the balance tilts in favor of the $CD4^+ CD5^{low}$ subset (Sakaguchi and Sakaguchi, 1994).

3. 2 Immunogenetics

The issue of what precipitates autoimmunity in some but not other individuals even within the same extended family continues to be an intriguing puzzle for medical researchers. The etiology of human autoimmune thyroiditis or of other autoimmune diseases including insulin-dependent diabetes mellitus, multiple sclerosis and rheumatoid arthritis etc., is considered to be multifactorial (Burek and Bresler, 1990). As mentioned earlier, HT was found to occur more frequently in HLA DR4- or DR5- positive individuals (Volpé, 1990). It is possible that the actual gene responsible might only be associated with these HLA genes by linkage disequilibrium. To date, gene(s) primarily responsible for the occurrence of thyroiditis have not been characterized. On the other hand, EAT studies have generated a wealth of information on how different H-2 loci can affect mouse susceptibility to the induced disease.

3. 2. 1 MHC genes

The early pioneering work of Vladutiu and Rose (1971) provided strong evidence associating EAT-susceptibility in mice with their MHC loci. EAT was induced in 33 different mouse strains of 11 different haplotypes using thyroid gland extract in CFA. Based on their susceptibility to thyroiditis development,

the mouse strains were classified as: high responders (H-$2^{s, k, or\ q}$ haplotypes), low responders (H-$2^{b, d, g, i, or\ v}$ haplotypes) and intermediate responders (H-$2^{a, m, r\ or\ p}$ haplotypes). Further, experiments using congenic strains (e.g. C3H.SW vs. C3H/HeJ) sharing the same background non-H-2 genes, but having different H-2 alleles (k or b) suggested that the susceptibility is linked to the H-2 haplotype. The susceptibility to EAT was shown to be autosomally dominant as F1 animals, of crosses between high- and low- responders, were good responders (Vladutiu and Rose, 1971).

Employing several intra-H-2 recombinant strains and MTg as inducing Ag, Tomazic et al., (1974) localized the immune response (Ir) -gene controlling the susceptibility to EAT to the K and/or I-A region of the H-2 complex. The first evidence that the I-A region of the H-2 complex was mainly responsible for susceptibility to the EAT induction by MTg, came from studies by Beisel et al., (1982) who found high responsiveness to MTg in both B10.A(4R) and B10.MBR strains that are only identical at their I-A^k genes of their MHC. Several other studies (Okayasu et al., 1981; Salamero and Charreire, 1983; Braley-Mullen et al., 1985; Okayasu, 1985; Simon et al., 1986) confirmed the importance of the I-A locus in susceptibility to MTg-induced responsiveness.

The importance of the D region within the H-2 complex was shown by Kong and co-workers who used four sets of intra-H-2 recombinant strains with the same K-end alleles but different D-end alleles. Within mice of high responder haplotypes k and s, thyroiditis was significantly reduced in mice expressing d, b and q but not f alleles at the D-end. Among the mice of poor responder haplotypes d and b, the D-end effect could not be appreciated due to the already

low level EAT that occurred in these strains (Kong et al., 1979). It was concluded that EAT severity depends on the interactions between I-A/I-E and D-end alleles.

The influence of K-region on EAT development was initially suggested by Maron and Cohen (1979) based on the finding that EAT developed significantly in B6.H-2^{ba} (HZ1) mice, a mutant strain of C57.BL (H-2^{b}) that carries a point mutation in the H-2K locus, and expresses K^{ba} instead of K^{b}. On the contrary, Beisel and colleagues found no effect on EAT -susceptibility in mice with H-$2K^{b}$ mutations. It became evident that the mutations in the K-locus had no effect on EAT severity unless high responder alleles (A^{k}) were present in the I-A subregion (Beisel et al., 1982). From these studies came the following conclusions: the primary genes controlling susceptibility seem to be those present in the I-A region; D and possibly K loci might regulate effector cell functions determining the incidence and severity of EAT.

In the above genetic studies Tg- or thyroid extract- had been used to induce EAT. It remained unclear, whether or not the pattern of EAT induction by individual Tg-peptides would be analogous to that induced by intact Tg. In our laboratory, an algorithmic approach was used to identify putative T-cell sites on rat Tg (mouse Tg sequence was not known at that time). Direct challenge with the 17mer peptide (2495-11)* in CFA induced thyroiditis in H-2^{k} (B10. BR, C3H) and H-2^{s} (SJL) mice but not in H-2^{d} (BALB/c) or H-2^{b} (B10) haplotypes. A

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* A C-terminal MTg a. a. sequence was recently reported (Kuppers et al., 1996), revealing complete identity at the sites corresponding to (2495-2511) and (2695-2713) sequences on RTg. The two RTg-peptides, reported previously as TgP1 and TgP2 will be referred in this study as MTg(2495-11) and (2695-13).

peptide-specific IgG response was observed in all the strains except B10 mice (Chronopoulou and Carayanniotis, 1992). Thus interestingly, the observed genetic pattern of mouse susceptibility to EAT induced by the peptide was similar to that of Tg-induced EAT as described in earlier work (Vladutiu and Rose, 1971). Subsequent studies, using intra-H-2 recombinant mice, suggested that, E^k gene was necessary but not sufficient for EAT induction, as the concomitant expression of K^k or A^k genes was required (Chronopoulou and Carayanniotis, 1993).

Using the same algorithmic approach a second pathogenic sequence (2695-2713)* was identified within RTg (Carayanniotis et al., 1994) that was pathogenic only in mice of H-2^s but not H-$2^{b, k \text{ or } d}$ haplotypes. Thyroiditis was induced in SJL mice by both direct peptide challenge and adoptive transfer of peptide-specific LNC. These data highlighted the fact that when pathogenic Tg-determinants are tested in isolation, they may not necessarily follow the genetic pattern of Tg-induced EAT. The same data also confirmed that Tg-determinants do not need to be iodinated in order to induce EAT, in contrast to what was envisaged earlier by others (Champion et al., 1987; Champion et al., 1991; Hutchings et al., 1992).

3. 2. 2 Non MHC-genes

Due to their highly polymorphic nature and their central role in Ag presentation, the MHC genes have been studied extensively in autoimmunity both in human and in animal models. However, it is becoming increasingly clear

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* for explanation see foot -note on the previous page

that non-MHC genes contribute significantly towards the incidence of autoimmune diseases (Garchon and Bach, 1991). In EAT, it became evident that non-MHC genes play a role in susceptibility when H-2 congenic mice with different background genes showed significant differences in disease incidence and Ab titers (Beisel et al., 1982a). For example, when C57B1/10 and C3H.SW mice (both H-2^b) were compared, the latter strain showed 20-60% higher incidence of thyroiditis and 1.6-5.8 $\log_2$ higher titer of Ab. It was suggested that the non-MHC genes of C3H background favored EAT development. In other studies using recombinant inbred strains a gene from the *Igh* locus was shown to influence the Ab response to Tg (reviewed in Kuppers et al., 1988). Recently, the magnitude of MTg-specific IgG2a response was linked to genes in or near the *Igh* locus (Kuppers et al., 1994).

Similarly, work on the OS chicken model of spontaneous EAT suggested that besides MHC, two other loci might play important role in the disease process, influencing thymus development and/or creating a defect in the thyroid gland (reviewed in Kuppers et al., 1988). In addition, genes that control macrophage function and those that co-segregate with the altered glucocorticoid response were also hypothesized to be important in the OS chicken model of spontaneous thyroiditis (reviewed in Wick et al., 1989).

3. 3 Host immune responses

The mouse, due to its well characterized immune system and the availability of reagents to many of its lymphocyte markers, has provided a great deal of knowledge not only on the immunogenetic aspects of thyroiditis but also in assessing the role of different lymphocyte sub-sets in disease pathogenesis.

that MTg-specific helper T cells alone can initiate thyroiditis although host PBMC cells might become involved secondarily in the disease process.

Another line of evidence in support of effector T-cell involvement in EAT came from studies by Kong and co-workers (reviewed in Kong and Lewis, 1990). Immunofluorescence studies on pooled intrathyroidal cells collected between 12-21 days after challenge with MTg in CFA, showed an early predominance of Lyt-1^+ cells which was followed by an increase of Lyt-2^+ cells resulting in ratios that were very much different from those in peripheral blood (Creemers et al., 1984). Flynn et al., (1989) attempted to delineate the contribution of $CD4^+$ (previously, $L3T4^+$) and $CD8^+$ (previously, Lyt-2^+) cells. Based on treatment of mice with anti-CD4 or anti-CD8 mAb prior to or following the transfer of cells, it was concluded that donor $CD4^+$ cells were the primary effector cells mediating the thyroiditis transfer while both the $CD8^+$ and $CD4^+$ cells influenced the severity of the disease. In a separate study, Stull et al., (1988) found that splenocytes from mice pretreated with anti-CD4 mAb did not proliferate *in vitro* to MTg nor transfer the disease to normal recipients. EAT was reduced in recipients of MTg-activated splenocytes, if they had been injected with mAb before or after 14-19 days following the cell transfer. These findings clearly emphasize the predominant role played by helper T cells in the effector phase of EAT development. Spontaneous models of EAT also suggested an essential involvement of a $CD4^+$ ($CD5^{dull}$) subset in the pathogenesis of thyroiditis (Sugihara et al., 1993).

To date, few studies have documented the cytokine-profiles of lymphocytes with pathogenic effector function in EAT. Sughihara et al., (1995) demonstrated that a thyroid epithelial cell (TEC)-reactive, $CD8^+$ line secreted IL-2, IFN-γ, and

TNFα but not IL-4 and IL-5, thus resembling a Th1-type cytokine profile. Further, indirect evidence such as: 1) treatment of mice with mAb against IFN-γ reduced the Tg-Ab response and severity of thyroiditis in mice (Tang et al., 1993); and 2) oral feeding of Tg (a regimen known to elicit a T-cell subset with Th2-type cytokine profile) (Guimaraes, 1995; Guimaraes, 1996) protected mice from Tg-induced EAT, support the notion that thyroiditis is mediated by Th1-type cells.

3. 3. 2 B cell response

Although the role of T cells is established in EAT, the contribution of specific Ab is unclear. A good correlation exists between the incidence of thyroiditis and the detection of Tg (or TPO) specific Ab in human sera, but Ab presence alone is not suggestive of disease condition (Burek and Bresler, 1990). In EAT, the adoptive transfer of immune serum from Tg-challenged donors has resulted in mild disease in mice (Vladutiu and Rose, 1971a; Tomazic and Rose, 1975) guinea pigs (Godal and karesen, 1967) and rabbits (Nakamura and Weigle, 1969) but not in monkeys (Roitt and Doniach, 1958) and rats (Rose et al., 1973). These disparate findings about the role of Ab in EAT may reflect variations in the detection-methodology and perhaps, the immunological heterogeneity of the species. However, even in the mouse model, the failure by others to show EAT transfer by immune serum (Okayasu, 1985) or the lack of a good correlation between Ab titers and severity of thyroid lesions (Vladutiu and Rose, 1972; Esquivel et al., 1978) suggests a minor role (if any) for Ab in Tg-induced EAT. Expectedly, the serum of Tg-challenged mice contains Abs with a mixture of specificities (some are directed to pathogenic determinants while others are not), and conceivably, there is a qualitative variation in the nature of epitopes targeted

by the immune responses and their influence on EAT development. Recent attempts to obtain high local auto-Ab concentration by perfusion of thyroids in situ (via the superior thyroid artery) with sera containing high titers of anti-Tg Ab (but not sera lacking anti-Tg Ab) led to induction of thyroiditis in rabbits (Inoue et al., 1993a). Further studies are necessary to confirm these findings in other models.

3. 3. 3 Other immune mediators

In a recent study the importance of late complement components was assessed in rabbits challenged with homologous thyroid extract (Inoue et al., 1993). Rabbits deficient in the sixth complement component (C6-D) exhibited less disease severity than normal controls. This effect was reversed by administering serum from normal rabbits, suggesting a role for C6 in EAT. Among the immune mediators, recombinant IFN-γ (rIFN-γ) has been assessed directly for its ability to mediate EAT (Remy et al., 1987). Mice injected intrathyroidally with one unit of rIFN-γ developed anti-Tg Ab in 70% of the animals and thyroiditis in all of them. It was suggested by Charreire and colleagues that lymphocyte infiltration of the thyroid could be initiated with the local production of IFN-γ by the resident lymphocytes of the thyroid gland following an unknown initial trigger in the disease cascade. The same group recently investigated the role of human rIL-10 and IL-4 on MTg-induced EAT. Compared to the control group, rIL-10-treated mice exhibited reduced proliferative and cytotoxic responses of splenocytes and a much less severe EAT. The protective effects of IL-10 were explained to be due to the activation induced cell death of T lymphocytes (Mignon-Godefroy et al., 1995a). Lastly, in a study

comparing the *in vitro* effects of murine rIL-4 and human rIL-10 on MTg-sensitized spleen cells prior to their adoptive transfer into syngeneic naive mice, IL-10- but not IL-4-treated cells showed decreased proliferative responses and mediated less severe thyroiditis (Mignon-Godefroy et al., 1995), findings consistent with a protective role of IL-10 in EAT.

4. T-cell determinants in murine EAT

In the mid-1980s it became clear that priming of mice with homologous MTg elicited T cells that proliferated *in vitro* against the heterologous HTg (Simon et al., 1985). Conversely, T cells from HTg-immunized mice could be activated and expanded *in vitro* with MTg to transfer thyroiditis or show cytotoxicity to thyroid monolayers (Kong et al., 1986). Parallel studies showed that pathogenic T-cell lines or clones specific for MTg (Romball and Weigle, 1987) or T-cell hybridomas (Champion et al., 1987) recognize Tg-determinants conserved across Tg of other species. However, the location of these determinants on the MTg was unknown until recently partly because the MTg sequence was not known and partly due to the large size of Tg molecule.

The prevailing notion in the field is that Tg harbors a limited number of T-cell determinants since Tg-induced EAT is controlled by the I-A locus as discussed earlier (see section 3. 2. 1). Processing of Tg by APC leads to presentation of the "dominant" determinants with thyroiditogenic properties. Dominant determinants meet two criteria: 1) following challenge with whole protein, specific T-cell responses can be demonstrated against these determinants *in vitro* , and 2) conversely, T-cell responses induced *in vivo* against these determinants can be recalled *in vitro* with the whole protein Ag. On the other

hand, some determinants can induce specific T-cell responses that can be demonstrated *in vitro* against themselves but not the whole protein Ag that they are part of; and in turn, priming with the whole Ag fails to induce T-cell responses against those determinants. Such determinants are said to be 'cryptic' (reviewed in Sercarz et al., 1993). Further, these cryptic determinants can be divided into facultative or absolute depending on whether the induced T cells will respond to large doses of native Ag or not.

4. 1 Epitope mapping

4. 1. 1 Thyroglobulin

Diverse strategies were used to identify pathogenic Tg epitopes. Roitt and co-workers first demonstrated a critical role for iodination in T-cell recognition of Tg employing two clonotypically distinct HTg-reactive murine T-cell hybrids ADA2 and CH9. Poorly iodinated HTg or MTg purified from aminotriazole (an inhibitor of TPO)-treated mice failed to activate the T-cell hybrids (Champion et al., 1987). Later, using synthetic T_4-containing peptides representing the four major hormonogenic sites in Tg, they identified a 9mer epitope (2551-2560) containing T_4 at position 2553 as the T-cell determinant seen by those hybrids (Champion et. 1991). And in a subsequent study, HTg(2549-2560)- primed LNC adoptively transferred EAT to naive syngeneic recipient CBA mice, following *in vitro* activation with the peptide (Hutchings et al., 1992). While these experiments clearly provided evidence that T_4-containing Tg-epitopes can induce T cells with a pathogenic role in EAT, it was not clear whether T-cell activation depended on the presence of iodine within T_4 or simply required the two phenyl-

ring side chain of T_4. A study by Dawe et al., (1996) addressed this issue. When two analog peptides containing T_4 or T_0 (that lacks iodine) were assessed for their capacity to activate the hybrids ADA2 and CH9 only the former was stimulatory suggesting the importance of the four iodine atoms for the antigenicity of HTg(2549-2560).

A recent study by Kong and co-workers (1995) reexamined whether or not iodine is important in T-cell recognition and EAT induction. They tested three HTg 12mer peptides (1-12, 2549-2560 and 2559-2570), carrying T_4 at the primary hormonogenic sites 5, 2553 and 2567. Peptides carrying T_0 were used as controls. It was found that both T_4 (2553)- and T_0 (2553)- containing peptides were immunogenic and activated splenocytes from CBA mice primed either with MTg or the respective peptide, to adoptively transfer thyroiditis to syngeneic hosts. This suggested that the presence of iodine was not necessary for pathogenicity. When the T_4 (5)- and T_4 (2567)- containing peptides were tested similarly, both exhibited poor immunogenicity and antigenicity. These findings demonstrate that the presence of T_4 within a hormonogenic site is not sufficient to impart immunogenic properties. Interestingly, LNC from mice primed with the T_4 (2553)-containing epitope could neither be cross-stimulated by the T_0 (2553)-containing analog, as evidenced by lack of a proliferative response nor EAT transfer. This suggested that iodine atoms sufficiently modify the peptide-MHC complex and induce a distinct subset of pathogenic T cells that exclusively recognize the T_4 (2553)-containing epitope. This result is consistent with the finding (Dawe et al., 1996) that T_4 (2553)-containing peptide-reactive hybrids cannot be activated by the T_0 (2553)-containing analog.

A different approach followed by Charreire and co-workers led to the characterization of a pathogenic 40mer Tg-peptide (Texier et al., 1992). They used a class I-restricted cytotoxic T-cell hybrid that lysed syngeneic CBA macrophages pulsed with tryptic porcine Tg fragments of < 10 kD. Subsequent purification and sequencing of the selected peptide revealed partial homology with the HTg and a 40mer HTg (1672-1711) peptide representing the region of homology was synthesized. Challenge of CBA/J mice with this peptide led to EAT in 4 out of 5 hosts (Texier et al., 1992). However, it remained unclear if thyroiditis developed because of a response to the class I-restricted determinant recognized by the hybridoma or because of a response to another unknown epitope within the 40mer sequence.

A third approach was based on earlier findings by McLachlan and Rapoport (1989) that HTg and human thyroid peroxidase (hTPO) share B-cell epitopes and it was hypothesized that they may also share T-cell epitopes. Hoshioka and co-workers (1993) identified two 14mer peptides HTg(2730-43) and hTPO(118-131) which had 5 identical consecutive a. a. in common and elicited weakly cross-reactive T-cell responses. Proliferative assays strongly suggested that the HTg peptide contained an immunodominant T-cell epitope but direct challenge of mice with this peptide, or adoptive transfer of peptide-specific spleen cells did not induce thyroid pathology. Extensive thyroiditis was observed only by adoptive transfer of cells that were primed *in vivo* with MTg and boosted *in vitro* with HTg(2730-43). The reasons for these discrepancies were not discussed.

In our laboratory, the search for putative T-cell epitopes made use of computerized algorithms (Margalit et al., 1987; Rothbard and Taylor, 1988) to scan the RTg sequence (the MTg sequence was unknown). A 17mer

nondominant peptide (2495-2511) was identified and shown to induce EAT in different mouse strains with a pattern similar to that of Tg-induced EAT (Chronopoulou and Carayanniotis, 1992). Subsequently, attempts were made to identify the MHC loci controlling the peptide-induced disease. Based on the EAT susceptibility of intra-H-2 recombinant B10.BR strains following peptide challenge, it was concluded that the I-E gene was necessary for disease induction but insufficient since the K^k and/ or D^k end genes were also required for the thyroiditis to occur (Chronopoulou and Carayanniotis, 1993). It remained, however, unclear whether the 17mer peptide harbored one or more T-cell determinants and what their MHC-restriction was. Also, these studies could not explain how the peptide-induced EAT is influenced by the I-E locus in H-2^k haplotype mice since the same peptide induced EAT in H-2^s haplotype mice that lack functional I-E molecules.

Via the same algorithmic approach a second peptide on RTg with coordinates (2695-2713) was identified and tested for EAT induction in different mouse strains (Carayanniotis et al., 1994). The peptide elicited EAT in SJL (H-2^s) but not C3H or B10.BR (H-2^k), BALB/c (H-2^d) and B10 (H-2^b) mice. These findings suggested a new genetic pattern of susceptibility to EAT (different from that of Tg-induced EAT) provided the first evidence of an A^s-restricted pathogenic Tg-determinant.

In the latter study (Carayanniotis et al., 1994), it was also shown that MTg(2695-2713) is identical with its counterpart within the HTg sequence at fifteen of the nineteen positions (79% homology) and carries three amino acid substitutions at positions 2703 (Q > S), 2704 (T > S), 2707 (D > T) and one insertion (S) at position 2708. The new questions generated from these data were:

(1) Is the HTg(2695-2713) immunogenic or pathogenic in SJL mice ?; (2) Are the human and mouse homologs mutually cross-reactive at the T- and/or B- cell level ? Answers to these questions (see Chapter 6) would illuminate what effect(s) (if any) these above a. a. substitutions have on the immunopathogenicity of the HTg-peptide, and thus, provide clues as to why some Tg (for example bovine Tg) fail to induce thyroiditis despite being immunogenic in mice (Romball and Weigle, 1984).

In summary, data from various laboratories support the notion that Tg T-cell determinants do not need to be either iodinated or immunodominant to be able to induce thyroiditis. The role of cryptic epitopes in autoimmunity remains unclear but increasing evidence suggests that a large number of cryptic self-determinants can be generated as a result of altered Ag processing by APC (Salemi et al., 1995; Lanzavecchia, 1995). In addition, the immune response can spread to the less dominant or cryptic self determinants (intra-molecular spreading) leading to a state of self-sustained chronically disregulated autoreactivity (Lehmann et al., 1993).

4. 1. 2 Thyroid peroxidase

Relatively little is known about T-cell determinants of TPO. Kotani et al., (1990) induced EAT in C57BL/6 (H-2^b) mice following immunization with immunoaffinity-purified porcine TPO (pTPO) in CFA or by adoptive transfer of a specific T-cell line. In a subsequent study, following proteolytic cleavage with cyanogen bromide, purification by reverse phase chromatography and analysis of synthetic peptides based on comparison with cDNA sequence of pTPO, Kotani and co-workers further identified an immunodominant T-cell determinant (774-

788). Direct challenge of mice with this 15mer peptide induced thyroiditis in 66-75 % of the mice. Also pTPO-reactive Ab was observed in these mice.

5. T-cell receptor variability in autoimmune thyroid disease

Since T cells recognize MHC-bound peptide determinants via their TCR, studies in recent years have focused on the diversity of TCR on autoreactive T cells. The assumption has been that if the TCR of autoreactive cells are of limited heterogeneity a therapeutic approach can be developed to eliminate those cells before the onset of autoimmunity or to reverse the pathogenic process in already affected subjects. Two approaches were used in animal studies to test this concept: the first relied on the *in vivo* administration of mAb specific for appropriate TCR Vβ families to eliminate autoreactive cells expressing these families (Zaller et al., 1990); the second depended on vaccinating animals with glutaraldehyde-fixed autoreactive T cells to induce anti-clonotypic immunity leading to induction of regulatory cells against the injected clones (Ben-Nun et al., 1981). The practicality of TCR-based immunotherapy in human autoimmune disease has been questioned because of the extensive polymorphism of HLA genes and high variability in TCR V-gene usage among patients (Kono and Theofilopoulos, 1993).

5. 1 In human studies

Two methods were used to study TCR variability in human AITD. The first method relied on the observation that after digestion with restriction enzymes, DNA from differentiated mature T cells gives rise to a clonotypic pattern when probed by TCR-β gene probe with a Southern blot. DNA from T cells of a monoclonal population will yield a new uniform-sized fragment in a pattern that can be distinguished from that of the germ line DNA. On the other hand, a similar analysis using DNA from T cells of a polyclonal population will reveal a pattern of multiple bands of varying length due to the nucleotide differences in the rearranged segments encoding Vβ, D and J regions of TCR. Therefore, the higher the degree of polyclonality of the sample T cells, the greater will be the number of differences in comparison to the host germline DNA.

The second approach was based on the amplification of sample T-cell cDNA by the polymerase chain reaction (PCR) method. The amplification is selective since it is achieved by using primers each specific for a different TCR-Vβ gene family and the sample clonality is, therefore, inversely proportional to the number of primers able to amplify the sample DNA. Once amplified using a given Vβ family specific primer, the product represents DNA from all the T cells present in sample that were using the specific Vβ genes regardless of their clonality and fine Ag specificity.

Kaulfersch and co-workers (1988) determined the clonality of ITL following the first described approach namely, TCR gene rearrangement analysis. On the basis of Southern blots of EcoR1 and BamH1 digests of circulating and infiltrating lymphocytes with a radiolabeled TCR probe, these workers suggested that the ITL were polyclonal in nature. Davies et. al., (1991) used the PCR

approach and showed that, ITL from patients with autoimmune thyroid disease had expressed restricted Vα gene usage although the pattern of the Vα chain usage varied from patient to patient. Based on the inconsistent restriction in the TCRα genes with T cells from each patient showing a different pattern of expression, it was hypothesized that cells with certain TCR-Vα genes might show preferential homing. In a second study, Davies and co-workers also observed that ITL obtained from six patients with AITD, expressed a widespread usage of Vβ - but not Vα- family genes (Davies et al., 1992). The mean utilization of the 18 Vα and 19 Vβ gene families (that they were tested for), were 4.7 (range = 2 to 8) and 14.4 (range = 8 to 18) respectively. Their interpretation was that, the observed selective utilization of Vα genes by T cells might result from some preferential recruitment that is antigen-unrelated, probably analogous to Vβ-based selection by superantigens operative at the target organ level. Further independent work is needed to support this hypothesis. Lastly, Davies and co-workers observed comparatively less TCR diversity in biopsied thyroid specimens from GD than those from HT (Davies et al., 1993). While confirming the previous observations, the study further suggested that GD patients early in the disease may have ITL with more restricted V-gene usage.

In contrast to the above findings from Davies group, McIntosh et al., (1993a) observed no Vα gene restriction in GD, since PCR amplification with 10-15 Vα families occurred in all of the twelve cases examined. No significant difference was found between the PCR amplification pattern of TCR with lymphocytes derived from peripheral blood of normal controls vs. patients or with the intra-thyroidal lymphocytes obtained from patients with GD or toxic nodular goiter. In a second study by McIntosh and co-workers, cDNA from lymphocytes from

peripheral blood or thyroid tissue were examined for TCR-Vα heterogeneity. In comparison to peripheral blood samples ITL showed no less heterogeneity, suggesting the polyclonal nature of these T cells (McIntosh et al., 1993).

In a recent study that examined the TCR V-gene usage in seven GD patients (treated with carbimazole), a widespread use of 12-18 V-gene families by the ITL was noted (Caso-Pelaez et al., 1994). Perfusion of one thyroid gland to avoid blood contamination made no difference in the results obtained.

The apparently conflicting evidence on the issue of TCR variability of AITD may be related to the differences in the etiology of AITD, determinant selection effects by HLA gene products, variation in the detection methodologies or the time of tissue sampling. To resolve the issue of TCR variability in AITD more studies are necessary. Since HLA is known to have profound effects on the TCR repertoire, comparison of Vβ-profiles between HLA-matched individuals with AITD must be undertaken. Even then it is difficult to interpret data on TCR variability in the absence of knowledge of TCR ligand and the restricting HLA molecule. Significant variations in the autoreactive TCR V-gene usage are likely to occur depending on whether class I or II MHC molecules present pathogenic determinants to such cells.

5. 2 In mouse EAT

In NOD mice spontaneously developing EAT, predominant expression of up to 3 TCR-Vβ gene products among ITL was interpreted by Matsuoka et al., (1993) to be consistent with a restricted TCR use. Sequencing of the amplified VDJ region further suggested that at least one of the overexpressed Vβ gene families was clonally expanded but the expanded Vβ family was different in each

mouse. Even within the same Vβ family products, VDJ region homology at the nucleotide or amino acid level was lacking. The interpretation given to these data was that the choice of the TCR Vβ rather than D or J segment determined the process of thyroid infiltration. On the other hand, in mice developing spontaneous EAT following elimination of regulatory T-cell subsets, Sugihara et al., (1993) observed use of Vβ 2, 4, 8.3, 14 and an undefined Vβ by ITL. Out of these, Tg-reactive T cells expressing Vβ 2, 8.3 and the undefined Vβ receptors were found to adoptively transfer EAT, while Vβ 4-expressing cells, reactive to some uncharacterized TEC-associated antigen, did not transfer EAT.

In Tg-induced thyroiditis in CBA/J mice, Matsuoka et al., (1994) stated that there was a biased, oligoclonal T-cell expansion because 10 out of 17 Vβ families (1, 2, 4, 5, 6, 7, 8, 11, 13 and 16) were expressed by the intrathyroidal T cells analyzed 28 d post immunization. Tg-specific T-cell lines were also reported as biased because they expressed 7/17 families (1, 2, 4, 8, 11, 21 and 16). The authors suggested that the TCR-Vβ influenced lymphocyte homing to the thyroid. In apparent contrast to these findings, Braley-Mullen and co-workers (McMurray et al., 1996) recently observed use of multiple TCR Vβ families including Vβ 1, 2, 4, 6, 8, 11, 13 and 14 by intrathyroidal MNC. Using RT-PCR, they have observed no differences in Vβ T-cell repertoires in thyroids with lymphocytic vs. granulomatous EAT.

It is clear that in both spontaneous and Tg-induced models of EAT described above there is considerable heterogeneity in the Vβ-gene usage by Tg-specific T cells in the periphery or ITL. The apparently contradictory interpretations given to these data stem partly from the number of families used to define ' diverse' vs. ' restricted' TCR usage. It is difficult to interpret data on

TCR variability purely on the basis of their thyroidal origin or specificity for Tg since the fine Ag specificity of such cells is not known. Diverse epitopes may be recognized by T cells with diverse TCRs even when the same MHC molecule is involved in Ag presentation. In EAT, no TCR V-gene families had been assigned to T-cell clones that recognize defined pathogenic Tg determinants prior to the present study (see Chapter 5). As mentioned earlier, the knowledge on TCR gene use among cells recognizing a thyroiditogenic determinant would be useful to design an approach for elimination of such cells.

6. Molecular mimicry and AITD

Infection by microorganisms, especially viruses, has long been suspected to predispose certain individuals for autoimmunity. One of the mechanisms put forth to explain how microbes cause autoimmunity is 'molecular mimicry'. Initially proposed by Damien (1964) to denote sharing of antigenic determinants between a host and parasite, molecular mimicry as a concept was developed by Fujinami et al., (1983) to explain the induction of autoimmune responses. They suggested that, Ags shared between parasite and host can be sufficiently similar to cross-react immunologically, but bear enough dissimilarity to allow breakdown of tolerance and generation of an autoimmune response. Expectedly, when the self epitope is part of an important host protein such as an enzyme or hormone receptor, a dysfunction may result during the immune response against the foreign microbe. They also suggested that the microbe's presence is not necessary for the autoimmune attack to progress or perpetuate.

6. 1 Molecular mimicry involving thyroidal antigens

Little evidence exists in the literature to clearly substantiate the molecular mimicry phenomenon in AITD. Although the thyroidal antigens are known in AITD, knowledge of a. a. sequence of thyroiditogenic peptides is necessary in order to search for sequences from microbial proteins with potential for EAT induction via molecular mimicry.

In human studies several examples have suggested an association between viral infections or anti-viral immune responses and thyroid disease (Tomer and Davies, 1995). For example, subacute thyroiditis was found to occur in epidemic proportions (Eylan et al., 1957; deBruin et al., 1990) in summers coinciding with the distribution of enteroviruses. Mumps (Eylan et al., 1957) and Epstein-Barr (Coyle et al., 1989) viruses were implicated in the causation of subacute thyroiditis. In an extensive study (Volpé, et al., 1967), serum Abs from patients with subacute thyroiditis reacted against a number of viruses including influenza, coxsackie, mumps, ECHO and adenoviruses were observed. In children with rubella syndrome, Abs reactive with TPO or Tg were demonstrated in 34% of the patients (Ginsberg-Fellner et al., 1985). Likewise, 20% of the patients (women) with chronic hepatitis C infection showed significant levels of thyroid autoantibodies (Tran et al., 1993).

These studies are based on data from serological cross-reactivity with viruses and it is difficult to relate the occurrence of the virus-reactive Abs with the events in the development of thyroid disease because of the presence of polyreactive Abs. Moreover, no particular virus has been clearly shown to be associated with etiopathogenesis of AITD in humans.

A. a. sequence homology between a host protein and a microbial Ag may not necessarily indicate that an autoimmune response will develop upon infection with the appropriate microorganism. To date there is no strong evidence to support molecular mimicry as an underlying cause of thyroid autoimmunity. In the past, several studies suggested an association between GD and *Yersinia enterocolitica* infection. Based on the higher frequency of *Yersinia* -reactive Ab in GD patients (Shenkman et al., 1976) and the demonstration of a TSH-binding site on *Yersinia* membranes (Weiss et al., 1983), it was hypothesized that GD might result due to molecular mimicry by *Yersinia* infection. This was further supported by Wenzel et al., (1988), who showed that GD patients had Abs reactive with plasmid-encoded *Yersinia* Ag. However, the role of *Yersinia* in precipitating AITD in humans is controversial (Volpé, 1990). A recent study by Arscott and co-workers examined reactivity of sera from GD and HT patients or healthy controls and found no unique pattern of serological reactivity with *Yersinia* Ag (Arscott et al., 1992). Further evidence against the role of *Yersinia* in the pathogenesis of AITD comes from the fact that patients with thyroid disease show clinically no evidence of *Yersinia* infection even when they have Abs reactive with the organism (Ingbar et al., 1987) and, from the observation by Wolf and co-workers (1988) that patients recovering from *Y. enterocolitica* infections show no signs of thyroid dysfunction despite having Abs with "Graves' like activity" and reactive with thyroid epithelium. In addition, immunization of rats with *Yersinia* protein elicited anti-TSH receptor Abs but completely failed to yield any evidence of thyroid disease (reviewed in Volpé, 1990).

In EAT, Srinivasappa et al., (1988) documented that mice infected with reovirus type 1 developed anti-Tg and anti-TPO Abs and lymphocytic infiltration

of their thyroids. These mice, however, showed no alteration in thyroid function. In contrast, a decrease in the Tg mRNA and circulating Tg hormones was observed in mice infected with lymphocytic choriomeningitis virus (Klavinski et al., 1988). Interestingly, no autoantibodies to Tg were observed, and despite the viral persistence, no signs of morphological changes or inflammation were observed in the thyroids.

Finally, the only study showing structural homology between viruses and TPO was by Dyrberg (1989). Within the human TPO sequence five stretches each of eight to ten residues were found to bear homology with proteins from three different viruses namely, adenovirus (types 2 or 5), hepatitis B and papillomavirus. Despite their impressive homology with TPO, none of the viral sequences have been investigated further. In the present study an attempt has been made to investigate the relevance of a sequence similarity between a pathogenic Tg T -cell determinant and murine adenovirus type 1 E1B protein (see Chapter 7).

CHAPTER 2

THESIS PROPOSAL AND OBJECTIVES

For almost three decades murine EAT, as a model for HT, has generated valuable data leading to a greater understanding of the immunogenetics, pathology and immunoregulation of thyroiditis. This information has played a major role in developing our current understanding of HT. It is well established that immunization of genetically susceptible mice with MTg in adjuvant (CFA) induces thyroiditis and is influenced by the host MHC class II molecules. However, most thyroiditogenic epitopes in Tg or other thyroidal Ags still remain unknown. Previous studies in our laboratory have shown that the 17mer MTg(2495-11) peptide induces thyroiditis in mice of k and s haplotypes, while a second peptide MTg(2695-13) mediates thyroid pathology only in mice of s haplotype. Therefore, it was felt important to accurately map pathogenic T-cell epitopes within these sequences and further investigate the profile of T cells that recognize them.

Objectives:

1) To determine which T-cell determinants within the pathogenic MTg(2495-11) peptide are responsible for EAT induction in mice of two different haplotypes

2) To examine the lymphokine profile and TCR Vβ-gene usage among cells specific for a minimal Tg-epitope within MTg(2495-11) capable of inducing EAT

3) To map pathogenic T-cell epitope(s) within MTg(2695-13) and further examine if the limited a. a. variations present in the homologous HTg sequence would affect its immunopathogenicity

4) To test the concept of molecular mimicry in EAT, working with a viral peptide of murine adenovirus type 1 E1B-protein showing high homology with MTg(2695-13).

CHAPTER 3

MATERIALS AND METHODS

3. 1 ANIMALS AND ANTIGENS

Female C3H/HeJ, CBA/J, B10.BR/SgSnJ, B10.A(2R), B10.A(4R), B10.A(5R) and SJL/J mice of age 6-10 weeks, were obtained from the Jackson Laboratories, Bar Harbor, ME, USA and used for immunizations at 6-10 weeks of age.

Frozen thyroids from Sprague-Dawley rats (Bioproducts for Science, Indianapolis, IN, USA) or frozen normal thyroid tissue from patients undergoing thyroidectomy due to thyroid carcinoma (kindly provided by Dr. A. Kwan) were used for the purification of rat and human Tg respectively, by gel filtration on Sepharose CL-4B columns (Pharmacia, Baie d'Urfé, Quebec, Canada) as described in **section 3. 1. 1.** Ovalbumin was purchased from Sigma (St. Louis. MO, USA). Tuberculin purified protein derivative (PPD, Seruminstitut, Copenhagen, Denmark) was purchased from Cedarlane (Ontario, Canada).

The peptides were synthesized at > 75% purity at the Alberta Peptide Institute on an Applied Biosystems (Foster City, CA) 430 A synthesizer or at the Medical Center Protein Chemistry Facility of the University of Pennsylvania using a general procedure for solid-phase synthesis outlined by Erickson and Merrifield (1976) with modifications by Hodges et al., (1988). Briefly, the procedure included hydrogen fluoride cleavage of peptide resin at -5 °C for one hour in hydrogen fluoride: anisole: dimethyl sulfoxide (DMSO):*p*-thiocresol: peptide resin (10 ml: 1 ml: 0.5 ml: 0.2 ml : 1 g). Peptide purity was assessed by HPLC and mass spectroscopic analysis. MTg(2495-2511) (acetyl-GLINRAKAVKQFEESQG-amide, termed previously as TgP1) and truncated

peptides thereof, similarly carrying an acetyl group at their N-terminal and an amide group at their C-terminal (synthesized at the Medial Center Protein Chemistry Facility of the University of Pennsylvania), MTg(2695-2713) (acetyl-CSWSKYIQTLKDADGAK-amide, termed previously as TgP2) as well as the three overlapping 12mer peptides representing its core, N- and C-terminal regions were completely soluble in aqueous media where as HTg(2695-06) was dissolved (10 mg/ml) in DMSO. For descriptive purposes, amino acid positions within MTg(2495-2511) are identified by the HTg sequence numbering, since the complete MTg sequence is not known. The murine adenovirus type 1 E1B(a. a. 368-91) and (368-78) peptides were synthesized at Alberta Peptide Institute whereas (368-77) peptide was synthesized by Synpep (Dublin, CA, USA).

3. 1. 1 Thyroglobulin purification

Frozen thyroid glands of outbred ICR mice or Sprague-Dawley rats were homogenized in phosphate buffer (pH 7.0). The clear homogenate was centrifuged thrice at 16,000 x g and the supernatant was passed through Sepharose CL-4B (Pharmacia, Quebec, Canada). Fractions corresponding to the second peak, previously shown during calibration of the column to overlap with BTg elution, were pooled, dialyzed and used as source of MTg or RTg. HTg purified similarly from frozen human thyroid gland was kindly provided by Dr. G. Carayanniotis.

3. 2 CULTURE MEDIA, CELL LINES AND MONOCLONAL ANTIBODIES

All assays were performed in DMEM medium (GIBCO) supplemented with 10-20% fetal bovine serum (FBS) (Bioproducts for Science, Indianapolis, IN, USA), 20 mM HEPES buffer, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin (all from GIBCO,) and 5 x 10^{-5} M 2-mercaptoethanol (2-ME) (Sigma, St. Louis, MO, USA). For cell washings HBSS (GIBCO) or PBS (pH 7.4) was used.

The BW5147$\alpha^-\beta^-$ variant (White et al., 1989) was a kind gift of Dr P. Marrack (National Jewish Centre for Immunology and Respiratory Medicine, Denver, CO, USA). The antigen presenting hybridoma cell line TA3 (H-2A$^{d/k}$, H-2E$^{d/k}$) (Allen et al., 1985) was a kind gift of Dr. L.H. Glimcher at Harvard Medical School, and was courteously provided by Dr. T. Watts at the University of Toronto. The IL-2-dependent CTLL-2 line (Gillis and Smith, 1977) was purchased from ATCC.

Anti-$\alpha\beta^+$-fluorescein isothiocyanate (FITC), anti-CD4$^+$-FITC and anti-CD8$^+$-phycoerythrin (PE) conjugates were purchased from GIBCO. Hybridomas specific for K^k (16-3-22S , Ig2a) (Ozato et al., 1980), I-A^k [k,r,f, or s] (10-3.6.2, Ig2a) (Oi et al., 1978), I-E^k (14-4-4S, Ig2a) (Ozato et al., 1980), D^k (15-5-5S, Ig2a, cross reacts with K^{d,f}) (Ozato et al., 1980), and influenza A nucleoprotein(H16-L10-4R5, IgG2a) (Yewdell et al., 1981) were obtained from the American Type Culture Collection, (Rockville, MD, USA) and the respective mAbs were purified from culture supernatants on protein G-Sepharose 4 Fast Flow affinity chromatography columns (Pharmacia, Baie d'Urfé, Quebec, Canada). Briefly, the harvested supernatant was centrifuged at 12,000 g for 20 min. and loaded on to the Protein G-sepharose column which had been equilibrated with phosphate

buffer (pH 7.0). After the supernatant had passed through, 100 ml each of acetate buffer pH 4.0 (elution buffer I) and glycine-HCl buffer pH 2.4 (elution buffer II) were passed. The antibody containing fractions were quickly pooled, dialyzed against distilled H_20 and concentrated on Diaflow membranes using Amicon ultrafiltration cell, model 8050 (Amicon, Danvers, MA, USA). The mAb preparation was filter-sterilized, and prior to storage its protein content was estimated by measuring the absorbance at 280 nm on a Beckman DU-64 spectrophotometer (Beckman, Fullerton, CA, USA). Antibody concentration (mg/ml) = absorbance @ 280 x dilution of the sample / 1. 45.

3. 3 THYROGLOBULIN PEPTIDE-SPECIFIC T-CELL HYBRIDOMAS

3 .3 .1 Generation of hybridomas

T-cell hybridomas were generated following a slightly modified method of Perkins et al., (1991). Mice were immunized s. c. at the base of the tail with 100 nmol peptide in 100 µl of CFA emulsion (with *Mycobacterium butyricum*, Difco Laboratories Inc., Detroit, MI, USA). Ten days later, the inguinal lymph nodes were collected aseptically and single cell suspensions (LNC) were prepared in DMEM 10% FBS culture medium. After centrifugation and washing, 4×10^6 cells/ml were cultured in the presence of 10 µg/ml peptide used for immunization, at 37° C in a 5% CO_2, 95% air, humidified incubator. After 4 days, viable lymphocytes were isolated on Ficoll Paque (Pharmacia, Canada) and fused with BW5147 $\alpha^-\beta^-$ cells using polyethylene glycol 1500 (Boehringer Mannheim Biochemicals, Indianapolis, IN, USA) at a 1:3 ratio of lymphocytes to tumour cells. After the fusion step, gradual addition of DMEM without FBS and

suspension of the cells in 40 ml of DMEM with 20% FBS were performed as described (Perkins et al., 1991). Hybridomas usually appeared in microwells after 7-10 days of culture and were gradually transferred from hypoxanthine/aminopterin/thymidine (Sigma)- to hypoxanthine/aminopterin (Sigma)- containing medium and finally to complete medium. Peptide-specific hybridomas were cloned by limiting dilution at 0.3 cell/well using 1% syngeneic RBC as filler cells.

3. 3. 2 Activation of hybridomas

3. 3. 2. 1 Mitomycin-C treatment of splenocytes

Spleens were collected aseptically from naive healthy mice and single cell suspensions were prepared by passing the spleen tissue through sterile stainless steel mesh (Sigma, St. Louis, MO, USA) and the resulting cell suspension was vigorously pipetted several times to obtain a uniform suspension of cells. The cells were washed three times with DMEM, adjusted to 5 x 10^7/ml and incubated with 100 µg of mitomycin-C (Sigma, St. Louis, MO, USA) for 20 min. at 37°C avoiding exposure to light. The cells were washed thrice with DMEM and finally resuspended in DMEM with 10 % FBS to be used as APC.

3. 3. 2. 2 Glutaraldehyde treatment of APC

TA3 cells were washed thrice in serum free DMEM and once in PBS and cell concentration was adjusted to 5 x 10^6 cells/ml. The cells were treated with glutaraldehyde (0.1% final concentration) for 30 seconds and an equal volume of freshly prepared 0.2M glycine in PBS was immediately added to the cells. The

cells were washed twice with serum free DMEM and once with DMEM with 10% FBS. The cell concentration was adjusted to 10^5 /ml and used in T-cell hybridoma activation assays.

3. 3. 2. 3 CTLL-2 proliferation assay

CTLL-2 were maintained in continuous culture by growing in DMEM -10% FBS supplemented with 10% culture supernatant from rat spleen cells stimulated with con A (2 μg/ml) for 72 hours. Residual con A in the supernatant was inactivated by α-methylmannoside (50 mM). In flat-bottomed wells of microtiter plates, 10^5 hybridoma T cells and an equal number of TA3 / LS 102.1 or 10^6 mitomycin-C treated spleen cells were cultured for 24 hours with or without MTg(2495-11) 10 μg/ml in a total volume of 200 μl culture medium per well. Then, 50 μl of supernatant was harvested from each well, transferred into new plates, and kept frozen for more than 2 h at -70 ^{o}C. Upon subsequent thawing, 10^4 CTLL-2 cells were added per well and 18 h later, ^{3}[H]-thymidine (1μCi/well) (Amersham, Canada) was added. The cells were harvested 6 hours later using a semi-automated cell harvester (Skatron Inc. Sterling, VA, USA) and incorporated thymidine was counted in a liquid scintillation counter (LS3801, Beckman Instruments, Palo Alto, CA, USA). Peptide-specific hybridomas were cloned by limiting dilution at 0.3 cell/well using 1% syngeneic RBC as filler cells.

3. 3. 3 FACS analysis

Expression of TCR αβ, CD4 and CD8 was assessed by direct labeling of T-cell hybridomas as described by Stewart (1990). Briefly, 1 x 10^6 cells were

washed twice in PAB (PBS containing 0.1% sodium azide (Sigma, St. Louis, MO, USA) and 0.5% BSA (Pharmacia, Canada), and were incubated with a control IgG (specific for influenza A nucleoprotein) (30 μg in 10-20 μl for 20 min.) to block potential Fc receptors. The cells were washed once in PAB, stained with 1μg (4μl) of labeled conjugates for 30 min., washed twice with PAB and finally suspended in 1 ml cacodylate buffer (pH 7.2) containing 1% paraformaldehyde (Sigma, St. Louis, MO, USA). FACS analysis was performed using a FACStar Plus analyzer (Becton-Dickinson Inc.,). Labeling procedures were carried out at 4^{o} C.

Phenotypic characterization of TCR-Vβ family gene expression by T-cell hybrids was carried out in a two-step method. 10^5 hybrid T cells were first incubated on ice for 30 min. in separate Eppendorf tubes with culture supernatant (in appropriate dilution) containing mAb-specific for TCR-Vβ 2 (B20, 3, 4, 5, 6, 7, 8.1/.2/.3, 9, 11, 13, 14 or 17 (kindly provided by Dr. C. Guidos, University of Toronto). Following 2-3 washes with PAB, the cells were incubated again for 30 min. with anti-hamster IgG (in case of Vβ 3), anti-rat IgM (in case of Vβ14) or anti-mouse or rat IgG (in case of all other Vβs) as a second Ab-FITC conjugate. Following two washes in PAB, the cells were fixed by suspending in 1.0 ml cacodylate buffer (pH 7.2) containing 1% paraformaldehyde and analyzed as mentioned earlier.

3. 4. ASSESSMENT OF PEPTIDE IMMUNOGENICITY

3. 4. 1 LNC proliferation assays.

Mice were injected s. c. with peptides (50 nmol) in 100 μl of CFA (with *Mycobacterium butyricum*, Difco Laboratories Inc., Detroit, MI, USA) emulsion at

the base of the tail. After 10-11 days, draining inguinal LNC were collected aseptically, washed with culture medium, and cultured (4×10^5 cells / well) for 4 days in the presence of appropriate antigen in 200 µl microcultures. Eighteen hours prior to harvesting, 1µCi of ^{3}[H]-thymidine was added to each culture well in 25 µl of medium. Harvesting and counting of the incorporated radioactivity was done as described earlier (section 3. 3. 2. 3). Stimulation index is defined as (cpm in the presence of Ag/ cpm in the absence of Ag).

3. 4. 2 Cytokine assays

The IFN-γ and IL-4 levels in culture supernatants were determined using Biotrak kits (Amersham International, Buckinghamshire, UK). Briefly, 50 µl of the culture supernatant was added to microwells for 2 h. After the prescribed number of washings, the wells received 100 µl of the appropriate IFN-γ- or IL-4-specific Ab-horseradish peroxidase conjugate and were incubated further for one hour. Following a wash, 100 µl of tetra-methyl- benzidine substrate was added to the wells and, 30 min. later, the optical density (OD) was measured at 450 nm using an automated microplate reader (Molecular Devices, Sunnyvale, CA, USA).

3. 4. 3 Assessment of antibody

The presence of specific serum IgG was determined by enzyme linked immunosorbent assay (ELISA). Microwells of polyvinyl chloride plate (Dynatech Laboratories, Chantilly, VA, USA) were coated overnight with 1 µg of MTg, 4 µg of MTg(2495-2513), or 8 µg of human or rat Tg(2695-2706) dissolved in

carbonate buffer pH 9.6. After emptying, the wells, they were blocked for one hour with 0.1% BSA in PBS. Sera samples were incubated for 1 hour with Ag and following three washings with PBS containing Tween-20, 100 µl of alkaline phosphatase-conjugated goat anti-mouse IgG (Sigma, St. Louis, MO, USA) as the second antibody was added to each well. One hour later, plates were washed thrice with PBS containing Tween-20, and p-nitrophenyl phosphate substrate (Sigma, St. Louis, MO, USA) was added (1 mg/ml in 10 % diethanolamine, 100 µl/well) and incubated for one hour. Absorbance of the p-nitrophenolate product was measured at 405 nm as described above.

3. 5 THYROIDITIS INDUCTION

3. 5. 1 Direct challenge with peptides

To induce EAT directly, mice were primed with 100 nmol of peptide in CFA (with *Mycobacterium butyricum*, Difco Laboratories Inc., Detroit, MI, USA) emulsion and boosted 21 days later with 50 nmol of peptide in IFA. Thirty five days after priming, thyroids and sera were collected from the mice.

3. 5. 1 Adoptive transfer

To induce EAT by adoptive transfer, donor mice were primed with 50 nmol of rat or human Tg-peptides or 50 µg of OVA in CFA. Ten days after priming, inguinal LNC were harvested and cultured for 72h in the presence of immunizing peptide (10 µg/ml). After washing twice with serum free DMEM and once with HBSS, 2 x 10^7cells suspended in HBSS were injected i. p. into each of the recipients. In some experiments, control mice received the same number of

con A-activated syngeneic splenocytes. Fourteen days later, thyroid glands and sera were collected.

3. 5. 3 Assessment of thyroiditis

Formalin-fixed thyroids were embedded in methacrylate and approximately 40 sections, 3.0 µm thick, were obtained at 36 µm intervals from each gland. The sections were stained with haematoxylin and eosin, and scored for the presence of mononuclear cell infiltration as follows: 1= interstitial accumulation of cells between 2 to 3 follicles; 2= one or two foci of cells at least the size of one follicle; 3= extensive infiltration, 10-40% of total area; 4= extensive infiltration, 40-80% of total area and 5= extensive infiltration, > 80% of total area.

3. 6 CHARACTERIZATION OF TCR-Vβ GENE USAGE BY RT-PCR

3. 6. 1 RNA extraction

Total RNA was extracted from 5 x 10^6 hybrid T cells or spleen cells using RNAzol (Biotecx Laboratories, Inc. Houston, TX, USA)) according to the manufacturer's instructions. Briefly, 5 x 10^6 hybrid T cells, were washed twice with serum-free DMEM and at least once with PBS, and were then completely solubilized in 1 ml of RNAzol. After adding 200 µl of chloroform, the tubes were vortexed and centrifuged at 12,000 g for 15 min. The upper aqueous phase containing RNA was transferred into a fresh Eppendorf tube and, after adding isopropanol (in 1:1 ratio) the tube was left overnight at 4°C. The RNA was precipitated by centrifuging at 12,000 g for 15 min., washed once with 75%

ethanol, and after air drying for 10 min., it was finally dissolved in diethyl-pyrocarbonate (DEPC)-treated water and stored at -70°C.

3. 6. 2 cDNA preparation

First strand cDNA synthesis was performed using a kit from Pharmacia (Uppsala, Sweden). Briefly, five micrograms of RNA (in 20 µl volume made up with DEPC-treated water) was placed in a Eppendorf tube and was denatured by heating at 65°C for 10 min. and then chilled on ice. In a separate tube, 11 µl of the reaction mix, 1µl of oligo $[dT]_n$ - primer, 1 µl of dithiothreitol solution and were completely mixed together and finally, the RNA was added. The cDNA synthesis reaction was allowed to proceed at 37°C for 1 h and at the end, it was terminated by heating the tube at 65°C for 10 min. The cDNA was stored at -70°C.

3. 6. 3 Amplification by RT-PCR

For PCR amplification, 2 µl of cDNA was incubated with 0.4 mM dNTP mix, 4 mM $MgCl_2$, 2.5 units of Taq DNA polymerase (*Thermus aquaticus* strain YT1) (GIBCO, BRL), 20 pmol of each primer, and PCR buffer in a total reaction volume of 50 µl. Forward primers specific for Vβ 17 (Waters et al., 1992), other Vβ families and a reverse Cβ primer (Casanova et al., 1991) were synthesized by the GSD Center for Biomaterials (Toronto, ON, Canada). Forward and reverse primers for glyceraldehyde-3-phosphate dehydrogenase (GADPH) were selected from (Ju et al., 1995). A 30-cycle step program (95°C for 1 min., 56°C for 1 min., and 72°C for 1 min.) was preceded by a 5-min. denaturation step at 94°C and

was followed by a 7-min extension step at 72^{O}C (Perkin-Elmer DNA Thermal Cycler, Cetus, Norwalk, CT, USA).

3. 6. 4 Analysis of PCR products

The amplified PCR products were subjected to electrophoresis on 1.5% agarose gels containing ethidium bromide (0.5 μg/ml). Gel images were photographed (film # 667, ISO 3000/36 from Polaroid Corp. Cambridge, MA, USA) using Photo-Documentation Hood (FisherBiotech Electrophoresis Systems FB-PDH-1314, Fisher Scientific, Pittsburg, PA, USA) or recorded using a IS-500 Digital Imaging system (Alpha Innotech Corp., San Leandro, CA, USA).

CHAPTER 4
MAPPING OF THYROGLOBULIN EPITOPES: PRESENTATION OF A PATHOGENIC 9mer PEPTIDE BY DIFFERENT MOUSE MHC CLASS II ISOTYPES

4.1 SUMMARY

The 17mer MTg(2495-11) peptide has been reported to induce EAT in H-2^k mice, a process requiring expression of E^k genes, and in H-2^s mice that lack functional H-2E molecules. To test whether this apparent discrepancy was due to recognition of distinct MTg(2495-11) determinants in each strain, we mapped minimal T-cell epitopes within MTg(2495-11) and examined their pathogenicity in C3H (H-2^k) or SJL (H-2^s) mice. Truncation analysis using MTg(2495-11)-specific, $CD4^+$ hybridomas from C3H mice identified two overlapping determinants, (2496-2504) and (2499-2507), that were restricted by the E^k and A^k molecules, respectively. Subsequent challenge of C3H and SJL mice with these 9mer peptides revealed that the E^k-restricted (2496-2504) determinant elicited EAT and specific proliferative LNC responses in both strains suggesting recognition in the context of A^s, since this is the only class II molecule expressed in SJL mice. This was further confirmed by blocking of the proliferative LNC response by an A^s-specific mAb. In contrast, the A^k-restricted (2499-2507) determinant induced weak EAT and no proliferative LNC reponses in either strain. These data: (1) delineate the 9mer (2496-2504) peptide as a minimal

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Some of the results presented in this chapter have been published as:

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Tg T-cell epitope with direct pathogenic potential in mice and (2) highlight the use of non allelic MHC class II molecules for the presentation of this peptide in mice of different H-2 haplotypes.

4. 2 INTRODUCTION

The MTg(2495-11) has been previously identified as a thyroiditogenic sequence in mice (Chronopoulou and Carayanniotis, 1992) and rats (Balasa and Carayanniotis 1993) and consists of nondominant determinants at the B or T-cell level. Despite its nondominant nature, MTg(2495-11) induces EAT with a genetic pattern similar to that obtained with intact Tg, and elicits strong specific T-cell responses as well as IgG responses that cross react with Tgs from different species (Chronopoulou and Carayanniotis, 1992). For these reasons, MTg(2495-11)-mediated EAT provides an excellent model for studying the immunoregulatory mechanisms leading to mononuclear infiltration of the thyroid and hypothyroidism - the main symptoms of HT (Weetman, 1992).

Earlier work (Chronopoulou and Carayanniotis, 1993) demonstrated that E^k expression is a necessary but not sufficient requirement for EAT induction with MTg(2495-11) and suggested involvement of the K^k and/or A^k loci in the disease process. In apparent contrast to these findings, however, SJL mice that lack H-2E molecules, were found to be EAT-susceptible after MTg(2495-11) challenge (Chronopoulou and Carayanniotis, 1992). Since fine epitope mapping in the MTg(2495-11) sequence had not been attempted, this discrepancy between k and s mice could be accounted for by recognition of either a) distinct pathogenic epitopes within MTg(2495-11) or b) identical pathogenic epitope(s) that could be presented in the context of both E^k and A^s molecules. In the present study we

have addressed this issue by mapping the minimal T-cell epitopes within MTg(2495-11) using cloned T-cell hybridomas and by examining the immunopathogenicity of these epitopes in C3H (H-2^k) and SJL (H-2^s) mice.

4. 3 RESULTS

4. 3. 1 Generation and phenotypic analysis of MTg(2495-11)-specific, cloned T-cell hybridomas

Following the fusion of MTg(2495-11)-primed C3H-derived LNC with BW5147 $\alpha^-\beta^-$ thymoma cells, 11 out of 788 seeded wells showed hybridoma growth and of those, cells from 8 wells were finally tested for peptide specificity using TA3 cells as APC. The results were shown in **Figure 4. 1.** Hybrids 4A2, 4A12 and 4B7 isolated from three different wells responded specifically to MTg(2495-11) and were cloned by limiting dilution (0.3 cells / well). The MTg(2495-11)-specific reactivity of two representative clones, one from each of the hybrids 4A2 and 4A12, is shown in **Figure 4. 2.** Both hybrid clones responded by secreting IL-2 upon incubation with MTg(2495-11) (1-20 μg/ml final concentration range) and mitomycin-C-treated syngeneic spleen cells (APC). No response was observed against ovalbumin (OVA) (control antigen). FACS analysis after direct labeling of the hybridomas with FITC- or PE-conjugated mAb, indicated that both the clones were derived from a TCR $\alpha\beta^+$, $CD4^+$ $CD8^-$ T-cell subset **(Figure 4. 3)**.

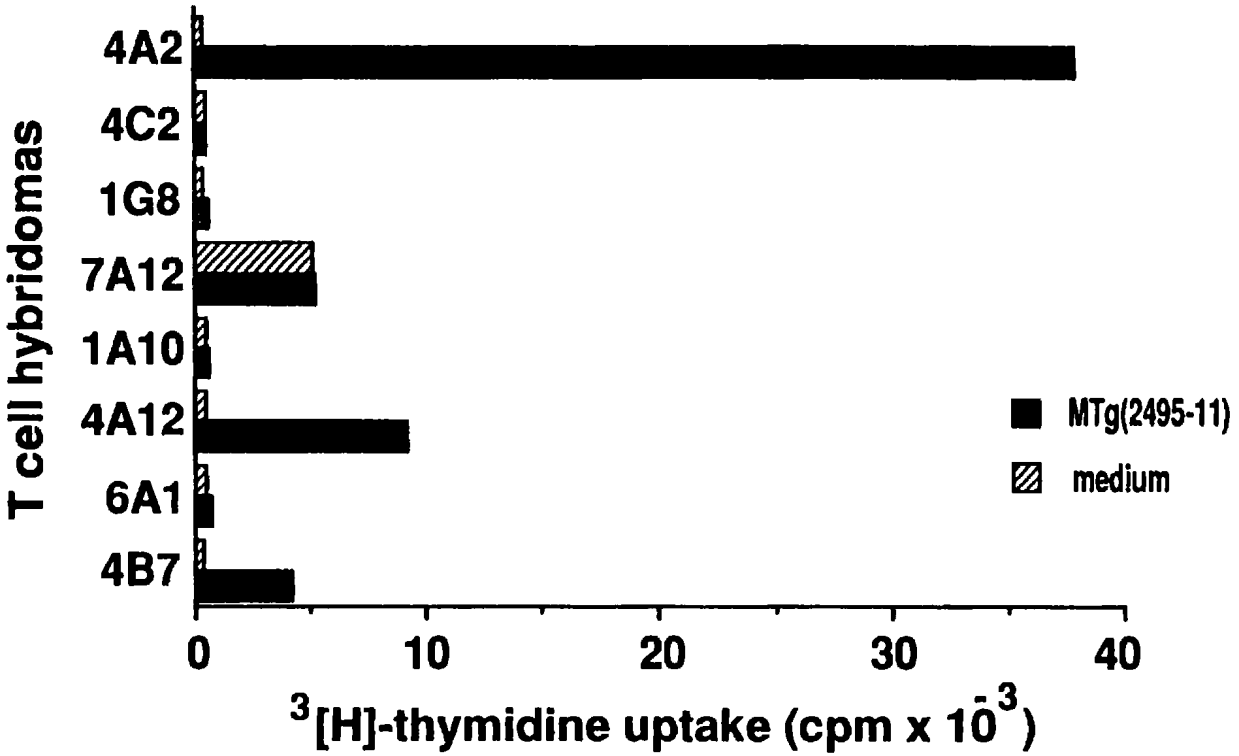

Figure 4. 1 Screening of C3H mice-derived T cell hybridomas for specificity to MTg(2495-11). Using TA3 cells as APC, peptide specific activation of T-cell hybrids was assayed by their IL-2 release which in turn was assessed by proliferation of CTLL-2. Data represent mean cpm values of triplicate wells. SD values were < 10% of the means.

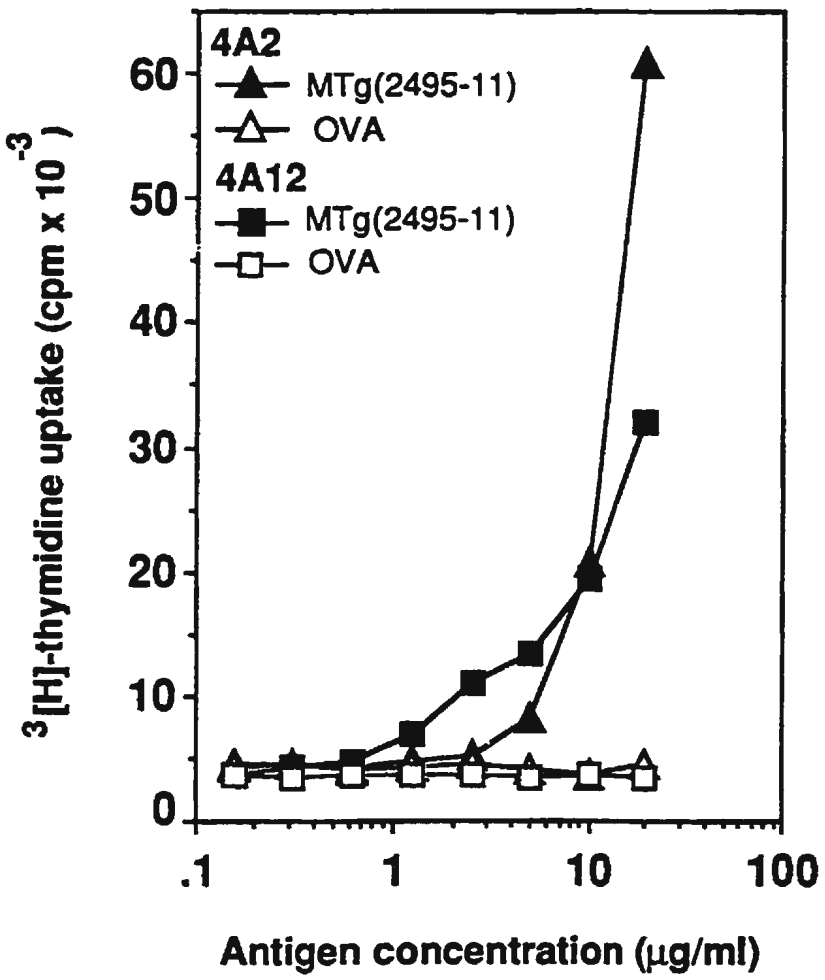

Figure 4. 2 IL-2 release by T-cell hybridoma clones 4A2 and 4A12 upon incubation with MTg(2495-11) or OVA (control). The hybridomas were cultured with mitomycin-C- treated syngeneic splenic APC and peptide at the concentrations shown. After 24 h of incubation, the IL-2 content in culture supernatants was assessed by the proliferation of the IL-2 dependent CTLL-2 line as described in *Materials and methods*. Data represent mean cpm values of triplicate wells. S.D values were <10% of the means. Background means of cpm in the absence of TgP1 were 4A2 = 4,205 and 4A12 = 3,519.

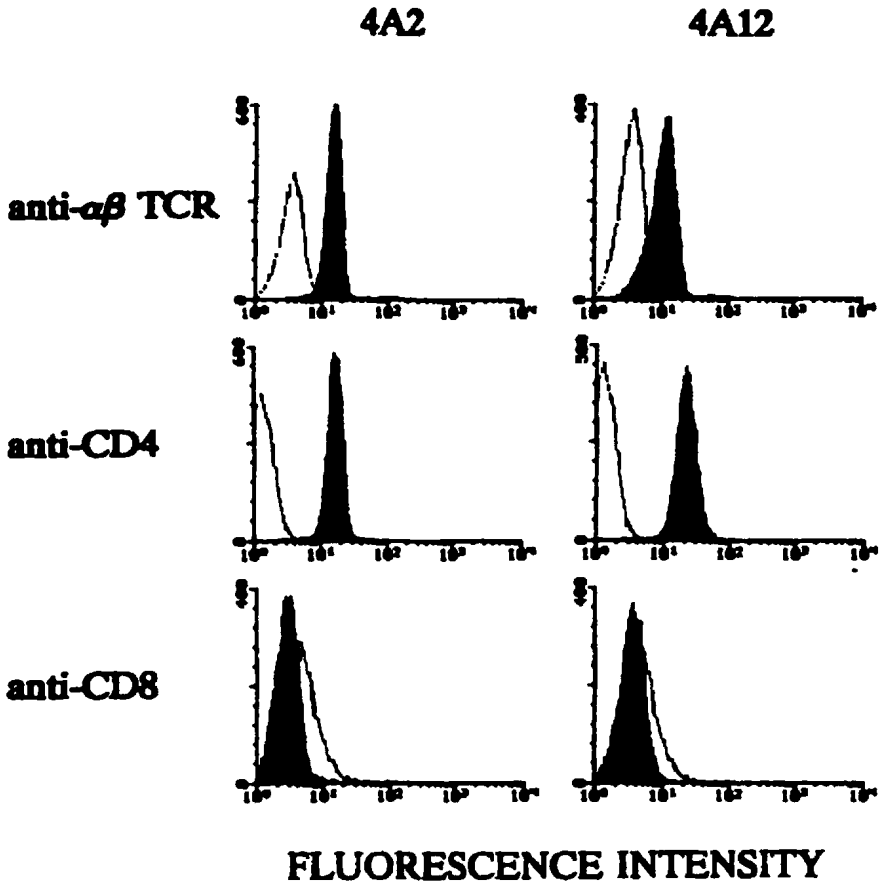

Figure 4. 3 Fluorocytometric profile of the 4A2 and 4A12 T-cell hybridoma clones after direct labeling with the indicated mAb. The phenotypic profile of the BW5147 $\alpha^-\beta^-$ fusion parent (control) is shown in the *background*.

4. 3. 2 MHC-restriction profile of MTg(2495-11)-reactive hybrids

The MHC-restriction profiles of the 4A2 and 4A12 clones were determined using a panel of blocking mAbs specific for different MHC products and the TA3 cell line as a source of APC. As shown in **Figure 4. 4**, the MTg(2495-11)-mediated activation of the 4A2 clone was blocked by an E^k-specific mAb but not by mAb directed against K^k, A^k, and D^k products. In contrast, the MTg(2495-11)-specific activation of the 4A12 clone was blocked only by the mAb directed against the A^k molecule. The observed MHC-restriction pattern of the C3H-derived hybrids was further confirmed by using splenic cells from intra-H-2 recombinant mice as APC (**Table 4. 1**). The E^k-restricted 4A2 clone recognized MTg(2495-11) on B10.BR and B10.A(2R) cells that express H-2E products but was unreactive with B10.A(4R) APC which do not express functional E molecules. In addition, the 4A2 hybrid was unresponsive with B10.A(5R) APC that express a hybrid H-2E heterodimer suggesting a critical role for the $E\beta^k$ chain in the recognition process. On the other hand, the A^k-restricted 4A12 clone responded to MTg(2495-11) only in the presence of APC expressing A^k products confirming the inhibition assay results (**Figure 4. 4**). These data raised the possibility that distinct MTg(2495-11) epitopes are recognized by T cells in C3H mice. We proceeded to test this by peptide truncation analysis.

4. 3. 3 Two overlapping 9mer T-cell epitopes are present within the 17mer MTg(2495-11) sequence

The clones 4A2 and 4A12 were initially tested against a peptide panel (**Figure 4. 5**) consisting of the MTg(2495-11) sequence (pep. 1) and 13 shorter peptides truncated by one a. a. at the N-terminal (peptide no. 2-6) or the C-

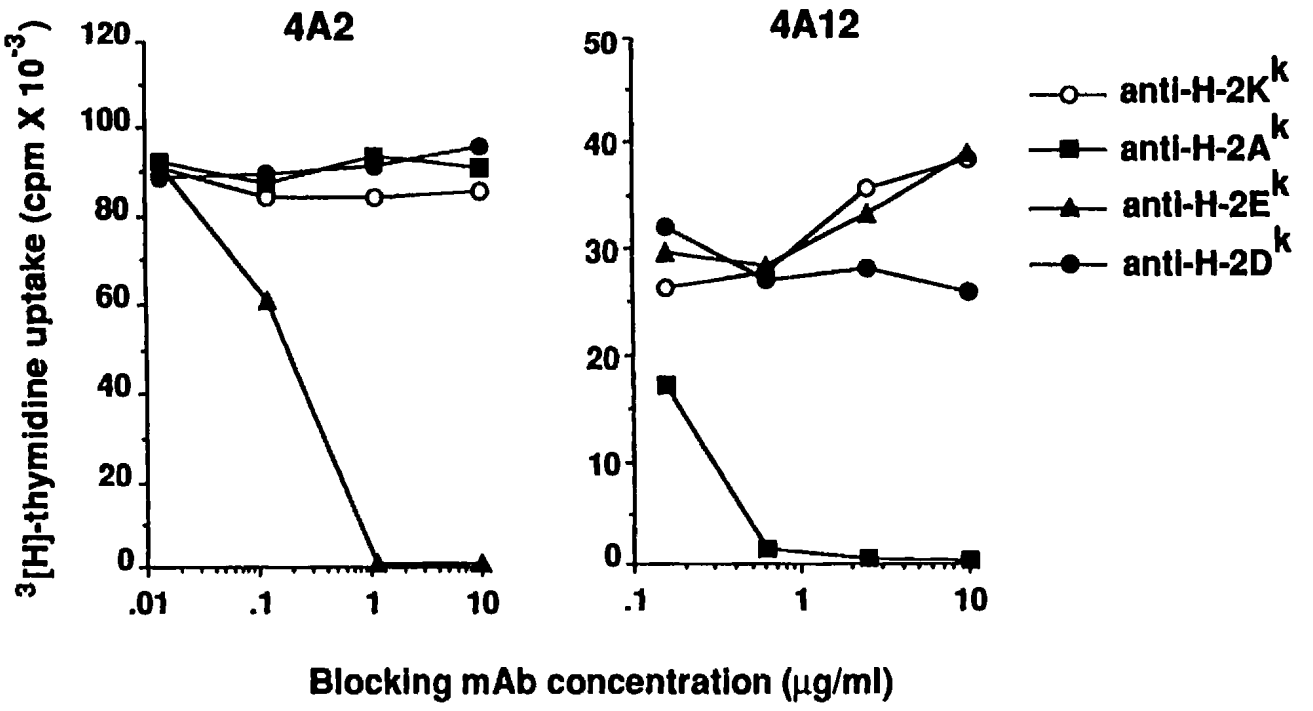

Figure 4. 4 mAb-mediated blocking of MTg(2495-11)-specific recognition by the cloned T-cell hybrids 4A2 and 4A12. In triplicate wells, 10^5 T cells and 10^5 TA3 cells, used as APC, were cultured with MTg(2495-11) (10 μg/ml) and the indicated mAb specific for class I or class II MHC. The concentration of IL-2 in the supernatant was assessed by the proliferation of the IL-2-dependent CTLL line. Background cpm means in the absence of antigen were 4A2 = 1,094 and 4A12 = 528.

Table 4. 1 Activation of T-cell hybrids 4A2 and 4A12 using splenic APC from intra- H-2 recombinant mice *

APC source	H-2 loci					4A2		4A12	
	K	AβAα	Eβ	Eα	D	MTg(2495-11)	medium	MTg(2495-11)	medium
B10. BR	k	k	k	k	k	28, 985 ¶	3, 603	19, 774	3, 639
B10. A(2R)	k	k	k	k	b	36, 289	3, 382	22, 509	3, 913
B10. A(4R)	k	k	(k)¥	- †	b	2, 750	4, 365	21, 336	3, 393
B10. A(5R)	b	b	b	k	d	3, 329	2, 943	2, 723	2, 139

* 10^6 mitomycin-C-treated splenocytes, used as APC, and 10^5 hybrids were cultured with or without peptide in triplicate microwells. After 24h, well supernatants (50 µl) were collected and frozen for at least 2h at -70° C before they were assayed for IL-2 activity.

¶ Mean cpm values of triplicate wells obtained from peptide titration curves represent the response to Tg-peptide at a final concentration 20 µg per ml of culture as measured through the ^{3}[H]-thymidine uptake of the IL-2-dependent CTLL line. Standard deviations did not exceed 10% of the mean values.

† No gene product; ¥ Not expressed at the cell surface.

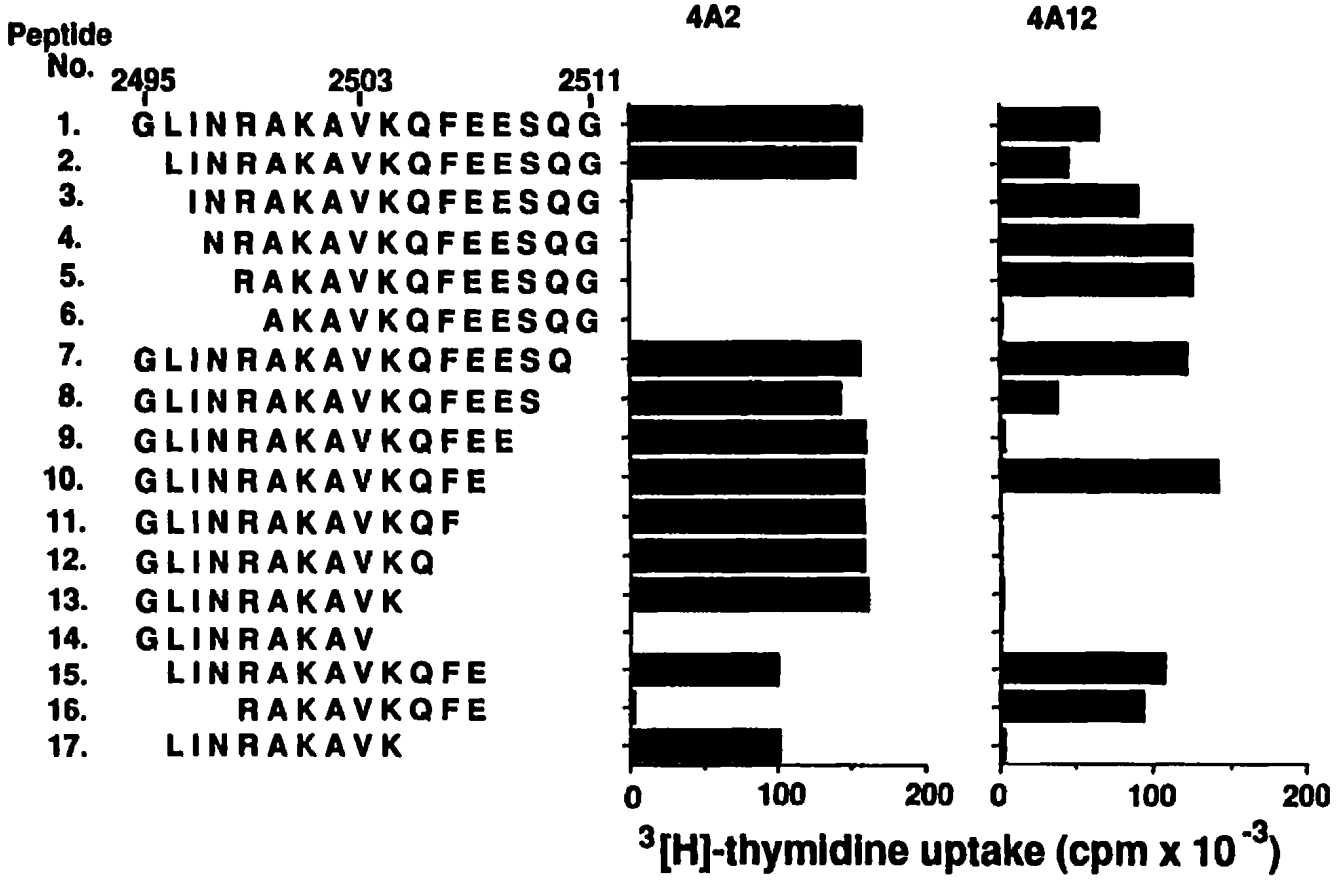

Figure 4. 5 Truncation analysis for T-cell epitope mapping within MTg(2495-11) using T cell hybrid clones 4A2 and 4A12 and TA3 cells as APC. Peptides were used at a final concentration of 10 μg/ml. Data represent means of triplicate cpm values of proliferating CTLL and reflect the relative IL-2 content of supernatants during T-cell activation. S.D. values were <10% of the means. The standard single letter code is used to designate amino acid residues. In the presence of medium alone background cpm were 4A2 = 426, 4A12 = 633.

terminal (peptide no. 7-14) of MTg(2495-11). TA3 cells were used as APC and final peptide concentration was kept constant at 10 μg/ml. It was found that reactivity of the E^k- restricted 4A2 clone was lost upon the removal of the Leu2496 at the N-terminal (pep. 3) or the removal of the Lys2504 at the C-terminal (pep. 14), thus delineating the H-2E-binding epitope as a 9mer sequence with a. a. coordinates (2496-2504). On the other hand, removal of the Arg2499 (pep. 6) or the Glu2507 (pep. 11) abrogated the activation of the A^k-restricted 4A12 clone, revealing the presence of a second 9mer T-cell epitope (2499-2507) within MTg(2495-11). The reason for the lack of response of 4A12 to pep. 9 is not clear but may reflect lack of A^k-binding due to the presence of two negatively charged Glu residues at the carboxyl end. Subsequent testing of the above clones against the 12mer peptide 2496-2507 containing the overlapping epitopes (pep. 15) or the 9mer peptide epitopes themselves (pep. 16 and 17) confirmed the distinct specificities of these clones. In dose-response experiments, subsequent comparison of MTg(2495-11) with the minimal epitopes showed that the peptides LINRAKAVK and RAKAVKQFE were not significantly different, on a molar basis, from the 17mer MTg(2495-11), in their capacity to activate the appropriate hybridomas (**Figure 4. 6**).

4. 3. 4 Presentation of E^k- but not A^k-restricted MTg(2495-11) epitope requires live APC

Since peptide antigens may also undergo processing prior to their presentation by APC to T cells (Fox et al., 1988), we ascertained whether presentation to T cells of the pathogenic epitopes within the 17mer MTg(2495-11) requires peptide internalization and processing by APC. To address this,

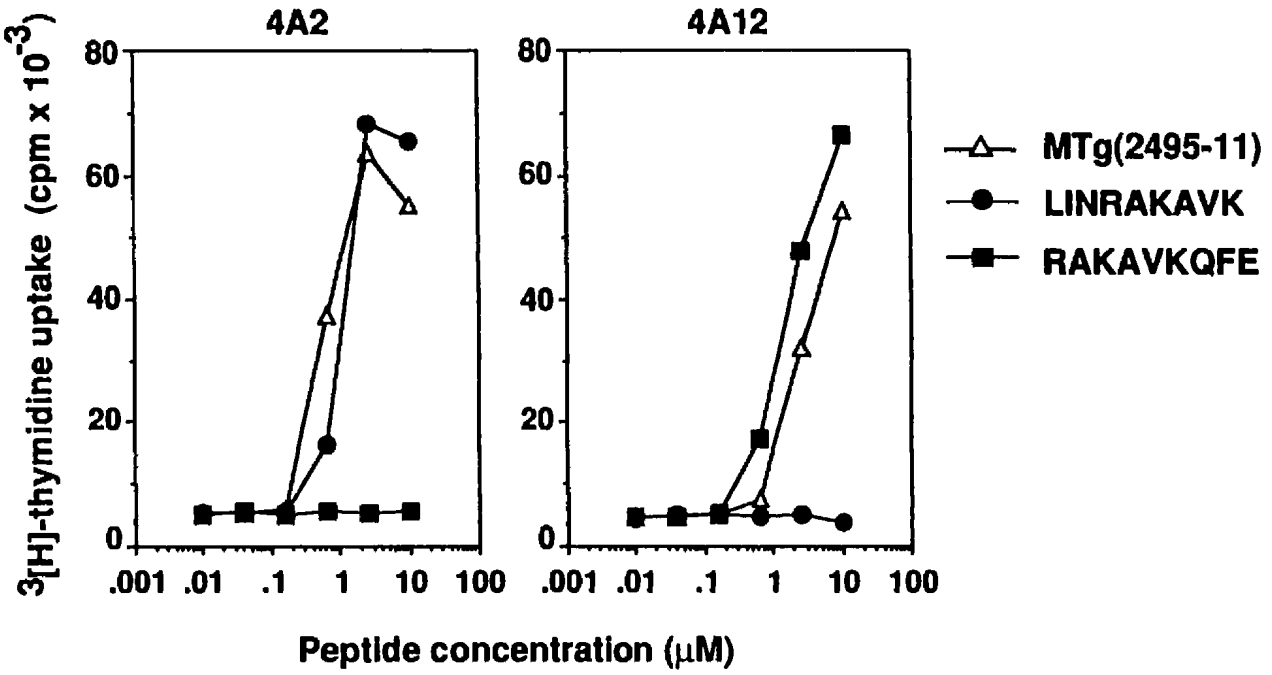

Figure 4. 6 Comparison between MTg(2495-11) and its 9mer T-cell epitopes for their capacity to activate the T-cell hybrids 4A2 and 4A12. TA3 were used as APC. Data represent mean cpm of triplicate wells in the CTLL assay. S.D. values were <5% of the means. Background means of cpm in the absence of antigen were: 4A2 = 5,155 and 4A12 = 5,143.

glutaraldehyde-treated TA3 cells (fixed APC) were used to activate the T-cell hybridomas 4A2 and 4A12 as described in *Materials and Methods*. The E^k-restricted clone 4A2 responds to the minimal peptide (2496-04) on fixed TA3 (**Figure 4. 7**) as well as on live TA3 (**Figure 4. 6**). On the other hand, the clone responded to the 17mer (2495-11) on live APC but did not respond to the 17mer or a 12mer peptide (2496-07) on fixed APC (**Figure 4. 7**), suggesting that presentation of the minimal peptide (2496-04) requires internalization and processing of (2495-11) or (2496-07) by APC. In contrast, the A^k-restricted hybrid clone 4A12 responded not only to the minimal peptide (2599-07) but also to the 17mer and 12mer peptides on fixed APC (**Figure 4. 7**). These data confirm that some peptide Ag need further processing by APC and show that two overlapping T-cell determinants present within a 17mer peptide can exhibit differential processing requirements.

4. 3. 5 Immunopathogenicity of MTg(2496-04) in H-2^k and H-2^s mice

T-cell recognition of the LINRAKAVK epitope in the context of E^k or A^s molecules could explain why MTg(2495-11) induced H-$2E^k$ - controlled EAT in k mice but was equally pathogenic in SJL mice lacking H-2E expression (Chronopoulou et al., 1992). To confirm the immunogenic properties of this 9mer peptide, C3H or SJL mice were immunized with 50 nmol of MTg(2495-11) or the 9mer peptides delineated above and 9-10 days later the proliferative responses of the inguinal LNC were examined. Results from two independent experiments (**Table 4. 2**) demonstrated that addition of the E^k-restricted LINRAKAVK (2496-04) epitope in culture induced significant responses in C3H and SJL LNC following *in vivo* priming with MTg(2495-11) or the 9mer peptide itself. On the

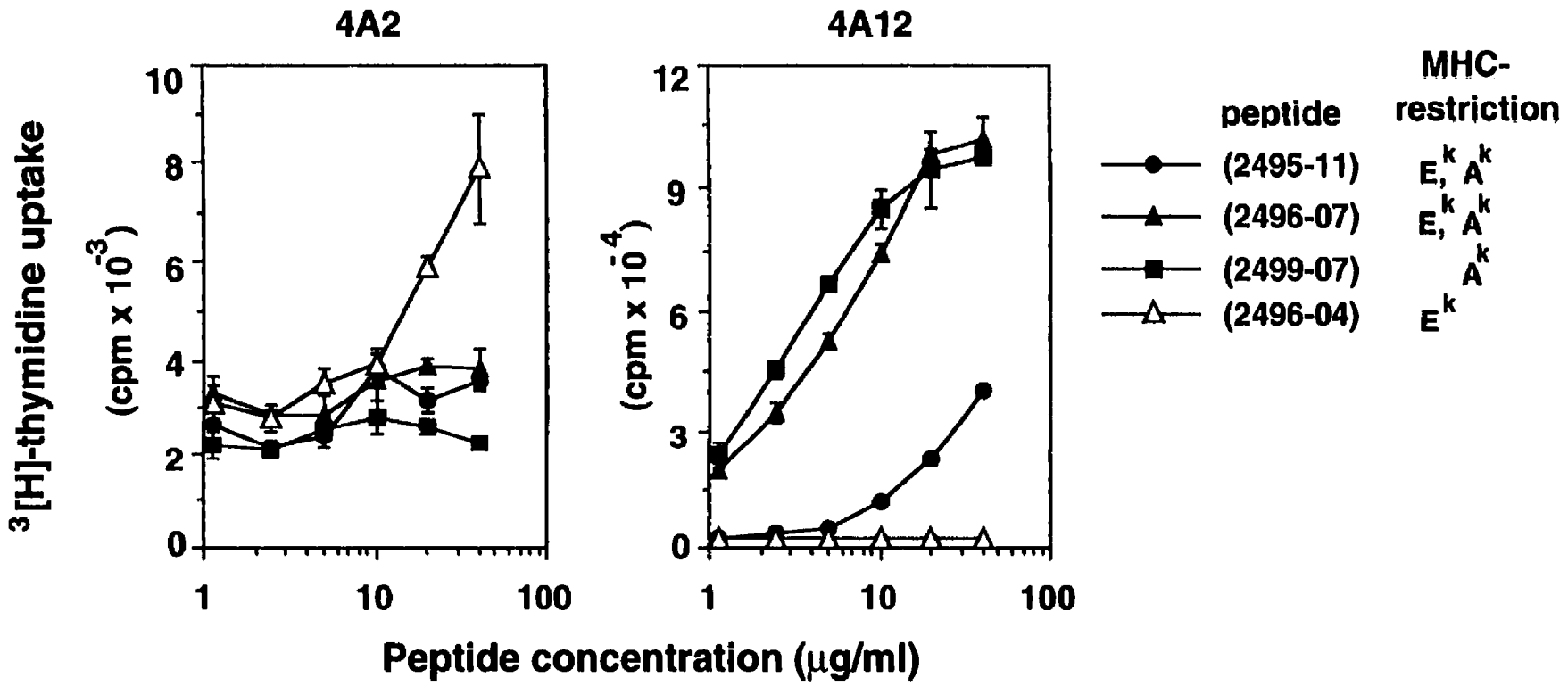

Figure 4. 7 E^k- but not A^k- restricted epitope within the MTg(2495-11) needs processing by APC. 10^5 glutaraldehyde (0.05%)-treated TA3 cells and an equal number of 4A2 or 4A12 hybrid cells were cultured in the presence of varying concentrations of above mentioned antigens. The IL-2 content of supernatants was measured by CTLL proliferation assay as described in **Materials and methods**. The data represent mean cpm values of triplicate wells. SD values were < 10% of the means.

Table 4. 2 Proliferative LNC responses (S. I.) to 9mer T cell epitopes within the MTg(2495-11)

Antigen *in vivo* *		Antigen *in vitro* (20 μg ml^{-1})					
		MTg(2495-11)		RAKAVKQFE		LINRAKAVK	
	Exp.	Strain: C3H	SJL	C3H	SJL	C3H	SJL
MTg(2495-11)	1	5.4 †	24.5	1.2	1.9	2.0	6.6
	2	8.1	19.9	1.2	1.1	3.2	3.2
RAKAVKQFE	1	1.8	1.6	1.4	1.8	1.1	1.5
	2	1.5	1.1	1.4	1.9	1.6	1.9
LINRAKAVK	1	1.6	10.5	1.0	1.9	2.1	18.8
	2	3.6	2.1	1.4	1.1	5.3	9.6

* Mice were s.c. primed with 50 nmol of peptide in CFA, 10 days before collecting the inguinal LNC.

† Stimulation index values >2 are considered significant and are underlined. Background cpm (no added antigen in culture) were: For C3H: MTg(2495-11), exp.1 = 11,721, exp.2 =524; RAKAVKQFE, exp.1 = 3,268, exp.2 = 7,808; LINRAKAVK, exp.1 = 5,188, exp.2 = 8,584. For SJL : MTg(2495-11), exp.1 = 2,870, exp.2 =2,396; RAKAVKQFE, exp.1 = 3,462 exp.2 =3,485; LINRAKAVK, exp.1 = 5,030, exp.2 = 9,736

other hand, the A^k-restricted RAKAVKQFE (2499-07) epitope did not induce significant LNC responses in either strain regardless of the priming antigen used. Also cross-reactivity, at the level of LNC proliferation, was not observed between the two minimal epitopes. These data provided strong evidence that the (2496-04) peptide is recognized in association with H-$2A^s$, since this is the only MHC class II molecule expressed by SJL mice. This was further confirmed by a blocking experiment in which the proliferative response of SJL LNC to the (2496-04) epitope was inhibited in the presence of a mAb specific for A^s determinants but not by an isotype-matched control mAb specific for influenza A nucleoprotein (**Figure 4. 8**)

Direct challenge of C3H or SJL (5 mice per strain) with the immunogenic epitope LINRAKAVK (2496-04) led to significant EAT induction in both strains to a degree equal to that induced by MTg(2495-11) (**Table 4. 3**). All C3H mice and 4 out 5 SJL mice developed EAT following challenge with the (2496-04) epitope; similarly, all C3H and 3 out of 5 SJL developed EAT with MTg(2495-11). On the other hand, the epitope RAKAVKQFE (2499-07) that failed to induce proliferative responses, caused only focal infiltration in 1 out of 5 C3H and 2 out of 4 SJL mice. This immunization regime also induced significant IgG responses to MTg(2495-11) in SJL but not C3H animals (**Table 4. 3**) primed with the (2496-2504) peptide. In contrast, MTg(2495-11)-specific antibodies were not induced to a significant extent by the (2499-07) peptide. Unlike MTg(2495-11), however, which elicits MTg-reactive IgG (**Table 4. 3**), reactivity to MTg was not observed by IgG induced by either minimal epitope. Thus, it is unlikely that such antibodies are involved in the disease process.

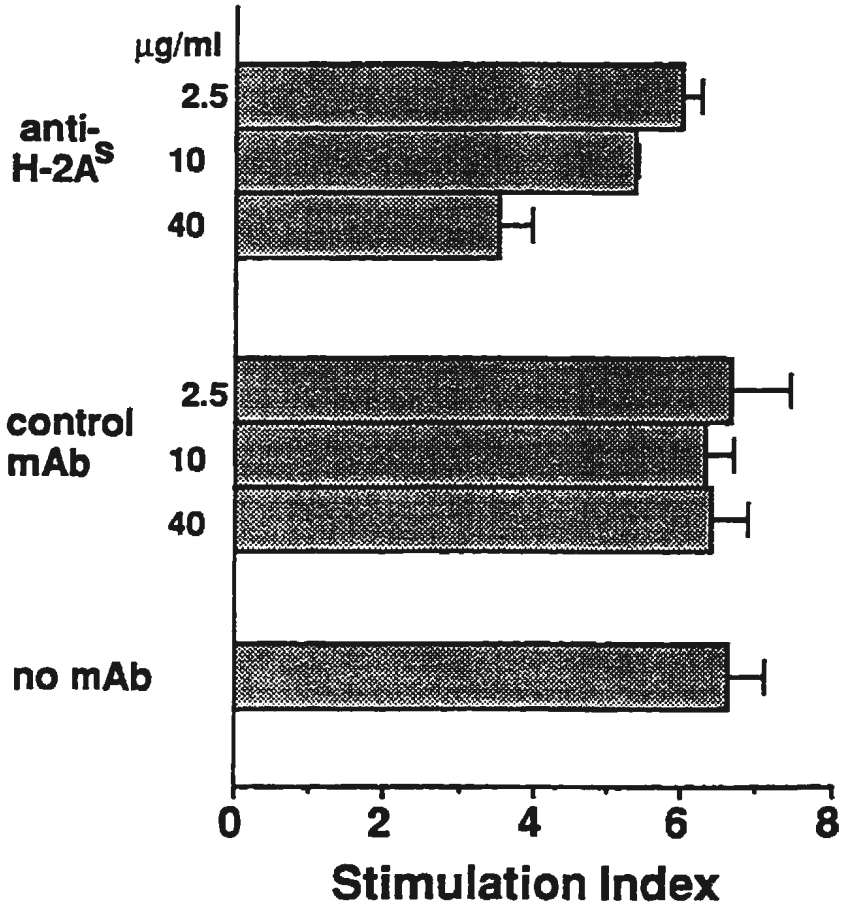

Figure 4. 8 Inhibition of the MTg(2496-04)-specific proliferative response of LNC from SJL mice, in the presence of anti-A^s or anti-influenza-NP mAb. Blocking mAb were used at the indicated concentrations. The final peptide concentration was 10 μg/ ml. A control mAb specific for influenza A nucleoprotein was used, of the same subclass (IgG2a) as the anti-A mAb. In the absence of antigen, background cpm were = 18, 469. Results represent cpm ± SD of triplicate values. Background stimulation (not shown) was obtained in the presence of another Tg peptide, MTg(2695-13).

Table 4. 3 EAT induction with the 9mer Tg T-cell epitopes in C3H and SJL mice

Peptide challenge in vivo *	Infiltration Index †				Mice with thyroiditis	Serum IgG response (O.D @ 405 nm)¥ to			
						MTg(2495-11)		MTg	
	0	0.5	1	2		1:20	1:80	1:20	1:80
C3H mice									
MTg(2495-11)	-	3	1	1	5/5	0.65	0.37	0.20	0.04
RAKAVKQFE	4	1	-	-	1/5	< 0.05	< 0.05	< 0.05	< 0.05
LINRAKAVK	-	3	1	1	5/5	0.04	< 0.05	0.02	< 0.05
SJL mice									
MTg(2495-11)	2	1	1	1	3/5	1.35	1.42	0.86	0.71
RAKAVKQFE	2	2	-	-	2/4	0.21	< 0.05	0.06	< 0.05
LINRAKAVK	1	1	2	1	4/5	0.71	0.20	0.06	0.02

* Mice were primed and boosted with appropriate peptide as described in Materials and Methods. EAT was assessed 5 weeks after the first challenge.

† Severity of thyroiditis: 0.5 = interstitial accumulation of inflammatory cells distributed between two or more follicles; 1= one to two foci of inflammatory cells at least the size of one follicle; 2= extensive infiltration, 10-40% of total area.

¥ Specific IgG assessed by an alkaline-phosphatase based ELISA in pooled sera from each group, tested at the dilutions shown. Microtiter wells were coated with 1 µg mTg or 4 µg MTg(2495-11) as described in *Materials and methods.*

4. 4 DISCUSSION

The present data delineate the 9mer (2496-04) determinant as the shortest Tg peptide known to directly elicit EAT in mice. This peptide is equal in length to the only other known minimal T-cell epitope of Tg (a. a. 2551-58) containing thyroxine (T_4) at position 2553 (Champion et al., 1991). However, the thyroiditogenic potential of the T_4-containing peptide has been shown only by adoptive transfers with peptide-specific LNC (Hutchings et al., 1992) and this peptide was described by Roitt and his colleagues, as being not directly pathogenic in mice (Hutchings et al., 1992). The (2496-04) epitope is recognized by the E^k-restricted 4A2 hybrid and this restriction pattern has been consistent within a large panel of peptide-specific T-cell hybridomas generated from various H-2^k mice such as C3H, B10.BR, and CBA (see Chapter 5, section 5. 3. 2). The same 9mer peptide induces significant proliferative LNC responses in C3H (H-2^k) as well as SJL (H-2^s) mice. Blocking of the specific proliferation of SJL LNC in the presence of an anti-A^s mAb confirmed recognition of this 9mer peptide in the context of A^s, the only functional class II MHC molecule in SJL mice.

Direct challenge of C3H and SJL by the (2496-04) peptide induces thyroid infiltration equivalent to that obtained after challenge with MTg(2495-11). Therefore, this determinant is likely to be the main pathogenic epitope within MTg(2495-11). This property of the (2496-04) determinant can explain why MTg(2495-11) was found earlier to be pathogenic in H-2^s mice which lack functional E molecules but induced E^k-controlled EAT in H-2^k mice (Chronopoulou and Carayanniotis, 1992; Chronopoulou and Carayanniotis, 1993). It should be emphasized, however, that mapping of minimal MTg(2495-

11) epitopes was not attempted with SJL T cells and at this stage it is unclear whether the LINRAKAVK sequence represents a minimal thyroiditogenic peptide in SJL mice. It is possible that a nested epitope present within (2496-04) may mediate H-2^s-restricted EAT. A rationale for such speculation can be derived from studies describing that T cells from different strains of mice recognize distinct determinants within the short myoglobin peptide [(110-121) Nanda et al., 1992)].

The lack of MTg(2495-11) pathogenicity in B10.A(5R) mice (Chronopoulou and Carayanniotis, 1993), which express hybrid (E_b^b: E_a^k) molecules can also be explained if such class II molecules are unable to present the pathogenic peptide. The requirement for the E_b^k chain for the presentation of the minimal (2496-04) epitope to the 4A2 T cells, selected in the context of (E_b^k: E_a^k), tends to support this view. Thus, MTg(2495-11) pathogenicity may not be influenced by the simple presence (B10.A(5R)) or absence (SJL) of E molecules but it may pivot on the expression of class II heterodimers suitable in presenting the (2496-04) determinant. Under this hypothesis, MTg(2495-11) pathogenicity in k mice may not require concomitant expression of A^k or K^k molecules as previously suggested (Chronopoulou and Carayanniotis, 1993).

The A^k-restricted (2499-07) epitope did not elicit LNC-proliferative responses in either strain but exhibited weak direct pathogenicity. This was a puzzling observation in view of the fact that both minimal peptides were mapped by IL-2-secreting T-cell hybrids isolated from the same fusion using MTg(2495-11)-reactive LNC. It is possible that T cells reactive with the (2499-07) determinant may proliferate in culture after they receive helper signals from E^k-restricted T cells responding to the (2496-04) determinant. In that regard,

MTg(2495-11)-specific proliferation of rat LNC was found earlier to be blocked by both A- or E-specific mAb (Balasa and Carayanniotis, 1993). Also, the (2499-2507) epitope may be recognized by Th cells that participate in the induction of humoral responses since strains such as B10.A(4R), do not mount proliferative LNC responses to MTg(2495-11) but do generate high titers of MTg(2495-11)-specific IgG (Chronopoulou and Carayanniotis, 1993). On the other hand, it has been shown that non-proliferative T cells can adoptively transfer disease upon *in vitro* incubation with autoantigens (Hunter, 1986; Gregerson et al., 1989) and it is possible that a pathogenic role for (2499-2507)-specific T cells may emerge in adoptive transfer studies. Preliminary results have clearly shown that MTg(2495-11)-elicited T cells can transfer EAT in both C3H and SJL mice.

The identified 9mer T-cell epitopes have identical a. a. sequences with their human homologues (Chronopoulou and Carayanniotis, 1992; Malthiery et al., 1987) and they both carry the tetramer KAVK, one of the Rothbard and Taylor (1988) motifs that contributed to the identification of MTg(2495-11). The coordinates of the E^k-restricted epitope coincide with residues that delineate a common motif for E^k-binding peptides (Leighton et al., 1991) with the amino-terminal Leu and Ile residues being, respectively, eight and seven positions away from the C-terminal. In addition, the same epitope has a structural motif (Ile 2497-Lys 2502) characteristic of peptides binding to HLA-DR molecules (Hill et al., 1991) as well as motif (Ile 2497-Ala 2501) found in naturally processed peptides bound to HLA-DR8 (Chicz et al., 1993). On the other hand, the E^k-restricted epitope does not obey a motif described for naturally processed A^s-binding peptides (Rudensky et al., 1992) even though it is recognized in the context of A^s. This is not an uncommon finding, since natural ligand motifs by

incorporating constraints of both processing and binding may differ significantly from binding motifs (Falk et al., 1994; Englehard, 1994). The basis for the inability of MTg(2495-11) to bind directly to E^k but not A^k molecules on fixed APC as assessed by the activation of T cell hybrids, is not clear, but might in part result from possible steric hindrance posed on the E^k-binding epitope (2496-04) by the adjacent C-terminal residues. The observation that MTg(2495-11) or the minimal peptides activate T-cell hybrids equally well, suggests that there is no significant interference of flanking residues on peptide recognition, as has been described in other studies (Vacchio et al., 1989). The present findings do not exclude, however, the possible presence, within MTg(2495-11), of other epitopes recognized by non IL-2-secreting T cells or epitopes restricted by class I MHC products.

The variable selection of A or E molecules for the presentation of a pathogenic Tg peptide in mice of different MHC haplotypes has important implications in the quest for possible linkage of class II HLA molecules with susceptibility to thyroid disease. It is reasonable to expect that several peptides from each of the major thyroid autoantigens (Tg, TPO, and thyrotropin receptor) can be recognized by autoimmune T cells. If the binding of a pathogenic peptide to different MHC class II isotypes is a frequent event, and recognition of several such peptides initiates or potentiates the autoimmune process, a strong association of disease with the allelic class II products of any region (DP, DQ or DR) will be difficult or impossible to emerge at the population level or in family studies. Such complexity becomes apparent when defined thyroid antigen epitopes are used for the screening of autoreactive T cells. For example, Dayan and co-workers (1991) have described that ITL from a DR3-positive patient with

GD recognize the TPO peptides (535-551) and (632-645) in the context of DP2 and DQ2 respectively. Epidemiological studies have associated neither of these molecules with GD but instead have shown a weak association with DR3 (Farid et al., 1979; Allannic et al., 1980). That different loci on different haplotypes may account for susceptibility to the same disease has been clearly shown in pemphigus vulgaris, where the DR4 association is attributed to DRB1 *0402 allele whereas the DR6 association is attributed to the DQB1 *0503 allele (Scharf et al., 1989; Nepom and Concannon, 1992). Our present data confirm this concept in the EAT model and further suggest that recognition of a single pathogenic T-cell peptide may account for such associations.

CHAPTER 5

RECRUITMENT OF MULTIPLE Vβ GENES IN THE TCR REPERTOIRE AGAINST A PATHOGENIC THYROGLOBULIN PEPTIDE

5.1 SUMMARY

In autoimmune thyroid disease, although $CD4^+$ T-cell subset(s) were clearly shown to be important, the question whether thyroid-infiltrating, autoreactive T cells are derived from a polyclonal or oligoclonal subset has been the subject of considerable debate. In the present study, we sought to address this issue while working with MTg(2495-11). In mice of both k and s haplotypes, adoptive transfer of MTg(2495-13)-specific syngeneic LNC mediated severe thyroiditis. T-cell hybrids generated from peptide-specific LNC from k mice (C3H, CBA, B10. BR and B10. A(4R) strains) showed expression of 4, 6, 8.2 and 14 plus some unknown Vβ family TCR upon FACS analysis. In parallel, we determined whether the pathogenic (2496-04) sequence constituted a minimal T-cell epitope in SJL mice. *In vitro* recall assays based on LNC proliferation and cytokine release demonstrated that (2496-04) was a minimal T-cell epitope inducing a Th1 type of response in SJL hosts. The TCR variability question was re-examined by testing the TCR Vβ profile of IL-2-secreting hybridomas generated from this Th1 subset. Ten clones derived from two independent fusions were found to utilize three Vβ gene families (Vβ 2, 4, and 17). To the extent that Tg or other

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Some of the results presented in this chapter have been published as:

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thyroid autoantigens encompass multiple pathogenic epitopes it appears unlikely from these data that a restricted TCR-Vβ chain usage will be a general characteristic of thyroiditogenic T cells.

5. 2 INTRODUCTION

The issue whether pathogenic autoreactive T cells utilize restricted or multiple TCR V genes has been intensely debated in autoimmune thyroid disease as in other autoimmune syndromes (Cooke, 1991; Gold, 1994; Kono and Theofilopoulos, 1993). TCR analysis of ITL in GD has been reported to yield evidence for (Davies et al., 1991; Davies et al., 1992; Davies et al., 1993) or against (McIntosh et al., 1993; Caso-Pelaez et al., 1995) oligoclonal expansion, whereas no restriction in Vα or Vβ TCR genes has been observed among ITL of patients with HT (Davies et al., 1993). Several parameters may contribute to such apparent discrepancies from different studies, including diverse etiology of disease, determinant selection effects by various MHC alleles and the presence of non-specific inflammatory T cells in the thyroid infiltrate.

Similar contrasting findings have been obtained in animal models of EAT. NOD mice developing spontaneous thyroiditis were reported to exhibit a thyroid T-cell infiltrate biased towards the use of a single TCR Vβ gene family, but there was no consistent use of a single TCR Vβ chain among the various animals tested (Matsuoka et al., 1993). On the other hand, considerable heterogeneity of TCR Vβ was found in thyroid-derived $CD4^+$ T cells of mice developing "spontaneous" EAT, following protocols that are likely to eliminate regulatory T-cell subsets (Sugihara et al., 1993).

Even in the well-controlled model of Tg-induced EAT, variable conclusions have been drawn from the analysis of intrathyroidal TCR Vβ profiles. The detection of 10 Vβ gene families of the 17 tested in the thyroid infiltrates of CBA/J mice challenged with human Tg (HTg) was presented by Matsuoka et al. as evidence for an early oligoclonal T-cell expansion (Matsuoka et al., 1994). In contrast, following the adoptive transfer of EAT in CBA mice with MTg-specific spleen cells, McMurray et al., (1996) reported the intrathyroidal expression of up to 12 TCR Vβ families as evidence for recruitment of polyclonal T cells in EAT. In such studies, it remains unclear whether the TCR V gene diversity among ITL reflects the recognition of multiple Tg epitopes or is an outcome of a response to a limited number of pathogenic peptides. Furthermore, many variables such as the antigen used, the method of EAT induction, the time of sampling, the choice of PCR primers and/or the method of detection may influence the results.

Since TCR Vβ gene families have not yet been assigned to T-cell clones that recognize defined pathogenic Tg epitopes, we have examined in this report the TCR Vβ diversity among clonal T cells responding to a single thyroiditogenic peptide-MHC class II complex. As a model antigen we have used the A^s-restricted, 9mer MTg peptide (2496-04) that has been previously shown to directly cause EAT in SJL mice in the presence of adjuvant (**Chapter 4, section 4. 3. 5**). Due to a genomic deletion, the SJL strain lacks the Vβ gene families 5, 8, 9, 11, 12 and 13 (Behlke et al., 1986). The rationale was that, in this strain, a limited TCR Vβ heterogeneity would be more easily discerned against a single pathogenic epitope than against the large multiepitopic MTg molecule.

5. 3 RESULTS

5. 3. 1 MTg(2495-11) specific LNC transfer thyroiditis in k and s haplotype mice

Following the observation that MTg(2496-11) induced EAT in mice of k and s haplotype, it was decided to test whether peptide specific LNC would transfer thyroiditis into syngeneic recipients. LNC from mice primed 10 days earlier with MTg(2496-11) were boosted with the peptide *in vitro* and 2 x 10^7 cells were subsequently injected i. p. into syngeneic recipients. Thyroids from all the recipient mice (three from each of the strains C3H and SJL) showed high degree of infiltration by MNC (**Table 5. 1**). In the control group that received equal numbers of con A-activated splenocytes obtained from normal mice, no infiltration was observed in their thyroids (**Table 5. 1**). Adoptive transfer of EAT by MTg(2495-11)-specific cells clearly highlighted the importance of T cells in this model and further raised the question whether the TCR on these effector cells are of high or low variability in nature, since TCR of limited variability would allow testing of future strategies aimed at intervention in the development of EAT.

5. 3. 2 MTg(2495-11)-specific T-cell hybrids express multiple TCR-Vβ genes

In earlier studies it was revealed that MTg(2495-11) was thyroiditogenic in C3H, B10. BR and SJL mice. To ascertain the TCR variability in MTg(2495-11)-specific cells in H-2^k mice, attempts were made to establish hybridomas in CBA, B10. A(4R) and B10. BR mice. Following the fusion of LNC from CBA mice, 112 microwells were plated, out of which 18 wells showed hybridoma growth. After screening six hybrids, one hybrid (3F1) was found to be peptide-specific (**Figure 5. 1**) and was cloned. Subsequent to the fusions involving cells from peptide-

Table 5. 1 MTg(2495-11)-specific LNC transfer thyroiditis

Group	Mouse strain	Cultured *in vitro* with		Infiltration Index					Mice with
		MTg(2495-11)	con A	0	0.5	1	2	3	EAT
1.	C3H	+	-	0	-	-	2	1	3/3
2.	SJL	+	-	0	-	1	-	4	5/5
3.	C3H	-	+	3	-	-	-	-	0/3

For EAT induction adoptive transfer of 2×10^7 peptide-specific cells was carried out as described in *Materials and Methods.* Fourteen days after cell transfer, thyroid glands were collected for histological examination.

primed B10.A(4R) and B10.BR mice, 576 and 614 microwells were seeded respectively. After screening, one out of nine hybrids from B10. A(4R) and ten out of eighteen hybrids from B10. BR mice were found to be MTg(2495-11)-specific (**Figures 5. 2 and 5. 3**). The screening data on C3H mice-derived hybridomas are presented in **chapter 4 section 4. 3. 1.**

Flow cytometric analysis of MTg(2495-11)-specific T-cell hybridomas was performed using Vβ-family-specific mAbs. The fusion partner BW 5147 TCR αβ $^-$ cell line served as negative control. The results of Vβ analysis of T-cell hybrids from B10.A(4R), C3H, B10. BR and CBA mice are presented in **Table 5. 2.** All hybrids responded specifically to MTg(2495-11) presented by TA3 cells (APC) that express E^k and A^k class II molecules. Interestingly, all three A^k-restricted clones 7A10, 4B7 and 4A12 were found to express TCR-Vβ 8.2 family genes whereas TCR-Vβ 6, 4 and some yet other unknown families were expressed among the E^k-restricted hybrids. Additional evidence on TCR-Vβ gene usage among clones specific to the E^k-restricted thyroiditogenic epitope (2496-04) has been sought and is presented later in this chapter (**see section 5. 3. 6**).

5. 3. 3 A^s-restricted T-cell epitopes within MTg(2495-11) are identical to the of E^k / A^k- restricted epitopes

From our earlier findings on epitope mapping within MTg(2495-11) (**Chapter 4, section 4. 3. 4)** it became clear that two distinct overlapping T-cell determinants exist within MTg(2495-11). Further, the E^k-restricted T-cell determinant (2496-04) could induce A^s-restricted T-cell responses and thereby mediate thyroiditis in SJL mice (**section 4. 3. 5**). These data on presentation of the

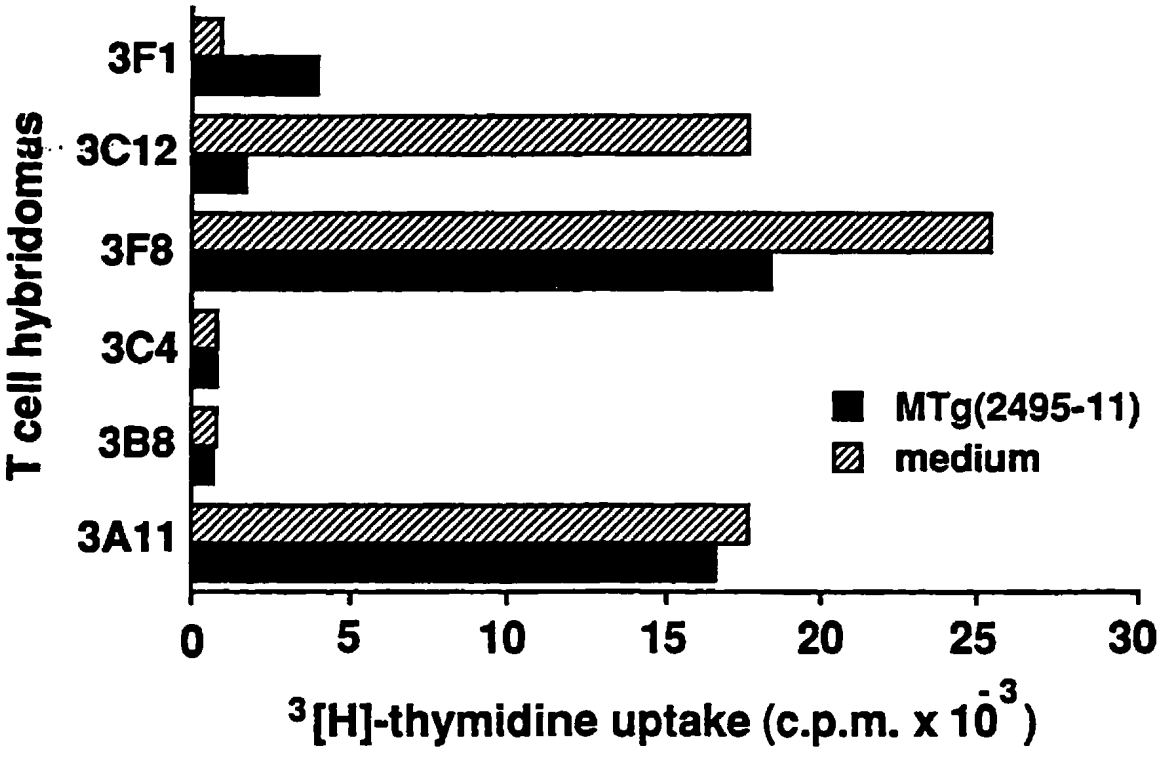

Figure 5. 1 MTg(2495-11)-specific screening of T-cell hybridomas derived from CBA/J mice. Using TA3 cells as APC, peptide-specific activation of the T-cell hybrids was assayed by their IL-2 release which in turn was assessed by proliferation of CTLL-2 as described in *Materials and Methods*. Data represent mean cpm values of triplicate wells. SD values were < 10% of the means.

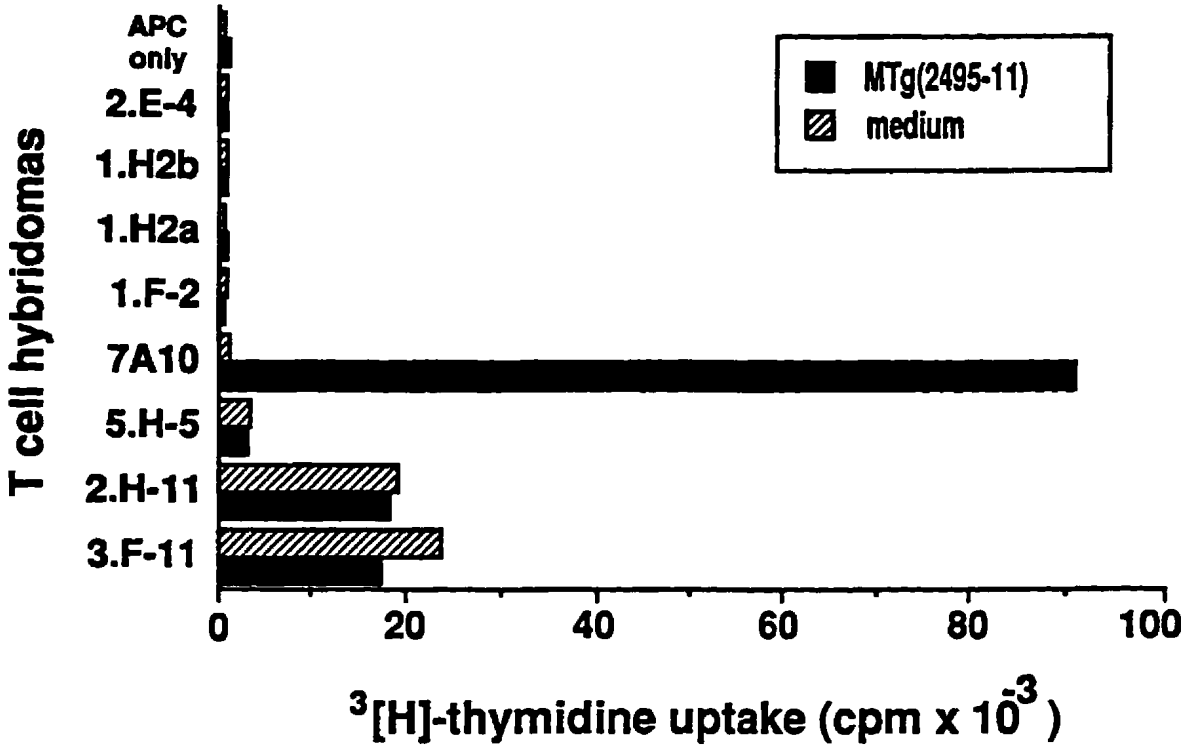

Figure 5. 2 MTg(2495-11)-specific screening of T-cell hybridomas derived from B10. A(4R) mice. Using TA3 as APC, peptide-specific activation of the T-cell hybrids was assayed by their IL-2 release which in turn was assessed by proliferation of CTLL-2 as described in *Materials and Methods*. Data represent mean cpm values of triplicate wells. SD values were < 10% of the means.

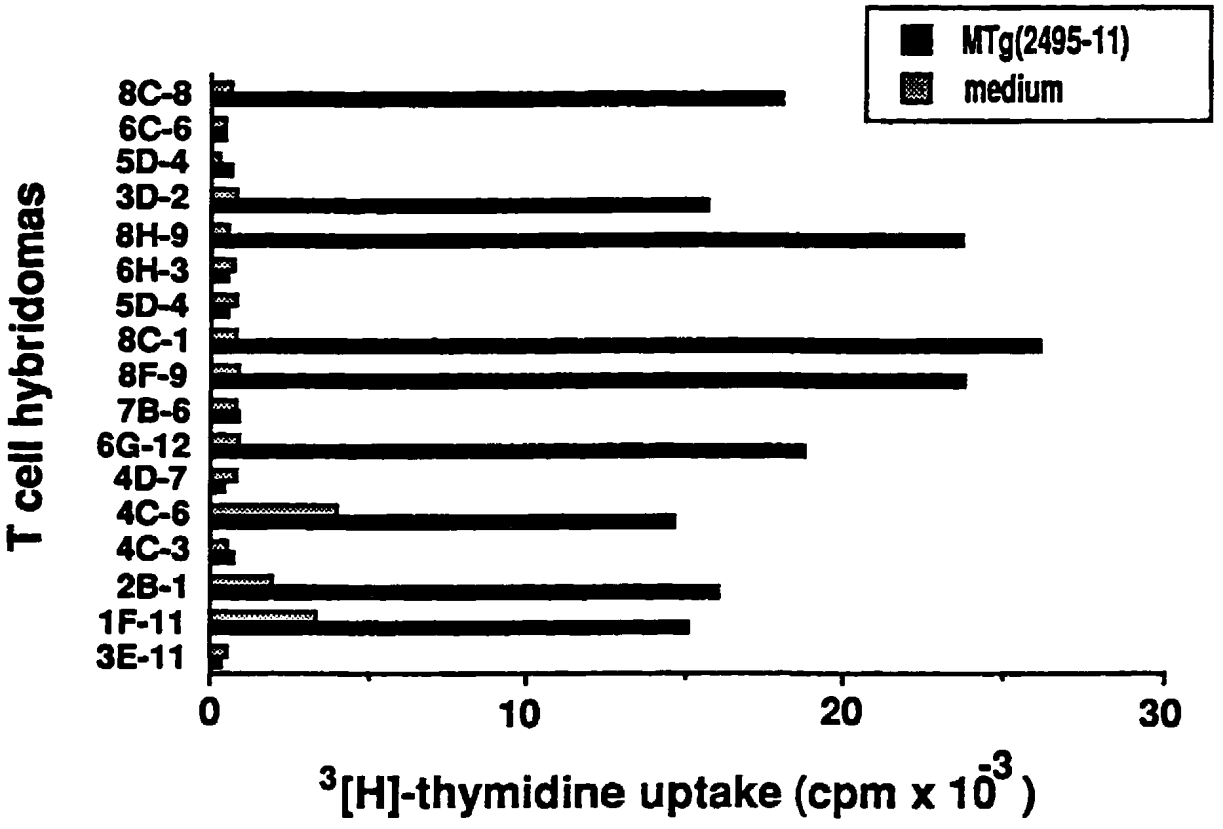

Figure 5. 3 MTg(2495-11)-specific screening of the T-cell hybridomas derived from B10.BR mice. Using TA3 (APC), peptide specific activation of T-cell hybrids was assayed by their IL-2 release which in turn was assessed by proliferation of CTLL-2 as described in *Materials and Methods*. Data represent mean cpm values of triplicate wells. SD values were < 10% of the means.

Table 5. 2 Multiple TCR-Vβ gene usage by MTg(2495-11)-specific T-cell hybridomas

Mouse strain	T cell [a] hybrid	Cloning status	MHC-restriction	CTLL proliferation (c.p.m.) MTg(2495-11)	CTLL proliferation (c.p.m.) medium	Vβ family
B10.A(4R)	7A10	-	I-A^k	90,626	1,070	8.2
C3H	4B7	+	I-A^k	43,265	417	8.2
	4A12	+	I-A^k	36,111	407	8.2
	4A2	+	I-E^k	29,437	244	6
B10.BR	8C8	-	ND	18,019	570	14
	8F9	-	ND	23,841	845	14
	8H9	-	I-E^k	23,781	815	?
	8C1	-	I-E^k	26,161	787	?
CBA	3F1	+	I-E^k	46,530	332	4

+: cloned, -: uncloned, ND: not done

Peptide-specific response and MHC-restricition data of hybrid clones 4A12 and 4A2 have been discussed earlier (see **Chapter 4, section 4. 3. 2).** MHC-restriction of the remaining T-cell hybrids was similarly determined in mAb-blocking assays using class I- (K and D) and class II (A and E molecules)-specific mAb as described in *Materials and Methods.* Phenotyping for TCR-Vβ gene expression was done using mAb specific for 2, 3, 4, 5, 6, 7, 8.1/.2/.3, 9, 11, 13, 14 and 17 TCR-Vβ families. All hybrids were stained positive with anti-TCRαβ-FITC conjugate.

T-cell determinant (2496-04) by non-isotypic class II molecules prompted further studies on localizing epitopes within MTg(2495-11) recognized by SJL T cells. The objective was to determine if more than one A^S-restricted T-cell epitope(s) exists within MTg(2495-11) and whether they map distinctly from E^k -/ A^k -restricted epitopes.

To establish peptide-specific clonal T cells, SJL mice were primed with the MTg(2495-11) in CFA and a T-cell fusion was performed as described in *Materials and Methods* (**section 3. 3. 1**). Following the fusion, a total of 768 microwells were seeded; 563 showed hybridoma growth; and finally 11 hybrids were screened for their peptide-specific response (**Figure 5. 4**). Of those specific to the immunizing peptide, two randomly selected hybrids, 5H3 and 5E8 were cloned. Two representative clones namely 5H3.18 and 5E8.9, one from each hybrid, were further selected for epitope mapping studies. In blocking experiments using anti-A^S mAb, both clones were characterized to be peptide-specific and A^S-restricted (**Figure 5. 5**).

Subsequently, in epitope mapping studies clones 5H3.18 and 5E8.9 were tested against a peptide panel (**Figure 5. 6**) consisting of the MTg(2495-11) sequence (pep. 1) and 13 shorter peptides truncated by one a. a. at the N-terminal (peptide no. 2-6) or the carboxyl terminus (peptide no. 7-14) of MTg(2495-11). LS 102.9 cells were used as APC and final peptide concentration was kept constant at 10 μg/ml. The reactivity of the 5H3.18 clone was lost upon the removal of the Leu2496 at the N-terminal (pep. 3). Peptides further truncated of their N-terminal residues (i.e., pep. 4-6) were clearly non-stimulatory. The response against pep. 6 was marginally higher than the cut-off value which is background mean cpm + 2 SD). When tested against a second set of peptides with truncations

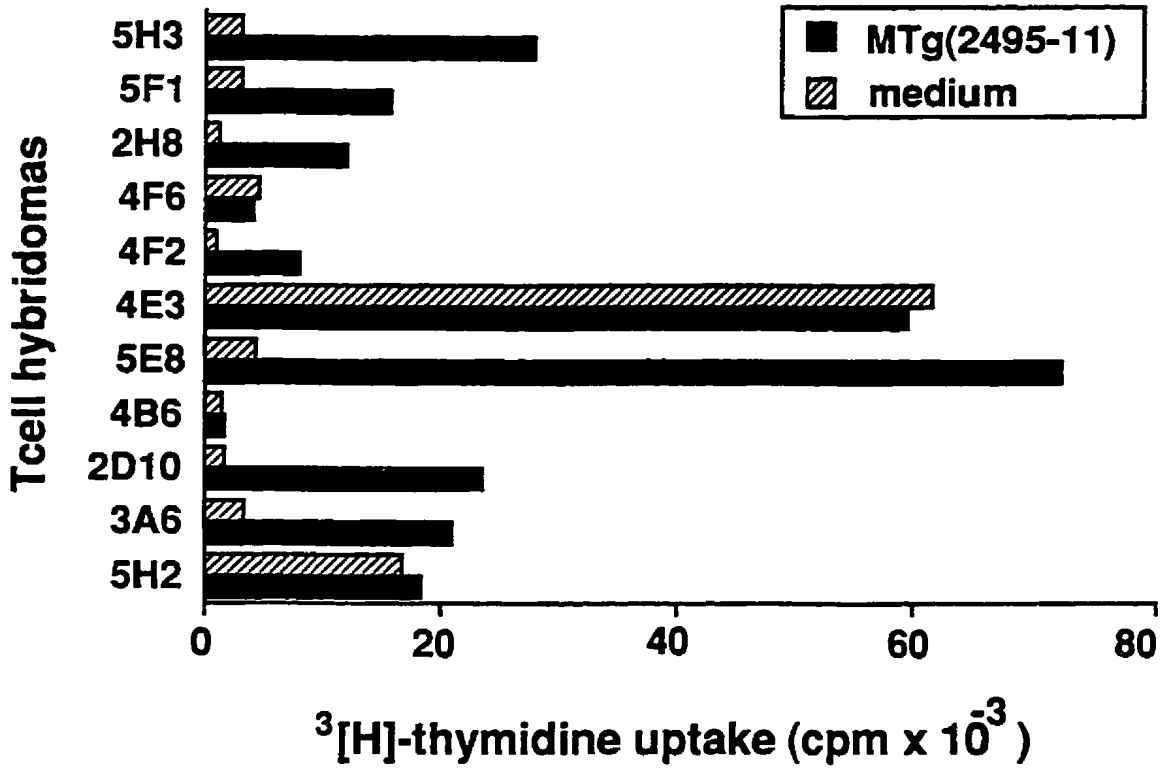

Figure 5. 4 MTg(2495-11)-specific screening T-cell hybridomas derived from SJL mice. Using LS 102.9 (APC), peptide-specific activation of the T-cell hybrids was assayed by their IL-2 release which in turn was assessed by proliferation of CTLL-2 as described in *Materials and Methods*. Data represent mean cpm values of triplicate wells. SD values were < 10% of the means.

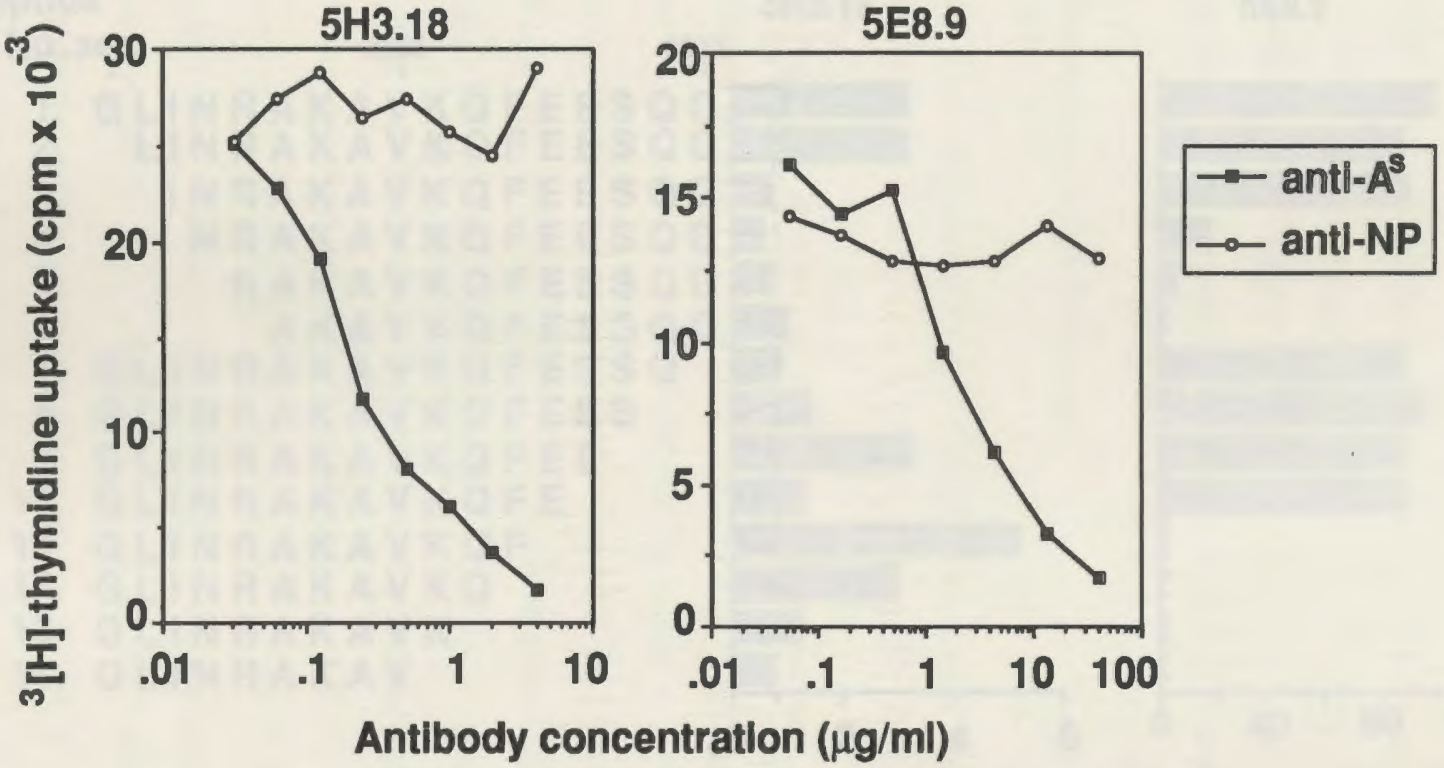

Figure 5. 5 mAb-mediated blocking of MTg(2495-11)-specific activation of the cloned T-cell hybridomas 5H3.18 and 5E8.9. In triplicate wells, 10^5 T cells and equal number of LS 102.9 cells (APC) were cultured with MTg(2495-11) (10 μg/ml) and the indicated anti-A^s (TIB 92) or anti-NP (HB 65). The concentration of IL-2 in the supernatant was assessed by the proliferation of the IL-2-dependent CTLL line. Background cpm means in the absence of antigen were 5H3.18 = 864 and 5E8.9 = 848.

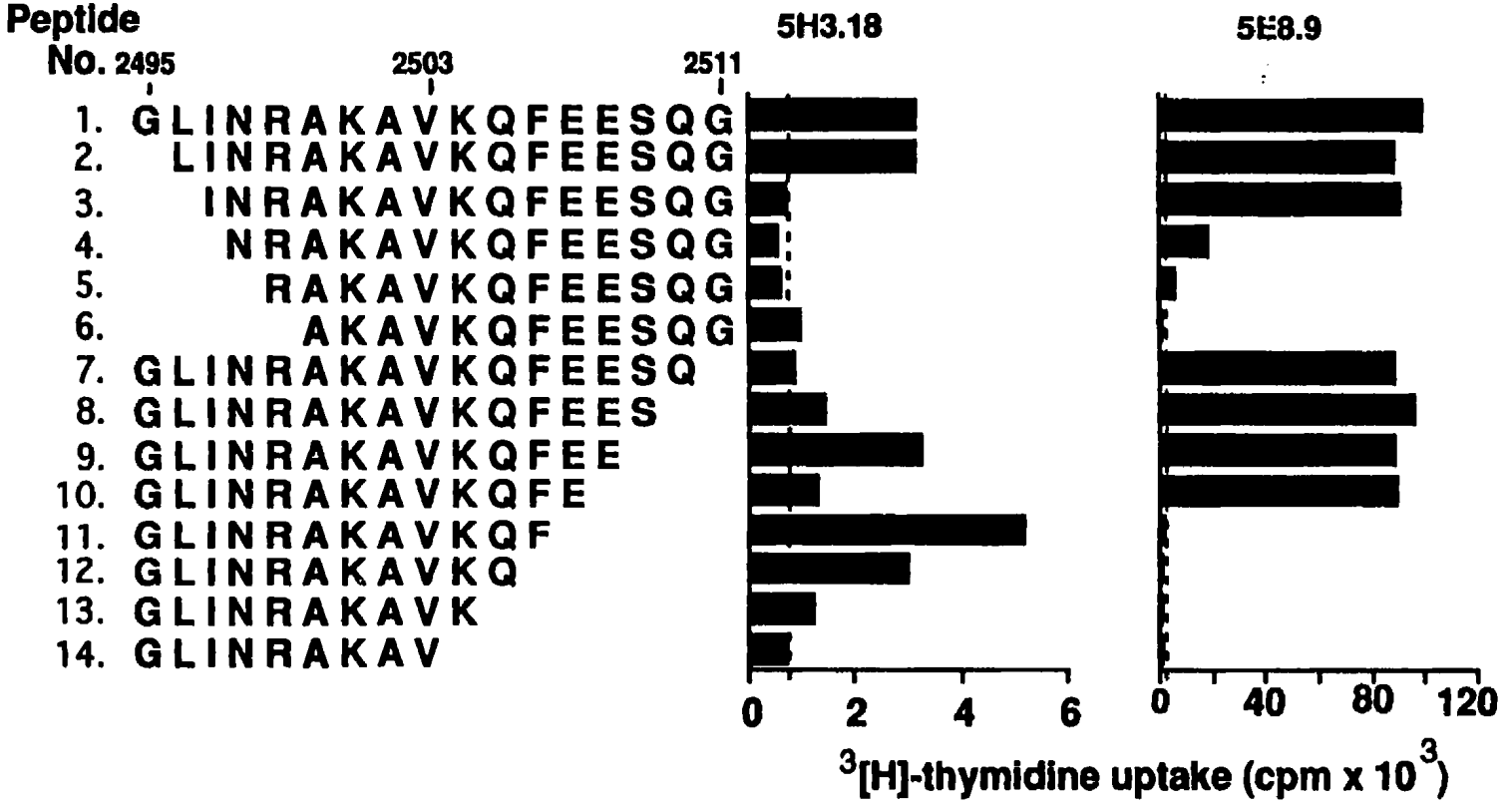

Figure 5. 6 Mapping of T cell epitopes within MTg(2495-11) using SJL T cell hybrids 5H3.18 and 5E8.9. LS 102.9 cells were used as APC and all the peptides were kept at a final concentration of 10 μg/ml. Data represent means of triplicate cpm values of proliferating CTLL and reflect the relative IL-2 content of supernatants during T-cell activation. S.D values were <10% of the means. The standard single letter code is used to designate amino acid residues. In the presence of medium alone background cpm were 5H3.18 = 527, 5E8.9 = 1706. Dotted line shows the cut-off value (background mean + 2 SD) for the activation of the hybrid.

in their C-terminal residues (pep. 7-14), the response of the 5H3.18 hybrid was lost upon removal of Lys2504 at the carboxyl terminus (pep. 14) (**Figure 5. 6**). These data delineated the 5H3.18 recognizing determinant by Leu2496 and Lys2504, a 9mer epitope (2496-04) that is identical to the one mapped earlier by E^k-restricted 4A2 clone (**Chapter 4, section 4. 3. 3**). On the other hand, removal of the N-terminal Arg2499 (pep. 6) or the C-terminal Glu2507 (pep. 11) abolished the activation of the 5E8.9 clone, revealing the presence of a second 9mer T-cell epitope (2499-2507) (**Figure 5. 6**), identical to the one mapped previously by the A^k-restricted 4A12 hybrid clone.

5. 3. 4 Lack of generation of MTg(2496-04) T-cell epitope from Tg processing by APC

Previous work in our laboratory revealed that, in H-2^k mice, MTg(2495-11) could not induce MTg- or RTg- reactive LNC-proliferative responses and vice versa i.e., priming mice with either Tg failed to induce peptide-reactive LNC-proliferative responses (Chronopoulou and Carayanniotis, 1992). In the present study, MTg(2495-11)-specific SJL hybrid clone 5E8.9 was used to examine whether MTg or RTg processing by APC *in vitro* would generate the peptide. The rationale was that the 5E8.9 hybrid, being a highly sensitive clone responding to nM concentration of its peptide-ligand on LS 102.9, would detect (2495-11) following Tg processing. Equal number of the hybrid T cells and APC were cultured for 24 h in the presence of 0.07 to 0.3 μM concentration of MTg(2495-11), mouse or rat Tg. The results presented in the **Table 5. 3** clearly suggested that the epitope in question was not a product of APC processing of mouse or rat Tg. As assayed by CTLL-2 proliferation, the clone did not respond to LS 102.9 pulsed

Table 5. 3 The MTg(2495-11) peptide is not generated during Tg processing by LS 102.9 cells

Antigen in culture	CTLL-2 proliferation (c p m x 10^{-3}) *		
	Ag concentration (μM)		
	0.30	0.15	0.07
MTg(2495-11)	133.17 ± 12.23	130.52 ± 6.06	111.12 ± 8.62
MTg	0.43 ± 0.02	0.62 ± 0.14	0.67 ± 0.19
RTg	0.92 ± 0.68	0.59 ± 0.31	0.70 ± 0.23
MTg(2695-13) (control Ag)	0.68 ± 0.27	0.45 ± 0.23	0.53 ± 0.09

10^5 LS 102.9 cells and an equal number of hybrid cells were cultured with above mentioned antigens at the indicated concentrations. Culture supernatants after 24h, were assayed for their IL-2 content using CTLL-2 as described in *Materials and Methods*.

*Data represent mean cpm ± SD of triplicate wells. Background c.p.m. (no added antigen in culture) = 511.

with MTg or RTg but mounted a strong response to LS 102.9 plus MTg(2495-11) peptide added at all the three doses (**Table 5. 3**). Therefore, these findings confirm the view that the MTg(2495-11) is a site of cryptic T-cell determinants.

5. 3. 5 MTg(2496-04) peptide constitutes a minimal T-cell determinant

To determine whether LINRAKAVK is a minimal T-cell epitope in SJL hosts, inguinal LNC from mice challenged with this epitope were tested for their proliferative capacity against a panel of truncated peptides from the immunopathogenic 17mer (2495-11) sequence. As shown in **Table 5. 4**, loss of Leu2496 or Lys2504 led to a diminished proliferative response, delineating the a. a. (2496-04) as the coordinates of a minimal T-cell epitope in H-2^S mice. These findings are in agreement with our previous studies that described (2496-04) as a minimal epitope recognized by E^k-restricted T-cell hybrids derived from C3H mice (**Chapter 4, section 4. 3. 3**) and also by A^S-restricted T-cell hybrids from SJL hosts (**Figure 5. 6**). Amino acids Leu2496 and/or Lys2504 may play an important role for peptide binding to A^S or they may act as key TCR-contacting residues.

5. 3. 6 MTg(2496-04) activates Th1 cells

To ascertain the cytokine profile of T cells responding to MTg(2496-04), inguinal LNC, from SJL mice primed ten days earlier with this epitope, were cultured for 72 hr in the presence of MTg(2496-04) or the 17mer MTg(2495-11). The culture supernatants were then assayed for IL-2, IFN-γ and IL-4. As shown in **Figure 5. 7**, both peptides induced the release of IL-2 from specific T-cells which was approximately 4-9-fold higher than that observed in control supernatants (medium alone). Similarly, IFN-γ was easily detectable (8.5-11.5 ng/ml) upon

Table 5. 4 MTg(2496-04) constitutes a minimal T-cell epitope

Antigen *in vitro*	Proliferative LNC-response * (cpm) Mean ± SD	(S. I.)
GLINRAKAVKQFEESQG	57, 115 ± 617	(11.35)
LINRAKAVK	80, 057 ± 6, 666	(19. 82)
INRAKAVKQFEESQG	10, 372 ± 1, 328	(2. 57)
NRAKAVKQFEESQG	6, 059 ± 159	(1. 50)
GLINRAKAVK	85, 055 ± 4, 184	(21. 05)
GLINRAKAV	6, 146 ± 1, 011	(1. 52)

* Recall *in vitro* assay of inguinal LNC from SJL mice primed s. c. at the base of the tail with 50 nmol MTg(2496-04) in CFA ten days earlier, and tested against the indicated peptides (10 µg/ml final concentration). Background cpm ± SD (no added antigen in culture) varied from 4, 040 to 5, 030. S. I. values > 3 are considered significant and are underlined.

Bold letters denote the a.a. residues of the priming peptide.

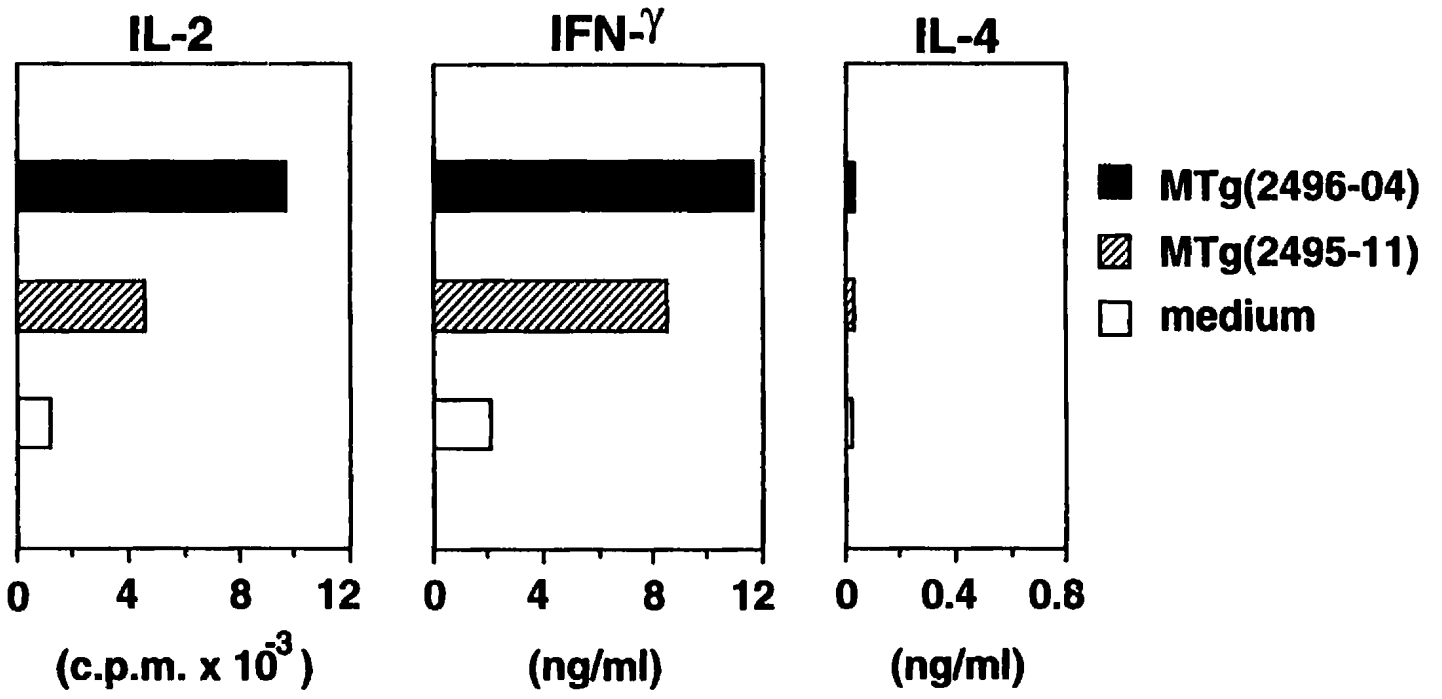

Figure 5. 7 Determination of IL-2, IFN-γ and IL-4 in the culture supernatants of MTg(2496-04)-primed LNC. Ten days after priming, inguinal LNC were cultured in the presence of 10 μg/ml of peptide MTg(2496-04) or MTg(2495-11) for 72 h. Relative IL-2 content was assessed by the proliferation of the IL-2-dependent CTLL-2. The values for IFN-γ and IL-4 were extrapolated from standard curves.

stimulation with either peptide. In contrast, IL-4 was detectable only at very low amounts (20-23 pg/ml) in the same cultures. These results strongly suggested that the MTg(2496-04) epitope induces Th1 cells.

5. 3. 7 Multiple TCR-Vβ gene usage by T-cell hybridomas specific for the minimal MTg-epitope: (2496-04)

To sample the Vβ gene expression among the MTg(2496-04)-specific Th1 cells in the above cultures, we proceeded to generate a panel of T-cell hybrid clones from two independent fusions between BW5147$\alpha^-\beta^-$ and peptide-specific proliferating LNC. A total of 21 hybrids (out of 82 tested) were found to be peptide-specific and were preserved. MTg(2496-04)-reactive, IL-2 secreting clones representing five randomly selected positive hybrids from each fusion were further tested for their gene expression by RT-PCR (**Table 5. 5**). Out of ten hybrids analyzed, five clones expressed Vβ 2, three expressed Vβ 4, and two were positive for Vβ 17.

The amplification products from the cDNA of three representative T-cell clones, 9-32.1, 9-13.6, and 10-25.2 expressing Vβ 2, Vβ 4 and Vβ 17, respectively, are shown in **Figure 5. 8**. For each clone, only a single Vβ product was amplified using primers for each of the 12 SJL Vβ families tested. All Vβ primers yielded detectable PCR products of the expected size when mRNA from SJL splenocytes was analyzed by RT-PCR (data not shown). These findings clearly show the presence of multiple Vβ genes in the MTg(2496-04)-specific TCR repertoire and suggest that Vβ 2, 4 and 17 are predominantly utilized by IL-2 secreting Th1 cells that recognize this pathogenic epitope in SJL hosts.

Table 5. 5 TCR-Vβ gene usage by MTg(2496-04)-specific hybrid T cell clones

Fusion	T cell hybrid	CTLL-2 proliferation (cpm) *		Vβ family †
		MTg(2496-04)	medium	
I	9-13.6	46, 608	383	4
	9-2.4	92, 903	1, 407	2
	9-6.10	63, 223	312	17
	9-32.1	115, 115	7, 092	2
	9-5.1	5, 875	349	2
II	10-1.6	69, 563	3, 931	2
	10-19.1	49, 194	2, 862	4
	10-25.1	33, 701	307	17
	10.26.1	20, 160	187	4
	10.28.1	30, 184	737	2

* Data represent mean cpm of duplicate wells. SD values were < 20% of mean values.

† TCR Vβ-family gene expression was determined by RT-PCR as described in *Materials and Methods*. Due to genetic deletions SJL mice do not express Vβ5, 8, 9, 11, 12 and 13 gene families (Behlke et al., 1986).

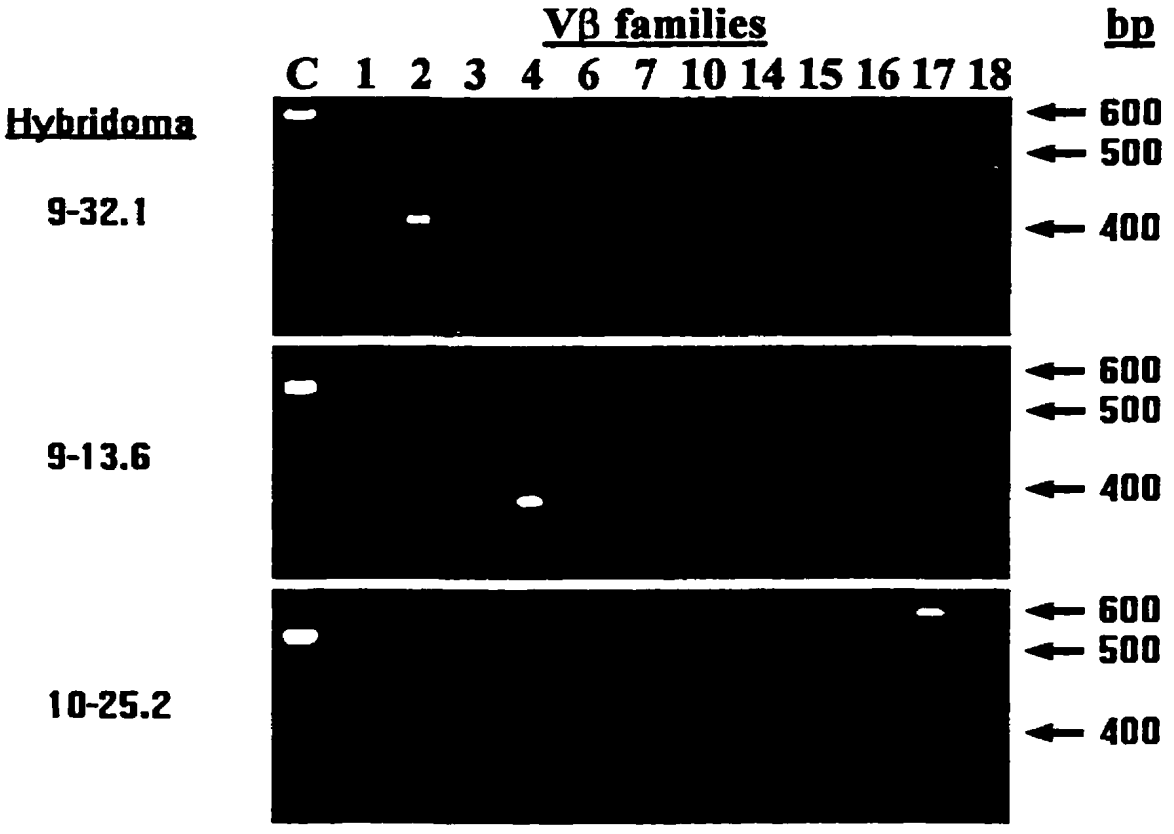

Figure 5. 8 Determination of TCR-Vβ gene expression in MTg(2496-04) specific T-cell hybridoma clones by RT-PCR. Data from three representative clones 9-32.1, 9-13.6 and 10-25.2 showing expression of TCR-Vβ 2, 4 and 17 respectively, are shown. C = GADPH control.

5. 4 DISCUSSION

The present data demonstrate for the first time in the field of EAT, the recruitment of multiple TCR Vβ genes in the recognition of a defined thyroiditogenic epitope. In SJL mice, which express only one functional MHC class II molecule, A^s, the utilization of three (i.e. 2, 4 and 17) out of twelve available Vβ families for the recognition of a single peptide-MHC complex is compatible with results from other EAT studies (Matsuoka et al., 1994; McMurray et al., 1996; Nakashima et al., 1996) showing involvement of many Vβ families in the response to the multiepitopic Tg molecule (Carayanniotis and Rao, 1997). Since the MTg(2496-04) epitope also binds to the non isotypic E^k molecule in H-2^k mice (**Chapter 4**), and the MHC haplotype is known to affect the thyroiditogenic TCR repertoire (Lomo et al., 1996), it is likely that T cells expressing other Vβ families in addition to 2, 4 and 17 may be involved in the recognition of this epitope at the population level. The use of three different TCR-Vβ gene familes (Vβ6, 4 and some unknown) by the E^k-restricted MTg(2495-11)-specific hybrids derived from C3H, CBA and B10. BR mice (with differing non-MHC background) also tends to support this view.

Because homing of lymphocytes to the thyroid gland can be influenced by many parameters (Weetman and McGregor, 1994), and the approach we have followed here selects only for IL-2-secreting MTg(2496-04)-specific T cells, we cannot exclude the possibility that the intrathyroidal TCR Vβ profile may not be identical to that observed in peripherally derived peptide-specific clonal T cells. In that regard, it has been hypothesized that selective expansion of intrathyroidal T cells may occur via mechanisms such as recognition of self-MHC or superantigen-like molecules (Davies, 1995). On the other hand, the assessment of

intrathyroidal TCR V genes can be complicated due to the presence of aberrant thymus tissue in the thyroids of 20-60% of mice from several inbred strains including CBA/J and SJL/J (Dunn et al., 1961; Vladutiu and Rose, 1972a). Unlike other organs targeted by autoimmune responses, the normal thyroid contains discrete masses of lymphoid tissue, that could lead to detection of many TCR V gene families that are unrelated to those used by pathogenic T cells. To date, this issue has not been adequately addressed in EAT studies that have focused on the assessment of intrathyroidal TCR V genes.

It remains to be seen whether T-cell subsets responding to other known pathogenic Tg epitopes (Carayanniotis and Rao, 1997) will similarly express multiple Vβ genes. In other autoimmune models, evidence for (Karin et al., 1993) or against (Su and Sriram, 1992; Bell et al., 1993; Kuchroo et al., 1992; Sobel and Kuchroo, 1992) limited TCR V gene usage has been reported for individual pathogenic peptides. All known pathogenic Tg epitopes, including the 9mer MTg(2496-04) used here, have been defined as non dominant (Carayanniotis and Rao, 1997) but, in general, multiple TCR Vβ genes can be recruited in the recognition of either cryptic or dominant epitopes (Kono and Theofilopoulos, 1993).

Non dominant but pathogenic Tg epitopes must be generated intrathyroidally to allow for specific homing of effector T cells. Anti-TCR based immunotherapy aimed at eliminating these cells could be potentially beneficial because it takes into account: a) the spreading of autoaggression from dominant to cryptic epitopes during the course of disease (Lehmann et al., 1993), and b) potential triggering of the autoimmune response by cryptic epitopes per se. e.g. via molecular mimicry with other determinants of microbial or self origin

(Oldstone, 1987). However, the documented involvement of multiple TCR V genes among ITL in autoimmune thyroid disease (Davies et al., 1993; McIntosh et al., 1993; Caso-Pelaez, 1985), among Tg-specific T cells in EAT (Matsuoka et al., 1993; Sugihara et al., 1993; Matsuoka et al., 1994; McMurray et al., 1996), and in T cells responding to a single pathogenic Tg epitope as shown in the present study, argue against the practicality of TCR-based immunotherapy in thyroid disease.

CHAPTER 6

CONTRASTING IMMUNOPATHOGENIC PROPERTIES OF HIGHLY HOMOLOGOUS PEPTIDES FROM MOUSE AND HUMAN THYROGLOBULIN

6. 1 SUMMARY

The current lack of a. a. sequence data for MTg necessitates mapping of pathogenic T-cell epitopes on heterologous Tgs in mouse EAT. A prevailing assumption has been that epitopes sharing a high degree of a. a. homology among heterologous Tgs are likely to exhibit the same immunopathogenic properties in the same host. In this chapter, I have examined this concept while working with a non dominant 18mer MTg(2695-13) peptide that was previously shown to elicit A^s-restricted T cells and EAT in SJL mice. A major immunopathogenic T-cell epitope was localized within the 12mer MTg(2695-06). It was found that the human 12mer homologue carrying two Ser substitutions at Gln2703 and Thr2704 exhibited contrasting properties: a) it failed to activate Th1 cells in lymphokine and proliferation assays, b) it did not cross react with MTg(2695-06) at the T-cell level and c) it induced only focal thyroiditis following adoptive transfer of specific LNC. These data highlight caution against extrapolating results of pathogenic T-cell epitope mapping across heterologous Tgs, even when such epitopes share a high degree of a. a. homology.

Some the results presented in this chapter have been published as:

i) Carayanniotis, G., Chronopoulou, E. and Rao, V.P. (1994). Distinct genetic pattern of mouse susceptibility to thyroiditis induced by a novel thyroglobulin peptide, Immunogenetics 39, 21-28

ii) Rao V.P. and Carayanniotis G. (1997). Contrasting immunopathogenic properties of highly homologous peptides from rat and human thyroglobulin, Immunology *90*, 244-249.

6. 2 INTRODUCTION

From studies that span the last four decades, it has been clearly shown that Tg, the most abundant antigen of the thyroid gland and the largest autoantigen known (homodimeric mol. mass = 660 kD), induces EAT, a T-cell mediated disease (Charreire, 1989; Rayner et al., 1993; Kong, 1994; Weetman and McGregor, 1994). In the mid-1980s, cDNA sequencing showed that the HTg and BTg consist of 2748 and 2750 a. a. residues respectively (Malthiery and Lissitzky, 1987; Mercken et al., 1985) and partial sequencing of the RTg gene delineated the last 967 a. a. at the C-terminal end (DiLauro et al., 1985). These studies revealed that, despite the enormous molecular size, Tgs from various species were evolutionarily conserved. For example, more than 77% of the a. a. residues in BTg and HTg are identical and more than 6% are conservative substitutions.

In recent years, efforts to map pathogenic Tg T-cell epitopes in mouse EAT (Hutchings et al., 1992; Texier, 1992; Chronopoulou and Carayanniotis, 1992; Carayanniotis et al., 1994; Hoshioka et al., 1993; Kong et al., 1995) utilized the a. a. sequences of heterologous Tgs, since the a. a. sequence of MTg remains unknown. A central assumption has been that heterologous pathogenic Tg epitopes would be easily identified because they would probably be themselves phylogenetically conserved and, therefore, their a. a. sequence would be identical to or very similar with pathogenic MTg determinants. This assumption seems plausible in view of the success of these early efforts but it does not take into account that limited a. a. substitutions at key residues contacting either the MHC or TCR can exert profound changes in the immunogenic properties of peptides (Windhagen et al., 1995; Evavold et al., 1993; Sette et al., 1994; Suhrbier, 1991). In addition, this assumption fails to explain the contrasting immunopathogenic

profiles of heterologous Tgs in mice. For example, BTg induces strong 2^o proliferative LNC responses but no direct thyroid pathology in CBA mice (Romball and Weigle, 1984), whereas RTg and HTg induce both EAT and considerable LNC proliferation in the same strain (Charreire, 1989; Romball and Weigle, 1984; Simon et al., 1985).

To the extent that these findings remain poorly understood at the molecular level, we sought, in the present study, to investigate the immunopathogenic properties of a HTg peptide sharing high homology with the A^s-restricted 18mer MTg (2695-13) sequence that was previously shown to induce EAT in SJL hosts (Carayanniotis et al., 1994). Our aim was to examine whether a limited number of a. a. substitutions across heterologous Tg epitopes would lead to large differences in thyroiditogenicity, a concept that has not so far been tested in the field of EAT. The results have important implications in the strategies devised to map pathogenic determinants in Tg.

6. 3 RESULTS

6. 3. 1 Characterization of MTg(2695-13)-specific A^s-restricted T-cell hybrids

Following *in vivo* priming, with MTg(2695-13) only mice of s haplotype developed thyroiditis. To further characterize the T-cell determinant(s), that are possibly responsible for inducing EAT, MTg(2695-13)-specific clonal T cells were generated in the form of T-cell hybridomas from a fusion between TCR $\alpha\beta^-$ BW5147 and peptide-specific lymph node blasts as described earlier (see section 3. 3. 1). Out of eight hundred and eight wells seeded, forty showed hybridoma growth. As shown in the **figure 6. 1** eleven out of twenty three hybridomas screened were found to be specific for MTg(2695-13). Out of these, hybrids 6E10

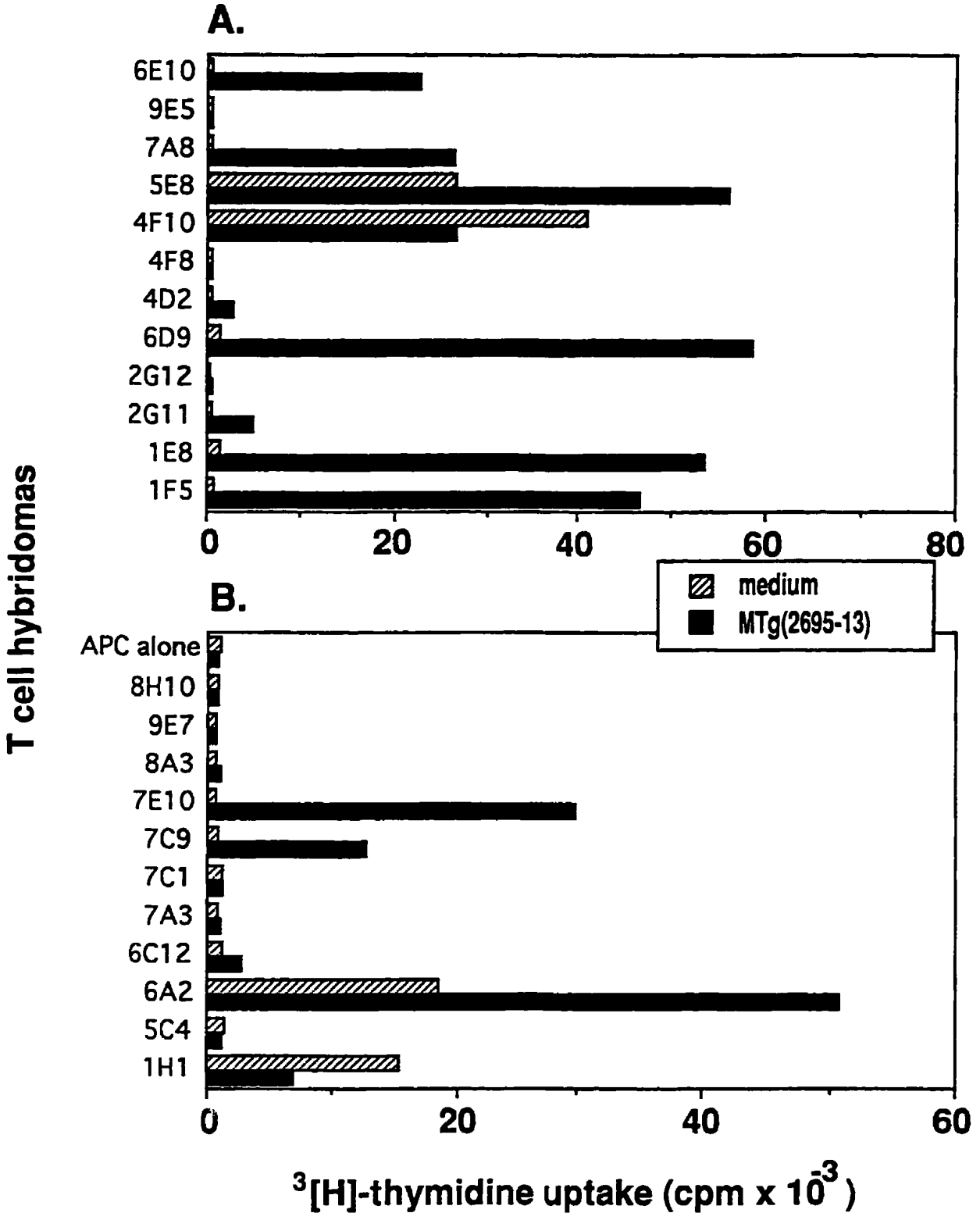

Figure 6. 1 MTg(2695-13)-specific screening of T-cell hybridomas derived from SJL mice (**A** & **B**). LS 102.9 were used as APC. Peptide (10 μg/ml)-specific activation of T cell hybrids was assayed by their IL-2 release which in turn was assessed by proliferation of CTLL-2. Data represent mean cpm values of triplicate wells. SD values were < 10% of the means.

screened were found to be specific for MTg(2695-13). Out of these, hybrids 6E10 and 6D9 were selected and cloned. FACS analysis revealed that clone 6E10.8 (derived from 6E10 hybrid) was $CD4^+$, $CD8^-$ and TCR $\alpha\beta^+$ (**Figure 6. 2A**). From mAb blocking experiment (**Figure 6. 2B**) it was clear that within MTg(2695-13) the epitope recognized by 6E10.8 was A^S-restricted since anti-A^S mAb (but not control Ab) inhibited the activation of hybridoma as assessed by IL-2 content of the culture supernatant.

6. 3. 2 MTg(2695-13) is not a product of *in vitro* processing of Tg by APC

It has also been speculated that for pathogenic effector cells to home to, and persist in the target organ they should be able to recognize specific antigen in that microenvironment. Since the immunization of mice with MTg(2695-13) in adjuvant induces thyroiditis in SJL hosts, it raised the possibility that the above peptide might be generated out of processing of Tg by APC - a property that is often shared by immunodominant peptides. To test if the MTg(2695-13) epitope(s) is expressed in association with class II molecules, LS 102.9 (APC) and 6E10 hybrid T cells were incubated in the presence of mouse or rat Tg. As shown in **Figure 6. 3A** the T-cell hybridoma responded strongly to MTg(2695-13) but not to the control peptide (MTg(2495-11) in the range of 0.3 - 0.07 μM. Interestingly, however, the 6E10 hybrid T-cell clone did not respond to the equimolar concentrations (i.e., 0.30, 0.15 and 0.07 μM) of MTg or RTg, a result compatible with the lack of presentation of MTg(2695-13) epitope(s) in the context of A^S-molecules (**Fig 6. 3A**). As APC are known to differ in the generation and presentation of T-cell epitopes (Vidard et al., 1992 and Sun et al., 1993), we asked if other APC such spleen cells could present the epitope in

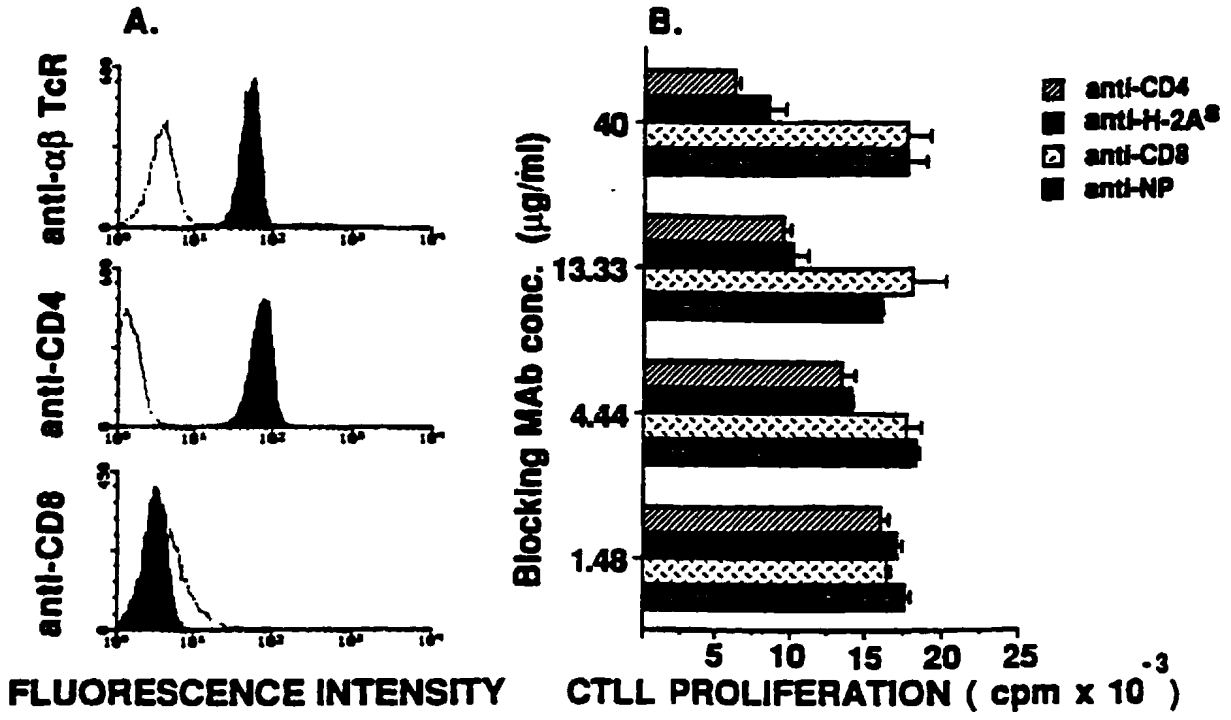

Figure 6. 2 A. Fluorocytometric profile of the MTg(2695-13)-specific T-cell hybridoma clone 6E10 after labeling with the indicated mAb. The phenotypic profile of the BW5147 $\alpha^-\beta^-$ fusion parent (control) is shown for comparison. **B.** mAb-mediated blocking of IL-2 release during MTg(2695-13)-specific activation of the clone 6E10. LS102.9 were used as APC. Results are expressed as (mean +/-SD) values of quadruplicate wells.

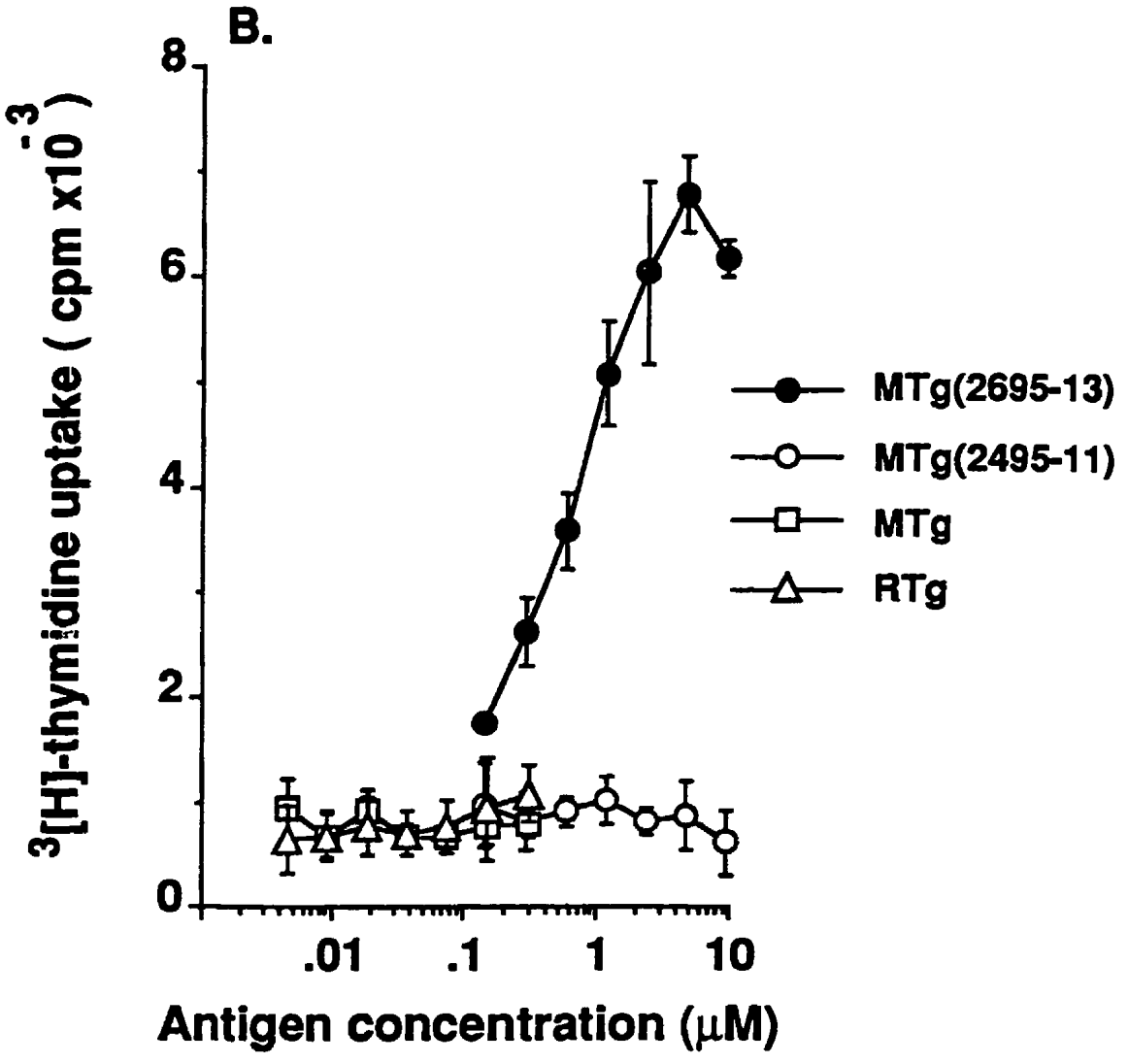

Figure 6. 3B The MTg(2695-13) is not generated during Tg processing by splenocytes.10^6mitomycin-C treated syngeneic splenocytes and 10^5hybrid T cells were incubated in the presence of indicated concentrations of the antigens. Data represent means of triplicate cpm values of proliferating CTLL and reflect the relative IL-2 content of the culture supenatants following T cell activation. Backgound (6E10 plus spleen cells alone) cpm were = 524.

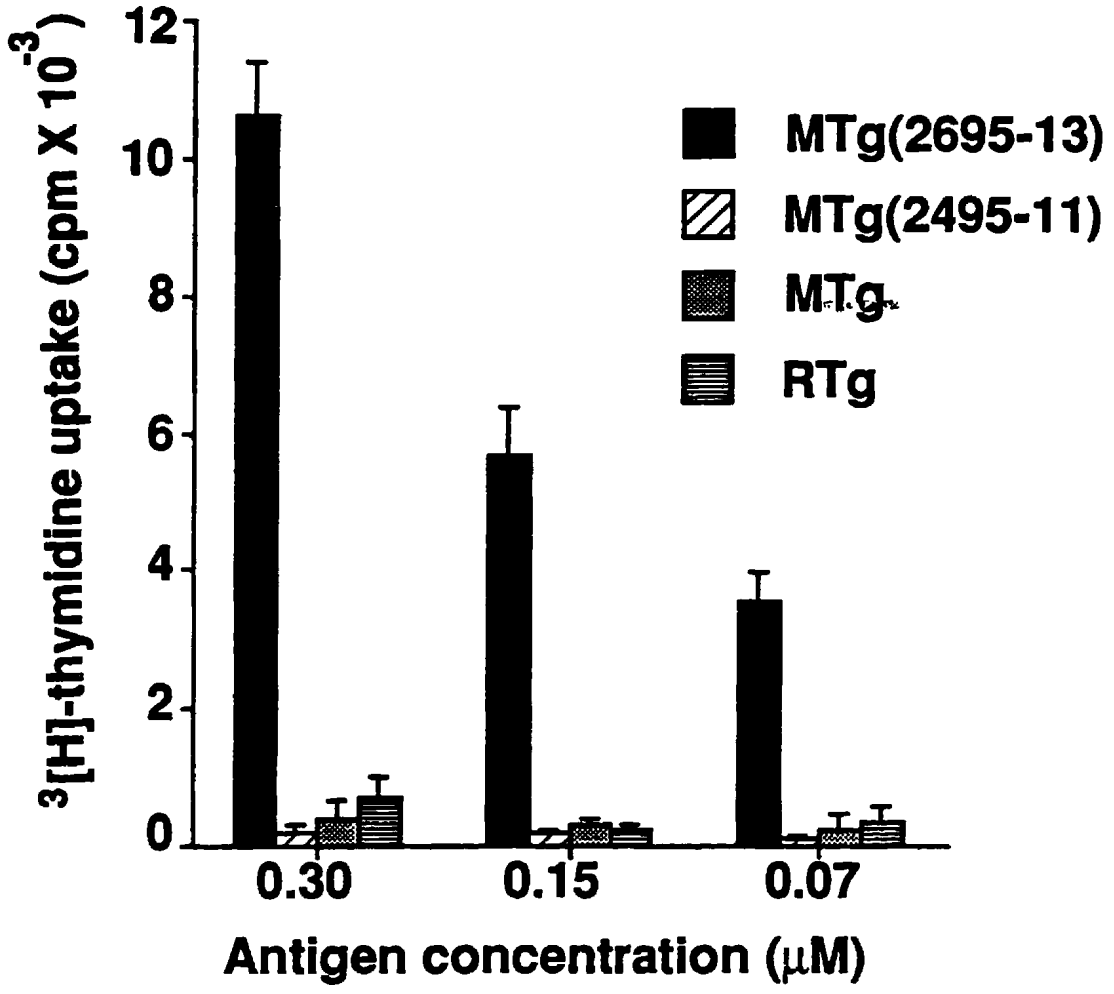

Figure 6. 3A The MTg(2695-13) is not generated during Tg processing by LS102.9. CTLL proliferation as an index of IL-2 release (mean c.p.m. of triplicate wells) by the MTg(2695-13)-specific T-cell hybrid 6E10, in the presence of the indicated Ag. Backgound c.p.m. (6E10 plus LS 102.9 alone) were = 230.

question. To test this, mitomycin-C treated SJL splenocytes were incubated with MTg and RTg as described earlier and used in the hybridoma activation assays. Activation of the clone by MTg(2695-13) but not MTg(2495-11) in all doses employed, confirmed the specificity of the clone and showed that mitomycin-C-treated splenocytes were competent APC. MTg(2695-13) is not a product of Tg-processing because of lack of activation of the clone by Tg at 0.3 and 0.15 μM concentrations, at which the peptide was clearly stimulatory (**Figure 6. 3B**).

6. 3. 3 Lack of T-cell cross-reactivity between homologous Tg epitopes

RTg and HTg are phylogenetically conserved proteins with 74.5% of their a. a. being identical and 7.14% conservatively substituted (Malthiery and Lissitzky, 1987). Intact RTg and HTg elicit strong cross-reactive proliferative LNC responses in mice (**Table 6. 1**), a phenomenon that is explained on the basis of shared T-cell epitopes. It was decided to examine if high a. a. homology between heterologous Tg peptides would be also predictive of similar immunogenic properties by comparing the known pathogenic MTg(2695-13) peptide (Carayanniotis et al., 1994) with its human analogue which is approximately 79% identical and carries 3 a. a. substitutions at positions 2703 (Q -> S), 2704 (T-> S), 2707 (D ->T) and one insertion (S) at position 2708 (**Figure 6. 4**). It was found that LNC from SJL mice primed with the mouse Tg peptide did not recognize the human analogue *in vitro* (**Table 6. 1**). Furthermore, the HTg(2695-13) peptide failed to prime SJL mice for a proliferative LNC response against itself (**Table 6. 1**). The lack of antigenicity and immunogenicity in a human peptide highly homologous to a pathogenic site of MTg prompted us to investigate the T-cell site(s) responsible for this contrasting response.

Table 6. 1 Lack of immunogenicity and antigenicity of the HTg(2695-13)

Antigen *		Proliferative LNC response *in vitro* (S. I. +/- S.D.) against †			
In vivo	*In vitro* (µg/ml)	RTg	HTg	MTg(2695-13)	HTg(2695-13)
RTg	10	<u>10.1</u>	<u>9.4</u>	1.0	1.0
	2.5	<u>8.5</u>	<u>8.0</u>	1.0	0.9
HTg	10	<u>4.8</u>	<u>8.5</u>	1.1	1.0
	2.5	<u>4.1</u>	<u>7.8</u>	1.1	1.1
MTg (2695-13)	10			<u>6.4</u>	1.1
	2.5			<u>5.6</u>	1.0
HTg (2695-13)	10	1.5	1.5	1.3	1.2
	2.5	1.0	1.0	1.1	1.1

* Two mice per group were challenged with 50 µg of RTg or HTg or 50 nmol of MTg(2695-13) HTg(2695-13) in CFA. Ten days later, inguinal LNC were allowed to proliferate against the above indicated Ag as descibed in *Materials and methods*.

† S.I. values > 3 are considered significant and are underlined. Background cpm (cultures with no added Ag) were RTg = 13,144, HTg = 16,943, MTg(2695-13) = 1,052 and HTg(2695-13) = 3,992. In all experiments, a strong proliferative response was observed against PPD @ 10 µg/ml (not shown).

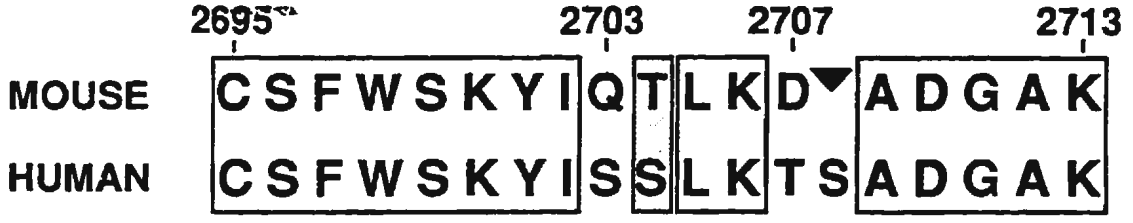

Figure 6. 4 Alignment of the pathogenic MTg(2695-13) sequence with its human homologue. Open box shows identical a.a. (79%), shaded box shows conserved a. a. substitutions, and ▼ indicates a. a. insertion. Numbers indicate relative positions within the HTg sequence.

6. 3. 4 Mapping of T-cell determinants within MTg(2695-13)

To localize T-cell epitope(s) within MTg(2695-13), we synthesized three overlapping 12mer peptides (2695-06), (2698-09) and (2701-13) spanning the MTg(2695-13) sequence. LNC from SJL mice, primed ten days earlier with MTg(2695-13), responded strongly against MTg(2695-13) and the N-terminal (2695-06) peptide (**Table 6. 2**). No response was observed against the "core" (2698-09) peptide and only marginal reactivity was demonstrable against the C-terminal (2701-13) 12mer. Conversely, the N-terminal MTg(2695-06) peptide was clearly immunogenic in SJL mice and elicited strong LNC responses against the 18mer MTg(2695-13) but not against the other overlapping peptides. A weak cross-reactive response against the (2698-09) peptide was detectable, reflecting the extensive a. a. sharing between these epitopes. These results delineated the MTg(2695-06) as the site responsible for the immunogenicity of the 18mer MTg(2695-13).

Further evidence that peptide(2701-13) harbored a distinct T-cell determinant when MTg(2701-13)-specific clone 6D9 was tested against the three overlapping peptides. The clone 6D9 strongly responded to (2695-13) as well as (2701-13) but remained unreactive to (2695-06) (**Figure 6. 5**). Taken together, these findings that 18mer MTg(2695-13) encompasses two distinct overlapping T-cell determinants.

6. 3. 5 Substitution of Gln2703 and Thr2704 by Ser influences immunogenicity of the Tg(2695-06) peptide

We next synthesized the 12mer human analogue of MTg(2695-06) which carries two Ser substitutions at positions 2703 and 2704 (**Figure 6. 6**). SJL mice

Table 6. 2 The Tg(2695-06) site delineates epitope(s) recognized by proliferative LNC in SJL mice

Peptide *in vivo* a)		LNC-proliferative response *in vitro* (S. I.) against †			
	(a. a.)	(2695-13)	(2695-06)	(2698-09)	(2701-13)
CSFWSKYIQTLKDADGAK	(2695-13)	10.7	12.9	1.9	2.7
CSFWSKYIQTLK	(2695-06)	12.1	11.3	4.3	1.1
WSKYIQTLKDAD	(2698-09)	1.9	1.5	2.0	1.7
YIQTLKDADGAK	(2701-13)	3.5	1.5	1.9	2.9

* Two mice per group were s.c. primed with 50 nmol of peptide in CFA and ten days later, their inguinal LNC were allowed to proliferate in the presence of the indicated peptide Ag (20 µg peptide /ml) *in vitro*.

† S.I. values >3 are considered significant and are underlined. Data representative of two experiments are shown. Standard deviations were less than 10% of the means. Background cpm were: (2695-13) group=1,052; (2695-06) group=1,706; (2698-09) group=4,149 and (2701-13) group=1,135.

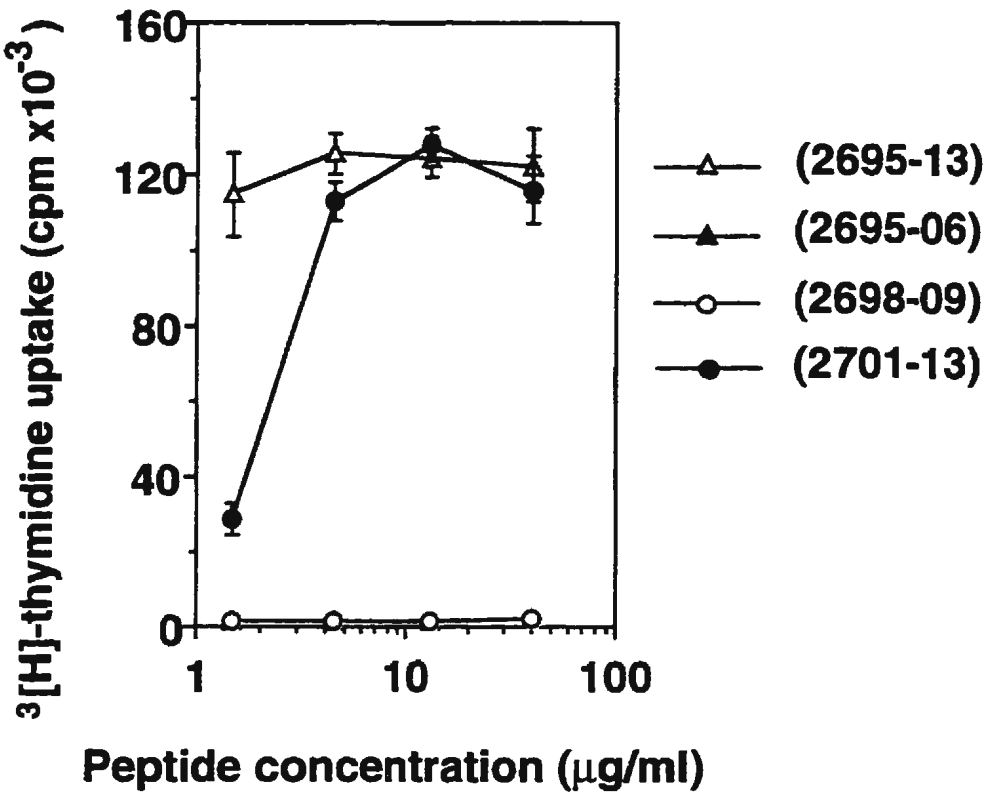

Figure 6. 5 Activation of MTg(2695-13)-specific T-cell hybrid clone 6D9 by the C-terminal 12mer sequence (2701-13). In triplicate wells, equal numbers of T cells and LS 102.9 were cultured separetely in the presence of 10 μg/ml peptide concentration. The data represent mean cpm values reflecting the IL-2 dependent proliferation of CTLL-2. Background cpm were = 1531.

were again primed with either the mouse or human (2695-06) peptides and, ten days following antigenic challenge, their inguinal proliferative LNC responses were assessed *in vitro*. It was observed that the human analogue was not recognized by MTg(2695-06)-primed LNC and, furthermore, it could not induce proliferative LNC responses in SJL mice (**Figure 6. 6**). To determine whether HTg(2695-06) primed non proliferative T cells, we tested by ELISA the supernatants of these cultures for the presence of lymphokines. After 72 h of culture, MTg(2695-06)-primed T cells released highly significant amounts of IL-2 and IFN-γ in response to the rat peptide - but not to the human 12mer analogue (**Figure 6. 7**). In contrast, supernatants from HTg(2695-06)-primed LNC cultures contained neither of these lymphokines, regardless of the antigenic challenge *in vitro*. IL-4 was undetectable in all cultures. Lack of proliferation or lymphokine release by the HTg(2695-06)-primed T cells highlights the importance of Gln2703 and Thr2704 in the recognition of MTg(2695-06) by Th1 cells.

6. 3. 6 In a homologous Tg peptide pair, a T cell clone recognizes only the mouse but not the human analogue

Confirmation of the above findings from the LNC proliferation assays was sought at the T-cell clonal level, using the A^S-restricted T-cell hybrid 6E10 that is specific for MTg(2695-13) (Carayanniotis et al., 1994). As shown in **figure 6. 8A** the 6E10 clone secretes high amounts of IL-2 in response to MTg(2695-13) but not against the 18mer HTg(2695-13). Similarly, 6E10 responded strongly to the 12mer MTg(2695-06) but not to equimolar concentrations of HTg(2695-06) (**Figure 6. 8B**). Lack of activation was demonstrable even at concentrations >100 μg/ml of the human 12mer peptide (data not shown). These data confirmed the

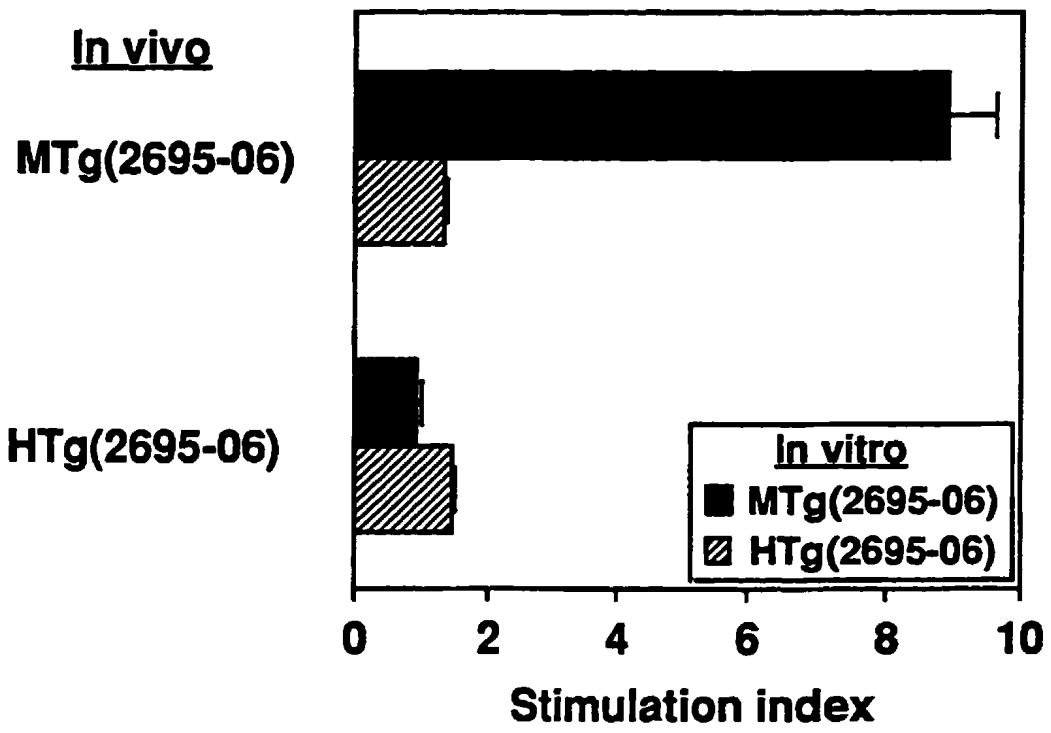

Figure 6. 6 HTg(2695-06) does not induce proliferative T-cell responses and is not recognized by MTg(2695-06)-specific cells. Inguinal LNC from mice (3 mice / group) primed s.c. with 50 nmol of MTg(2695-06) or HTg(2695-06) 10 days earlier, were cultured for 96 h in the presence of varying concentrations of the indicated peptides. Data represent the S. I. values (against 50 μg/ml antigen concentration) of triplicate wells obtained from peptide titration curves. Background cpm were: MTg(2695-06) group = 5838; hTg(2695-06) group = 2601.

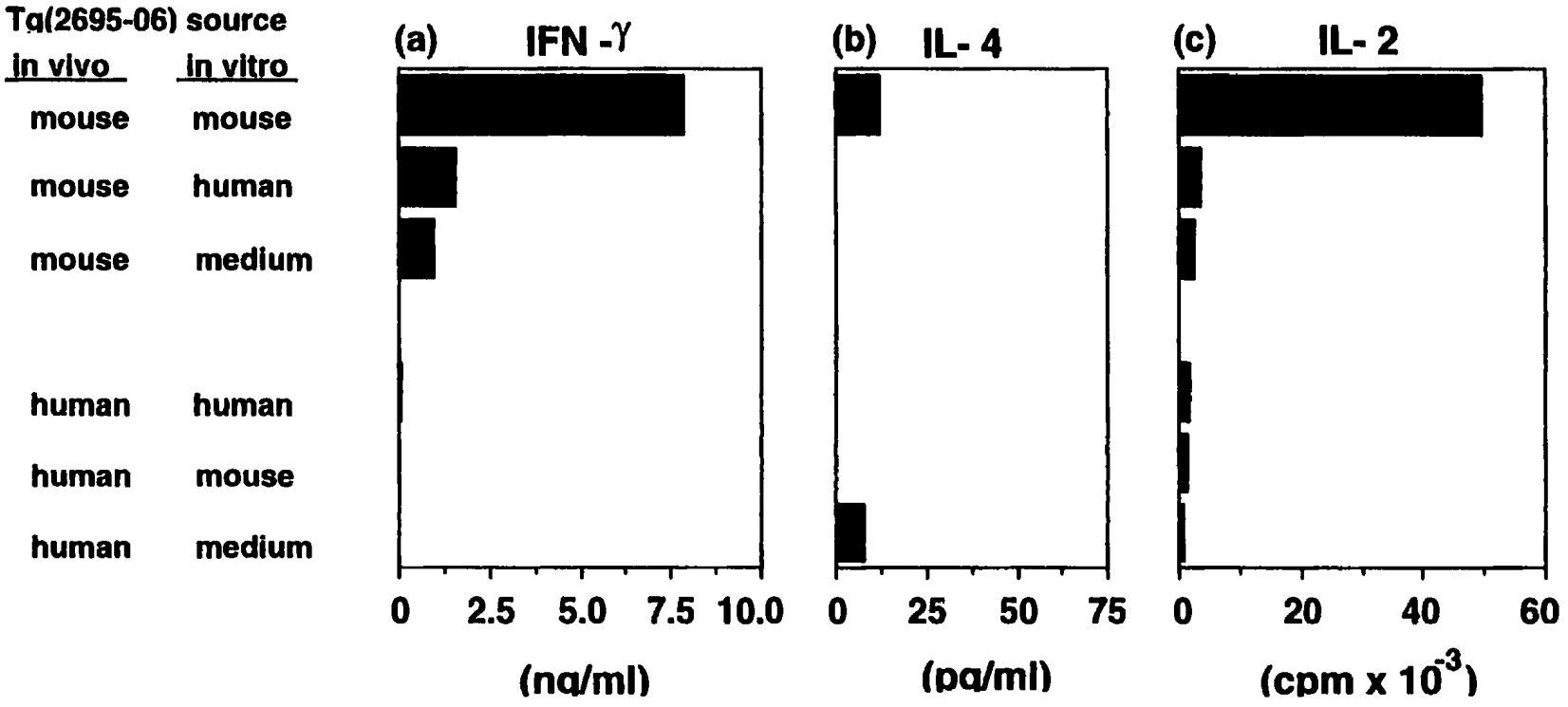

Figure 6. 7 Lymphokine assays of culture supernatants of LNC primed *in vivo* (50 nmol peptide in CFA/mouse) and restimulated *in vitro* (at 3 μM final peptide concentration) with the indicated combination of peptides. Data represent mean concentration values of IFN-γ (a) and IL-4 (b) extrapolated from ELISA standard curves, constructed according to the manufacturer's instructions. Data for IL-2 (c) represent mean cpm reflecting 3[H]-thymidine uptake by proliferating CTLL. S. D. < 10% of means.

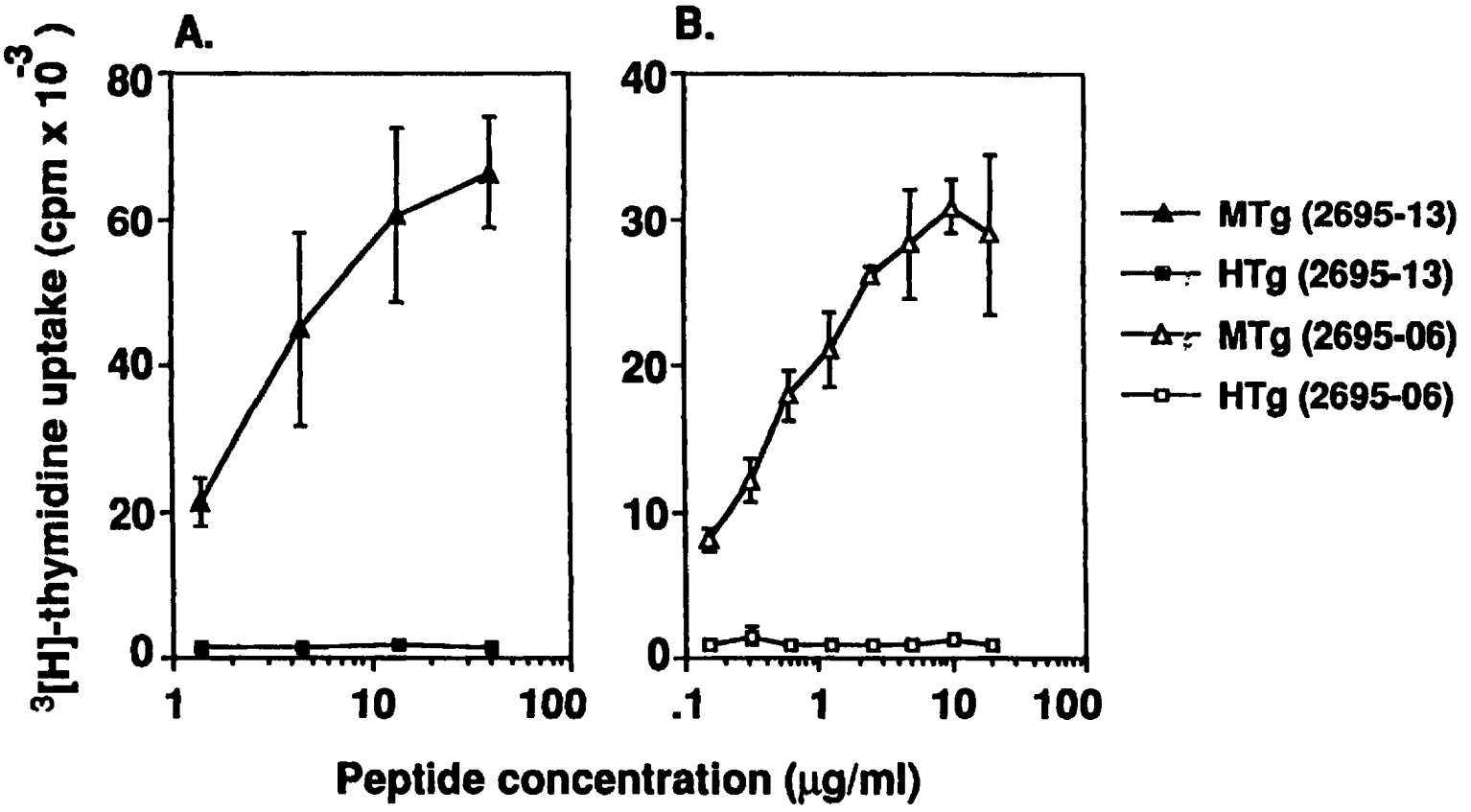

Figure 6. 8 The human homologue of MTg(2695-13) or its amino terminal 12mer sequence (2695-06) fails to activate the T cell clone 6E10. In triplicate wells, equal numbers of T cells and LS102.9 were cultured separately in the presence of mouse or human Tg(2695-13) (a) or mouse and human Tg(2695-06) (b). The data represent mean cpm values reflecting the IL-2 dependent CTLL proliferation. Background cpm in these experiments were 1514 in (a) and 932 in (b).

importance of positions 2703 and 2704 for peptide recognition by T cells suggesting that the serine substitutions affected either binding to MHC and/or interfered with TCR contact.

6. 3. 7 Comparison of EAT induction and IgG response by homologous MTg and HTg peptides

To determine pathogenicity, five SJL mice were directly primed and boosted with HTg(2695-13) in adjuvant as described in *Materials and Methods*. Five weeks after the initial challenge with HTg(2695-13), MNC infiltration could not be detected in the thyroids of any of the five mice (**Table 6. 3**). In contrast, similar challenge of mice with MTg(2695-13) was shown previously to cause significant thyroid infiltration (Carayanniotis et al., 1994). Interestingly, however, pooled sera from the HTg(2695-13)-primed mice contained peptide-specific IgG. These IgG bound to the 12mer HTg (2695-06) and also cross-reacted with MTg(2695-06) (**Table 6. 3**). Thus, at the serological level, the human 18mer homologue is clearly immunogenic and contains B-cell epitopes that cross-react with their counterparts in the rat sequence.

Adoptive transfer of $2x10^7$ HTg(2695-13)-primed LNC into syngeneic naive SJL hosts resulted in detectable thyroiditis in three out of four mice (**Table 6. 3**). However, the degree of infiltration induced by HTg(2695-13)-specific cells was low and is in contrast to the severe EAT previously induced with MTg(2695-13)-specific LNC (Carayanniotis et al., 1994). Analogous results were observed when LNC primed to the rat or human (2695-06) 12mer peptides were used in adoptive transfer studies. Extensive infiltration was observed in all 6 mice that received MTg(2695-06)-specific LNC, whereas only small perivascular foci of MNC were

Table 6. 3 EAT and serum IgG-responses in SJL mice challenged with homologous mouse and human Tg peptides

Method of induction *	Infiltration Index					Mice with EAT †	Serum IgG response (O. D. @ 405 nm) to ¶			
	0	1	2	3	4		HTg(2695-06)		MTg(2695-06)	
							1:20 ¥	1:80	1:20	1:80
<u>By direct challenge</u>										
HTg(2695-13) + CFA	5					0/5	2.50	1.11	2.84	1.50
CFA (control)	5					0/5	0.12	0.14	0.11	0.10
<u>By adoptive transfer</u>										
HTg(2695-13)	1	2	1			3/4	0.44	0.16	0.34	0.19
HTg(2695-06)	1	0	3			3/4	0.19	0.11	0.14	0.10
MTg(2695-06)				5	1	6/6	0.45	0.19	0.64	0.23
OVA (control)	7					0/7	0.23	0.08	0.37	0.09

* Direct EAT was induced by two s. c. injections of HTg(2695-13) as described in *Materials and Methods*. For adoptive transfer, 2 x 10^7 inguinal LNC primed *in vivo* and restimulated *in vitro* with the indicated antigen were i.p. injected.

† Thyroids and sera were collected from mice 5 weeks after direct challenge with peptide or 2 weeks after cell transfer.

¶ Peptide-specific IgG response in pooled sera from each group was assessed by an alkaline-phosphatase based ELISA. Microtiter wells were coated with 8 µg of human or mouse Tg(2695-06) peptide as described in *Materials and Methods*.

¥ Serum dilutions tested.

observed in 3 out of 4 mice that received lymphoid cells primed with HTg(2695-06). These effects were antigen-specific because none of the 7 mice tested developed thyroiditis following transfer of OVA-specific LNC. Low titers of peptide-specific IgG were detected in some recipient mouse sera probably due to the co-transfer of activated B-cells in the LNC inoculum.

6. 4 DISCUSSION

The reliance on heterologous Tg sequences in our search for pathogenic T-cell epitopes in mouse EAT has so far yielded successful results (Hutchings et al., 1992; Texier, 1992; Chronopoulou and Carayanniotis, 1992; Carayanniotis et al., 1994; Hoshioka et al., 1993; Kong et al., 1995; also, **Chapter 4**). The search strategies have involved testing of conserved peptides surrounding hormonogenic sites (Hutchings et al., 1992; Kong et al., 1995), peptides from the RTg sequence which are, on a phylogenetic basis, likely to be identical with mouse pathogenic epitopes Chronopoulou and Carayanniotis, 1992; Carayanniotis et al., 1994; **Chapter 4**) or peptides from the HTg sequence which are similar to other heterologous Tg fragments (Texier et al., 1992) or to peptides from other molecules such as hTPO (Hoshioka et al., 1993). The results of the present study, however, demonstrate that the thyroiditogenic RTg(2695-06) peptide and its human homologue, carrying two Ser substitutions at Gln2703 and Thr2704, do not have similar immunopathogenic properties. The human peptide is not detectable in proliferation or lymphokine assays and exhibits very weak pathogenicity demonstrable only by adoptive transfer of peptide-specific LNC. This raises caution in extrapolating pathogenic sequence data from one heterologous Tg to another even when EAT is induced within the same strain

and, in fact, when the given epitopes are presented in the context of the same H-2-restriction molecule - in this case A^s (Carayanniotis et al., 1994).

The two Ser substitutions at the carboxyl end of the human analogue may interfere with TCR engagement resulting in the triggering of non proliferative, autoreactive T-cell subsets that do not secrete IL-2 or IFN-γ, unlike those activated by MTg(2695-06). This hypothesis can explain the lack of cross stimulation by the two peptides as assessed by proliferation or by the activation of the IL-2-secreting 6E10 clone. It may also account for the differences in the severity of the adoptively transferred EAT, as a result of qualitative and/or quantitative differences at the level of inflammatory effector cells elicited by HTg(2695-06). Single a. a. substitutions in peptides of basic myelin protein are known to modify pathogenicity (Kibler et al., 1977), to modulate cytokine pattern secretion in autoreactive T cells (Windhagen et al., 1995), or to prevent EAE induction by the encephalitogenic analogue (Smilek et al., 1991).

The capacity of the HTg(2695-06) to elicit weak EAT by adoptive transfer of non proliferative LNC is in agreement with similar observations in experimental uveoretinitis showing that pathogenic peptides of the S-Ag do not stimulate proliferative responses (Gregerson et al., 1989) and with results of experimental autoimmune encephalomyelitis studies using MBP (Hunter, 1986). Other Tg peptides, such as the A^k-restricted RTg(2499-07), also exhibit weak thyroiditogenicity but fail to elicit proliferative LNC responses (**Chapter 4** section **4. 3. 5**). In addition, the pathogenicity of Tg(2549-60) peptides, containing either T4 or T0 at position 2553, has been shown only by adoptive transfer of peptide-specific lymphoid cells and not by direct challenge (Hutchings et al., 1992; Kong et al., 1995). These observations are reminiscent of earlier results

describing EAT induction by adoptive-transfer of BTg-specific T-cell lines (Maron et al., 1983) but not by intact BTg in adjuvant (Romball and Weigle, 1984). The mechanisms underlying these phenomena remain elusive but more extensive lymphokine analysis of clonal T-cell populations responding to minimal pathogenic Tg peptides should elucidate the nature of non proliferative effector cells in EAT.

Taken together, these data strongly suggest that screening for EAT-causing T-cell epitopes should combine several approaches including *in vitro* assays of proliferation and lymphokine analysis, direct challenge of mice with candidate peptides, as well as *in vivo* adoptive transfer of peptide-specific T cells, before conclusions concerning the pathogenicity of a given epitope can be made. In epitope mapping studies, the emphasis needs to be placed in EAT induced by homologous antigens and rat EAT currently provides an ideal choice for this; in that regard, the only peptide that has been tested for pathogenicity in a homologous host is the 17mer Tg(2495-11) that induced EAT in several strains of rats (Balasa and Carayanniotis, 1993). Because of its phylogenetic proximity, the partial RTg sequence also offers a good choice for epitope mapping in mouse EAT but, by extrapolation from other models (Zamvil et al., 1986), disparity at single a. a. positions may still lead to detection of RTg T-cell determinants of no relevance to mouse EAT.

CHAPTER 7

THYROIDITIS INDUCTION BY ADENOVIRAL PEPTIDE THROUGH MOLECULAR MIMICRY

7.1 SUMMARY

A computer search of SWISS-PROT data bank was undertaken to identify sequences highly homologous with pathogenic Tg epitopes: MTg(2496-04) and MTg(2695-06). Since adenovirus infections were previously suspected in patients with subacute thyroiditis, we examined in the present study if a peptide from murine adenovirus type-1 E1B sequence (a. a. 368-81) (AVP) exhibiting a high degree of homology with the MTg(2695-06) mediates EAT in mice. The AVP was highly antigenic *in vitro* as it induced strong recall proliferative responses in MTg(2695-13) or MTg(2695-06)-primed LNC. In addition, AVP stimulated the A^S-restricted T-cell hybrid clone 6E10 and, in ELISA it significantly bound to MTg(2695-13)-specific serum IgG. AVP, however, was a poor immunogen failing consistently to induce LNC-proliferative responses or mediate EAT directly in SJL hosts. Nevertheless, in preliminary experiments, it was found that MTg(2695-06)-primed LNC upon culture with AVP acquired the ability to adoptively transfer severe EAT in SJL hosts. In light of these findings, we propose that viral peptides such as AVP, when generated during viral infections, may mimic host Tg-epitopes leading to activation and amplification of autoreactive T cells in AITD.

7. 2 INTRODUCTION

Despite the impressive progress made in the last two decades in molecular characterization of the immune system, the etiopathogenesis of many human autoimmune diseases still remains elusive. Growing evidence points to infection by microbes as an important cause of AITD (Tomer and Davies, 1995) and several reports on association of viral infections with the development of thyroiditis exist (Eylan et al., 1957; McArthur, 1964; Hung, 1969; Volpé, 1975; Martino et al., 1987 and deBruin et al., 1990). However, it remains speculative as to why only some of the infected individuals develop thyroid autoimmunity during the post-infection period (Tomer and Davies, 1993).

Several studies point to T cells having a pivotal role in the pathogenesis of autoimmune thyroiditis and the characterization of thyroiditogenic T-cell epitopes has strongly supported this view (Carayanniotis and Rao, 1997). Peptide recognition by T cells seems to be highly degenerate (Bharadwaj et al., 1993) and often involves TCR contact with a few a. a. residues of the peptide (Gautam et al., 1992; Wraith et al., 1992; Boehncke et al., 1993). Thus it is possible that molecular mimicry involving only partial identity between host and microbial Ag is sufficient to drive autoreactive immune responses. For example, a hepatitis B virus polymerase-derived peptide identified based on its homology with a pathogenic site on MBP was shown to induce infiltration of central nervous system in rabbits, a characteristic sign of EAE (Fujinami and Oldstone, 1985). Molecular mimicry by microbial proteins was similarly incriminated in the etiopathogenesis of many other autoimmune diseases including myasthenia gravis (Dieperink and Stefansson 1989), autoimmune oophoritis (Tung, 1994; Garza and Tung, 1995), celiac disease (Kagnoff, 1989; Tuckova et al., 1995),

juvenile rheumatoid arthritis (Albani, 1994), insulin dependent diabetes mellitus (Atkinson et al., 1994) and experimental autoimmune uveitis (Singh et al., 1990).

Our previous work in EAT has demonstrated that MTg(2695-06) is a pathogenic T-cell site and mediates EAT (Chapter 6, section 6. 3. 6). In this chapter I have examined the concept of molecular mimicry using a viral peptide (AVP), identified based on its homology with MTg(2695-06). Data are presented in support of the hypothesis that peptides such as AVP, can activate, via molecular mimicry, autoreactive T cells reactive with host Tg-epitopes thereby leading to induction of thyroiditis.

7. 3 RESULTS

7. 3. 1 Homology comparison of MTg(2496-04) and MTg(2695-13) with sequences in SWISS-PROT data bank

To identify "molecular mimics" between microbial antigens and pathogenic Tg epitopes, we scanned SWISS-PROT data bank for sequences that show significant homology with pathogenic Tg T-cell epitopes: (2496-04) and (2695-13). The search results comprising the top five sequences along with their homology scores against the appropriate Tg-peptide are presented in **Table 7. 1**. Out of the five sequences that scored >40% homology with Tg(2496-04), four showed an overlap of all nine residues whereas the sequence from human myosin heavy chain precursor presented an overlap of only eight residues. The sequences with the highest % homology were from the yeast cytoplasmic transport protein and human myosin heavy chain precursor. Of the other three sequences scoring same degree of homology (i.e., 44. 4%), one was derived from *E. coli* while the

Table 7.1 Homology comparison of MTg(2496-04) and MTg(2695-13) with sequences in SWISS-PROT data bank

Sequence [a]	Protein	% Homology	Overlap	Reference
L I N R A K A V K	Mouse thyroglobulin	100	9	(Kuppers et al., 1996)
L I G H **A** S **A V K**	Cytoplasmic transport protein (yeast)	66.7	9	(Hicke and Schekman, 1989)
L I T Q V R S **V K**	Diaminopimelate decarboxylase (E. coli)	44.4	9	(Stragier et al., 1983)
L I T **R** T Q **A V C**	Human fast skeletal muscle myosin	62.5	8	(Eller et al., 1989)
L I Q P D S S **V K**	Human fibrinogen beta chain precursor	44. 4	9	(Watt et al., 1979)
L I S M **I** Q V **V K**	Human leukocyte common antigen precursor	44. 4	9	(Streuli et al., 1987)
C S F W S K Y I Q T L K D A D G A K	Mouse thyroglobulin	100	18	(Kuppers et al., 1996)
K Y I Q A **LK** G	DNA-directed RNA pol	75.0	8	(Oeser and Tudzynski, 1989)
S F Y **S** S **Y I Q T L** T **V A Q**	Adenovirus E1B-protein	64.3	14	(Ball et al., 1988)
S F W G E **A** V **Q T**	Retrovirus *pol* protein	55.6	9	(Grandbastien et al., 1989
C S F W N D **Y** L P K V R	Acetyl cholinesterase precursor	41.7	12	(Fournier et al., 1989)
T **F W Q** Q F **G L** V **L K** E G	Heat shock protein	30.8	13	(Bardwell and Craig, 1987)

[a] A total of 26, 706 sequences in the data base were searched.

Bold letters denote residues identical with the Tg peptide.

Underlined letters denote conservative substitutions.

other two were human proteins (fibrinogen β-chain precursor and CD 45) (**Table 7. 1**).

Out of five different sequences that scored >30% homology with Tg(2695-06), two sequences were from viruses (adenoviral E1B protein and retroviral *pol* protein), one is from bacteria (*E. coli* heat shock protein), another one is from DNA-directed RNA pol while the last sequence is from acetylcholinesterase precursor, another autoantigen (**Table 7. 1**). Interestingly, the adenoviral E1B(368-381) sequence shows significant homology (64.3% in a overlap of 14 residues) with the N-terminal 12 a. a. sequence of the MTg(2695-13). As described in **chapter 6**, the N-terminal region (2695-06) encompasses an immunopathogenic T-cell epitope(s) in SJL hosts. Hence, we proceeded to test whether the adenoviral E1B-peptide (AVP) sharing sequence homology (64.3% in a overlap of 14 residues) with the epitope MTg(2695-06) would be immunologically cross-reactive at B- or T-cell level. Studies with AVP are also relevant, since an increased incidence of adenovirus infections was noted previously in patients with subacute thyroiditis (Volpé et al 1967).

7. 3. 2 AVP is recognized by MTg(2695-13)-specific LNC or T-cell clone 6E10

Initially, we sought to determine whether or not the 14mer AVP can be recognized by Tg(2695-13)- or Tg(2695-06)- primed LNC *in vitro*. Mice were primed with Tg(2695-13) or Tg(2695-06) in CFA and proliferation assays of inguinal LNC were subsequently done as described earlier. AVP was found to be recognized by Tg(2695-13)-specific LNC as shown in **Figure 7. 1**. Expectedly, AVP was also recognized by MTg(2695-06)-primed LNC (in 3 out of 4 experiments) **Figure 7. 2**. In addition, AVP was recognized by the T-cell hybrid

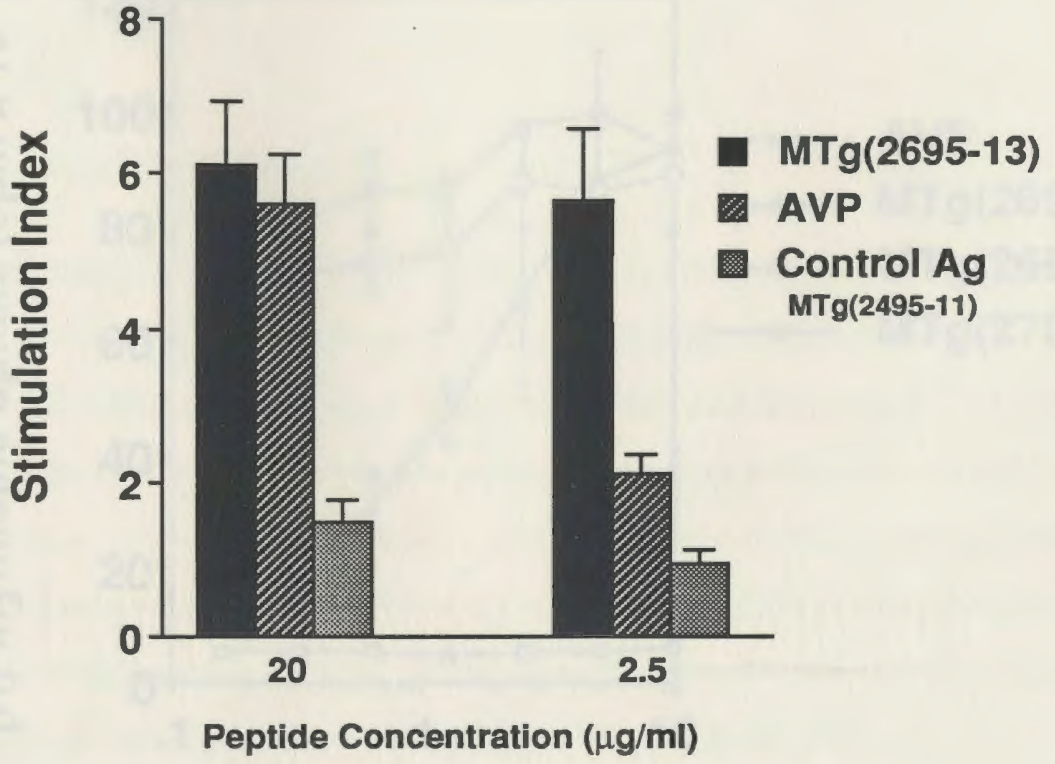

Figure 7. 1 Specific activation by AVP *in vitro* of MTg(2695-13)-primed LNC from SJL mice. Inguinal LNC from mice (2/group) primed s. c. with 50 nmol of MTg(2695-13) 10 days earlier, were cultured in triplicate microwells in the presence of varying concentrations of antigen. Data (S. I. values) obtained from full titration curves are representative of two independent experiments. Background cpm were = 12, 500 ± 2,100.

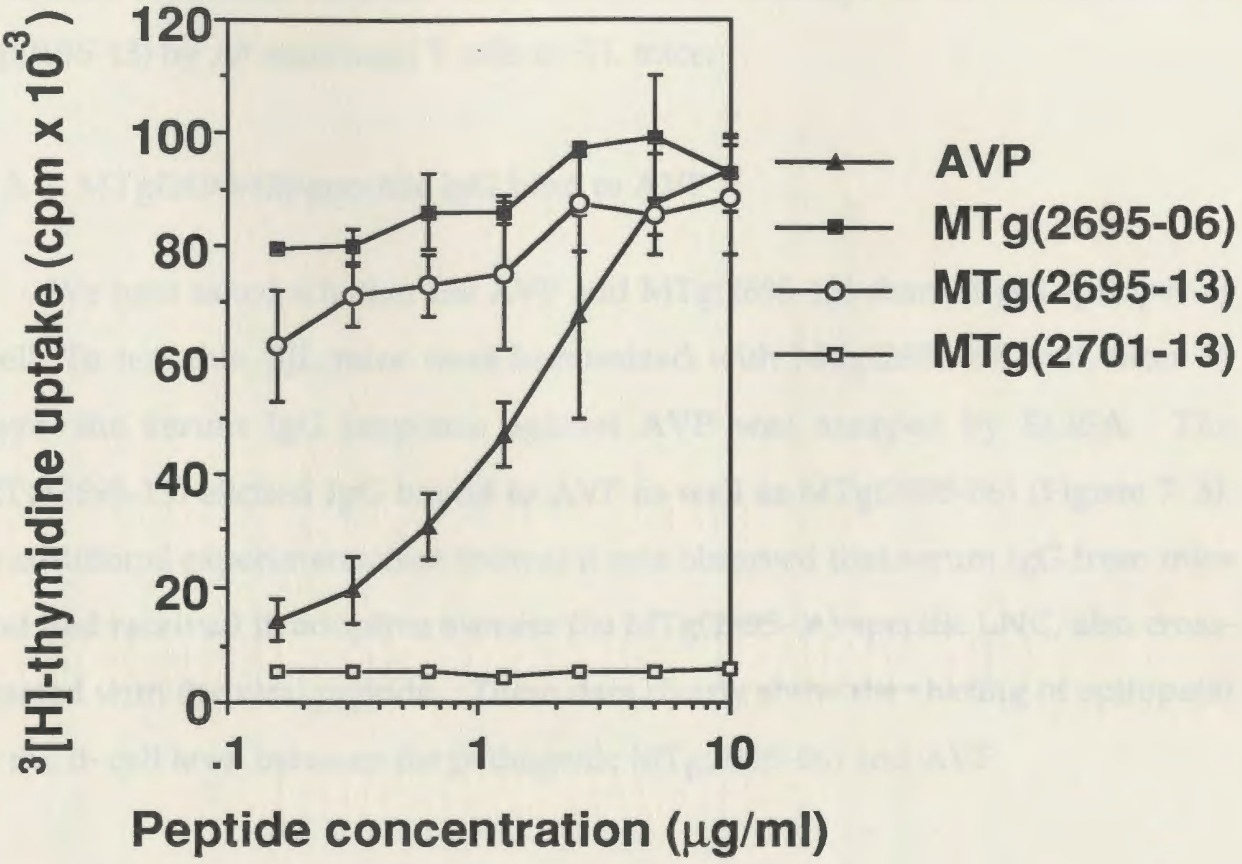

Figure 7. 2 LNC from MTg(2695-06)-primed SJL mice proliferate against AVP. Mice (2/group) were primed s. c. with 50 nmol of peptide and 10 d later, their inguinal LNC were cultured in the presence of varying concentrations of the antigens shown above. Data (mean cpm) representative of three independent experiments are shown. Background cpm were = 3,930.

6E10 (**Table 7. 2**) confirming cross-reactivity between MTg(2695-06) and AVP at the clonal T-cell level. These data suggest that the three conservative a. a. substitutions i.e., Trp -> Tyr, Lys -> Ser, and Lys -> Thr at positions 2698, 2700 and 2706 constitute residues that can be well tolerated in the recognition of Tg(2695-13) by A^S -restricted T cells in SJL mice.

7. 3. 3 MTg(2695-13)-specific IgG bind to AVP

We next asked whether the AVP and MTg(2695-13) share B-cell epitopes as well. To test this, SJL mice were immunized with MTg(2695-13) and, after 35 days, the serum IgG response against AVP was assayed by ELISA. The MTg(2695-13) elicited IgG bound to AVP as well as MTg(2695-06) (**Figure 7. 3**). In additional experiments (not shown) it was observed that serum IgG from mice that had received in adoptive transfer the MTg(2695-06)-specific LNC, also cross-reacted with the viral peptide. These data clearly show the sharing of epitope(s) at the B- cell level between the pathogenic MTg(2695-06) and AVP.

7. 3. 4 AVP is poorly immunogenic at T- and B- cell level

The immunogenicity of AVP was next examined in SJL mice. Due to its high hydrophobic nature, AVP was not completely soluble in PBS and thus, the partially solubilized preparation was used initially for immunization. Ten days after priming, the *in vitro* proliferative response of inguinal LNC against AVP (14mer), MTg(2695-06) and MTg(2695-13) was assessed. In two out of four experiments, the LNC responded weakly to AVP 14mer but not to MTg(2695-06) or (2695-13). The data presented in **Figure 7. 4** illustrate the data from one such

Table 7. 2 AVP is recongized by MTg(2696-06)-specific T cell hybrid clone 6E10

Peptide *in vitro*	CTLL proliferation (cpm x 10^{-3}) Antigen concentration (μg/ml)			
	40	13.3	4.4	1.5
MTg(2696-06)	59.67 ± 6.3	49.10 ± 2.7	40.21 ± 4.9	24.83 ± 3.9
AVP	12.67 ± 14	4.56 ± 0.6	2.92 ± 0.3	2.64 ± 0.2
MTg(2701-13) (Control Ag)	1.32 ± 0.1	1.41 ± 0.1	1.41 ± 0.1	1.41 ± 0.1

10^5 LS 102.9 cells and an equal number of hybrid cells were cultured with the above mentioned antigens at the indicated concentrations. Culture supernatants after 24 h were assayed for IL-2 content as described in *Materials and methods*.

Data represent mean cpm ± SD of triplicate wells. Background cpm were = 1, 444 ± 0.10.

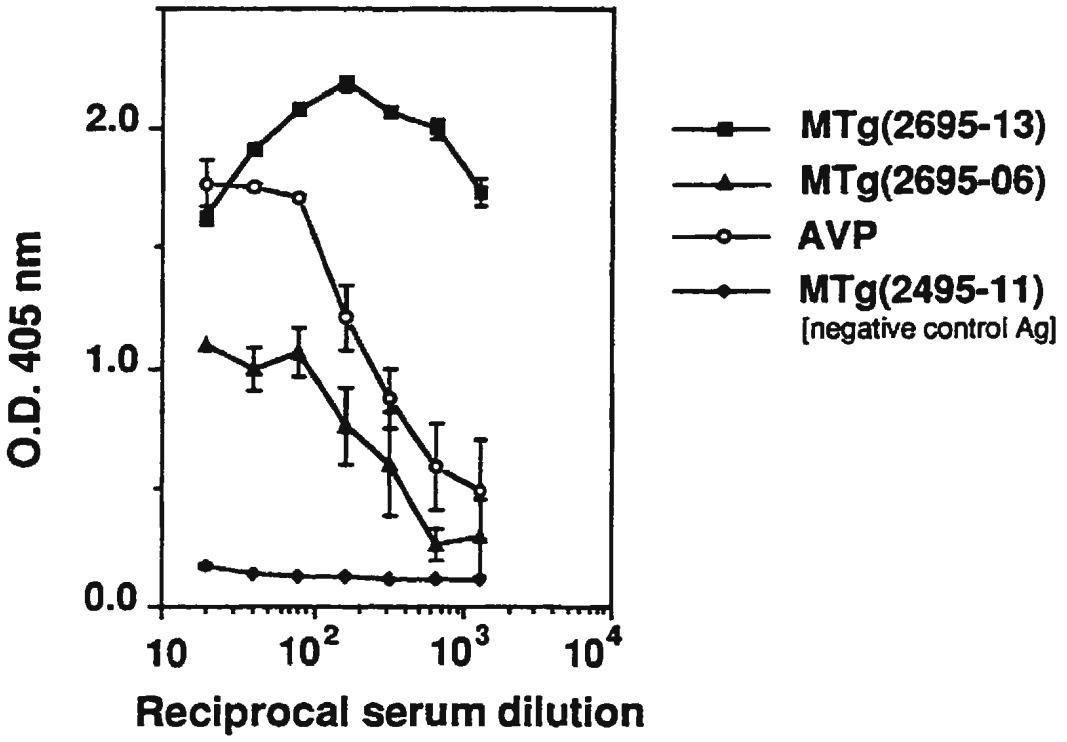

Figure 7. 3 AVP is recognized by MTg(2695-13)-specific IgG. Three mice were primed with MTg(2695-13) in CFA and their pooled serum was tested for specific-IgG against the indicated peptide Ag in an alkaline phosphatase-based ELISA (see *Materials and Methods* section 3. 4. 3 for details) Microtitre wells were coated with 8 μg of peptide /well. Data represent mean O.D. of duplicate wells, while vertical bars represent SD.

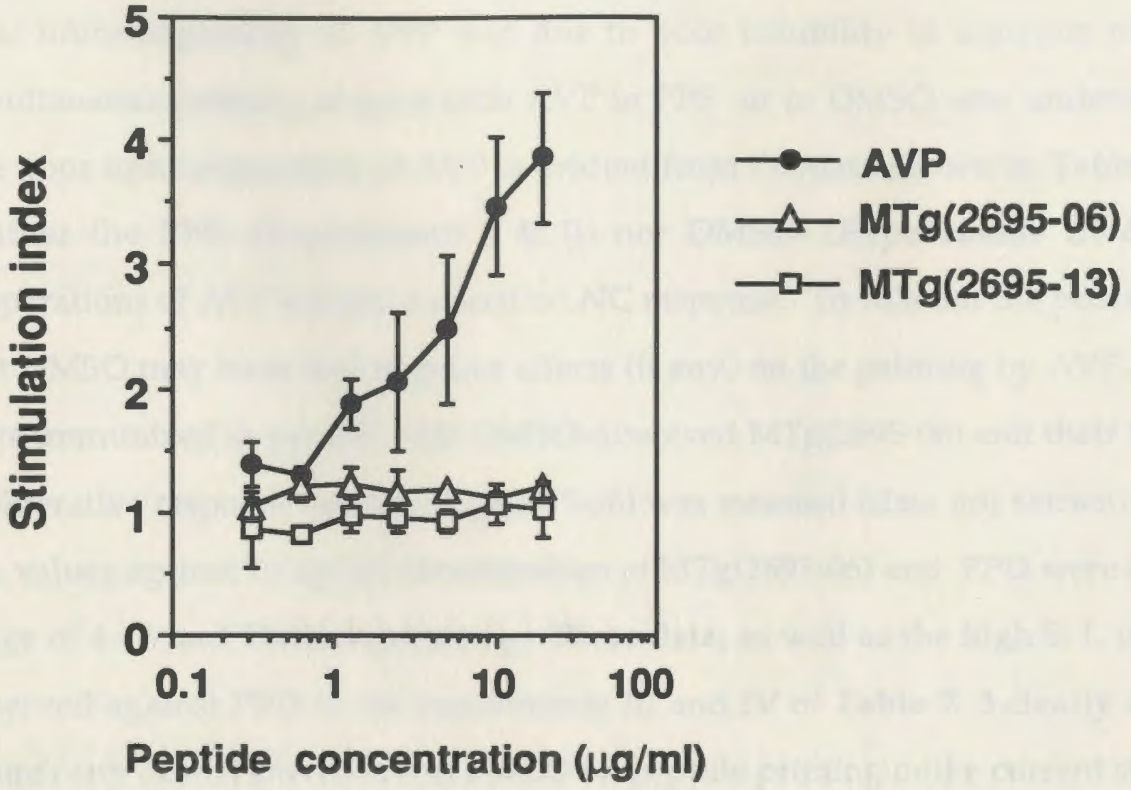

Figure 7. 4 AVP can prime for proliferative LNC-reponse in SJL mice. Mice were primed s. c. at the base of the tail, with 50 nmol of AVP 14mer in CFA and, 10 d later, their inguinal LNC were cultured in the presence of varying concentrations of peptide Ag as shown above. Data represent S. I. values of triplicate wells. Background cpm were = 6, 605.

experiment. The LNC proliferation is only moderate against AVP (S. I. ≈ 4.0) and is completely lacking against MTg(2695-13) or MTg(2695-06). To examine if the poor immunogenicity of AVP was due to poor solubility in aqueous media, simultaneous priming of mice with AVP in PBS or in DMSO was undertaken. The poor immunogenicity of AVP is evident from the data shown in **Table 7. 3.** Neither the PBS- (Experiments I & II) nor DMSO- (Experiments III & IV) preparations of AVP elicited a specific-LNC response. To rule out the possibility that DMSO may have had negative effects (if any) on the priming by AVP, mice were immunized in parallel with DMSO-dissolved MTg(2695-06) and their LNC-proliferative response against MTg(2695-06) was assessed (data not shown). The S. I. values against 10 μg/ml concentration of MTg(2695-06) and PPD were in the range of 4 - 6 and 12-15 respectively. These data, as well as the high S. I. values observed against PPD in the experiments III and IV of **Table 7. 3** clearly argue against any deleterious effects of DMSO on peptide priming in the current study.

To test whether AVP-priming might induce specific IgG in SJL hosts, sera were collected from 5 mice that were primed and boosted with AVP-dissolved in DMSO. ELISA performed on sera revealed no specific IgG to the viral peptide (data not shown).

7. 3. 5 Adoptive transfer of EAT

Some Tg peptides can mediate EAT by adoptive transfer of specific LNC, although they fail to induce proliferative LNC responses or pathology following direct challenge in mice with peptide (Hutchings et al., 1992; **Chapter 6, section 6. 3. 7**). Therefore, we examined the pathogenicity of AVP in adoptive transfer

Table 7. 3 Lack of specific proliferative response of LNC from SJL mice primed with AVP

Experiment [a]	Ag dose (μg/ml)	Proliferative response [b] against AVP	MTg(2695-06)	MTg(2695- 13)
AVP in PBS + CFA				
Exp. I	40	1.31	ND	ND
	10	1.55	1.61	1.32
	2.5	ND	1.44	1.58
Exp. II	40	1.08	0.99	1.32
	10	0.87	1.42	1.08
	2.5	ND	1.05	1.04
AVP in DMSO+ CFA				
Exp. III	5	1.49	0.95	1.11
	0.5	1.15	1.01	0.94
	0.05	0.86	0.99	0.89
Exp. IV	5	1.22	0.89	0.93
	0.5	1.02	0.73	0.76
	0.05	0.89	0.83	0.89

[a]Mice (2/group) were injected s. c. at the base of the tail with 50 nmol of AVP 14mer in CFA and ten days later their inguinal LNC were allowed to proliferate against the indicated doses of Ag as mentioned above.

[b]Data represent S. I. values of triplicate wells. Background cpm were: exp. I = 1906; exp. II = 1140; exp. III = 1995 and exp. IV = 2536; Against PPD (10 μg ml^{-1}) the S. I. values were: exp. I = 27. 27; exp. II = 25.17; exp. III = 20.99 and exp. IV = 16.28.

Table 7.4 AVP* mediates adoptive transfer of EAT

Group	*In vivo* immunogen	*In vitro* stimulation with	Infiltration Index					Mice with EAT
			0	0.5	1	2	3	
1.	AVP	AVP	6	1	-	-	-	1/7
2.	MTg(2695-06)	AVP	1	-	1	3	1	5/6
3.	MTg(2695-06)	-	5	-	-	-	-	0/5

For EAT induction adoptive transfer of 2-3 x 10^7 peptide-specific cells was carried out as described in *Materials and Methods*. Fourteen days after the transfer, thyroid glands were collected for histological examination.

* AVP 10mer was used.

Table 7. 5 Physico-chemical characteristics of AVP and MTg(2695-06) *

Parameter	AVP		MTg(2695-06)
	14mer	10mer	
Molecular weight	1608	1208	1504
Isoelectric point	5.25	6.12	10.03
Half-life *in vitro*			
mammalian reticulocytes	1.9 hr	1.9 hr	1.2 hr
Half-life *in vivo*			
yeast	> 20 hr	> 20 hr	> 20 hr
E. coli	> 10 hr	> 10 hr	> 10 hr

* Physicochemical characteristics estimated according to the PHYSICOCHEM program of PC/GENE (IntelliGenetics, Mountain View, CA, USA)

experiments. A 10mer peptide was used in these experiments, since work in parallel had established that deletion of the four C-terminal residues (Thr, Val, Ala and Gln) within the 14mer AVP did not affect the peptide-specific activation of the T cell hybrid 6E10 (data not shown). Of the three groups of recipient mice, the first group received LNC primed *in vivo* and boosted *in vitro* with AVP, while the other two groups received MTg(2695-06)-primed LNC after culturing in the presence of either AVP (group 2) or medium alone (group 3). The data on adoptive transfer of EAT are presented in **Table 7. 4.** One out of seven mice (group 1) that received AVP-primed and -boosted LNC exhibited focal thyroiditis. On the other hand, EAT was seen in six out of seven mice that received MTg(2695-06)-primed LNC following a boost *in vitro* by AVP. In contrast, all five control mice (group 3) that received MTg(2695-06)-primed LNC without AVP-boosting *in vitro* did not develop EAT. These findings highlight the potential of viral peptides such as AVP to induce thyroiditis, by virtue of their cross-reactivity at T-cell level and, provide an example of EAT induction via molecular mimicry.

7. 4 DISCUSSION

We have shown that a viral peptide can amplify pathogenic T cells specific for a cryptic Tg-peptide leading to induction of thyroiditis in mice. The relationship between viral infections and autoimmunity is complex: viruses can unveil privileged self-antigens or modulate antigen processing leading to expression of cryptic epitopes and in genetically susceptible hosts they may induce autoimmunity (Barnaba, 1996). It is not known if adenovirus type 1 infection in mice can lead to development of thyroid autoimmunity. In human

studies, mumps (Eylan et al., 1957) and Epstein-Barr (Coyle et al., 1989) viruses were implicated in the causation of subacute thyroiditis, while the presence of antibodies to influenza, coxsackie, mumps, and ECHO and adenoviruses was recorded in sera from patients with proven subacute thyroiditis in another study (Volpé et al., 1967). However, to date no particular virus has been clearly shown to be associated with the etiology of autoimmune thyroiditis in humans or animal models.

Previously we noted that the human homologue of MTg(2695-06) differing at only two residues Gln2703 and Thr2704 showed no cross-reactivity at T-cell level and exhibited contrasting immunopathogenic properties (**Chapter 6**). We hypothesized that these residues may act as important contact sites for the cross-reactive TCR or for binding to MHC. Such a view is supported by the present study because 10mer AVP is identical to MTg(2695-06) at eight positions including Gln2703 and Thr2704. Grafting of residues individually from the MTg(2695-06) sequence on to a backbone of alanine residues, may reveal within the pathogenic peptide the key positions for MHC and TCR, as earlier reported in EAE by Gautam and co-workers (1992). Knowledge of the structural requirements for both MHC binding and TCR recognition has further allowed identification of several molecular mimicry peptides by Wucherpfennig and Strominger (1995), and facilitated the construction of combinatorial peptide libraries as tools by Hemmer and co-workers (1997) to identify multiple cross-reactive ligands for MBP-specific T cells.

The present study delineates the E1B(368-81) sequence of murine adenovirus type 1 as a T-cell epitope site that mimics a pathogenic cryptic Tg epitope. AVP is cross-reactive at B- and T- cell level with MTg(2695-06) but is a

poor immunogen. The reasons behind this puzzling observation are not well understood. Lack of affinity to MHC does not seem to explain the poor immunogenicity of AVP since the T-cell clone (6E10) responded equally well to both AVP and MTg(2695-06). It is, however, possible that inclusion of additional (N- or C-terminal) residues may enhance the immunogenicity of AVP. Analogous data on the importance of flanking sequences on the immunogenicity of a measles epitope were reported by Partidos and Steward (1992). In contrast to MTg(2695-06), AVP may have a shorter half-life *in vivo* but this is rather unlikely, since AVP was able to prime for specific LNC proliferative response in one experiment (**Figure 7. 4**) and as shown in **Table 7. 5**, the half-life values of AVP 14mer or 10mer are not significantly different from those of MTg(2695-06).

The present study provides evidence for the first time in the field of EAT, that thyroid pathology in mice can result due to molecular mimicry by a viral peptide. It remains to be tested if the other sequences that scored significant homology with MTg(2695-06) (Table 7. 1) will be cross-reactive and/or thyroiditogenic as well. Based on the pathogenicity data of AVP we hypothesize that T-cell peptides, that are cross-reactive with and can mimic pathogenic epitopes on autoantigens, need not necessarily induce detectable immune responses to themselves and, that the pathogenic potential of such peptides could become apparent following infection in disease-susceptible subjects with pre-existing autoreactive immune responses. Regardless of the mechanisms involved, the present data underscore the possibility of induction of thyroiditis via molecular mimicry by non-self peptides.

CHAPTER 8

FUTURE DIRECTIONS

The present study was initiated following the observation that the 17mer Tg(2495-11) induces thyroiditis in H-2^k mice. The questions raised at the beginning of the study were: (a) what is the phenotypic profile and MHC-restriction property of MTg(2495-11)-specific T cells ? (b) where are the pathogenic T-cell determinant(s) located within MTg(2495-11) ? (c) do T cells recognizing a minimal pathogenic peptide:MHC complex belong to Th1 or Th2 phenotype and are TCR on such cells genetically restricted ? In a later phase of the study, the observation that the Tg-peptide (2695-13) induces thyroiditis only in SJL mice, spurred a different set of questions: (a) Is a human homologue of MTg(2695-13) immunopathogenic as well ? and (b) can a viral sequence showing homology with MTg(2695-06) contribute to EAT development via molecular mimicry? The data obtained have answered these questions but generated a set of new questions.

For example during the present study Tg(2496-04) has been characterized as minimal T-cell epitope that can elicit autoreactive T cells in C3H and SJL mice. Also it was shown that such T cells resemble Th1 cells by secreting IL-2 and IFN-γ but not IL-4 although it is possible that such cells secrete other lymphokines (not assayed for in the present study). Alternatively, using RT-PCR, the lymphokine gene expression among thyroid infiltrating lymphocytes (at different time points) in mice challenged with Tg(2496-04) can be assessed. Once the knowledge of the key lymphokine(s) secreting T-cell subset(s) becomes available, whether or not mice lacking genes for a particular (set of) lymphokine(s) will

develop EAT after peptide challenge will further confirm their role in EAT.

The studies of Braley-Mullen and co-workers showed that granulomatous, as opposed to lymphocytic EAT, develops in mice following adoptive transfer of Tg-primed LNC that are boosted *in vitro* with Tg in cultures containing anti-IL-2R Abs (Braley-Mullen et al., 1991; McMurray et al., 1994). It is not clear in their studies whether or not the granulomatous EAT-inducing LNC subset(s) is different from the subset(s) responsible for inducing lymphocytic EAT, in terms of their antigen fine specificity. Anti-IL-2R treatment of LNC might selectively promote activation and growth of a subset of LNC whose antigen fine specificities are different from those inducing lymphocytic thyroiditis. Whether or not the granulomatous EAT phenotype is a true reflection of the anti-IL-2R treatment on the effector T cells, can be easily clarified by adoptively transferring Tg(2496-04)-specific LNC treated with or without anti-IL-2R mAb. Finally studies focused on the intracellular signaling events in the T cells of a defined specificity, which participate in the granulomatous as opposed to lymphocytic thyroiditis, may aid in our understanding of the immunoregulation of EAT.

The findings that epitopes of MTg(2499-07) or (2695-06) are cryptic in the context of whole Tg , as shown by lack of activation of the appropriate specific T-cell hybridomas by APC pulsed with MTg (see sections **5. 3. 4 & 6. 3. 2**) confirms the previous results (Chronopoulou and Carayanniotis 1992; Carayanniotis et al., 1994). The hybridomas generated and characterized in the present study are invaluable tools for future antigen presentation studies. For example, the assumption that these apparently cryptic epitopes (2496-06) or (2695-06) must be generated and expressed by intra-thyroidal APC to allow thyroid specific

homing of the effector cells, can be tested using the T-cell hybridomas. Activation of the T cells by thyroidal epithelial cells or thyroid-derived APC such as dendritic cells (of course, all expressing MHC class II plus appropriate co-stimulatory ligands) will be a direct evidence in support of the above assumption. Iodination of Tg has been shown to alter its structure and possibly its immunogenicity. It will be worthwhile to examine if varying degrees of iodination of Tg would make a difference in the generation these epitopes. Alternatively, extracts of limited digestion by thyroid lysosomal cathepsins of Tg that is iodinated to a varying degree, can be tested for their capacity to activate the hybridomas. Another approach to test whether or not processing of Tg by APC *in vivo* will generate the cryptic epitopes, could be to immunize mice with whole Tg in adjuvant and 6-7 days later use the draining LNC as APC to activate the hybridomas in culture without adding Tg or peptide exogeneously.

The inability of homologous but not heterologous Tg to induce proliferative LNC responses has been attributed to suppressor T-cell determinants. This can be tested now by co-culturing of (2496-04)- or (2695-06)- primed LNC with homologous (mouse) or heterologous (rat) Tg -primed LNC in medium containing the immunizing peptide and Tg. Peptide-specific LNC proliferation should not be suppressed in cultures that had rat Tg-primed cells, but on the other hand, should be suppressed in cultures where mouse Tg-primed LNC were added. As a control, cultures should be set up where only peptide-specific LNC are allowed to respond to the immunizing peptide in doses equivalent to those

used in LNC-mixing experiments as described above. In additional experiments, culture supernatants from mouse or rat Tg-activated LNC cultures can be assayed similarly for suppressive effects (if any) on peptide-specific LNC proliferation assays.

In the present study it has been demonstrated that (2695-06) constitutes an A^S-restricted, pathogenic site on MTg encompassing highly immunogenic epitope(s) that elicit cells of Th1 type. The contrasting findings, however, that the human homologue of this sequence is non-immunogenic in SJL hosts raise some interesting questions: Is the human Tg(2695-06) capable of inducing proliferative LNC responses in mice of other (k, d, b or q) haplotypes ? Is one or both serine substitutions in the human homologue at positions 2703Gln and 2704Thr responsible for the observed lack of immunogenicity and/or pathogenicity in mice. What are the key residues within (2695-06) that are responsible for MHC binding? Are they distinct from those contacting the TCR on pathogenic Th1 cells? These studies eventually may help in designing and testing of peptide analogues with therapeutic potential in EAT. Also, it remains to be tested if HTg(2695-13) or (2695-06) can be recognized by sera or T cells from HT patients.

Finally, the work involving identification of an adenoviral E1B-derived peptide (AVP) that is immunologically cross-reactive at B- and T -cell level with a Tg-epitope present in the peptide (2695-06) raises new interesting questions. Can adenovirus infection in mice lead to thyroiditis and an AVP-specific B- or T- cell response ? Is AVP a site for dominant T-cell epitope on the E1B protein ? Also the question whether a cryptic Tg-epitope can be mimicked by a dominant viral epitope can now be addressed by assaying the activation of T-cell hybridoma

(6E10) in cultures containing APC incubated with E1B protein. Alternatively, APC expressing E1B protein, following transfection with E1B-cDNA, may be used in the hybridoma activation assays.

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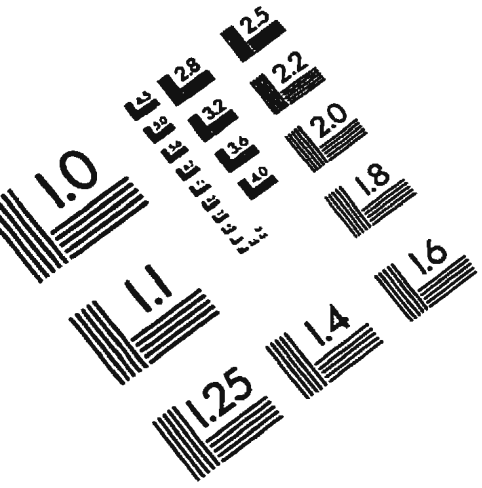
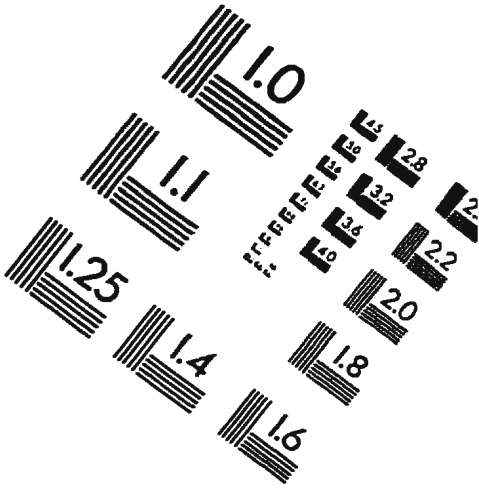
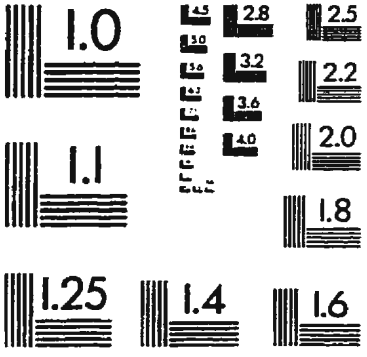
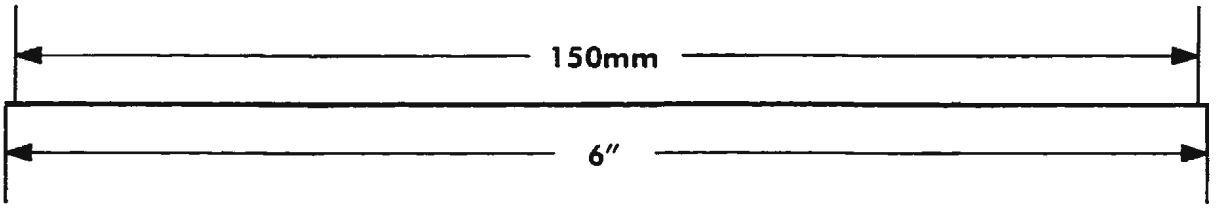
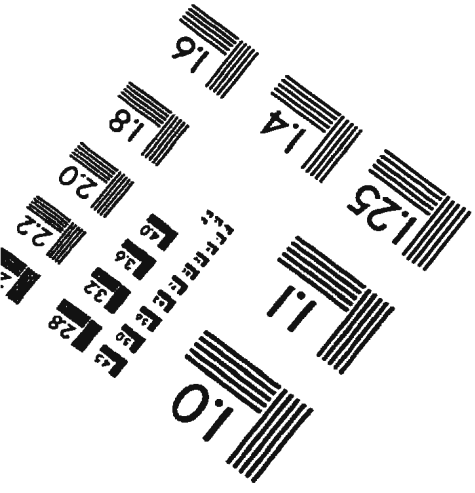
IMAGE EVALUATION TEST TARGET (QA-3)

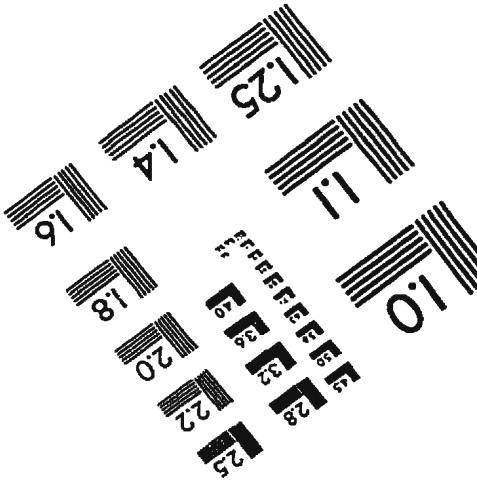












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