STUDIES ON EXPERIMENTAL AUTOIMMUNE THYROIDITIS IN MICE AND RATS WITH THYROGLOBULIN AND THYROGLOBULIN PEPTIDES

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STUDIES ON EXPERIMENTAL AUTOIMMUNE THYROIDITIS IN MICE AND RATS WITH THYROGLOBULIN AND THYROGLOBULIN PEPTIDES

by

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dedicated to

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Smt. Krishnavenamma

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ABSTRACT

Experimental autoimmune thyroiditis (EAT) induced in mice or rats with thyroglobulin (Tg) emulsified in complete Freund's adjuvant (CFA) has been a model system to understand the immunopathogenesis of Hashimoto's thyroiditis (HT).
I have demonstrated, by the immunotargeting approach, that adjuvant-free challenge of mice with small doses of mTg conjugated to monoclonal antibodies (MAbs) specific for class II MHC ($I\text{-}A^k$) expressed on APC, induces an mTg-specific IgG response in CBA ($H\text{-}2^k$) but not in B6 ($H\text{-}2^b$) mice. Priming of CBA mice with mTg conjugated to an irrelevant MAb (control) did not elicit an autoimmune response. Despite the induction of an mTg-specific IgG response, mononuclear cell infiltration of the thyroid was not detected in CBA mice thus indicating a clear divergence in the requirements for autoantibody (AAb) production and the requirements for disease. These findings may help elucidate the role of various APC subsets in autoimmunity and facilitate study of the initial events that trigger autoreactivity outside a CFA-induced granuloma site.

Investigation in rats of the immunopathogenicity of a 17mer homologous (rat) Tg peptide, rTgP1 (2495-2511), revealed that TgP1-priming of F334, WKY and WF rats elicits lymph node cell (LNC) proliferative responses to the peptide *in vitro* without concomitant specific primary IgG responses. LNC

proliferative assays demonstrated that specific $CD4^+$ T cells recognize TgP1 in the context of class II MHC and that TgP1 contains cryptic T cell epitope(s). Strong TgP1-specific IgG responses were not observed despite the presence of EAT in rats. These results provide the first evidence that a self-Tg peptide, TgP1, is immunopathogenic in rats. In contrast to 17mer TgP1, examination in rats of the immunopathogenicity of an 18mer rTg peptide, rTgP2 (2695-2713) revealed that TgP2-priming of rats induced specific LNC proliferative responses in F344, WKY but not in WF rats, although concomitant specific IgG responses were elicited in all three rat strains. Thyroiditis was readily induced in F344 rats both by direct challenge with TgP2 and by adoptive transfer with TgP2-specific T cells ($CD4^+$, $CD8^-$, TCR α/β^+) indicating that peptide-induced rat EAT is a $CD4^+$ T cell-mediated disease. Specific T cells recognize TgP2 in the context of class II molecules in an MHC-unrestricted fashion and TgP2 contains non-dominant T cell determinants. The TgP2-specific IgG (day 28) readily binds intact rTg and such binding could be abrogated by free peptide indicating the absence of "determinant spreading". These results indicate that TgP2 is pathogenic in rats but differs in its immunogenicity from that of TgP1.

Class II^+ rat thyrocytes (IFN-γ treated), to address their putative APC function in TgP2-mediated EAT, were

examined by testing their ability to present endogenous Tg epitope to the TgP2-specific cloned T cells. Both unpulsed or peptide pulsed class II^+ thyrocytes failed to activate the hybridoma (assessed by IL-2 release) suggesting that thyrocytes by themselves do not function as APC in vitro. The findings that both glutaraldehyde fixation and irradiation of spleenocytes abolished their capacity to present TgP2 leading to activation of the 8F7-5 hybridoma raised the possibility that thyrocytes may be deficient in the expression of certain costimulatory molecules. These findings have implications for understanding Ag-presentation in thyroid autoimmunity.

Examination of the relative serological immunodominance of the pathogenic TgP1 and TgP2 peptides revealed contrasting findings. Priming of F344 rats either with homologous or heterologous Tgs did not elicit TgP1-specific IgG responses indicating the non-dominance of TgP1. In contrast, homologous Tg-priming elicited TgP2-specific IgG response. The immunogenicity of the TgP2 epitope(s) on heterologous Tgs varied dramatically, being highest on bovine Tg, intermediate on mouse Tg and undetectable on human and porcine Tgs although peptide-specific IgG readily cross-reacted with Tgs of various species. Epitope analysis of heterologous Tg-primed sera revealed that TgP2-reactivity is directed to distinct B epitopes within TgP2. These data provide the first evidence in EAT that variable immunodominance may partially explain why

distinct Tg epitopes are recognized by MAbs in various species/Tg combinations and may have implications in serological screening of pathogenic Tg epitopes.

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a. a.	amino acid
AA	Adjuvant arthritis
Ab	Antibody
AAb	Autoantibody
AChR	Acetylcholine receptor
ADCC	Antibody-dependent cell-mediated cytotoxicity
Ag	Antigen
ALV	Avian leukosis virus
AITD	Autoimmune thyroid disease
APC	Antigen-presenting cell
ATCC	American type tissue culture centre
BB	Bio-Breeding
BSA	Bovine serum albumin
bTg	Bovine thyroglobulin
BUF	Buffalo
cAMP	Cyclic AMP
cDNA	Complementary DNA
CFA	Complete Freund's adjuvant
CHO	Chinese hamster ovary
CIA	Collagen-induced arthritis
Con A	Concanavalin A
cpm	Counts per minute
^{51}Cr	^{51}Chromium
CTL	Cytotoxic T lymphocyte
CTLL	Cytotoxic T lymphocyte line
Cys	Cysteine
DMEM	Dulbeccos' modified Eagle's medium
DTH	Delayed-type hypersensitivity
EAE	Experimental autoimmune encephalomyelitis
EAO	Experimental autoimmune oopharitis

EAT	Experimental autoimmune thyroiditis
EAU	Experimental autoimmune uveitis
EBVL	Epstein barr virus transformed B lymphoblastoid cell line
EC	Endothelial cell
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-activated cell sorter
FBS	Foetal bovine serum
FITC	Fluorescein isothiocyanate
FRTL-5	Fisher rat thyroid epithelial cell line-5
HA	Haemagglutination
HAT	Hypoxanthine, aminopterine, thymidine
HBSS	Hanks' balanced salt solution
HEPES	N-2-hydroxyethylpiperzine-N'-2-ethane-sulfonic acid
HPLC	High-performance liquid chromatography
HRF	Homologous restriction factor
hsp	Heat shock protein
HT	Hashimotos' thyroiditis
hTg	Human thyroglobulin
hTgP2	Human thyroglobulin peptide 2
hTPO	Human thyroid peroxidase
GD	Graves' disease
ICAM-1	Intercellular adhesion molecule-1
Id	Idiotype
IFA	Incomplete Freund's adjuvant
IFN-γ	Interferon γ
Ig	Immunoglobulin
I.G.F-1	Insulin-like growth factor-1
i. p.	intraperitroneal
i. v.	intravenous

LAK cell	Lymphokine activated killer cell
LCMV	Lymphocytic choriomeningitis virus
LFA-1	Lymphocyte function associated antigen-1
L. F. I	Logfluorescence intensity
LNC	Lymph node cell
LPS	Lipopolysaccharide
m. w	Molecular weight
MAb	Monoclonal antibody
MAC	Membrane attack complex
2-ME	2-Mercaptoethanol
MBP	Myelin basic protein
MDP	Muramyl dipeptide
MHC	Major histocompatibility complex
Mic Ag	Microsomal antigen
MLR	Mixed leucocyte (lymphocyte) reaction
MNG	Multinodular goitre
mTg	Mouse thyroglobulin
MS	Multiple sclerosis
NK cell	Natural killer cell
NOD	Non-obese diabetic
NP	Nucleoprotein
NTx	Neonatal thymectomy
O. D.	Optical density
OS	Obese strain
OVA	Ovalbumin
PAB	PBS with 0.5% BSA and 0.1% sodium azide
PBS	Phosphate-buffered saline
PBMCs	Peripheral blood mononuclear cells
PEG	Polyethylene glycol
PHA	Phytohemagglutinin
PLP	Proteolipid protein
PMA	Phorbol myristate acetate
PPD	Purified protein derivative

pTg	Porcine Tg
pTPO	Porcine TPO
PVC	Polyvinyl chloride
RBC	Red blood cell
RFLP	Restriction fragment length polymorphism
RPMI	Rosewell Park-Memorial Institute
RT	Room temperature
rTg	Rat thyroglobulin
rTgP1	Rat thyroglobulin peptide 1
rTgP2	Rat thyroglobulin peptide 2
rTPO	Rat thyroid peroxidase
SAT	Spontaneous autoimmune thyroiditis
s. c.	Subcutaneous
SCID	Severe combined immunodeficient
SD	Standard deviation
SDS	Sodium dodecyl sulfate
S. I	Stimulation index
SLE	Systemic lupus erythemetosus
SSO	Sequence-specific oligonucleotides
SSCP	Single strand conformational polymorphism
ST	Subacute thyroiditis
TCC	Terminal complement component
TCF	Tissue culture flask
TcR	T cell receptor
TdR	Thymidine deoxyribose
TEC	Thyroid epithelial cell
TGF-α	Transforming growth factor-α
TGF-ß	Transforming growth factor-ß
Th	T helper
Tg	Thyroglobulin
TLI	Total lymphoid irradiation
TNF-α	Tumor necrosis factor-α
TPO	Thyroid peroxidase

TRH	Thyrotropin-releasing hormone
Ts	T suppressor
TSAB	Thyroid stimulating antibody
TSBAb	Thyroid stimulating blocking antibody
TSH	Thyroid stimulating hormone
Tx-X	Thymectomy and sublethal irradiation
Tyr	Tyrosine
VCAM-1	Vascular cell adhesion molecule-1
VLA-4	Very late activation antigen-4

CHAPTER 1

Introduction

Recently, Rose and Bona (1993) put forward the following criteria to establish whether a disease is actually autoimmune in origin: (1) induction of T cell and antibody (Ab) responses to the self-antigen(s), (2) identification of Abs and T cells within the lesions of the target organ, (3) transfer of disease to syngeneic animals by either lymphocytes or Abs, (4) reproduction of similar autoimmune disease in experimental animals by immunization with autoantigen(s) or its synthetic peptides, and (5) transfer of disease by lymphocytes (collected from patients affected with autoimmune disease) to severe combined immunodeficient (SCID) mice. Autoimmune thyroid disease (AITD) in humans fulfils many of the above criteria.

AITD is an organ-specific autoimmune disorder which affects approximately 5% of the human population (Weetman and McGregor, 1984a). The thyroid gland is the target organ that is affected in AITD. Based on the spectrum of clinical manifestations, AITD is classified into Hashimoto's thyroiditis (HT) or Graves' disease (GD). HT is a chronic form of autoimmune thyroiditis characterized by hypothyroidism, autoantibodies (AAbs) to thyroglobulin (Tg) and thyroid peroxidase (TPO), infiltration of thyroid with lymphocytes,

monocytes, and plasma cells and the formation of germinal centres leading to destruction of thyroid follicles (Weetman and McGregor, 1994). On the other hand, GD is characterized by chronic and excessive stimulation of thyroid gland by "thyroid stimulating antibodies" (TSAb) to thyroid stimulating hormone receptor (TSHR) simulating the effects of thyroid stimulating hormone (TSH) leading to hyperthyroidism (Zakarija et al, 1980; Weetman and McGregor, 1994). Frequently, high titers of AAbs to Tg and TPO are also detected (Mullins et al, 1995). Histological examination of GD thyroids revealed that thyroid follicles are small and are lined by both hypertrophied and hyperplastic columnar epithelium. In addition, a marked loss of colloid with neo-follicle formation and infiltration with lymphocytes and plasma cells which form aggregates or follicles have also been observed.

The initial triggering events and subsequent immunopathogenic mechanisms leading to AITD in humans are not yet fully understood. This is partly because the mechanisms of abrogation of natural tolerance to self-antigen(s) are not well understood. Understanding of the immunopathogenesis of AITD was further complicated because the analysis of molecular structure of thyroid antigens (Tg, TPO and TSHR) was slow. In addition, this was technically difficult because most of the autoantigens (TPO and TSHR except Tg) occur at very low concentrations and their purification is extremely laborious. To study the immunopathogenic mechanisms of HT, various animal

models have been developed (**Section 1.2.1**). On the other hand, to study the immunopathogenic mechanisms of GD, good animal models have not yet been established. However, recent efforts focused in that direction have shown promising results (Costagliola et al, 1994; Hidaka et al, 1995).

1.1 Thyroid gland and thyroid antigens

Our understanding of the immunopathogenesis of human thyroid disease has been dramatically improved in the last few years due to major developments in recombinant DNA and T cell cloning technology. A few of the autoantigens that are thought to be responsible for AITD have now been characterized. The autoimmune response has been shown to be directed against three distinct thyroid Ags: Tg, TPO, and TSHR. Recently, Two thyroid Ags, a 70 kDa (Chan et al, 1989) and a 64 kDa (Dong et al, 1991) molecules, have been shown to be the additional target Ags in GD. More recently, Na^+/I^- cotransporter has been identified as a potential autoantigen in HT (Raspe et al, 1995). It has been well established that Tg and TPO are recognized by autoreactive T- and B-cells in HT patients, whereas TSHR is recognized by autoreactive T- and B-cells in GD patients (Weetman and McGregor, 1994). The presence of AAbs to Tg and TPO has also been described in many GD patients (Mullins et al, 1995). At present, whether or not the expression of heat shock proteins (HSP) in the thyroid

(Heufelder et al, 1992) will serve as additional targets remains conjectural. Before describing the molecular structure of thyroid antigens: Tg, TPO and TSHR, I will discuss the salient features of the thyroid gland.

1.1.1 Normal Thyroid gland

The thyroid gland (the term thyroid comes from Greek meaning oblong shield) consists of two lobes connected by an isthmus wrapped around the trachea in the anterior compartment of the neck. It is the largest of all the endocrine glands.

The thyroid is composed of innumerable discrete follicles clustered together. The cells lining the follicles produce thyroid hormones, triiodothyronine (T3) and thyroxine (T4). These hormones are stored in the lumen of the follicles for subsequent controlled release. In humans, the gland begins to sy thesize thyroxine from about the third month of fetal life. The follicles are filled by a structureless semifluid protein, the colloid, which contains Tg. The gland is highly vascular and is essential for growth of the body and physical and mental well-being.

1.1.2 Thyroglobulin (Tg)

Tg is a homodimeric 660-kDa glycoprotein and serves as a prohormone for the biosynthesis of thyroid hormones. Tg is available in large quantities. It is synthesized by thyroid

epithelial cells (TEC) or thyrocytes. Tg is iodinated (0.3-0.5% of the molecule) and phosphorylated (10-12 phosphate groups/molecule) (reviewed in Charreire, 1989). Tg is stored as colloid in the lumen of thyroid follicles and is an extracellular glycoprotein (Vassart et al, 1975). Tg reenters TEC in the form of colloid droplets.

Tg that reenters TEC has been shown to undergo proteolysis under the influence of lysosomal enzymes (Cathepsins B, D, and L) resulting in the generation of Tg peptides (Dunn et al, 1991). In that study, the authors identified three cleavage sites for the enzymes Cathepsin B, D, and L. The Tg peptide fragments thus generated have been shown to contain three of the four major hormonogenic sites. The authors suggested that generation of these smaller Tg peptides is one of the steps in the biosynthesis of thyroid hormones. Thyroid hormones form at discrete sites within the Tg. Four hormonogenic sites in Tg have been identified (Fassler et al, 1988). Two of these sites occupy terminal location in the polypeptide chain: Site A, the major T4-forming site, near the NH2-terminus corresponding to human residue 5, and site C, the major T3-forming site, corresponding to human residue 2746 near the COOH-terminus. Sites B and D are more internal. These two sites correspond to human residues 2553 and 1290.

Production of thyroid hormones involves the iodination of tyrosine (Tyr) residues within the Tg molecule by TPO. Enzymatic iodination of Tg has revealed that both initial iodine concentration and native structure of Tg influence the hydrolysis of Tg (Lamas et al, 1986). It has been shown that when Tg is highly iodinated, three out of four hormonogenic sites localized at the COOH-end of the molecule, are most susceptible to proteolysis (Marriq et al, 1982; Dunn et al, 1983; Lejeune et al, 1983). In contrast, excessive iodination of Tg has been shown to inhibit the hydrolysis of Tg and subsequent thyroid hormone secretion (Bagchi et al, 1985b; Becks et al, 1987).

The follicular activity and Tg production are under the physiological control of TSH. TSH is released from pituitary gland in a typical "feedback" manner when the serum levels of T3 and T4 are decreased. Transcription of Tg is under the positive control of TSH which is probably mediated by cyclic AMP (cAMP) following TSH binding to TSHR (Van Heuverswyn et al, 1985). It has been suggested that TSH and its cAMP signal are not the primary policemen directing the Tg synthesis, but rather that this is controlled by insulin-like growth factor-1 (IGF-1) i.e., TSH/cAMP may amplify and regulate more than they initiate the Tg synthesis (Santisteban et al, 1987). It has been shown that Tg contains approximately 120 Tyr residues. Only 25-30 of these Tyr residues are available for

iodination and only 8 residues can couple to synthesize hormone.

The primary structure of bovine Tg (bTg) (Mercken et al, 1985a), of human Tg (hTg) (Malthiery and Lissitzky, 1987) and of a part of rat Tg (rTg) (Musti et al, 1986) derived from the sequence of their complementary DNA (cDNA) have been reported. Comparison of their primary a.a. sequences indicated that Tgs of various species are highly conserved. The presence of structural differences near the thyroxine-forming sites has been demonstrated using MAbs to Tgs (Chan et al, 1986). Availability of Tg in large quantities has made easier to establish animal models for studying the immunopathogenesis of HT. It should be mentioned here that although the molecular structure of Tg molecule has been known for a while, the putative autoantigenic T- and B-cell epitopes largely remain undefined. This was mainly because of its large size.

1.1.3 Thyroid peroxidase (TPO) or Microsomal antigen (Mic Ag)

Since the first description of a "thyroid microsomal antigen" (Mic Ag) by immunofluorescent studies using sera from patients with AITD (Trotter et al, 1957), the precise molecular nature of this autoantigen remained elusive for decades. Mic Ag is expressed at low levels in the thyroid. It has now been shown by immunochemical and biochemical studies that Mic Ag is identical to TPO in that the reactivity

of TPO was immunoprecipitated by sera containing anti-microsomal Abs (anti-Mic Abs) and, conversely, TPO was able to absorb anti-Mic Abs (Czarnocka et al, 1985; Portmann et al, 1985). The identity between TPO and Mic Ag was once again confirmed using MAb specific for Mic Ag (Portmann et al, 1988). It is well documented that purified TPO protein is visualized on gel electrophoresis as a doublet of approximately 107 kD and 100kD (Czarnocka et al, 1985; Kajita et al, 1985). The TPO is involved in two important steps in the biosynthesis of the thyroid hormone: Iodination of Tyr residues on Tg, and intramolecular coupling of iodotyrosines, leading to the formation of T3 and T4 (DeGroot and Niepomniszcze, 1977).

The TPO enzyme has been shown to be expressed on the cell surface (apical membrane) of the TEC (Khoury et al, 1981; Chiovato et al, 1985). On the other hand, the possible expression of the TPO on the basal membrane of the TEC (Hanafusa et al, 1984), a region normally exposed to the effectors of the immune system, has been a matter of considerable debate (Khoury et al, 1984). TPO is a transmembrane glycoprotein and contains 5 potential glycosylation sites (for a review, see Banga et al, 1991). The complete nucleotide sequence of pig TPO (pTPO) cDNA that codes for a 926 a.a. protein has been reported (Magnusson et al, 1987). Three different groups independently reported the

nucleotide sequence of human TPO (hTPO) cDNA, which codes for a 933 a.a. protein (Kimura et al, 1987; Magnusson et al, 1987; Libert et al, 1987). The nucleotide sequence of cDNA for rat TPO (rTPO) was also reported (Derwahl, 1989). The analysis of molecular structure of TPO and its availability in recombinant forms hastened the progress in understanding the immunopathogenesis of autoimmune thyroid disease and will be discussed later.

1.1.4 Thyroid stimulating hormone (TSH) receptor (TSHR)

TSHR is expressed in very low abundance on TEC (10^3-10^4 receptors/cell) (Rees Smith et al, 1988). Knowledge of TSHR at the molecular level began when Parmentier et al (1989) isolated a dog TSHR cDNA (cDNA) from a thyroid cDNA library. Simultaneously, cDNA clones for a human (Nagayama et al, 1989; Libert et al, 1989; Misrahi et al, 1989) and for a rat (Akamizu et al, 1990) TSHRs were reported.

The TSHR is a 120 kDa glycoprotein consisting of 764 amino acids including a 20mer signal peptide. The molecular weight of the N-terminal TSHR is approximately 84.5 kDa (Nagayama and Rapoport, 1992). The extracellular region (398 amino acids) of TSHR of human, dog, and rat bears 85-90% a.a sequence homology. The transmembrane/intracellular C-terminal half of the TSHR consits of 346 amino acids with 7 hydrophobic transmembrane segments. It has been shown that TSH does not

bind chinese hamster ovary (CHO) cells transfected with a TSHR cDNA in which the N-terminal a.a. 38-45 sequence is deleted or substituted suggesting that a.a. 38-45 sequence is important in TSH binding (Wadsworth et al, 1990). Recently, using mammalian cells transfected with deletion mutants of TSHR cDNA, it has been shown that amino acids at positions 390, 385, 301 of the N-terminal (extracellular) TSHR are involved in TSH binding (Kosugi et al, 1991). It has also been shown that TSH interacts with multiple discrete regions of TSHR since anti-peptide Abs of distinct specificities inhibit the binding of TSH to its receptor (Dallas et al, 1994). The extracellular region of TSHR has also been shown to be important in signal transduction (Nagayama et al, 1991). By site-directed mutagenesis of the TSHR cytoplasmic regions, a 10mer (441-450) sequence of the cytoplasmic loop of C-terminal TSHR has been shown to be important in cAMP generation (Chazenbalk et al, 1990).

1.2 Experimental autoimmune thyroiditis (EAT)

EAT is an animal model for HT and has been widely used for decades to elucidate the mechanisms of self-tolerance and of immunopathogenesis in human thyroid disease. EAT is characterized by autoreactive T- and B- cell responses, a marked mononuclear cell infiltration of the thyroid gland, and the occurrence of circulating Abs to thyroid Ags (Rose et al,

1971).

There are four main EAT models. The first model depends on immunization with thyroid antigens (Ags) in the presence or absence of adjuvants, whereas the second model depends on the manipulation of the T cell population either by thymectomy and irradiation (Tx-X) or by treatment with Abs to T cells. In the third model, the disease spontaneously develops in experimental animals and the fourth model involves xenografting of thyroid tissue from HT patients into SCID mice.

1.2.1 EAT induction in various species

EAT has been induced in various animal species by immunization with crude thyroid extract or Tg emulsified in complete Freund's adjuvant (CFA). Initially, it was not possible to decide whether establishment of autoimmune response to thyroid Ags is responsible for the development of thyroid lesions or is merely a non-pathogenic consequence of tissue destruction. This ambiguity in thyroid autoimmunity was resolved when EAT was induced for the first time in rabbits by subcutaneous (s.c.) immunization into four footpads with CFA emulsion containing syngeneic thyroid gland homogenate (Witebsky and Rose, 1956; Rose and Witebsky, 1956). On the other hand, challenge of rabbits with CFA emulsion only did not result in the development of an immune response to

thyroid Ags. The authors demonstrated the presence of AAbs to thyroid Ags by complement fixation, precipitation, and hemagglutination (HA) tests. These pioneering studies have provided the first evidence that thyroid Ag(s) is the target Ag in thyroid autoimmunity.

Later, EAT was induced in various species: rats (Jones and Roitt, 1961), guinea pigs (Mc Master et al, 1961), dogs (Terplan et al, 1960), and mice (Rose et al, 1971). The thyroid disease induced in these various species closely resembles the characteristic features of HT in humans in terms of lesions generated, T-cell and Ab responses and genetic makeup. However, the pathological changes induced by active immunization with crude thyroid extract in CFA differ in certain respects from that of HT. In EAT, assessment of hypothyroidism by thyroid hormone production has rarely been done. These animals often develop severe mononuclear cell infiltration of the thyroid and subsequent hypothyroidism due to their thyroid gland destruction, but do not contain the germinal centres in the thyroid that is a hallmark of human HT.

One may wonder why induced EAT has been widely used to study the immunopathogenesis of HT when the latter disease develops spontaneously in humans. The answer to this question is multifaceted. EAT induced with thyroid Ags will allow the researchers to study the regulation of the immune response at

the levels of both the central lymphoid and target organs, the kinetics of the disease development, T- and B-cell responses, analysis of the respective specificities and functions of lymphocyte subsets found during thyroiditis, and the site of initial priming and the inductive signals required to induce thyroiditis. However, it should be noted that EAT induced under controlled experimental conditions is quite different from that found in spontaneous thyroid disease in humans because in the latter the triggering events in terms of the immunogen, the site of initial-priming and the site of chronic immunization remain a black-box. Nevertheless, EAT studies have provided valuable information concerning the nature of autoantigens, initiation, progression, and immuno-pathogenesis of autoimmune thyroiditis (Charreire, 1989).

1.2.1.1 EAT induction with thyroid Ags using adjuvants

In contrast to a spontaneous autoimmune disease, induction of EAT with thyroid Ags in susceptible strains of mice and rats often requires the use of a potent adjuvant. It is assumed that inflammatory responses induced by the complete Freund's adjuvant (CFA) emulsion may have an influence on the pathological manifestations of the disease. Even then, CFA has been the adjuvant of choice to induce EAT in various species. However, CFA has been shown to induce undesirable effects such as a chronic inflammatory response at the site of

injection (Wiedmann et al, 1991; Yamanaka et al, 1992).

EAT was induced in mice for the first time with Tg + CFA, employing inbred mice of various strains (Vladutiu and Rose, 1971a). These authors demonstrated variations in the onset of Ab production: the strains that had delayed onset of Ab response showed little or no mononuclear cell infiltration of the thyroid, whereas the mouse strains that had rapid onset of Ab production showed extensive mononuclear infiltration of the thyroid.

In addition to CFA, various other adjuvants have also been used to enhance the immunogenicity of Tg and to induce EAT. These adjuvants include incomplete Freund's adjuvant (IFA) (Esquivel et al, 1977; Vladutiu and Rose, 1972), silica (Rose et al, 1965), alhydrogel (Twarog et al, 1970), latex particles (Esquivel et al, 1977), lipopolysaccharide (Esquivel et al, 1977), polyadenylic-polyuridylic acid complex (Poly A:poly U) (Esquivel et al, 1978). Moreover, adjuvants such as Bordetella pertussis (Twarog and Rose, 1969), Legionella pneumophilia (McMaster et al, 1986) and muramyl dipeptide (MDP) (Kong et al, 1985) have also been used either to enhance the immunogenicity of Tg or to accelerate the onset of autoimmune thyroiditis. It should be noted that the response of a given mouse strain to mTg varies depending upon the type of adjuvant used. For example, the use of LPS, a weak adjuvant, for EAT studies has demonstrated a distinction

between inbred mouse strains in terms of both Ab titer and thyroid pathology (Esquivel et al, 1977). More interestingly, Tg-induced EAT persists transiently in mice (Braley-Mullen et al, 1994a) possibly due to induction of regulator cells ($CD8^+$ T cells). In that study, it has been shown that depletion of $CD8^+$ T cells in the recipient mice exacerbated severity of thyroid lesions whereas their presence resulted in the resolution of thyroid lesions.

EAT was induced for the first time in Lewis rats by injecting intradermally at the base of the tail with CFA emulsion containing either Tg or thyroid extract (Jones and Roitt, 1961). In that study, the antigenic challenge of rats was done on day 0 and 21. The rats were examined for EAT characteristics on various time points after the first injection itself. It was found that injection of rats with two doses of Tg (on days 0 and 21) increases the severity of the thyroiditis compared with a single injection of Tg on day 0. Subsequently, Twarog and Rose (1969) demonstrated that injection of Lewis rats with CFA emulsion containing homologous thyroid extract followed by concomitant injection of Bordetella pertussis organisms (Pertussis vaccine) induces marked thyroiditis. In contrast, thyroids collected from rats that received CFA emulsion containing rat thyroid extract only showed minor thyroid lesions. In that study, the importance of CFA was also highlighted in its ability at enhancing the

immunogenicity of Tg because the use of other adjuvants such as alhydrogel, IFA, or pertussis vaccine in the place of CFA did not result in the production of either thyroid lesions or Abs to thyroid Ags. In another study, it has been shown that the need for a booster Tg injection to induce thyroiditis was circumvented if rats were given Bordetella pertussis organisms on the day of first injection of Tg (Paterson and Drobish, 1968). This accelerated form of EAT might be attributed to the histamine sensitization factor "pertussigen", the active ingredient of Bordetella pertussis, that increases vascular permeability (Bergman et al, 1978). Finally, the putative pathogenic self-Tg epitopes that cause EAT in rats have not yet been demonstrated.

1.2.1.2 Induction of EAT with thyroid Ags in the absence of adjuvants

Though CFA has been known at enhancing the immunogenicity of Tg, it has also been shown to induce inflammation at the site of priming. To avoid this influence on Tg-induced EAT and to understand how the tolerance to autoantigens is abrogated, EAT studies have been carried out with Tg in saline. In that direction, Weigle, (1965) has demonstrated that chemical modification of Tg enhances its immunogenicity. In that study, the author modified the homologous Tg by coupling to chemically defined haptens. Injection of rabbits

with chemically modified soluble homologous Tg ((Tg is coupled to diazonium derivatives of arsanilic acid and sulfanilic acid (arsanil-supfanil Tg)) but not unaltered homologous Tg in the absence of adjuvants, readily circumvented the natural tolerance to Tg and resulted in the induction of thyroid lesions and Abs to thyroid Ags. In contrast, injection of native heterologous Tg (unaltered Tg) in soluble form resulted in the induction of Ab response to homologous and heterologous Tgs. No thyroid lesions were induced. These findings suggested that pathogenic autoimmunity can be triggered through modification of autoantigens. Similar results were obtained in rabbits with altered heterologous (bovine and human) Tgs. Subsequently, it was shown that this immunization regimen induces in rabbits not only thyroid lesions but also chronic glomerulonephritis (Weigle and Nakamura, 1969). Moreover, priming of rabbits with fragments of Tg in saline (the Tg fragments were obtained either with proteolytic digestion of Tg with papain or with pepsin) (Anderson and Rose, 1971; Stylos and Rose, 1968; Weigle et al, 1969) elicited anti-Tg Abs.

It has also been shown that in some highly susceptible strains of mice, repeated injection of unaltered homologous Tg in larger doses in the absence of adjuvants, induced both thyroid lesions and Abs to Tg in good responder (CBA) mice (ElRehewy et al, 1981). In that study, it was shown that 57%

of the CBA mice had thyroid lesions. In contrast, no thyroid lesions were observed in poor responder BALB/c mice. These authors suggested that pathogenic autoreactive lymphocytes normally exist in the body. A marked shift in circulating Tg levels temporarily perturbs normal homeostatic controls and activates the self-Tg reactive lymphocytes leading to the initiation of an autoimmune response. Although Tg-specific Abs can be elicited in animals by repeated immunization with homologous or heterologous Tg(s) without adjuvants (EIRehewy et al, 1981), the use of adjuvants dramatically increased the vigour of Ab responses.

1.2.2 EAT induction using T cell depletion

EAT can also be induced in mice or rats by <u>in vivo</u> manipulation of the T cell population. The first set of experiments performed by Penhale et al (1973) demonstrated that depletion of T lymphocytes in rats by thymectomy and irradiation (Tx-X) [thymectomy was done at 5 weeks of age and was followed by four doses of sublethal whole body irradiation (200 rad each at 2 weeks intervals)] induces typical EAT in Wistar ($RT1^u$) strain of rats. These findings indicated that self-reactive T cells with the capacity to elicit autoimmune thyroiditis are neither deleted nor rendered anergic in these animals. Thymectomy combined with a series of sublethal irradiations has been suggested to cause a selective depletion

of regulatory T cells involved in the suppression of pathogenic thyroid autoimmunity. It has been directly demonstrated that (Tx-X)-induced EAT can be prevented by reconstitution of Tx-X rats with lymphocytes from healthy, syngeneic rats (Penhale et al, 1976). These data further supported that a role for a subset of T cells in the suppression of pathogenic autoimmunity. Later it was demonstrated that irradiation per se and irradiation sensitivity of the thyroid are not responsible for the thyroid lesions in this animal model (Ahmed and Penhale, 1981). These conclusions stem from the experimental data in which post-irradiation transplantation of syngeneic thyroids under the renal capsule of Tx-X rats led to the development of thyroiditis in the grafted thyroids. This was further supported by recent data showing that high dose irradiation of the thyroid alone does not lead to mononuclear cell infiltration whereas, whole body irradiation, except for thyroid, resulted in thyroid lesions (Sakaguchi et al, 1994).

It should be noted that EAT induced in rats by Tx-X resembles HT in several important respects. The disease is of a chronic nature and persists for at least 18 months. The lesion develops spontaneously without necessity for immunization with autoantigens in CFA. However, germinal centres have not been observed in the thyroids of Tx-X rats. The mechanisms of immunoregulation in the (Tx-X)-induced rat

EAT model were not investigated. The immuno regulatory mechanisms in this EAT model can be investigated by performing experiments similar to those that were demonstrated in PVG/c rats that develop diabetes following Tx-X. Injection of $CD4^+$ $CD45RC^{low}$ T cells that secrete IL-2, IL-4 and IL-10, but not IFN-γ prevented the development of diabetes in these animals (Fowell and Mason, 1993). Thus, the delicate balance between pathogenic and regulatory T cells appears to determine the tolerance status of host to self-antigens responsible for these autoimmune diseases.

By adopting a different experimental approach, Sugihara et al (1988) have induced EAT in T-cell-depleted B-cell-present mice (referred to as B mice). In this study, the authors have demonstrated that adoptive transfer of normal syngeneic $CD5^{dull}$ $CD4^+$ T cells, obtained by pretreatment of lymphoid cells with anti-CD5 MAb plus complement, into syngeneic "B mice" readily induced EAT. These results indicate that thyroiditis-inducing T cells are present in the peripheral lymphoid tissues of normal healthy mice. By immunohistochemical studies, it has been shown that the thyroid lesions contain both $CD4^+$ and $CD8^+$ T cells. These results raise the possibility that both of these T cell populations may act as effector cells in mediating thyroid damage (Sugihara et al, 1989). Interestingly, both $CD4^+$ and $CD8^+$ (CTL) T cell clones, that recognize Tg and some other

unknown thyroid antigen, have been isolated from thyroid lesions of these mice. Moreover, fractionated $CD5^{bright}$ $CD4^{+}$ T cells but not unfractionated T cells prevented the induction of EAT (Sugihara et al, 1990), showing the coexistence of both regulatory and disease causing T lymphocytes in the normal peripheral lymphoid cell population. EAT induction by depletion of regulatory T cell subsets has furthered our understanding of the immunoregulation in AITD.

1.2.3 Spontaneous Autoimmune Thyroiditis (SAT)

SAT has been reported in Non-Obese diabetic (NOD) mice, Bio-Breeding (BB) and Buffalo (BUF) rats and Obese strain (OS) chicken. A few of the studies performed on such spontaneous thyroiditis models are described below.

1.2.3.1 The NOD mice

As cited in the literature, NOD mice have been shown to develop SAT, but with some variability in percentage of incidence with age of the animals. However, the severity of thyroiditis did not vary with the age of the animals (Bernard et al, 1992). Thyroiditis and Abs to TPO have been demonstrated in these NOD mice at 1 month of age.

1.2.3.2 The BB and BUF rats

Silverman and Rose (1971) reported a low incidence of

spontaneous thyroiditis in 9-12 week old female Buffalo (BUF) rats and the incidence rose to 48% in ex-breeders. Disease prevalence was shown to be increased in both males and females by neonatal thymectomy (NTx) (Silverman and Rose, 1974a), by immunization with Tg in various adjuvants (Silverman and Rose, 1975b), and by subcarcinogenic doses of 3-methylcholanthrene (Silverman and Rose, 1975a). In these animals, an important MHC influence on susceptibility to thyroiditis was demonstrated. Animals with the $RT1^{b/b}$ genotype had the highest risk of developing thyroiditis and those with the $RT1^{u/u}$ genotype had the lowest risk (Colle et al, 1985). In contrast to the report of Silverman and Rose (1971), Cohen and Weetman (1987) did not observe SAT in their colony of BUF rats, but they were able to induce EAT following NTx. The inability to develop SAT was attributed to environmental influence on the immune system.

1.2.3.3 The OS chicken

OS chicken, among which over 95% develop SAT, were selected from Cornell strain chickens on the basis of phenotypic symptoms of hypothyroidism. SAT in OS chickens occurs during the first 3 weeks after hatching and is characterized by massive lymphoid cell infiltration of the thyroid gland with hypothyroidism and circulating AAbs to Tg after 4 weeks of age. The SAT in OS chicken has been a

particularly useful animal model to study the immunopathogenesis of HT for two reasons (Wick et al, 1982). First, germinal centres replace the functional thyroid tissue and result in fibrosis of the thyroid glands. Second, autologous C3 and IgG deposits forming immune complexes consisting of Tg and anti-Tg Abs are present in the basal lamina of thyroid glands in newly hatched birds (Katz et al, 1981). These Abs have been shown to precipitate the thyroid disease (Katz et al, 1986). Three groups of genes are believed to control susceptibility of OS chickens to SAT: (1) MHC, (2) an immunoregulatory locus outside the MHC, (3) genes for target organ abnormality (Wick et al, 1989). These findings suggested that autoimmune thyroid disease is multifactoirial in origin.

1.3 Immunogenetics of EAT in mice and rats

1.3.1 MHC control of mouse EAT

Classic studies in thyroid autoimmunity demonstrated that mouse susceptibility to Tg-induced EAT was closely linked to the MHC i.e., H-2 complex (Vladutiu and Rose, 1971a). In that study, the authors used 35 different strains of mice representing eleven different H-2 haplotypes. Based on the susceptibility to Tg-induced EAT, mouse strains were classified as good ($H-2^{s, k, q}$ haplotypes), fairly good ($H-2^{a}$) and poor ($H-2^{b, d}$ haplotypes) responders. Using F1 animals ($H-2^{s, d}$), these authors further demonstrated that mouse

susceptibility to Tg-induced EAT was a dominant trait. Using an *in vitro* proliferative response to mTg and employing intra-H-2 recombinant strains of mice, the Tg-Ir gene controlling the susceptibility to EAT was more precisely mapped to the K and/or I-A regions of the H-2 complex (Tomazic et al, 1974).

Using intra-H2 recombinant mouse strains (derived from recombination events between the good responder k haplotype and poor responder b haplotype), in was shown that the I-A subregion within the H-2 complex controls the susceptibility of mice to Tg-induced EAT (Beisel et al, 1982a). The T cell proliferative response was also shown to be under the control of genes located within the I-A subregion (Salamero and Charreire, 1983b). Tomazic et al (1974) suggested that besides Ir-Tg gene (I-A) control, the D-end also exerts an influence on the severity of thyroiditis. Moreover, Kong et al (1979), using H-2 recombinant mice, demonstrated that MHC class I at the D-end may also influence the incidence and/or severity of thyroiditis. It was found that animals carrying D^k have the highest degree of thyroid pathology, while those bearing D^d have markedly reduced infiltrations. It is apparent from these investigations that the I-A gene products determine a good or poor response to Tg as such, whereas the D region gene products modify the severity of the disease possibly through governing the effector mechanisms necessary for thyroid inflammation. It is possible that these MHC class

I and class II alleles from low responders may favour the generation of regulatory cells (Th2 or $CD8^+$ T cells) similar to those results that have been shown in myelin basic protein (MBP) peptide (63-88)-induced experimental autoimmune encephalomyelitis (EAE) in intra-MHC recombinant rat strains (Mustafa et al, 1994).

Like the D-end, the K-end has also been shown to influence the penetrance of the Ir-Tg by modifying the severity of the disease. Experiments performed using B6 ($H-2^b$) and B6.C-$H-2^{bml}$ demonstrated a higher incidence of thyroiditis in B6.C-$H-2^{bml}$ than B6 ($H-2^b$) mice (Maron and Cohen, 1979); B6.C-$H-2^{bml}$ has a point mutation in the H-2K molecule (Brown and Natheson, 1977). A second line of evidence implicating the K-end in regulating the severity of thyroid lesions comes from the studies of intra-H-2 recombinant mice (Beisel et al, 1982b). In these studies, it was shown that the presence of K^k reduces, whereas K^q or K^b increases the incidence of thyroiditis in mice to mTg but the mechanisms by which these MHC class I molecules influence the severity of thyroiditis have not yet been clarified. This would be possible by performing EAT studies with defined pathogenic Tg peptides in intra-H2 recombinant mice.

The susceptibility of mice to mTg-induced EAT controlled by MHC also extends to heterologous Tg-induced EAT (Tomazic and Rose, 1976; Maron et al, 1983; Simon et al, 1986; Romball

and Weigle, 1984). In contrast to homologous Tg (mTg), these heterologous Tgs are poor inducers of EAT by active immunization, although strong Tg-specific Ab titers were induced (Kong, 1986). In contrast to Tg-induced EAT, Kotani et al (1990) demonstrated that for TPO-induced murine EAT, H-2^b mice are good responders, whereas H-$2^{a,d,k,\text{and } s}$ mice are poor responders. Using congeneic and recombinant mouse strains, the authors further demonstrated that the I-A subregion had a weak association with the induction of thyroiditis and at least one non-H-2-linked gene controls the development of thyroid lesions; Ab production to TPO did not correlate with the incidence of thyroiditis in any given strain.

1.3.2 MHC control of rat EAT

Genetic control of EAT in rat strains was studied by two different investigators (Rose, 1975; Penhale et al, 1975a). In these studies, a wide divergence in response to homologous Tg in different rat strains both in the extent of thyroid lesions and in the level of AAbs to Tg, was been demonstrated. These studies suggested a linkage between EAT susceptibility and the MHC type. However, definitive experimental evidence to show the role for rat MHC in EAT has not been demonstrated.

Susceptibility of inbred rat strains to homologous Tg-induced EAT has also been classified into three categories:

high, intermediate, and low (Penhale et al, 1975b; Rose, 1975; Lillehoj and Rose, 1982). The classification was done based on the strain susceptibility to the induction of thyroiditis, AAb and LNC proliferative responses. The results obtained in these three studies are pooled and are shown here. High responder rat strains include F344 ($RT1^l$), AO ($RT1^w$), WF ($RT1^u$), AUG ($RT1^c$) and LH ($RT1^c$). Intermediate responder rat strains include AS ($RT1^l$), LEW ($RT1^l$), HL ($RT1^l$), BVF ($RT1^l$), and WKY ($RT1^k$). Low responder rat strains include CAM ($RT1^w$), B1 ($RT1^n$), DA ($RT1^a$), AC1 ($RT1^a$), HO ($RT1^c$), PVG/c ($RT1^c$), and SHR ($RT1^k$). The studies of Penhale et al (1975a) suggested a relationship between the RT1 complex (rat MHC complex) and susceptibility of rats to thyroiditis. These authors observed that strains with the $RT1^c$ (AgB5) haplotype were the most susceptible, whereas strains with the $RT1^w$ (AgB2) haplotype were least susceptible. However, no linkage studies were done to confirm this relationship between RT1 complex and EAT. Penhale et al (1975b) further demonstrated that, using a thymectomy-induced rat EAT model, rat strains were graded as high (AUG and PVG/c), intermediate (WAG and LIS), and low (CAM) responders. The incidence of AAbs to Tg correlated closely with the strain incidence of thyroiditis. Therefore, the classification of rat strains into high and low responders depends on the method of EAT induction. Subsequently, Lillehoj et al (1981) have investigated whether or not the

genetic factors (MHC) influence the susceptibility of inbred rat strains to rTg-induced EAT. The authors demonstrated that immune response to rTg is under polygenic control. The main factor controlling immune response to Tg is associated with X-chromosome. No evidence of linkage between the rat MHC (RT1) and immune response to Tg was documented. In contrast to the findings reported by Lillehoj et al (1981), a subsequent study strongly implicated a putative role for rat MHC in Tg-induced EAT (de Assis-Paiva et al, 1989). In the latter study, the authors employed both inbred (AOG and AO) and congenic PVG ($RT\text{-}1^c$, $RT\text{-}1^a$, and $RT\text{-}1^u$) rat strains. The results indicated that animals with $RT\text{-}1^c$ haplotype being high responders, whereas animals of $RT\text{-}1^u$ and $RT\text{-}1^a$ haplotypes were poor responders. It should be noted that this study has employed only few congeneic rat strains and needs a reinvestigation by employing a larger number of congeneic rat strains to definitely demonstrate that MHC influences the susceptibility of rat strains to homologous Tg-induced EAT.

1.3.3 Intrinsic abnormalities of the thyroid gland

Several investigations have been made to address whether or not intrinsic abnormalities or sensitivity of the target organ contributes to susceptibility of mouse or rat strains to thyroiditis.

The genetic susceptibility of mouse strains to Tg-induced EAT has been shown to depend on both the effective interaction of the immune system with the thyroid and intrinsic features (genetic background) of the target tissue (Ben-Nun et al, 1980; Okayasu and Hatakeyama, 1983). Ben-Nun et al (1980) examined the susceptibility to Tg-induced EAT of thyroid tissue from EAT-resistant ($H\text{-}2^b$) and -susceptible ($H\text{-}2^k$) mice grafted to histocompatible, but EAT-susceptible semi-allogeneic ($H\text{-}2^{kb}$) F1 hybrid mice [(susceptible ($H\text{-}2^k$) x resistant ($H\text{-}2^b$))F1 mice]. These authors found that thyroids from the donors of the resistant genotype was relatively resistant, whereas thyroids from susceptible strains were relatively susceptible to the development of thyroiditis. Similar results were reported when LPS + Tg was used to induce EAT (Okayasu and Hatakeyama, 1983).

The respective contributions of the immune system and the target organ to EAT development were reexamined in rat EAT by employing fully allogeneic, but immunologically tolerant rats (Eishi and McCullagh, 1988a). It was found that thyroid tissue transplanted from the donors of resistant genotype (PVG/c rats) to fully allogeneic, tolerant recipients of susceptible genotype (DA rats) invariably developed thyroiditis similar in severity to that of the host's thyroid. These findings indicate that variation in the genetically determined susceptibility or resistance to EAT is determined

by the effective interaction of the immune system with the target tissue but not by the intrinsic features of the thyroid gland. The cellular and molecular basis for the observed results are not understood. However, it is possible that, following Tg-priming, specific $CD4^+$ T cells elicited in the allogeneic, tolerant rats might be MHC-dependent but MHC-unrestricted and these T cells might have destroyed the transplanted thyroid from genetically EAT-resistant rats. Evidence for the existence of MHC-unrestricted rat $CD4^+$ T cells specific for acetylcholine receptor (AChR) has been documented in rats (Tami et al, 1987).

In vitro experiments performed by Lahat et al (1989) demonstrated a strain effect on IFN-γ induced class II expression on thyrocytes. In that study it was shown that IFN-γ treatment of thyroid monolayers from BUF (high responder) but not from F344 (low responder) rats resulted in a consistent and a greater degree of class II MHC expression. These authors failed to give a molecular explanation for the observed results, but proposed and supported the hypothesis that end-organ sensitivity to, or abnormality in MHC class II Ag expression, may be an important contributing factor in the pathogenesis of AITD. Previous studies performed in murine models of multiple sclerosis have indicated that inducibility of MHC class II on astrocytes but not on macrophages from susceptible and resistant strains of rats or mice differed

drastically when cultured in the presence of IFN-γ (Massa et al, 198[illegible]). Hence, strain-specific susceptibility to MBP-induced EAE has been linked to hyper-inducibility of MHC class II on astrocytes.

Kotani et al (1981) proposed that radiation sensitivity of the thyroid gland contributes to the susceptibility of various rat strains to (Tx-X)-induced EAT. These authors observed a decrease in serum levels of T3 and T4 in susceptible (K/BF1) but not in resistant (K/AF1) strain of rats. The thyroid morphology remained normal in both the high and low responder rat strains. These studies were contradicted recently by Sakaguchi et al (1994) who demonstrated that high dose (42.5 Gy) fractionated (2.5 Gy 17 times) total lymphoid irradiation (TLI) caused thyroiditis including other organ-specific autoimmune diseases in various mouse strains. They further showed that irradiation of the target organ alone did not result in thyroiditis development, whereas irradiation of peripheral lymphoid organs or thymus induced various autoimmune diseases including thyroiditis. These results indicate that radiation sensitivity of the target organ may not determine the murine susceptibility to thyroiditis.

Sospedra et al (1995) provided a direct evidence that intrinsic abnormalities of the target organ may influence susceptibility of individuals to thyroid disease. The authors

demonstrated that IFN-γ hyperinduces HLA class II expression on thyrocytes from Graves' disease (GD) but not from multinodular goitre (MNG) patients. These results are specific to thyrocytes only because they did not observe any differences in the sensitivity of macrophages (from GD and MNG patients) to IFN-γ-induced class II expression. The higher inducibility was parallel to a faster and stronger induction of HLA class II mRNA in GD thyrocytes but did not correlate with the levels of HLA class II or class I originally expressed by thyrocytes. These findings indicated that intrinsic abnormalities in the target organ contributes to the susceptibility of individuals to human thyroid disease. This differential class II hyperinducibility seems to be attributed to allelic polymorphisms in the upper regulatory regions of HLA class II genes as has been shcwn for DQB (Anderson et al, 1991) and DRB (Louis et al, 1993) genes. In both of these studies, the authors suggested such polymorphism may confer allelic differences in expression, inducibility, and/or tissue specificity of class II molecules. In conclusion, the findings reported in animal models and in patients with AITD favor the concept that genetic differences between animals or humans in the expression of MHC at level of the target organ contribute to susceptibility to AITD.

1.3.4 Non-MHC genes

The importance of genes outside the H-2 region of mice in responsiveness to Tg, as assessed by Ab titers and thyroid pathology, has been demonstrated. By examining a large panel of independent H-2 haplotypes on B10, BALB/c, C3H, and A/J background for their responsiveness to Tg using LPS as adjuvant, it was found that non-MHC genes influence the Ab titer and the incidence and severity of thyroiditis in mice (Beisel, 1982b). Till now, the putative non-MHC genes that influence the induction of EAT have not been characterized.

1.4 Autoimmune responses

1.4.1 T cell responses

1.4.1.1 Thyroid Ag-specific T cell responses

Using lymph node cell (LNC) proliferative assays, Okayasu et al (1981) have demonstrated that EAT susceptible ($H\text{-}2^{k, s}$), but not EAT resistant ($H\text{-}2^{b, d}$) mice can be distinguished by the capacity of their T cells to proliferate in response to mTg <u>in vitro</u>. The proliferative response to Tg clearly does not reflect T-helper (Th) function in aiding Ab production because Tg-priming elicits Ab responses in both susceptible and resistant mice. The authors suggested that Tg-specific proliferative T cells from EAT susceptible strains of mice contain both Th and Te (T effector) cells. Similar results were obtained either with homologous (mTg) or with

heterologous (hTg)-primed LNC in that LNC from C3A ($H\text{-}2^k$) but not from BALB/c ($H\text{-}2^d$) mice proliferate to respective Tg used for priming. The results of Okayasu et al (1981) were confirmed later by Romball and Weigle (1984) by using bovine and porcine Tgs to prime the mice. The T cell proliferative responses of Tg-primed LNC from CBA mice were inhibited by the addition of $I\text{-}A^k$- but not by $I\text{-}A^d$-specific (control) MAbs (Beisel et al, 1982a) indicating that specific T cells recognize Tg epitopes in the context of $I\text{-}A^k$ class II MHC. These results confirmed that that I-A subregion of the H-2 complex controls the Tg-induced EAT in mice.

1.4.1.2 Shared Tg epitopes versus EAT induction

Okayasu et al (1981) have shown that mTg-primed LNC from EAT susceptible mice proliferate to hTg and have indicated that specific T cells recognize shared T cell epitopes of Tg. These results were further confirmed by studies from various investigators. Simon et al (1985) demonstrated that the hTg-primed LNC proliferate in response to hTg as well as to mTg *in vitro*. Similarly, Romball and Weigle (1984) also demonstrated that bTg-primed LNC proliferate in response to bTg as well as to mTg. Simlar to mouse EAT, a role for shared Tg epitopes has been demonstrated in rat EAT (Jones and Roitt, 1961). In that study, it was shown that challenge of rats either with homologous (rat) or heterologous (human and sheep) Tg(s)

readily induced thyroid lesions. However, higher thyroiditis score was observed in rats that received homologous Tg only. Overall, these data in both mice and rats demonstrated that Tgs of various species contain shared epitopes recognized by T cells.

The role of shared Tg determinants in causing EAT was addressed either by direct or adoptive transfer EAT experiments. It has been shown that the direct transfer of spleen cells from mTg + LPS primed, EAT susceptible mice does not induce thyroid lesions in recipient syngeneic mice (Simon et al, 1986). Adoptive transfer experiments employing mTg-primed LNC that were first boosted in vitro for 3 days either with mTg (specific) (Simon et al, 1986) or with Con A (polyclonal activator) readily induced thyroid lesions in recipient mice (Maron et al, 1983; Okayasu, 1985; Simon et al, 1986). A role for shared pathogenic Tg T cell epitopes was demonstrated with mTg-primed (in vivo) and hTg-boosted (in vitro) LNC by adoptive transfer of thyroiditis in mice (Simon et al, 1986). Similarly, hTg-primed LNC or spleen cells upon an in vitro boost with mTg or hTg, readily transferred autoimmune thyroiditis to recipient mice. These data further highlighted a pathogenic role for shared Tg T cell epitopes in EAT. However, the precise location and nature of these putative T cell epitopes remain unknown.

1.4.1.3 Evidence for involvement of thyroid antigens in AITD

In order to address whether thyroid Ags are involved in AITD in humans, lymphocyte proliferative assays have been performed to stimulate peripheral blood lymphocytes (PBLs) *in vitro* by driving them with putative autoantigens such as Tg, TPO or TSHR. Although there were occasional reports of a positive response to Tg (Ehrenfeld et al, 1971; Delespesse et al, 1972), the consensus has been that Tg does not give proliferative responses in autoimmune thyroiditis (Calder and Irvine, 1975). Subsequent studies have corroborated these negative findings (Fukuma et al, 1990). The reasons for unresponsiveness or weak proliferative responses to Tg are not clear but warrant further investigation.

In order to identify the T cell specificity, computerized algorithms were used to map T cell epitopes on TPO (Fukuma et al, 1990; Tandon et al, 1991b). It was found that three TPO peptides (415-432, 439-457, and 463-481) significantly stimulated the PBLs from HT patients but not healthy individuals. However, proliferative responses to some other TPO epitopes were observed in both HT patients and healthy individuals. More interestingly, a heterogenous response to TPO peptides with PBLs from different HT patients was observed, i.e. different TPO epitopes were recognized by T cells of diverse HT patients. Thus, it is likely that T cells from HT patients and healthy individuals recognize distinct

epitopes (Tandon et al, 1991b).

The finding that weak T cell proliferative responses to thyroid Ags was observed in PBLS of both thyroid disease patients and healthy individuals makes it difficult to determine whether the T cells identified are ever activated at the site of disease and involved in its pathogenesis or are simply a part of the normal precursor pool and activated in vitro. Moreover, a recent study indicated a clear reduction in circulating $CD8^+$ T cells in active HT, with a slight increase in the number of double positive T cells ($CD4^+$, $CD8^+$) (Iwatani et al, 1992). The infiltrating T cells are mainly $CD4^+$ and MHC class II^+ indicating that the cells are in an activated state. About 20% of the mononuclear cells infiltrating the thyroid are monocytes or macrophages; the rest are roughly equal numbers of T and B cells (Totterman, 1978). In conclusion, the immuno-histochemistry studies have indicated that thyroid infiltrates of HT patients contain activated T cells raising the possibility that they may recognize thyroid Ags.

Cloning of thyroid Ag-specific T cells from diseased thyroid tissue and the demonstration of their reactivity to Tg, TPO and thyroid epithelium have directly demonstrated that thyroid Ags are involved in the pathogenesis of AITD (Londei et al, 1985; Dayan et al, 1991). Dayan et al (1991) made use of an antigen-independent T cell cloning technique to

establish T cell clones *in vitro* from thyroid tissue of a GD patient and demonstrated in conventional T cell proliferative assays that these T cell clones recognize Tg, TPO, and TEC. Recently, Martin et al (1993) established an *in vitro* model system in which hTPO cDNA transfected EBV-transformed autologous B lymphoblastoid cell lines (EBVL) were used to present endogenous Ag and to stimulate PBLs from a GD patient. By this approach the authors were successful in isolating hTPO-specific T cell clones *in vitro* from the thyroid of this GD patient. These results clearly indicated that thyroid Ag-specific T cells do exist in the PBLS of GD patients. The specificity of the clones was not assessed and so no correlation could be drawn between the T cell clones in the target organ and in the periphery (Dayan et al, 1991). Mullins et al (1994) utilized the above screening protocol to analyze the specificity of intrathyroidal T cell clones from GD patients. The authors established a panel of T cell clones established *in vitro* from the thyroid gland by an antigen-independent T cell cloning technique and then tested the specificity of these T cell clones against either TPO- or TSHR-transfected EBVL. The TPO- but not the TSHR-transfected EBVL could stimulate four out of five T cell clones tested indicate that TPO rather than TSHR is the major autoantigen recognized by autoreactive T cells in GD. However, following a similar methodology, the same group (Mullins et al, 1995)

demonstrated that 10% of the $CD4^+$ T cell clones isolated from the thyroid of a different GD patient were TSHR-specific and belonged to either the Th0 or the Th2 subsets. In conclusion, these data indicate that thyroid Ags are directly involved in the activation of autoreactive T cells in AITD patients.

1.4.1.4 T cell epitope mapping: thyroid antigen(s)

1.4.1.4.1 EAT studies in mice

It is well established that a T- or B-cell response usually is limited to a small proportion of the potential epitopes/determinants on a protein Ag. T cell determinants are classified into dominant, subdominant and cryptic epitopes (See review Sercarz et al, 1993). By definition, a dominant determinant induces a strong <u>in vitro</u> T cell response with LNC obtained from animals immunized with a native Ag in adjuvant. The T cell response induced <u>in vivo</u> by the dominant determinant can also readily be recalled <u>in vitro</u> with the native Ag. On the other hand, a cryptic determinant readily induces a strong T cell response to itself but not to the intact Ag; priming with the native Ag in adjuvant does not elicit T cell responses to the cryptic peptide. The cryptic deteterminants are further classified into "absolute" and "facultative" cryptic epitopes. The T cell responses induced by "absolute" cryptic T epitopes cannot be recalled <u>in vitro</u> by its native Ag. On the other hand, T cell responses induced

by the "facultative" cryptic determinants can be recalled at high doses of native Ags. T cell responses to "facultative" cryptic determinants are also induced in vivo under special circumstances only. Intermediate determinants inducing a weak response are referred to as "subdominant" determinants. Subdominant determinants induce a strong response when injected in the peptide form, a response which can be recalled in vitro by the peptide or by the protein itself.

Investigators from different laboratories have focused their research on identifying pathogenic, immunodominant Tg epitopes within the large Tg molecule. Roitt and his group (Champion et al, 1987b) have demonstrated that iodination-dependent T cell epitopes may be important in the induction and/or perpetuation of autoimmune thyroiditis. Two clonotypically distinct I-A^k-restricted Tg-specific T cell hybridomas (derived from T cell line MTg12B and clone MTg9B3, respectively) responded to normal, but not thyroxine lacking Tg peptides. In a different study, Champion et al (1987a) have also shown that these T cell hybridomas have closely similar but not identical epitope specificities since the hybridomas exhibited differential responsiveness to a panel of Tgs of diverse species. Later, Champion et al (1991), using a spectrum of synthetic T4-containing truncated peptides (corresponding to four hormonogenic domains of Tg) and the above Tg-specific T cell hybridomas, identified a nonameric

sequence (2551-2559) containing thyroxine (T4) at position 2553. Moreover, an absolute requirement for T4 at position 2553 was demonstrated for its recognition: replacement of thyroxine with its precursor residue Tyr (or any other a.a.) produced a non-stimulatory sequence. Hutchings et al (1992) demonstrated the relevance of this epitope to pathogenic thyroid autoimmunity by adoptive transfer experiments in which peptide-primed (in vivo) and peptide-boosted (in vitro) LNC adoptively transferred severe thyroiditis to naive syngeneic mice. These elegant studies, apart from identifying an immunodominant and pathogenic Tg sequence, directly demonstrated that the iodination of Tg influences its immunogenicity and also confirmed the results shown previously in OS chickens (Bagchi et al, 1985a) and BB/W rats (Allen et al, 1986).

Subsequently, Kotani et al (1992), following a traditional biochemical approach, identified an immunodominant 15mer T cell epitope (774-788) within the pTPO (926 a.a.) molecule that can readily induce specific LNC proliferative and Ab responses and causes thyroiditis in C57BL/6 ($H\text{-}2^b$) mice. Although this 15mer TPO peptide is pathogenic, it does not contain characteristic features of T cell epitopes such as amphipathic α-helical structure (Margalit et al, 1987) and/or the tetramer motif (Rothbard and Taylor, 1988). Thus, if one uses the above computerized algorithms, this immunopathogenic

TPO sequence would not have been identified. Subsequently, Texier et al (1992), using a Tg-specific murine cytotoxic T cell (CTL) hybridoma identified a 40mer (1672-1711) (4.5 kDa) immunopathogenic peptide within the hTg molecule. However, further truncation of the peptide was not attempted to localize the minimal T cell epitope within this 40mer peptide. More recently, Hoshioka et al (1993) demonstrated the pathogenic potential of a common T cell epitope of hTg (2730-2743) and hTPO (118-131), that was predicted earlier by McLachlan and Rapoport (1989), by its ability to stimulate mTg-primed LNC that can transfer thyroiditis in naive CBA mice. In conclusion, the Tg or TPO epitopes that are thyroiditogenic are immunodominant and have been examined in heterologous species. However, the role of these T cell epitopes in a homologous species remains unexplored. In addition, the Tg epitopes recognized by T cells from thyroid tissue of patients suffering from human AITD have not yet been identified.

1.4.1.4.2 AITD studies in human

Fisfalen et al (1988) described hTPO-reactive T cell lines with low stimulation index (S.I). They could not exclude the possibility of T cell reactivity to Tg because a pure TPO preparation was not used. Using recombinant hTPO, hTPO-specific T cell clones from the thyroid of a GD patient

were established by Dayan et al (1991). This study utlized an antigen-independent T-cell cloning technique that involves the stimulation of T cells with anti-CD3 MAb and IL-2 in the presence of syngeneic PBMCs as APC. In addition, some of these T cell clones recognized an unknown thyroid Ag and one clone recognized Tg. Further epitope analysis revealed that the TPO-reactive T cell clones recognized two distinct epitopes (535-551 and 632-645) in the context of DP2 or DQ6 and DQ2, respectively. A third TPO epitope has not yet been localized. These findings demonstrated a marked heterogeneity of autoantigen recognition by T cells at the active site of disease with respect to number of epitopes on a single protein involved and the number of HLA restriction elements used. The TPO peptide (535-551)-specific T cell clones derived from the diseased thyroid were found to be resistant to tolerance induction introduced by supraoptimal concentrations of the peptide or superantigen (SAg) (Staphylococcus enterotoxin D) *in vitro* in the absence of APC. This further indicates a role for thyroid Ags in driving T cell autoreactivity *in vivo* (Dayan et al, 1993).

In conclusion, the advent of T cell cloning techniques and the availability of recombinant thyroid Ags have dramatically increased the progress in the analysis of epitopes within thyroid Ags (in particular TPO) important in GD. However, similar progress has not yet been made with

respect to the generation of T cell clones from thyroid gland and their epitope specificities in HT patients.

1.5 Antigen-presentation in thyroid autoimmunity

Various types of APC, such as dendritic cells and B cells, have been shown to play a crucial role in the induction and/or maintenance of thyroid autoimmunity (Knight et al, 1988; Hutchings et al, 1987). Transfer of quite small numbers (10^5) of dendritic cells from the spleens of mice with EAT has been shown to induce both thyroiditis and Tg-specific Abs in more than 50% of the recipients. Similarly, dendritic cells pulsed exogenously with Tg and adoptively transferred, could also induce thyroiditis in recipient mice (Knight et al, 1988). Tg-primed B cells from mice have been shown to present Tg at very low concentrations to specific T cell hybridomas suggesting that specific autoreactive B cells may play a role in maintenance of T cell autoreactivity (Hutchings et al, 1987).

The observation that MHC class II Ags were expressed on thyrocytes in patients with AITD led to the hypothesis that these cells could function as APCs, capable of triggering autoreactive T cells by presentation of T cell epitopes of endogenous thyroid Ags and contributing perhaps to the initiation or propagation of thyroid destruction (Botazzo et al, 1983). Demonstration of the activation of T cell lines

and clones isolated from thyroids of HT and GD patients by class II$^+$ thyrocytes (Londei et al, 1985; Dayan et al, 1991) provided support for this hypothesis. However, the role of class II$^+$ thyrocytes as APC remains controversial because the primary thyroid cell cultures used to activate T cells were contaminated with APC such as dendritic cells and it has been suggested that these dendritic cells either assisted thyrocytes or presented autoantigens by themselves leading to the activation of specific T cells.

1.5.1 Factors that induce or modify MHC class II antigen expression on thyrocytes

Extensive studies in thyroid autoimmunity have been aimed at delineating the factors that can induce or modify class II expression on thyrocytes. A characteristic feature of class II expression on thyrocytes is its induction by numerous factors that are described below. Pujol-Borrell et al (1983) demonstrated the induction of class II expression on thyrocytes after the addition of phytohemagglutinin (PHA) to thyrocyte culture. IFN-γ was shown to be the most potent class II inducer described to date (Todd et al, 1985), and incubation of human thyrocytes <u>in vitro</u> with IFN-γ has been shown to result in the hierarchial expression of class II (DR > DP >> DQ), a finding similar to the one that was observed in thyroids of patients afflicted with AITD (Todd et al, 1987b).

Various other agents have also been used as inducers of class II Ags on murine or human thyrocytes such as leukoagglutinin (Iwatani et al, 1986), culture supernatants from syngeneic (Salamero et al, 1985b) or allogeneic stimulated T cells (Iwatani et al, 1986), thyrotropin (Todd et al, 1987a) and dibutyryl cyclic AMP (Todd et al, 1987a), tumor necrosis factor (TNF) (Todd et al 1987c), and Abs to TSH receptor (Bodolay et al, 1987; Ropars et al, 1994). TSH and TSAb (thyroid stimulating Ab) can also enhance IFN-γ-induced class II expression (Ropars et al, 1994). Methimazole at very low concentrations caused inhibition of IFN-γ induced HLA-DR expression on thyrocytes and that this inhibition could be abrogated by the addition of T3 and T4 suggesting that methimazole interferes with the synthesis of T3 and T4 (Atta et al, 1995).

MHC class II Ag expression, therefore, seems to be a result of T cell infiltration and IFN-γ release, but other factors may modify this. TNF-α is produced by the mononuclear cell infiltrate in autoimmune thyroiditis and it may synergize with IFN-γ and increase class II Ag expression on thyrocytes, although it does not induce class II Ag expression by itself. Both cytokines individually increase constitutive class I Ag expression (Weetman and Rees, 1988; Bucasema et al, 1989). In contrast to TNF-α, epidermal growth factor (EGF), which is structurally related to TNF-α, stimulates thyroid growth in

culture and suppresses TSH-stimulated processes, causes a 50% decrease in class II Ag expression in TEC cultured with IFN-γ in the presence or absence of TSH. Transforming growth factor-α (TGF-α) has also been shown to partially suppress IFN-γ-induced class II Ag expression (Todd et al, 1989). Recently, Takorabet et al (1995) demonstrated induction of class II Ags on human and murine thyrocytes upon treatment with a neuroleptic and anti-depressant drug, alimemazine. Previous studies using indirect immunofluorescence, have also shown that reoviruses type-1 and type-2 can enhance the class II Ag expression on mouse (Gaulton et al, 1989) and rat (Neufeld et al, 1989) thyrocytes indicating a direct role for these viruses in the modulation of class II Ag on thyrocytes. The viruses were less effective in inducing class II Ag on human thyrocytes than on murine thyrocytes (Atta et al, 1995). The reoviruses can also hyperinduce class I Ag expression on human thyrocytes. This class I expression was shown to be dependent on the cytokines (IFN-α and IFN-ß) released after the infection of thyrocytes. In conclusison, these various investigations suggest that expression and modulation of MHC class II and class I on thyrocytes may aid in amplification rather than initiation of autoreactivity.

1.5.2 Thyrocytes as APC

The concept that aberrant MHC class II Ag expression on thyrocytes is the initiating event in autoimmune thyroiditis has been a subject of various investigations and controversial results. The following reports do not support the concept that class II Ag expression on thyrocytes initiate thyroid autoimmunity but rather that class II expression on thyrocytes is a secondary event and is dependent on thyroid-infiltrating T cells and the cytokines they secrete. First, in kinetic studies of EAT in rats, it was demonstrated that the appearance of the lymphocytic infiltrate in thyroids always precedes class II Ag expression on thyrocytes (Cohen et al, 1988a). Immuno-histochemical analysis has revealed that the distribution of class II^+ thyrocytes in autoimmune thyroid disease is strongly correlated with the presence of nearby IFN-γ containing T cells (Margolick et al, 1988; Hamilton et al, 1991). MHC class II Ag expression, therefore, seems to be a result of T cell infiltration and IFN-γ release, but other factors may modify this (see **Section 1.5.1**).

It has been shown that IFN-γ treated (class II^+) mouse and rat thyrocytes failed to present a variety of Ags including Tg to appropriate T cells, and this was not improved by the addition of IL-1 or phorbol esters (Ebner et al, 1987; Minami et al, 1987). The class II^+ mouse TEC line (M5) also failed to activate T cells but this cell line readily does so

upon treatment with phorbol ester but not upon exogenous addition of IL-1 (Stein and Stadecker, 1987). However, the same M5 line acquires allostimulatory function after a two-step procedure of irradiation and class II induction, suggesting that irradiation of thyrocytes induces the expression of costimulatory molecules (Czirjak et al, 1990).

The abnormal expression of class I and class II Ags on target cells per se may not be sufficient to induce T lymphocyte activation, and other signals or factors may be required to initiate an autoimmune attack on a target cell. Thyrocytes have been shown to express ICAM-1, which is responsible for enhanced T cell binding to thyrocytes (Weetman et al, 1989b). However, two other groups failed to detect ICAM-1 expression on thyrocytes from GD patients (Bagnasco et al, 1991), thus the role of ICAM-1 in activation of thyroid Ag-specific T cells remains undefined. The expression of B7, a major costimulatory signal in T cell activation, on thyrocytes, even after their incubation with IFN-γ, has not been demonstrated (Tandon et al, 1994). However, phorbol ester treated thyrocytes from patients with AITD have been shown to trigger T cell alloreactivity (Tandon et al, 1992a). Overall, the findings presented in this section suggest that thyrocytes by themselves can activate paticular subset of T cells and in association with other APC may activate another subset of T cells in the target organ.

1.6 Etiology of autoimmune thyroid disease

It has been suggested that both genetic and non-genetic factors contribute to the etiopathogenesis of AITD in humans.

1.6.1 Genetic factors

1.6.1.1 Major histocompatibility complex (MHC)

The MHC products in humans, encoded by genes within human leukocyte antigen (HLA) complex, have been shown to control in part the immune responsiveness to a variety of Ags. In many autoimmune diseases, associations have been reported with certain MHC alleles encoded by either class I (HLA-A, -B, and -C) or class II (HLA-DR, -DP, and -DQ). Most analyses of HLA in thyroiditis to date have used serologically defined specificities in which a single DR or DQ serological specificity represents a broad public specificity, present on multiple DRB1 or DQB1 genes. Using serological typing, an association of HLA-DR5 with goitrous HT has been demonstrated (Farid et al, 1981). HT was associated with DR4 or DR5 in one study from Newfoundland (Farid et al, 1981; Thomson and Farid, 1985), with DR5 in patients from Toronto (Vargas et al, 1988) and with DR3 in Hungarian patients (Thomsen et al, 1983). Using the RFLP method of DR typing, an association between DR3 and HT was demononstrated in British patients (Tandon et al, 1991a). Although associations appear to be strongest with DR, other HLA-D genes such as DQ and DP, might increase the

susceptibility of individuals to HT. Thus, while it appears that genetic variation in the HLA-D region confers susceptibility to AITD, it is unclear which gene(s) carries the primary genetic predisposition. Using allele-specific oligonucleotide probes, an association between DQ7 (DQB1*0301) and HT was demonstrated in a population from Toronto and London (Badenhoop et al, 1990). In an RFLP study, using a Caucasian population in Chicago, it was demonstrated that HLA-DR3 in association with HLA-DQ2 was significantly increased in GD patients. In contrast, another study showed no association of HLA-DR or DQ with HT (Mangklabruks et al, 1991).

Transracial studies have been useful in determining susceptibility genes in some autoimmune disorders but have not yet been conducted in sufficient detail to clarify the HLA associations in autoimmune thyroiditis. HLA-Bw46 and DR9 are increased in Chinese HT patients (Hawkins et al, 1987; Wang et al, 1988), whereas B16 is increased and DR2 is decreased in Japanese HT patients (Nakao et al, 1978; Sakurami et al, 1982). In a more recent report, an association with HT was reported for HLA-DR53 (encoded by the DRB4 gene) which is present only on a restricted number of haplotypes (DR4, 7 and 9). The association of HT with DR9 being the weakest (Honda et al, 1989).

An increase in DQA1*0501 has been reported in HT and GD patients (Badenhoop et al, 1995). In this study, the alleles

were defined by hybridization with sequence-specific oligonucleotides (SSO) and single strand conformational polymorphism (SSCP). DQA1*0501 is found on DR3- and DR-5 positive haplotypes. DR5 is associated with HT (Farid and Balazs, 1988) and GD (Farid and Stenszky, 1988) patients. Both DR3 (associated with GD) and DR5 (associated with HT) are increased in family members with AITD, although no formal linkage has been demonstrated (Roman et al, 1992). Yanagawa et al (1993) recently described a positive association of GD with DQA1*0501 which is in linkage disequilibrium with DQB1*0201 (DR52 = Dw25) in Caucasian DR3 haplotypes. These findings suggested a role for DQ genes in GD susceptibility. In HT-affected patients, both DR4 and DR5 are in positive linkage disequilibrium with DQB1*0301, but not with DQB1*0302 (DQ8). This is compatible with a role for DQ genes (or closely linked genes) in susceptibility to HT, as previously suggested (Jenkins et al, 1992; Shi et al, 1992; Tandon et al, 1991a; Badenhoop et al, 1990). Interestingly, it has been shown in family studies that the risk for AITD in IDDM patients was conferred by the presence of DQB1*0201 (DQ2). Among the subjects with DQB1*0201, there was a weak negative association between the presence of DQB1*0302 on the second haplotype and HT. On the other hand, this allele favoured GD development (Santamaria et al, 1994). The findings in this study suggested that in families, certain alleles or

haplotypes in some IDDM patients may produce a risk for developing AITD, whereas other IDDM patients may lack that susceptibility. Thus, in families with IDDM and AITD, the HLA polymorphism modulates the susceptibility to each disease in a complex fashion, with each haplotype playing a complementary role. Interestingly, Badenhoop et al (1995) have shown that IDDM-affected subjects are at risk for AITD, especially those carrying DQA1*0501 and arginine at position at 52 of DQA1 alleles, and protection against IDDM and GD is conferred by DQB1*0602 (DQ6).

1.6.2 Non-genetic factors

These may be endogenous (age and sex hormones) or exogenous (chemicals, novel immunotherapeutic agents or drugs, iodine intake, and infection).

1.6.2.1 Age

It has been shown that the incidence of AITD clearly increases with age probably due to chronic exposure to environmental factors and to possible changes in immunoregulation. Phillips et al (1993) showed a relationship between fetal growth and the later (adult) appearance of anti-thyroid Abs. The proportion of women with elevated anti-Tg and anti-TPO Ab titers correlates with their lower birth weight. The prevalence of these Abs rose with increasing

adult body mass particularly in those individuals who did not have a central pattern of fat deposition. These effects were modest and unrelated to clinically overt HT. These results suggest that early nutritional factors and possibly hormonal consequences of body composition may influence susceptibility. In this regard, in mouse EAT, it has been shown that old mice (20 to 30 months) have an increased susceptibility to Tg-induced EAT with a concomitant decrease in the titer of Tg Abs (Romball and Weigle, 1987).

1.6.2.2 Sex hormones

There is a strong female to male predominance (5-20 : 1) for AITD in the adult population and a peak incidence between 30 and 50 years of age. This may be an effect of sex hormones. Several reports have documented the influence of sex hormones on the development of AITD: Hyperprolactemia has been shown to increase the risk of individuals of developing thyroid Abs (Ferrari et al, 1983). It has also been shown that women with postpartum thyroiditis go on to develop permanent hypothyroidism (Oathman et al, 1990). However, whether postpartum thyroiditis serves as a permissive factor for development of HT is unknown. The role of sex hormones has been verified in a rat model of SAT (Ahmed and Penhale, 1982). These authors demonstrated that prepubertal orchidectomy potentiated the development of (Tx-X)-induced

EAT in male PVG/c strain rats (this strain is normally resistant to EAT development if prepubertal orchidectomy is not performed). The authors also showed that administration of testosterone to these rats completely suppressed the development of thyroiditis as well as anti-Tg-Ab titers. Using thyroid grafting and extirpation techniques, Ahmed and Penhale (1986) have demonstrated the therapeutic potential of testosterone by reversing the established thyroiditis in (Tx-X)-PVG/c rats without altering the Tg Ab titers.

1.6.2.3 Iodine

Adverse effects of dietary iodine on the development of AITD has been a topic of several investigations both in humans and in various experimental animal models. Early studies in humans indicated a link between iodine intake and development of AITD (Beirwalters, 1969). In this study, the authors demonstrated that keeping hypothyroid-patients on an iodine-restricted diet has an ameliorative effect on the disease state with patients becoming euthyroid. When iodine was reintroduced, several of these patients relapsed to a hypothyroid state.

In the rat EAT model, it has been shown that excess dietary iodine enhances the severity or incidence of disease in genetically predisposed BB and BUF rat strains, whereas low iodine intake results in amelioration (Allen et al, 1986;

Cohen and Weetman, 1988b). This effect of iodine seems to be related to the iodine content of Tg (de Paiva-Assis et al, 1988; Ebner et al, 1992). High dietary iodine intake has also been shown to exacerbate thyroiditis in the OS chicken and also seems to be due in part to the iodination state of Tg (Bagchi et al, 1985a; Sundick et al, 1987). These results contrast with the findings reported by Allen et al (1986) in which, it has been shown that high dietary iodine intake did not result in hypothyroidism and the rats remained euthyroid. The findings of Allen et al (1986) were confirmed by the studies of Voorby et al (1990) in which the authors showed that a similar regimen induced only minor signs of thyrocyte destruction. Though the initial experiments suggested that iodine was not essential for the development of thyroiditis, subsequent experiments involving radical iodine depletion, begining in ovo, revealed that uptake or metabolism of iodine, in fact, is essential for the development of SAT (Brown et al, 1991).

It is believed that iodination of Tg results in the generation of neoantigenic epitopes. In that regard, an iodinated but not non-iodinated synthetic Tg peptide (2546-2571) has been shown to induce thyroiditis (Hoshioka et al, 1994). Interestingly, both forms of the Tg peptide readily induced T cell responses, but only iodinated Tg peptide induced Ab responses. Thus synthesis of mono- and di-

iodotyrosine residues, is necessary for the induction of thyroiditis and Ab responses. These findings are in contrast to the report of Hutchings et al (1992) in which the authors demonstrated that only T4 containing peptides induce pathogenic T cell responses. In addition to altering the immunogenicity of Tg, iodine has been shown to have additional effects on thyroiditis, e.g., by interacting with oxygen radicals to produce highly reactive oxidized iodine forms capable of tissue injury (Bagchi et al, 1990).

1.6.2.4 Infection and inflammation

1.6.2.4.1 Mechanisms of infection-mediated autoimmune disease

As in the majority of autoimmune diseases, viruses have also been implicated in the development of autoimmune thyroiditis (Tomer and Davies, 1993), but no clear correlation has been found in human thyroid disease. Such viruses could affect either the immune system or the target organ. It has been demonstrated that viruses can induce autoimmunity by a process called "molecular mimicry" as a result of structural or primary a.a. sequence similarities between autoantigens and viruses (Oldstone, 1987). However, to date, no sequence similarities have been reported between Tg, TPO and infectious agents that are associated with AITD.

Infection can induce autoimmunity through the induction of MHC class II expression on target cells and subsequent

presentation of T cell epitopes from autoantigens. The regulation of MHC gene products by viruses that infect thyroid tissue (directly or indirectly) represents a significant immunological phenomenon because of the central role of the MHC in Ag-presentation. The increased expression of MHC class II on thyrocytes by IFN-γ released by T cells (Bottazzo et al, 1983) may aid in the presentation of novel (cryptic or non-dominant) peptides above the critical threshold level required for recognition by T cells (Sercarz et al, 1993). In addition, the proteases released during the inflammatory process may also alter Ag processing and presentation (Opdenakker and Van Damme, 1994). It is possible that cytokines (IFN-γ, TNF-α, IL-1, and IL-8) released during the inflammatory response induced by infection can augment the expression of accessory and/or costimulatory molecules such as ICAM-1 and B7, thereby enhancing the presentation of thyroid Ags. This upregulation of adhesion molecules may lead to increased leukocyte adhesion, release of nitric oxide and oxygen radicals, thereby resulting in tissue destruction, and exposure of intracellular proteins.

The inflammatory process can also increase the expression of heat shock proteins (hsp) which can alter the presentation of self-antigens by modulating the transport and processing of intracellular peptides (Michalek et al, 1992). Thus, during infection abnormal processing and presentation of autoantigens

can take place leading to the induction or propagation of autoaggression.

1.6.2.4.2 Association of viral infections with AITD in humans

The role of infectious agents in the pathogenesis of AITD remains purely hypothetical and has been reviewed recently by Tomer and Davies (1993). Different viruses such as measles, influenza, adeno, epstein-barr, and coxsackie have been reported to be associated with subacute thyroiditis (ST) (Volpe, 1979). The presence of Abs to coxackie, adeno, influenza, and mumps viruses was demonstrated in ST patients, although these patients had no specific viral disease. The presence of anti-TPO and anti-Tg Abs were also detected in 42-64% of ST patients (Volpe et al, 1967). In contrast, Weetman et al (1987) demonstrated in ST patients the persistent levels of Abs that are polyclonal in nature, to several uncharacterized thyroid antigenic determinants. These Abs are believed to be secondary to the thyroid damage (endogenous priming) caused by viral infection. Apart from Abs to thyroid Ags, transitory presence of autoreactive T cells to thyroid Ags was demonstrated (Wall et al, 1976; Totterman et al, 1978). These findings were attributed to the inflammatory release of Ag rather than to specific autoimmune disease induced through molecular mimicry induced by viral infection

(Volpe, 1978).

An association between congenital rubella virus infection and thyroid dysfunction has been demonstrated. The mechanisms responsible for thyroid dysfunction in these patients are poorly understood. Anti-TPO or anti-Tg Abs were found in 34% of the patients (Ginsberg-Fellner et al, 1984). A recent bacterial or viral infection has been demonstrated in HT patients by Tonooka et al (1978). It was suggested that retroviral infection may be a triggering factor leading to the development of thyroid autoimmunity (Josephson et al, 1989). Using western blotting, these authors demonstrated the presence of Abs to non-HIV retroviruses that cross-reacted with HIV proteins. However, restricted antigenic responses by T lymphocytes from HT patients led to the suggestion that these autoreactive T cells might have been triggered by a specific Ag stimulus such as infection (Katzin et al, 1989). Ciampolillo et al (1989) reported the presence of HIV-related genomes in thyrocytes from patients with GD. More interestingly, a homology between a 161-base pair region of the gene encoding TSHR to the gene encoding the HIV regulatory protein Nef has been documented (Burch et al, 1991). Rabbit Abs to a peptide representing part of this TSHR sequence showed cross-reactivity with the recombinant Nef protein; sera from patients with GD also appear to recognize Nef weakly (Burch et al, 1991). These findings have sparked speculation

regarding retroviral involvement in the pathogenesis of GD. In a recent study, a higher prevalence of HT in patients with human T-lymphotropic virus type 1 (HTLV-1) infection than in the general population was demonstrated (Kawai et al, 1992). Thus, it is tempting to speculate that the HTLV-1 virus infects thyrocytes and elicits autoreactivity leading to HT. Isolation of either infecting organism from thyroid tissue or isolation of T cells to viral mimics of thyroid Ags have not been made but such studies will clarify the role of viral infections in the etiopathogenesis of HT. In one study, it was demonstrated that 20% of patients (women) with chronic hepatitis C infection had high titers of thyroid Abs and some of the patients developed hypothyroidism and HT (Tran et al, 1993). However, the cause and effect relationship between the viral infection and HT is not understood.

A large proportion of patients with GD and autoimmune thyroiditis have Abs to Yersinia enterocolitica (Shenkman and Bottone, 1976). Further, Weiss et al (1983) demonstrated a saturable binding site for TSH on Yersinia enterocolitica and such binding was shown to be inhibited by Abs present in patients with GD (Heyma et al, 1986). Murine Abs to extracellular TSHR react with Yersinia enterocolitica and vice-versa (Luo et al, 1993) and these authors have identified two low m. w (5.5 kDa and 8kDa) envelope proteins of Yersinia that are cross-reactive with TSHR (Luo et al, 1994).

1.6.2.4.3 Animal models to study the role of infection in thyroid disease

Direct evidence for viral involvement in the etiology of AITD comes from animal models in various species such as rats, mice, and chicken. Penhale et al (1988) have demonstrated that the composition of the gut microbial flora influences the susceptibility of female PVG/c strain rats to thyroiditis induced by Tx-X. Rats maintained under specific pathogen-free conditions until weaning were found to be significantly less susceptible to induction of thyroiditis than conventionally reared rats of the same strain. It is unclear at present how intestinal bacterial flora influence the susceptibility of rats to thyroiditis but a role for antigenic cross-reactivity with thyroid tissue has been suggested. Differences in the susceptibility to thyroiditis of BUF rats from different sources or from different colonies have been reported and have been attributed to environmental changes, including possibly viral infection (Silverman and Rose, 1971; Cohen and Weetman, 1987).

Klavinskis et al (1988) have demonstrated that in the absence of apparent thyroid cell destruction, there is a drop in the circulating levels of thyroid hormone following a neonatal viral infection of mice with lymphocytic choriomeningitis virus (LCMV). The virus has been shown to persist mainly in the thyrocytes, but signs of either necrosis

or inflammation were not demonstrated. In another study, Srinivasappa et al (1988) demonstrated that mice infected with reovirus develop thyroiditis characterized by lymphocytic infiltration, and anti-Tg and anti-TPO Abs, in the absence of alteration in thyroid function. Onodera and Awaya (1990) have shown that a polypeptide encoded by the S1 segment of reovirus type-1 but not type-3 was responsible for induction of anti-Tg and anti-TPO Abs.

Ziemiecki et al (1988) by employing southern blotting technique, demonstrated the presence of an endogenous retrovirus (ev 22) in OS chicken with SAT but not in healthy normal inbred strains. Ev 22 therefore was considered a very likely causative agent in SAT. Elaborate classical genetic studies on F1 hybrids of OSB15 and the higly inbred normal CB (B12) strain, F12 and backcross generations showed that the only correlation with the presence of the ev 22 locus was found with altered immunoendocrine feedback regulation, but not the absolute degree of SAT or the frequency and titer of anti-Tg Abs (Kroemer et al, 1988). Therefore, ev 22 has only a modulatory, effect, if any, on the course of the SAT pathogenesis. In contrast, a report by Carter and Smith (1983) implicated retroviral infection in the development of thyroiditis in chickens. These authors have demonstrated that inoculation of 10-day old chicken embryos with avian leukosis virus (ALV) strain RAV7 resulted in hypothyroidism within 3

weeks of hatching. Histological analysis of thyroids from these birds revealed an extensive lymphocytic infiltration. The hypothyroidism was manifested by marked stunting of growth and hyperlipidemia.

1.6.2.5 Drugs and chemicals

1.6.2.5.1 Amiodarone

Other exogenous factors that are associated with thyroid autoimmunity includes drugs and chemicals. It has been shown that ingestion of amiodarone (an iodinated derivative containing two iodine atoms/molecule of drug), an antiarrythmic and antianginal drug, by patients suffering from heart failure resulted in either hyper- or hypothyroidism with Abs to Tg, TPO and occasionally to TSHR (Singh, 1983). Similarly, an increase in thyroid Abs was reported after amiodarone treatment in people on low-iodine intake in UK (Monteiro et al, 1986). It is believed that the iodine released from amiodarone (a 200 mg oral dose generates 6 mg of free iodine/day) is partly responsible for the precipitation of the autoimmune response. Recently, Takorabet et al (1995) have demonstrated that drugs can modulate the autoimmune response by their effect on target cells. The authors in that study have shown that culturing thyrocytes in the presence of alimemazine, a neuroleptic and anti-depressant drug, results in the induction of MHC class II Ag on thyrocytes and these

class II^+ murine thyrocytes triggered an mTg-specific LNC proliferative T cell response. However, the significance of the proliferative T cell response to the induction of thyroiditis was not demonstrated.

1.6.2.5.2 Lithium

Development of both thyroiditis and thyrotoxicosis has been associated with lithium therapy (Calabrese et al, 1985). In this stduy, it has been demonstrated that some patients on lithium therapy exhibited baseline thyroid Abs or thyroid disease, whereas those patients with pre-existing thyroid disease all developed an increase in the autoimmune response to the thyroid Ags as seen by increased Ab titer or overt hypothyroidism. The mechanisms by which lithium mediates its effect are unknown, but it is believed that lithium affects immuno-regulatory mechanisms by interfering with the function of Ts cells (Shenkman et al, 1981).

1.6.2.5.3 Other chemicals

Various exogenous factors have been shown to modify the expression of SAT in rats. Trypan blue, and anthracene derivatives have been shown to enhance the low incidence of SAT in BUF rats, thereby producing an extensive dendritic cell and macrophage infiltration within the thyroid gland as well as the production of anti-Tg Abs (Bernard et al, 1992).

1.6.2.6 Cytokines

Several reports in humans and experimental animals have indicated that an iatrogenic cause of AITD is the long term administration of various cytokines. It was demonstrated earlier that long term treatment with IFN-α of patients suffering from breast cancer (Fentiman et al, 1985; Burman et al 1986) or from myelo-proliferative and myeloplastic syndromes (Gisslinger et al, 1992) resulted in development of thyroid dysfunction and clinical hypothyroidism. In some of these patients, thyroid dysfunction was suggested to be due to contamination of the leucocyte-derived IFN-α preparation with IFN-γ (Burman et al, 1986). However, this speculation seems unlikely because similar effects were observed when patients were treated with recombinant IFN-α (Gisslinger et al, 1992). Interestingly, IFN-γ given to patients afflicted with a malignant disease or chronic active hepatitis, who had no thyroid-specific Abs prior to treatment, did not result in thyroid dysfunction and autoimmunity (Kung et al, 1990).

It has also been shown that administration of recombinant IL-2 and granulocyte macrophage-colony stimulating factor (GM-CSF) to cancer patients with pre-existing thyroid Abs precipitates thyroid dysfunction (Atkins et al, 1988; Van Liessum et al, 1989; Hoekman et al, 1991). However, the thyroid dysfunction induced by GM-CSF was reported to be reversible (Hoekman et al, 1991). In some of these studies,

the results suggest that thyroid dysfunction is mediated by the combination of both IL-2 and LAK cells with no effect by either of these alone (Atkins et al, 1988). Pichert et al (1990) also showed that when cancer patients were treated with IL-2 and INF-α, the patients developed transient mononuclear infiltration of the thyroid, hypothyroidism and an increase in class II expression on thyrocytes.

In contrast to the iatrogenic effects of various cytokines on thyroid function in cancer patients, a pathogenic effect of IFN-γ has been demonstrated in mice. Injection of recombinant IFN-γ into the thyroids of CBA mice has been shown to induce thyroiditis and anti-Tg Abs (Remy et al, 1987). These results were further supported by transplantation studies in which grafting under the kidney capsule of *in vitro* IFN-γ treated but not control thyroids into syngeneic mice resulted in mononuclear infiltration of the grafted thyroid (Frohman et al, 1991). Thus, these results suggest that local concentrations of IFN-γ compared to its circulating concentration may have different effects.

Injection of a high dose of IL-1ß (8.0μg/ml/kg/d) in BB rats increased the severity of insulitis and lymphocytic thyroiditis (Vertrees et al, 1991). Thus, administration of large dose of cytokines exacerbates preexisting thyroiditis. Studies to demonstrate the immune mechanisms by which these cytokines mediate their effects were not done.

1.7 Pathogenic mechanisms in AITD

The precise cellular and humoral mechanisms leading to the manifestation of human thyroid disease are not completely understood (Weetman, 1992) although an autoimmune pathogenesis of thyroid disease is supported by (1) the production of AAbs to thyroid Ags, (2) the presence of lymphocytic infiltration in the thyroid, and (3) the association of the disease with HLA class II gene products. A diversity of mechanisms contributing to the tissue injury in thyroid disease in human and experimental animals have been shown and will be discussed in this section.

1.7.1 T cell-mediated injury

Infiltration of the thyroid gland by lymphocytes is a hall-mark of AITD; it is particularly evident in HT but it is also seen in most of the GD patients (Charreire, 1989). It has been suggested that both $CD4^+$ Th cells and $CD8^+$ CTLs have a pathogenic role and that these cells may destroy the target thyrocytes through DTH and cytotoxicity mechanisms, respectively. No definitive evidence of T lymphocyte-mediated thyroid damage in HT is available to date. Attempts have been made to establish CTL clones from HT thyroids using IL-2, but these experiments led to the generation of $CD8^+$ T cells that exhibited non-specific cytotoxic functions (Canonica et al, 1985; MacKenzie et al, 1987a; del Prete et al, 1986; del Prete

et al, 1987). These cytotoxic $CD8^+$ T cells were not specific to Tg or other thyroid Ags, and exhibited natural killer (NK) activity (del Prete et al, 1986) or lectin-dependent cytotoxicity (Bagnasco et al, 1987). Their role in the pathogenesis of thyroid disease remains unknown. Only one thyroid-specific T cell clone, derived from an HT patient, has so far been described; the nature of the autoantigen recognized by the T cell clone has not been elucidated (MacKenzie et al, 1987b). Recently, Wu et al (1994) using immunofluorescence staining demonstrated that HT thyroids were infiltrated with perforin containing $CD4^+$ and $CD8^+$ T cells. These findings raise the possibility for a perforin-mediated cytotoxic mechanism in the pathogenesis of HT.

The strongest arguments favouring $CD4^+$ T cells playing a role in thyroiditis induction have come from adoptive transfer studies in EAT, in which disease has been transferred with $CD4^+$ T cell lines and clones (Maron et al, 1983; Romball and Weigle, 1987; Sugihara et al, 1993). The precise mechanisms by which these T cells mediate thyroid pathology remain unknown. Recently, biased TCR Vß gene usage has been reported in the intrathyroidal infiltrate of hTg-immunized CBA/mice (Matsuoka et al, 1993). Of the 17 TCR Vß gene families examined by PCR, 6 families (Vß 1, 2, 4, 8, 13 and 16) were utilized by the hTg-specific T cell lines. The relative importance of each of these TCR Vß bearing T cells was not

addressed by adoptive EAT (Matsuoka et al, 1994). However, it has been shown that TCR Vß8$^+$ T cells do not play a major role in Tg-induced EAT since depletion of Vß 8$^+$ T cells from mTg-primed LNC does not abrogate the adoptive transfer of thyroiditis in mice (Fuller et al, 1994).

The role of CTLs in thyroid destruction was first suggested by the H-2-restricted damage of thyrocytes (Creemers et al, 1983). In these studies, the protocols used were similar: after <u>in vivo</u> priming with mTg and <u>in vitro</u> boosting either with mTg or with TEC, respectively, stimulated T cells were deposited on labelled, syngeneic TEC targets. These CTLs (CD8$^+$) mediated the lysis of TEC targets and were shown to be specifically directed against Tg epitope(s) of the TEC cultures and to be class I-restricted. These data were further supported by the demonstration of cytotoxicity against labelled syngeneic but not allogeneic TEC with mTg-specific CD8$^+$ CTL hybridoma (Remy et al, 1989) and CTL clones (Sugihara et al, 1995).

T cells, by virtue of the cytokines they secrete, can mediate thyroid damage indirectly and thereby affect thyroid function. Direct injury of thyrocytes in a primary culture is difficult to prove, but, cytokines could alter functional responses thus contributing to the development of hypothyroidism. A large proportion of CD4$^+$ and CD8$^+$ T cell clones derived from thyroids of HT patients has been shown to

release IFN-γ *in vitro* (del Prete et al, 1987) and $CD4^+$ clones established *in vitro* from the thyroids of both GD and HT patients produce most or all of the following cytokines: IL-2, IFN-γ, IL-6, TGF-ß, TNF-α and lymphotoxin (Grubeck-Loebenstein et al, 1989) suggesting the lack of clear distinction of clones into Th1 and Th2 subsets. In contrast, Paschke et al (1993) failed to detect TNF-α in the thyroids of GD patients.

It has been shown that TNF-α is cytotoxic for rat thyrocytes *in vitro* and its effects are potentiated by pre-incubation with IFN-γ (Taverne et al, 1987), whereas IFN-γ exhibits inconsistent inhibitory effects on thyrocyte survival (McLachlan et al, 1990). However, IFN-γ (Nagayama et al, 1987) and IL-6 (Weetman et al, 1990a; Tominaga et al, 1991) have been shown to inhibit the T3 and Tg release in response to TSH. Wilson et al (1990) have shown the pathogenic potential of IL-1ß, as demonstrated by its ability to enhance the severity of thyroiditis in BB rats. If these findings are confirmed in HT, then macrophages may be important effector cells in thyroid damage as the major producers of IL-1 and TNF-α. Both IFN-γ and TNF-α will also modulate MHC class I and class II expression and contribute to the autoantigen presentation and T cell reactivity (**Section 1.5**). Moreover, the potential role of proinflammatory molecules released by macrophages, such as oxygen metabolites, prostaglandins, and leukotrienes, needs to be investigated.

1.7.2 Antibody-mediated tissue injury

The presence of Tg-specific Abs is a hallmark of EAT in animals and HT in humans. The role of these anti-Tg Abs in thyroid disease is not completely understood. Both TPO- and TSHR-specific Abs are also found in HT patients (Weetman and McGregor, 1994). It is likely that these Tg- and TPO-specific Abs may mediate complement-mediated tissue injury, and antibody-dependent cell-mediated cytotoxicity (ADCC), whereas TSHR-specific Abs may mediate their pathogenic effect by blocking the binding of TSH to TSHR.

1.7.2.1 Role of Tg-specific Abs

Tg-specific Abs may differ in affinity, ability to activate the complement system or to cooperate with effector cells in an ADCC system. Jones and Roitt (1961) have demonstrated, using a tanned red cell hemagglutination (HA) technique, the existence of low Tg-specific Ab titers in rats with severe thyroid lesions. Later, Lillehoj and Rose (1982) confirmed these findings and demonstrated that the titer of Tg Abs, as measured by the chromium chloride red cell HA technique, does not significantly correlate with the severity of thyroiditis. In contrast, a close correlation was shown between the titer of anti-Tg Abs and the severity of thyroid lesions in rats in which autoimmune thyroiditis was induced by Tx-X (Ahmed and Penhale, 1981). Using the same rat model of

thyroiditis, Kotani et al (1981) showed a dissociation between the titer of Tg Abs and the extent of thyroid lesions in backcrosses of F1 hybrids (of high- and low-responder parental strains) to either parental strain. Overall, these experiments suggest that Tg Abs may comprise both pathogenic and non-pathogenic Abs since Tg Ab titers correlated with severity of thyroid lesions in some but not in other experiments. Furthermore, the passive injection of high titer homologous Tg Abs did not produce thyroid lesions in rats (Rose et al, 1973a). A role for anti-Tg Abs was further excluded by adoptive transfer experiments in rats in which the transfer of rTg-primed LNC to naive syngeneic rats induced thyroiditis without concomitant appearance of anti-Tg Abs (Rose and Molotchnikoff, 1973b).

Similarly, in the mouse EAT model using high responder mouse strains, a lack of correlation between the titer of Tg Abs and severity of thyroid lesions has been demonstrated (Vladutiu and Rose, 1972; Esquivel et al, 1977). Interestingly, Vladutiu (1982) showed that cyclophosphamide treated low-responder C57BL/6 mice compared to untreated control mice had suppressed Tg Ab titers with a concomitant increase in the severity of thyroid lesions. In contrast to the serum transfer experiments in rats (Rose et al, 1973a), passive immunization of mice with anti-Tg antiserum (Vladutiu and Rose, 1971b; Clagget et al, 1974a; Tomazic et al, 1975)

induced thyroid lesions. These data indicate that this organ is accessible to anti-Tg Abs and autoantibodies to Tg play a role in the induction of thyroid lesions.

It has been shown in Tg-induced mouse EAT that 63% of anti-IgM treated (B-cell depleted) mice had moderate to severe thyroid lesions when compared to 88% of irrelevant Ab-treated control mice (Vladutiu, 1989). These B-cell depleted mice showed a reduction in Ab titers. These data suggested that Tg Abs are not necessary for the development of thyroid lesions and further raised doubts about the pathogenic role for Tg Abs. However, it should be noted that B cell depletion in these mice was not complete. In contrast, Rayfield et al (1989) has induced a full B-cell deficiency in two CBA mice by injecting anti-IgM serum from birth. These mice developed less severe thyroiditis without concomitant induction of Tg Abs after immunization with Tg. These results suggested that thyroid lesions can be induced in the absence of B cells. However, B cells may play a role in increasing the severity of the disease. Another possibility is that Tg Abs may cooperate with Tg-primed LNC and enhance/potentiate the severity of thyroiditis in rats and mice.

More recently, a slight reduction in the severity of lymphocytic EAT has been shown by adoptive injection either of B cell depleted mTg-primed LNC cultures or of mTg-primed LNC into mice that were pretreated with anti-I-A^k Abs. In

contrast, the same approach drastically reduced the severity and incidence of granulomatous EAT in recipient mice. In the same study, it has been shown that challenge of mice that were treated with anti-I-A^k Abs developed lowered Tg Ab titers and less severe granulomatous thyroid lesions. These data suggested that anti-Tg Abs having a unique function/specificity or thyroid-infiltrating B cells are required for the development of granulomatous but not lymphocytic EAT (Braley-Mullen et al, 1994b). In that regard, recently it has been shown that anti-Tg Abs from HT patients possess catalytic activity as assessed in SDS electrophoresis gels by their ability to lyse radiolabelled Tg into several smaller sized fragments. Thus, by virtue of catalytic activity, these Abs may affect the generation of thyroid hormones (Li et al, 1995).

1.7.2.2 Thyroid cytotoxic antibodies: Complement-mediated injury

One potential mechanism for thyroid cell damage is Ab-mediated complement activation. It is thought to be a particular property of TPO-specific Abs (Khoury et al, 1981).

In experimental animals: It should be noted that cytotoxic Tg Abs have not been demonstrated in AITD, and in fact, only cytotoxic thyroid Abs specific for TPO have been demonstrated (Pulvertaft et al, 1959). Thus, whether or not

complement plays a role in Tg-induced EAT is controversial. Nakamura and Weigle (1968) showed that complement deficient mice developed EAT after immunization with a mixture of heterologous Tg without adjuvant, suggesting that complement was not necessary for the development of EAT. In contrast, the absence of Tg-specific Abs in recipient rats that were injected with Tg-primed LNC has been demonstrated by adoptive transfer experiments (Rose et al, 1973b). However, the complement level in the recipient rats decreased 7 days post-transfer, but this decrease was not observed after transfer of albumin-primed LNC, suggesting that a complement-mediated cytotoxic mechanism may be invoved in thyroid destruction. Clagget et al (1974a) demonstrated a correlation of amount and temporal appearance of Tg AAbs with the occurrence of immune complexes at the basal area of thyrocytes in A/J mice that received a mixture of heterologous Tg without adjuvant. Histological examination of the thyroids revealed neutrophil and mononuclear cell infiltration. Recently, Inoue et al (1993) demonstrated the importance of late complement components in Tg-induced EAT in rabbits. Rabbits deficient in sixth complement component did not develop severe thyroiditis, whereas normocomplementic rabbits developed severe thyroiditis subsequent to Tg + CFA challenge.

In HT and GD patients: Several lines of evidence suggest a major role for complement-fixing Abs against TPO in

mediating thyrocyte damage. Anti-Tg Abs do not fix complement, probably due to widely spaced epitopes on the Ag which prevent IgG cross-linking (Adler et al, 1984). The complement fixing properties of TSAbs are unknown. The presence of immune complexes associated with complement deposition has been observed on the thyroid follicular basement membrane (Werner et al, 1972) and has been confirmed by Weetman et al (1989a). The latter authors also demonstrated elevated levels of serum terminal complement component (TCC) concentrations, a factor associated with disease activity. Chiovato et al (1993) using ^{51}Cr-release assay in which rabbit serum was used as a complement source, demonstrated that TPO is the target for thyroid-cytotoxic Abs and suggested the involvement of other thyroid Ags because preadsorption of HT patients' sera with TPO did not completely abrogate the cytotoxic activity.

It should be noted that, like most nucleated cells, thyrocytes are resistant to homologous complement-mediated lysis (Morgan, 1989). This was shown to be mediated in part due to the expression of CD59 and the membrane attack complex (MAC) inhibitory protein/homologous restriction factor (HRF) by thyrocytes which prevent the insertion of the final complement components into the cell membrane. Blocking CD59 greatly increased thyrocyte killing by complement <u>in vitro</u> (Tandon et al, 1992b). In contrast, the release of cytokines

such as IL-1, IFN-γ and TNF-α can increase the expression of CD59 and HRF in HT patients and, by preventing MAC formation, may render the thyrocytes resistant to complement-mediated cell lysis. However, nonlethal MAC formation (sublethal complement attack) has been shown to inhibit thyrocyte function, causing the suppression of the cAMP response to stimuli such as TSH and TSAb (Weetman et al, 1990b). This sublethal complement attack on thyrocytes causes them to release a range of proinflammatory molecules such as IL-1, IL-6, prostaglandins E2, and oxygen radicals. These may be important in causing injury to thyrocytes leading to further infiltration by and activation of lymphocytes. It is possible that scavenging of such oxygen radicals by antithyroid drugs and by antioxidants administered experimentally explains the amelioration of thyroiditis observed with these agents (Weetman et al, 1984b; Bagchi et al, 1990).

1.7.2.3 Antibody-dependent cell-mediated cytotoxicity (ADCC)

Abs may also participate in ADCC by binding to cell surface Ags, thereby allowing NK cells to kill target cells via Fc receptor interaction with the bound Ab. ADCC is measured by ^{51}Cr-release assay. There are conflicting results regarding the role of ADCC in the pathogenesis of AITD. Bogner et al (1984) demonstrated that normal lymphocytes,

incubated with thyrocytes and HT patient sera killed thyrocytes at least two times more effectively than control sera. The authors also have shown a strong positive correlation between the degree of lysis produced and the anti-TPO Ab titer and that anti-Tg Abs do not participate in ADCC. In contrast, subsequent studies from the same research group did not demonstrate a correlation between the degree of lysis and anti-TPO Ab titers suggesting that Abs specific for unknown thyroid Ags also contribute to ADCC in a high proportion of patients (Bogner et al, 1990). In one study, 39% of HT patients contained thyroid cytotoxic Abs in their sera (Bogner et al, 1995), whereas others have failed to detect ADCC using HT patient sera (Sacks et al, 1986). Thus, it is likely that differences in ADCC observed by different research groups might be attributed to variation in the degree of thyroid Ag expression by the thyrocytes used in the assays. Therefore, the exact role of the ADCC mechanism in the pathogenesis of HT is uncertain.

1.7.2.4 Thyroid stimulating blocking antibodies (TSBAbs) or Thyroid Stimulating Antibodies (TSAbs)

The third mechanism by which AAbs can participate in the thyroid disease pathogenesis is to directly modify cell function. Anti-TPO Abs have been shown to inhibit the biological activity of TPO by binding to a catalytic site

(Weetman et al, 1987). Blocking the TSH binding site on TSHR by TSBAb is another notable mechanism. The existence of these Abs in HT patients has been documented (Tamai et al, 1991). Anti-Gal, a natural Ab, may contribute to the pathogenesis of GD by its ability to bind α-galactosyl moities on the TSHR and to chronically stimulate thyrocytes from GD patients. These Abs cannot stimulate the thyrocytes from thyrotoxicosis patients or healthy individuals because the α-galacosyl moities are absent on TSHR in these individuals (Winand et al, 1994).

1.7.3 Thyrocytes and Cytokines

It is likely that thyrocytes, by their ability to secrete various inflammatory and proinflammatory cytokines, may either stimulate T- and/or B- cells or exacerbate the mononuclear cell infiltration of the thyroid. These cells may participate more actively in the pathogenesis of thyroid disease than previously thought. It has been demonstrated by *in situ* hybridization of mRNA that thyrocytes from HT and GD patients secrete IL-1α and IL-6, although the IL-1α synthesis by GD thyrocytes is not a constant feature (Miyazaki et al, 1989; Zheng et al, 1991). Cytokines such as IFN-γ and TNF-α have been shown to increase the IL-6 production *in vitro* (Kennedy and Jones, 1990). Thus, the local release of IL-6 by thyrocytes seems likely to stimulate both T and B cells.

Thyrocytes produce IL-8 and the production of IL-8 is stimulated by IL-1 and is inhibited by IFN-γ (Weetman et al, 1992). At low concentrations, IL-8 is a chemoattractant for lymphocytes, and its local release by thyrocytes may aid in exacerbating mononuclear cell infiltration of the thyroid.

1.7.4 Thyrocytes and Adhesion molecules

Lymphocytic infiltration of the thyroid gland in autoimmune thyroid disorders requires, as a first step, attachment of lymphocytes to endothelial cells (EC) and, subsequently, their interaction with thyrocytes and extracellular proteins. Cytokines (IL-1, IFN-γ, and TNF-α) secreted by thyrocytes as well as by infiltrating mononuclear cells aid in the induction of adhesion molecules such as ICAM-1, which serves as a ligand for LFA-1 (Weetman et al, 1989b). This ICAM-1 expression is absent on thyrocytes under normal conditions but is induced in thyroid disease (Weetman et al, 1989b: Bagnasco et al, 1991; Tandon et al, 1992a). In contrast, the ICAM-1 expression on thyrocytes in GD patients has not been demonstrated (Bagnasco et al, 1991). The functional consequence of ICAM-1 expression by thyrocytes has been demonstrated by increased binding of lymphocytes to ICAM-1$^+$ thyrocytes (Weetman et al, 1989b) and increased NK and T cell-mediated cytoxicity against such targets (Weetman et al, 1990c). These data raise the possibility that thyroid cells

may be killed by cell-mediated cytotoxicity. Interaction of thyroid cell ICAM-1 with LFA-1 on lymphocytes will also lead to activation of the latter. Recently, it has been demonstrated by flow cytometry and immunohistochemical techniques, that TEC express ICAM-1 and VCAM-1, suggesting that the LFA-1/ICAM-1, and ICAM-3 and VLA-4/VCAM-1 pathways could play a relevant role in localizing and perpetuating the autoimmune response in autoimmune thyroid disorders (Marazuela et al, 1994). The expression of these adhesion molecules on thyrocytes and on endothelium will aid in migration and homing of autoreactive lymphocytes to the target organ.

1.7.5 Thyrocytes and heat shock proteins (hsp)

In addition to the secretion of cytokines and expression of adhesion molecules, thyrocytes in human thyroid disease (HT and GD) also express hsp-72 (Heufelder et al, 1991). In that study, it has been shown that hsp-60 and hsp-70 do not play a role in autoimmunization, since T cell reactivity to hsp-60 and hsp-70 on TEC does not appear to occur.

In conclusion, it appears that the pathogenesis of HT and GD is a complex interaction of several mechanisms and needs further extensive investigation.

1.8 Immunotherapy of AITD

Various attempts, thyroid Ag-specific and non-specific, have been made to modulate the host's immune response in order to treat thyroid disease in susceptible animals and in humans. Specific methods of EAT prevention include injection, before active immunization or adoptive transfer, of soluble thyroid Ags, irradiated Tg-specific T cells, or anti-idiotypic Abs (anti-Id Abs) specific to the TCR of Tg-specific T cells or to Tg Abs, or anti-TCR Vß Abs. Non-specific methods include the use of anti-class II MHC Abs, anti-thyroid drugs, cytokines or anti-cytokine Abs, sex hormones, anti-CD4 and anti-adhesion molecule Abs. Therapies that inhibit the hormonal secretion of the target organs, and/or modulate immunity by therapy with isohormone, have also been attempted in the suppression of AITD.

1.8.1 Specific Immunosuppression

1.8.1.1 Thyroid Ags as immunotherapeutics

This approach entails the use of organ-specific Ags such as Tg or thyroid extract. An initial set of experiments revealed that injection of guinea pigs with soluble thyroid extract in saline before or after immunization with thyroid extract in CFA delayed the onset of thyroiditis but did not influence its incidence or severity (Jankovic and Flax, 1963). The efficiency of this approach has been reexamined by

Silverman and Rose (1974b) who demonstrated that i. v. injection of soluble thyroid extract suppressed the development of SAT in BUF rats. These authors suggested that suppression might have been due to the induction of either tolerance or immune deviation. Later, Whitmore and Irvine (1977) were also able to prevent the development of Tx-X-induced EAT by i. p. injection of a crude thyroid extract in saline in PVG/c rats. The protection was Ag-specific because rats that were injected with liver extract as the tolerogen developed thyroid disease subsequent to Tg challenge. The treatment was effective only when rats were injected with soluble Tg extract during the course of irradiation or before but not after the onset of thyroiditis. These authors suggested that this immunization might have evoked suppressor (Ts) T cells. The differences observed in the results reported in guinea pigs and rats may be attributed to the dose or route of Ag or variations in the immunization schedules followed.

EAT prevention with soluble Tg has also been achieved in genetically susceptible strains of mice through augmentation of circulatory Tg by i. v. injection of deaggregated exogenous mTg (Kong et al, 1982) or through physiological stimulation of the thyroid gland by infusing with TSH or thyrotropin-releasing hormone (TRH) before immunization with Tg (Lewis et al, 1987; Kong et al, 1989). The suppression was shown to be

linked to a ≥ 2-3 day increase in circulatory Tg levels and was transferred by $CD4^+$ but not $CD8^+$ T cells of tolerant animals since pretreatment of splenic cells with anti-CD4 but not anti-CD8 Abs could abrogate the adoptive transfer of this suppressor function of splenic cells (Parish et al, 1988; Kong et al, 1989). Interestingly, Tg- and TSH-induced suppression was effective in preventing the afferent- (EAT induced by direct challenge with mTg in CFA) but not the efferent- (EAT induced by adoptive transfer of mTg-primed spleen cells) phase of EAT (Fuller et al, 1993). These findings support the prevailing contention in thyroid autoimmunity that circulatory mTg serves a physiologic role in maintaining self-tolerance by sustaining low levels of Ts activation and that additional elevation above baseline increases and prolongs resistance to EAT induction. It is unclear, whether the disease prevention by elevated Tg levels in the circulation occurs via the generation of the Th2 cells that secrete suppressive/anti-inflammatory cytokines such as IL-10/IL-4 (Burstein et al, 1991) or regulatory/suppressor T cells that secrete TGF-β, or through the induction of clonal anergy i.e., the tolerization of Th1 cells that secrete IL-2/IFN-γ (Dewit et al, 1992; Burstein et al, 1991). For that matter, it is not established whether EAT in mice or rats is a Th1- or Th2-mediated disease. The disease is transferrable by IL-2 secreting T cells (Hutchings et al, 1992; Sugihara et al,

1993). On the other hand, the disease is aggravated by anti-IL-2 receptor MAb (Braley-Mullen et al, 1991) or anti-IFN-γ MAb (Stull et al, 1992). Interestingly, Zerubavel-Weiss et al (1992) demonstrated that Tg-specific Th2 cell lines can adoptively transfer EAT to naive recipient mice. Though these methods have not yet been applied to other thyroid Ags such as TPO and TSHR, it is possible that induction of tolerance to one autoantigen may cover others through bystander suppression, a finding observed in the EAE model. If this is correct, this approach might even be successful in a disease in which the exact autoantigen is unknown if another protein from the target tissue is administered (Al-Sabbagh et al, 1994). Moreover, it solves a major conceptual problem related to designing Ag- or T-cell specific therapy of inflammatory autoimmune diseases such as human thyroid disease in which autoreactivity is directed to multiple Ags (Tg, TPO, TSHR) in the target tissue. More recently, it has been demonstrated that oral administration of Tg prior to challenge with Tg in CFA reduced the intensity of both humoral and cellular immune responses and severity of thyroiditis but did not completely protect the mice from thyroiditis (Guimaraes et al, 1995). However, the mechanisms of immunosuppression were not studied. It was suggested that TH0 cells might have been tolerized.

It has also been shown that EAT suppression could be induced by pretreatment of animals either with guinea pig Tg

(gpTg) in IFA (Braley-Mullen et al, 1978) or with i.v. injection of Tg-coated syngeneic spleen cells (Braley-Mullen et al, 1980). The mechanism(s) by which these regimens confer protection in mice has not been investigated, but the involvement of T cell anergy analogous to the one reported in the MBP-induced EAE model has been suggested (Gaur et al, 1992; Kennedy et al, 1990a and 1990b). The efficiency of these various methods remains to be tested in patients with AITD.

1.8.1.2 Monoclonal antibodies (MAbs) to CD4

Stull et al (1988) have shown by adoptive transfer experiments that EAT in susceptible strains of mice can be abrogated by injecting depleting anti-CD4 MAb either at the time of or 19 days after the transfer of mTg-primed LNC to recipient mice (only two time points were studied). More recently, Hutchings et al (1993) using the murine EAT model, demonstrated that injection of non-depleting anti-CD4 MAb at the time of immunization with Tg and adjuvant prevented the induction of both thyroiditis and AAb response. This protection was transferable and appeared to be both Ag-specific and Ag-dependent. This regimen did not affect the immune response to exogenous Ags. The kinetics and immunobiology of the therapy suggest that this approach has great therapeutic potential for AITD. It would be interesting

to use a synthetic analogue of the CD4 protein surface to inhibit EAT development since such a regimen has been shown to suppress EAE in mice effectively without depletion of the $CD4^+$ subset and without inherent immunogenicity of an Ab (Jameson et al, 1994).

1.8.1.3 TCR-based strategies

It has been shown that rats can be vaccinated against subsequent induction of active EAE (Ben-Nun et al, 1981) or adjuvant arthritis (AA) (Holoshitz et al, 1983) by injecting them with irradiated autoreactive pathogenic T cell lines under suitable conditions. Subsequently, Maron et al (1983) have extended these findings to prevent Tg-induced EAT in mice. In that study, it has been demonstrated that injection of mice with irradiated, activated mTg-specific $CD4^+$, $CD8^-$ T cell clones/lines can protect them from subsequent EAT development produced either by active immunization with Tg or by adoptive transfer of a Tg-specific pathogenic T cell line. However, anti-Tg Ab levels in vaccinated mice were not altered, demonstrating the lack of a correlation between EAT and anti-Tg Ab levels. The mechanisms of resistance that are activated by vaccination have not been elucidated fully, although, a role either for anti-clonotypic AAbs directed against the TCR or for Ts cells has been suggested (Maron et al, 1983).

Remy et al (1989) have employed a cloned MHC class I-restricted Tg-specific CTL hybridoma that recognizes determinants within a 40mer pathogenic Tg peptide as therapy in mice (Texier et al, 1992). Vaccination of mice with irradiation- attenuated CTLs was shown to prevent development of EAT, through the induction of anti-idiotypic Ab (Ab2ß) that recognizes the paratope of an anti-Tg MAb (Ab1) specific to the pathogenic epitope of the Tg molecule or through the generation of anti-clonotypic Abs (Roubaty et al, 1990). In contrast, Flynn and Kong (1991) demonstrated that the T-cell vaccination induced protection to Tg-induced EAT in mice operates through the generation of both suppressor/regulatory $CD4^+$ and $CD8^+$ T cells. Recently, Charriere's group has described one anti-clonotypic Ab that reacts with the TCR of the class I-restricted Tg-specific CTL hybridoma and is able to protect mice against the development of EAT when given parenterally one day before immunization with tryptic fragments of Tg (Texier et al, 1992). These data indicate that induction of anti-clonotypic Abs to Tg-specific T cells is one of the potential mechanisms of the protective immunity against EAT. Analogous Abs directed against specific V regions are protective in EAE, and this may be a potential approach to immunotherapy in situations such as human thyroid disease although the feasibility of this approach has been a matter of debate (Davies et al, 1991; Davies et al, 1992). It

should also be noted that irradiated thyroiditogenic Tg-specific T cells failed to protect C57BL/6 mice against subsequent challenge with Tg + CFA (Zerubavel-Weiss et al, 1992) thus further raising the questions regarding the feasibility of T cell vaccination as an approach to treat thyroid disease.

1.8.2 Cytokine-directed immunotherapy

Some recombinant cytokines, by virtue of their immunosuppressive properties, may provide an alternative to specific immunotherapy of autoimmune diseases. It has not been established which cytokines are of prime importance either in human or in animal models of thyroid disease, although various types of cytokines have been shown to play a crucial role in the immunopathogenesis of autoimmune thyroiditis (reviewed in Weetman and McGregor, 1994). Several reports have addressed the immunotherapeutic potential of cytokines in AITD. Injection of neutralizing MAb to IFN-γ has been shown to prevent Tg-induced EAT by decreasing the number of Tg-specific $CD8^+$ T cells (Tang et al, 1993). These findings are an extension of earlier studies where <u>in vivo</u> treatment with anti-IFNγ MAb exerted beneficial effects on the development or course of autoimmune diseases in animal models of type-1 diabetes (Cockfield et al, 1989) and of systemic lupus erythematosus (SLE) (Jacob et al, 1987). In contrast, anti-

IFNγ MAb treatment exacerbated the symptoms of MBP-induced EAE (Biliau et al, 1988) and heterogeneously modulated the course of adjuvant-induced arthritis (AA) (Jacob et al, 1989; Boissier et al, 1995).

Th2 cytokines, IL-4 and IL-10, under identical experimental conditions exhibit diverse effects by exacerbating or weakening of cytotoxic mTg-specific T cell reactivity and maintenance or attenuation of subsequent transferred EAT (Mignon-Godfrey et al, 1995a). The immunotherapeutic potential of IL-10 has been demonstrated by its ability to treat both early-phase and established-phase EAT in mice (Mignon-Goefroy et al, 1995b). These findings are extensions of previous results reported in various animal models of autoimmune diseases such as EAE in rats (Rott et al, 1994), and diabetes in NOD mice (Pennline et al, 1994). The therapeutic effect of IL-10 was reported to be through enhanced T cell death (apoptosis), since a reduction of up to 40 to 50% in $CD4^+$ and $CD8^+$ lymphoblastoid spleen cells was observed in IL-10-treated mice (Mignon-Goefroy et al, 1995b). In contrast, no reduction in Tg-specific Ab levels was reported in either experimental or control groups indicating that the Th cell compartment at least was not affected. These findings contrast with the results obtained in anti-IFNγ treated mice wherein Tg-specific Ab titers were reduced (Tang et al, 1993) indicating differences in the cytokine-mediated

immunoprotection mechanisms.

In contrast to the studies of Tang et al (1993), it has been shown that culture of mTg-primed $CD4^+$ splenic T cells in the presence of mTg and anti-IFNγ or anti-IL-2 receptor MAb augmented the development of thyroiditis and anti-mTg Ab responses in mice on adoptive transfer, although this treatment had been anticipated to neutralize Th1 cytokine and there by exert protective effects (Braley-Mullen et al, 1991; Stull et al, 1992). These various studies indicate that IFN-γ possesses either pathogenic or protective role. Thus, the effects of cytokine therapy may not be as simple as predicted and the observed capacities of cytokines to exacerbate disease limits their use as immunotherapeutics for human thyroid disease. In thyroid autoimmunity, Widder et al (1991) have shown that TGF-ß suppresses the <u>in vitro</u> proliferation of intrathyroidal T cells from GD patients in response to lectin, IL-2, or autologous thyrocytes. TGF-ß has been shown to exert a direct inhibitory effect on autoantigen and class II expression by thyrocytes. However, its utility as an immunotherapeutic agent has not been tried <u>in vivo</u> in HT patients or in animal models of AITD.

1.8.3 MHC-based strategies

Based on the findings that the I-A subregion of the H-2 complex has a major influence on Tg-induced EAT (**Section**

1.3.1), Vladutiu and Steinman (1987) have demonstrated that injection of anti-I-A MAbs of appropriate MHC specificity before antigenic challenge with Tg and CFA, results in the total prevention of the development of thyroid lesions and anti-Tg AAbs. When mice were injected with anti-class II MHC Abs after Tg challenge, the severity of EAT and the titers of anti-Tg AAbs were decreased but not completely suppressed. These findings were attributed to B cell depletion since these Abs seem to exert cytotoxic effects on B cells (Vladutiu and Steinman, 1987).

1.8.4 Sex hormone(s)-based strategies

Based on the findings that sex hormones influence the susceptibility of various animal species to thyroiditis (reviewed in **Section 1.6.2.2**), their capacity to treat thyroid disease has been tested. Ahmed et al (1986) demonstrated the beneficial effect of testosterone injected into PVG/c rats with chronic thyroiditis. There was a reduction in the severity and incidence of thyroiditis but not in the anti-Tg Ab titers.

1.8.5 Isohormonal therapy

Therapeutic approaches directed at modulating the feedback regulation of target cell activity have been reported. Takasu et al (1990) reported that those patients

with HT treated with thyroxine (which lowers TSH levels) remained euthyroid for 1-8 years after the thyroxine treatment was stopped. Over 20% of the patients with hypothyroidism after HT may recover satisfactory thyroid function, and can be identified during thyroxine treatment by their thyroid response to TSH in a TRH test. Kampe et al (1990) also reported that administration of L-thyroxine in women with postpartum thyroiditis (positive for anti-TPO Abs) prevented hypothyroid symptoms, but did not alter the course of postpartum thyroiditis. It has further been shown that supranormal serum T4 concentration has a significant suppressive effect on peripheral blood NK cell activity, and on IL-2 production by T cells to PHA in humans (Papic et al, 1987). Excess T4 interfered with the release of lytic factors from NK cells in mice (Stein-Streilein et al, 1987).

Convincing data on the immunosuppressive effect of T4 has also been demonstrated in BB rats that are known to develop SAT. Treatment of BB rats with thyroxine for 3-4 months resulted in a reduction in Ab titers to Tg and TPO, and significantly lowered the frequency of lymphocytic thyroiditis (13% versus 45% in treated versus untreated controls) (Banovac et al, 1988). However, treatment of BB rats with T4 and iodide has been shown not to significantly change the incidence of lymphocytic thyroiditis (Reinhardt et al, 1987). These results are in contrast with the previous findings

reported by Weetman et al (1982) wherein the authors demonstrated the absence of any therapeutic effect of T4 on Tg + CFA induced EAT in AUG rats except that TSH levels were brought to normal levels. This suggested that pathogenesis of autoimmune thyroiditis is independent of thyroid hormone secretion.

1.8.6 Monoclonal antibodies (MAbs) to adhesion molecules as immunotherapeutics

Accumulating evidence suggests that cytokine-induced expression of adhesion molecules on endothelium and thyrocytes is an essential step in the migration and homing of autoreactive T cells to the target organ of autoimmune attack (reviewed in Weetman and McGregor, 1994). Metcalfe et al (1993) demonstrated the efficacy of MAbs with specificity for ICAM-1 and LFA-1. In that study, the authors showed that administration of either of these MAbs significantly reduced the severity of lymphocytic infiltration in Tg-induced EAT in rats suggesting that these MAbs appear to inhibit cell-mediated autoimmunity <u>in vivo</u>.

1.8.7 Global immunosuppressive regimen

Recently, it has been shown that treatment of CBA mice with a novel immunosuppressive drug from day -2 to day +28 after immunization with Tg suppressed the development of

thyroiditis and Tg-specific Ab titers in a dose-dependent manner (Nicoletti et al, 1994). The immunosuppressive effects of FK 506 was shown by Tamura et al (1993) in Tx-X-induced EAT in PVG/c rats and was suggested to be due to its capacity to interfere with $CD4^+$ T lymphocyte function and adhesion molecule-dependent cytotoxic effector mechanisms.

Overall, the Ag-driven peripheral tolerance may serve as an effective immunotherapeutic approach to treat EAT. This model subsequently may provide the basis for immunotherapy of human autoimmune thyroid disease.

1.9 Summary and statement of objectives

1.9.1 Summary

Major achievements have been made in the molecular characterization of the three major autoantigens that may be involved in autoimmune thyroid disease and this in turn has facilitated the identification of T- and B-cell epitopes of these three thyroid Ags. Specific data on T cell epitope mapping within Tg so far have come from mouse EAT studies, whereas Tg epitopes recognized by intrathyroidal T cells from HT patients have not been identified. In contrast to the progress made in Tg-induced EAT, little progress has been made in identifying the epitopes recognized by autoreactive T- and B-cells in the animal models of spontaneous thyroid disease. Some progress has been made with respect to T- and B-cell epitope mapping within TPO in both human thyroid disease and animal models of EAT. After cloning of TSHR, rapid progress has been made in delineation of both T- and B-cell epitopes within TSHR associated with Graves' disease and animal models.

Heterologous epitopes of either human, porcine or rat Tg origin have been used to induce EAT in mice because the molecular structure of mouse Tg is not known. It is not clear if the disease induction was due either to the true recognition of the conserved Tg determinants or to the presence of xenogeneic determinants. Attempts have not been made to address the immunopathogenicity of these Tg epitopes

in a homologous species, for example in rat EAT. Though it has been shown that Tg contains dominant B epitopes, their nature and location remain mostly undefined. Moreover, the factors that influence Tgs to elicit IgG responses to distinct epitopes are unknown, although iodination of Tg has been shown to influence the T cell autoreactivity. Tg-specific $CD4^+$ and $CD8^+$ T cells have been shown to play a major role as mediators of thyroid disease in mice. However, it has not yet been clearly established in Tg-induced rat EAT whether the disease can be mediated by $CD4^+$ or $CD8^+$ T cells alone or in combination. In addition, the role of these T cells in the immunopathogenesis of human thyroid disease is unknown and needs further extensive investigation. The role for Tg-specific Abs in thyroid disease remains controversial but their contribution towards the thyroid pathology warrants further investigation.

The etiopathogenesis of human thyroid disease remains elusive. Despite molecular developments in HLA analysis, there have been no really major improvements in our understanding of the immunogenetics of thyroid autoimmunity. The existing data suggest that non-MHC genes play an important role in susceptibility. In addition, the importance of environmental and endogenous factors in modulating the thyroid disease is recognized, and the role of viruses in thyroid pathology is circumstantial.

Several studies have demonstrated that thyrocytes can activate autoreactive T cells. It is not known whether class II^+ thyrocytes by themselves or in association with dendritic cells by presenting endogenous T cell epitopes of thyroid Ags can activate specific $CD4^+$ $CD8^-$ TcR α/β^+ T cells. Presentation of immunodominant Tg epitopes by primary thyroid culture (thyrocytes) has been reported, but little is known on the presentation of non-dominant or cryptic Tg epitopes. Antigen-presentation studies have been hampered by the lack of knowledge on the nature and location of pathogenic Tg epitopes and by the lack of availability of appropriate tools such as T cell hybridomas or clones specific for these pathogenic epitopes and pure thyrocyte populations.

It is now known that TCR Vß usage, as demonstrated in induced and spontaneous models of thyroid disease, by intrathyroidal T cells specific for Tg or other thyroid Ags is highly heterogenous. These findings indicate that therapeutic approaches using peptide analogs or anti-TCR Vß Abs are unlikely to be helpful in the treatment of AITD. Oral tolerance has been recently considered as a potential immunotherapeutic approach based on the knowledge that T cell suppression is likely to be mediated by distinct T cell subsets that secrete different pattern of cytokines. Continued basic and applied research in human thyroid disease and in animal models in all of the aspects discussed above

will help in our understanding of the etiopathogenesis of AITD and of appropriate intervention.

1.9.2 Statement of objectives

The project originated with the general aim of inducing experimental autoimmune thyroiditis (EAT) with homologous Tg in mice in the absence of oil-based adjuvants (such as CFA), in order to avoid the adverse effects on the immune system of these adjuvants. Meanwhile, work was in progress in this laboratory on inducing EAT with rat Tg peptides in heterologous species (mice). Since knowledge of the immunopathogenicity of these Tg peptides in rats is unknown, I focused my research on the induction of EAT with these rat Tg peptides in homologous species (rats). Based on the findings during the course of EAT studies in rats, I subsequently focused on the delineation of immunodominant B-cell epitopes among Tgs of various species, and finally examined the ability of class II^+ thyrocytes to activate the Tg peptide-specific T cells in rats.

The particular aims or objectives of the studies reported in this thesis are:

A. To reexamine the adjuvant free-induction of EAT in mice by targeting soluble Tg onto antigen-presenting cells (APC) using monoclonal antibodies (MAbs), as delivery vehicles, specific for MHC class II molecules expressed on APC.

B. To investigate the immunopathogenicity in rats of a 17mer homologous Tg peptide (2495 -2511) that has been previously shown to cause EAT in heterologous species (mice) (Chronopoulou and Carayanniotis, 1992).

C. To examine the immunopathogenicity in rats of an 18mer homologous Tg peptide (2695-2713) (with respect to its ability to induce thyroiditis and T- and B-cell responses, and to determine if rat EAT is a $CD4^+$ T cell disease) that has recently been shown to cause EAT in mice (Carayanniotis et al, 1994).

D. To identify the nature and location of immunodominant Tg epitopes associated with thyroid disease in rats and to examine the factors contributing to variation in relative immunodominance of these epitopes among heterologous Tg of various species.

E. To reexamine the original concept proposed by Botazzo et al (1983) that class II^+ thyrocytes trigger thyroid antigen-specific T cells i.e., to examine specifically whether class II^+ thyrocytes, in the absence of professional APC, can present an endogenous Tg epitope of a pathogenic Tg peptide (2695-2713) to specific rat T cells.

CHAPTER 2

Materials and Methods

2.1 Animals

Female CBA/J ($H\text{-}2^k$), and C57B6/6J ($H\text{-}2^b$) inbred mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and were used for immunizations at the age of 6-10 weeks. Female F344 ($RT\text{-}1^l$), WF ($RT\text{-}1^u$), and WKY ($RT\text{-}1^k$) inbred rats were purchased from The Harlan Sprague Dawley Inc. (Indianapolis, IN) and were used for immunizations at the age of 6-7 weeks. Animals were acclimatized to the animal holding facilities at least for a week before immunization. All animal care procedures were carried out according to institutional regulations.

2.2 Cell culture media, cell lines, instruments, monoclonal antibodies, and other reagents

Cell culture media RPMI-1640 medium (Flow Labs), Dulbecco's modified Eagle's medium (DMEM) (GIBCO BRL, Burlington, Ontario, Canada), Ham's modified F-12 medium (GIBCO BRL), RPMI-10 [RPMI-1640 medium supplemented with 10% heat inactivated fetal bovine serum (FBS), 20 mM HEPES, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 5×10^{-5} M 2-mercaptoethanol], DMEM-10 is similar to RPMI-10 except that it contains DMEM instead of RPMI 1640, DMEM-20 is similar to DMEM-10 except that it contains 20% FBS instead of

10% FBS, Expansion medium (DMEM-10 containing 10% of filtered supernatant of Concanavalin A activated F344 rat splenocytes).

Cell lines CTLL-2 cell line (TIB 214) (Gillis et al, 1977), FRTL-5 cells (CRL 8305) (Ambesi-Impiombato et al, 1980), HB 64 [(M2-1C6-4R3 clone secreting IgG1 MAb specific for matrix protein of the influenza type A virus) (Yewdell et al, 1981)], HB 65 [(H16-L10-4R5 clone secreting IgG2a MAb specific for nucleoprotein of influenza type A virus) (Yewdell et al, 1981)], and TIB 92 [(10-3.6.2 clone secreting IgG2a MAb specific for determinants expressed on I-A^k) (Oi et al, 1978)] were obtained from American Type Culture Collection (ATCC Rockville, MD). BW5147 α^-/β^- variant (White et al, 1989) was a kind gift of Dr. P. Marrack (National Jewish Centre for Immunology and Respiratory Medicine, Denver, CO).

Instruments ß scintillation counter (Beckman Instruments Inc., Mississauga, Ontario, Canada), Brinkman homogenizer (Brinkman Instrument, Westbury, NY), FACStar Plus analyzer (Becton Dickinson, Mountain view, CA), Microplate reader (Biorad Model 3550), Semi-automated cell harvester (Skatron Inc., Sterling, VA), TSK 4000SW size-exclusion column (Beckman Instruments, Toronto, Canada) were used.

Monoclonal antibodies (MAbs) The following mouse MAbs (IgG1) specific for various rat T lymphocyte surface markers were purchased from Serotec Ltd., Toronto, Canada: OX-6 (MCA 46) specific for monomorphic determinants on RT1-B antigens

(I-A equivalent), OX-17 (MCA 50) specific for monomorphic determinants on RT1-D antigens (I-E equivalent), W3/25 (specific for CD4 determinants), OX-18 (MCA 51) specific for monomorphic determinants on RT1-A antigens (MHC class I equivalent), OX-19 (MCA 52) specific for CD5 antigen expressed on all T cells, MRC OX-8 (MCA 48) specific for CD8 determinants, OX-22 (MCA 53) specific for $CD45RC^{high}$ expressed on B and T cells, and R73 (MCA 453) specific for constant determinant of rat TcR Vß.

Other reagents The following reagents were used. Alkaline phosphatase-conjugated goat anti-rat IgG (Fc specific) (Sigma Chemical Company, St. Louis, MO), alkaline phosphatase-conjugated goat anti-mouse IgG (FC specific) (Sigma), heat-killed Bordetella pertussis organisms (Difco Laboratories, Detroit, Michigan), bovine serum albumin Fraction V (BSA) (Sigma), complete Freund's adjuvant (CFA) (containing Mycobacterium butyricum) (Difco, Detroit, Michigan), concanavalin A (Con A) (Boehringer Mannheim, Laval, Quebec, Canada), diethanolamine (BDH Chemical Ltd., England), EDTA (GIBCO BRL), fetal bovine serum (FBS) (Bioproducts for Science, Indianapolis, IN.), ficoll-paque (Pharmacia LKB Biotechnology, Baie d'Urfe', Quebec, Canada), $F(ab')_2$ fragment (Fab specific) of goat anti-mouse IgG-FITC conjugate (Sigma), fungizone (GIBCO BRL), glycine (Fisher Scientific, Toronto, Canada), glutaraldehyde (Sigma), hypoxanthine,

aminopterin, thymidine (HAT) (Sigma), Hank's balanced salt solution (HBSS) (GIBCO BRL), HEPES (GIBCO BRL), hypoxanthine, thymidine (HT) (Sigma), incomplete Freund's adjuvant (IFA) (Difco), polyethylene glycol 1500 (PEG 1500) (Boehringer Mannheim, Indianapolis, IN), 24-well, flat-bottomed cell culture plates (Nunc, Denmark), 96-well, flat-bottomed polyvinyl chloride (PVC) microtiter plates (Dynatech Laboratories, Chantilly, VA), 96-well, flat-bottomed microtiter plates (Nunc), L-glutamine (GIBCO BRL), 2-Mercaptoethanol (Sigma), ovalbumin (Sigma), penicillin (GIBCO BRL), purified protein derivative (PPD) (Statens Seruminstitute, Copenhagen, Denmark), propidium iodide (Sigma), protein G Sepharose 4 Fast flow affinity chromatography columns (Pharmacia), recombinant rat IFN-γ (Sigma), sepharose CL 4B (Pharmacia), sodium azide (BDH Chemical Ltd, England), streptomycin (GIBCO BRL), 3H-thymidine (6.7 Ci/mmol, ICN Radiochemicals, Missisauga, Canada), ICR mouse or Sprague-Dawley rat thyroid glands (Bioproducts for Science, Inc., Indianapolis, IN), bovine thyroglobulin (bTg) and porcine thyroglobulin (pTg) purchased in lyophilized form (Sigma), trypsin (GIBCO BRL), and tween-20 (BDH).

2.3 Thyroglobulin (Tg) peptides

Tg peptide	Amino acid sequence	Sequence position
Rat TgP1 (rTgP1)	(Ac-GLINRAKAVKQFEESQG-amide)	2495-2511
Rat Tg peptide	(Ac-TDDYASFSRALENATRDY-amide)	2550-2567
Rat TgP2 (rTgP2)	(Ac-CSFWSKYIQTLKDADGAK-amide)	2695-2713
N-terminal 12mer	(Ac-CSFWSKYIQTLK-amide)	2695-2706
C-terminal 12mer	(Ac-YIQTLKDADGAK-amide)	2702-2713
Human TgP2 (hTgP2)	(Ac-CSFWSKYISSLKTSADGAK-amide)	2695-2713

The peptides were synthesized at >70% purity at the Alberta Peptide Institute on an Applied Biosystems (Foster City, CA, USA) 430A synthesizer as described earlier (Chronopoulou and Carayanniotis, 1992; Carayanniotis et al, 1994). These peptides were used for in vivo or in vitro experiments without further purification. For descriptive purposes, amino acid positions within rat TgP1 or TgP2 are identified by the hTg sequence numbering, since the complete rat Tg (rTg) sequence is unknown (Di Lauro et al, 1985).

2.4 Purification of monoclonal antibodies and thyroglobulins

Monoclonal antibodies (MAbs) were purified on protein G-sepharose affinity chromatography columns from culture supernatants of the hybridoma cell lines TIB 92, HB 64, and HB

65. The details of the hybridoma cell lines were given under Section 2.2. After extensive dialysis against double-distilled water, MAbs were lyophilized and stored at -20^{0}C until further use. The protein (Ab) concentration was estimated spectrophotometrically at 280 nm as 1.4 O.D. units = 1.0 mg/ml.

Mouse thyroglobulin (mTg), rat thyroglobulin (rTg), and human thyroglobulin (hTg) were purified from frozen thyroids of outbred ICR mice, Sprague-Dawley rats, and humans, respectively as described (Chronopoulou and Carayanniotis, 1992) (the iodination status of Tgs were not assessed). The thyroids were homogenized in phosphate buffer, pH 7.0 and the supernatant was centrifuged three times at 16,000 X g. After extensive dialysis against double-distilled water, the Tgs were lyophilized and stored at -20^{0}C until further use. Bovine thyroglobulin (bTg) and porcine thyroglobulin (pTg) were purchased in lyophilized form (Sigma). The protein (Tg) concentration was estimated spectrophotometrically at 280 nm as 1 O.D. unit = 1 mg/ml.

2.5 Preparation and purification of thyroglobulin-monoclonal antibody (Tg-MAb) immunoconjugates

The Tg-MAb immunoconjugates were prepared as follows. 0.5 mg of mTg was mixed with MAb at 1:8 molar ratio (this ratio found to be optimal) in 1 ml PBS (pH 7.4). Later, an

equal volume of freshly made 0.3% (w/v) glutaraldehyde was added drop wise to the protein solution. This was followed by gentle stirring for 2 hr at room temperature. The reaction was stopped by the addition of 0.25 ml of 2 M glycine. Later, the reaction mixture was extensively dialysed against PBS at 4^0C, and centrifuged at 16,000 X g for 5 min to remove possible aggregates. Conjugates were purified by HPLC gel filtration on a 7.5 x 600 mm TSK 4000SW size-exclusion column and were stored at 4^0C until further use.

2.6 Immunization of mice

Priming of female CBA or B6 mice (6/group) was done by subcutaneous (s.c.) injection at the base of the tail of 0.1 ml PBS containing 5 μg of the appropriate mTg-MAb conjugate. In addition, a group of mice (2/group) were injected with 0.1 ml of complete Freund's adjuvant (CFA):PBS (1:1) emulsion containing 50 μg mTg. After 3 weeks, these mice received booster injection intraperitoneally (i.p.) with 50 μg free mTg in 0.1 ml PBS. Two weeks after the second injection, mice were bled from the retrobulbar sinus and their sera were collected and stored in 0.05% NaN_3 at -20^0C until further analysis by enzyme-linked immunosorbent assay (ELISA). The serological analysis of the mice sera for mTg-specific IgG was done by ELISA as described (Chronopoulou and Carayanniotis, 1992).

2.7 Lymphocyte proliferation assay

Rats were injected into one hind footpad with 100 µl of CFA:PBS (1:1) emulsion containing TgP1 (200 nmol = 380 µg) or TgP2 (200 nmol = 420 µg) or Tg (200 µg). Fourteen days later, the animals were sacrificed and popliteal lymph node cells (LNC) were collected aseptically, washed with RPMI-10 (culture medium) (Section 2.2). After two rounds of washing, 4 x 10^5 cells/200 µl/well were cultured with or without antigen in 96-well, flat-bottomed microculture plates for 4 days at 37^0C in a 5% CO_2, 95% air humidified incubator. Eighteen hours before harvesting, 1 µCi of ^{3}H-thymidine (^{3}H-TdR) was added to each well in 25 µl of medium. The cells were harvested onto glass fibre filters using a semi-automated cell harvester. The incorporated ^{3}H-TdR was counted in a ß scintillation counter. Proliferation data were expressed in Δ cpm +/- SD or stimulation index (SI) +/- SD. Δcpm was defined as (mean cpm in the presence of antigen minus mean cpm in the absence of antigen). Stimulation index (SI) was defined as (mean cpm in the presence of antigen / mean cpm in the absence of antigen).

2.8 Antibody blocking studies

TgP1-primed LNC were obtained by priming of different rat strains with 12.5 nmol TgP1. The method of immunizations and lymphocyte proliferation assays were as described under **Section 2.7.** TgP2-specific T line cells were obtained after

12 days of second *in vitro* boosting with TgP2 as described under Section 2.12. To inhibit the rat LNC proliferative response, MAbs such as MRC OX-6, MRC OX-17 and W3/25 were used. As a control, HB 65 or HB 64 MAb were used. The details of the MAbs were given under **Section 2.2.** All Serotec MAbs were used at nontoxic concentrations derived from MAb titration curves on inhibition of Con A stimulation of rat splenocytes. Inhibition of proliferation by specific blocking MAbs was expressed as % inhibition or Δ cpm +/- SD. The % inhibition was defined as (1-(cpm in the presence of MAb) / (cpm in the absence of MAb)) x 100.

2.9 EAT induction with Tg peptides (TgP1 and TgP2)

Rats were injected subcutaneously (s.c.) with 0.2 ml CFA:PBS (1:1) emulsion containing either 200 nmol (380 μg) TgP1 or 200 nmol (420 μg) TgP2. This 0.2 ml emulsion was divided equally and 100 μl emulsion was injected at the base of the tail and the rest of the 100 μl emulsion was injected into one hind footpad. Immediately, these rats were injected intravenously (i.v.) with 3 x 10^9 *Bordetella pertussis* organisms in 0.5 ml PBS since the additional injection of rats with *Bordetella pertussis* has been reported to enhance the severity of EAT caused by Tg plus CFA (Eishi and McCullagh, 1988b). One week later, the rats were boosted s.c. in the right flank with 100 nmol (190 μg) TgP1 or TgP2 in 100 μl

IFA:PBS emulsion. Three weeks after the second challenge, rats were ether anaesthtized and bled (2-3 mls) by cardiac puncture. The sera were collected and stored in 0.05% NaN_3 at -20^0C until further use. Later, the rats were sacrificed and thyroids were removed and fixed in 10% buffered formalin for histopathological examination.

2.10 Histological examination of thyroids

Serial sections (300-500/thyroid) of rat thyroids were stained with hematoxylin and eosin by Ed Evelly (Department of Anatomy, Faculty of Medicine at this institute). The sections were scored by the author for mononuclear infiltration according to a modified method of Twarog and Rose (1970). Scoring of mouse thyroids for mononuclear infiltration was performed as described (Chronopoulou and Carayanniotis, 1992). The slides were coded as unknowns until the results were tabulated. The scoring for mononuclear infiltration was expressed as infiltration index and was as follows.

Infiltration index	% area of thyroid infiltrated with mononuclear cells
0	No infiltration
0.5	Interstitial accumulation of inflammatory cells distributed between two or more follicles

1	One or two foci of inflammatory cells at least the size of one follicle
2	Extensive infiltration, 10-40% of total area
3	Extensive infiltration, 40-80% of total area
4	Extensive infiltration, >80% of total area

2. 11 Immunization with thyroglobulin and Tg peptides to study the serological immunodominance of Tg peptides

F344 rats (2/group) were primed by injecting s.c. with 200 nmol (324 μg) N-terminal (2695-2706) or 200 nmol (280 μg) C-terminal (2702-2713) 12mer peptides of rTgP2 or with 200 nmol (438 μg) 19mer hTgP2 (2695-2713) or with 1 mg Tg in 0.2 ml of CFA:PBS (1:1) emulsion. 100 μl of 0. 2ml emulsion was injected at the base of the tail and the rest was into one hind footpad. A week later, the rats were boosted s.c. in the right flank with 100 μl IFA:PBS (1:1) emulsion containing 100 nmol of respective peptide or 0.5 mg of respective Tg. Three weeks after the second challenge, the animals were anaesthetized and were bled by cardiac puncture. The sera were collected and stored in 0.5% NaN_3 at -20^0C until further use.

2.12 Generation of a TgP2-specific T cell line

The protocol for the generation of a rTgP2-specific T

cell line(s) was established and was as follows. The T cell line was generated from draining popliteal LNC of rTgP2-primed rats. Briefly, F344 rats (2/group) were injected into one hind footpad with 100 µl of CFA:PBS (1:1) emulsion containing 100 nmol (210 µg) TgP2. Fourteen days later, the popliteal LNC from these rats were collected and washed thrice with DMEM-10 medium (**Section 2.2**). The LNC were boosted in vitro by culturing 4 x 10^6 cells/ml with 10 µg/ml TgP2 in 24-well flat-bottomed cell culture plates for 9 days at 37^0C in a 10% CO_2, 95% air humidified incubator. After the first in vitro boost, the viable T cells were separated from cell debris by Ficoll-Paque density gradient centrifugation (400g for 30 min). These Ficoll-cut T cells were washed three times with DMEM-10 and boosted (2nd round) again in vitro by incubating T cells at 2.5 x 10^5/ml with 8 x 10^6/ml to 10 x 10^6/ml of irradiated (2000 rads) syngeneic thymocytes as antigen-presenting cells (APC) and 10 µg/ml TgP2 in 24-well, flat-bottomed plates for 4 days. Later, the cell population was expanded at 1:4 ratio in expansion medium (**Section 2.2**). After 8-10 days of expansion, the viable T cells were separated from cell debris by Ficoll-Paque density gradient centrifugation and washed 3 times with DMEM-10. Stimulation and expansion cycles were followed as above. Ficoll-cut T cells were subjected to specificity and MHC-restriction assays.

2.13 FACS analysis

The T cell line of **Section 2.12** was stimulated with TgP2 for 96 hr, then centrifuged over Ficoll-Paque to remove APC debris. The T cells were washed three times and stained by indirect immunofluorescence technique using mouse MAbs specific for CD5 (pan T cells) (OX-19), CD4 (W3/25), CD8 (OX-8), TCR-α/β (R73), RT1-B (I-A equivalent) (OX-6), RT1-D (I-E equivalent) (OX-17, RT1-A (OX-18) and OX-22 ($CD45RC^{high}$) according to a modified protocol of Caspi et al (1986). Briefly, 8 x 10^5 T cells were washed once with sterile PAB (phosphate buffer with 0.5% BSA and 0.1% sodium azide), suspended in 50 ul of 2% goat serum in PAB (to avoid subsequent non-specific binding of goat anti-mouse IgG-FITC conjugate) and incubated on ice for 30 min. Later, the cells were washed twice and incubated with the above MAbs at 1/100 dilution in PAB for 30 min on ice. A control group consists of T line cells incubated with isotype (IgG1)-matched MAb (HB 64). The cells were washed twice and incubated with 50 μl of 1/80 diluted $F(ab')_2$ fragment of FITC-conjugated goat anti-mouse IgG on ice for 30 min. After giving two washes, the cells were suspended in 0.5 ml of cacodylate buffer (pH 7.2) containing 1% paraformaldehyde. A similar protocol was followed for surface phenotyping of T cell hybridomas. The cells were then subjected to flow cytometric analysis of a FACStar plus analyzer. A minimum of 10,000 cells were

counted. The results were expressed as histograms of relative cells number versus log fluorescence intensity (L. F. I).

2.14 The T cell line specificity and MHC-restriction assays

The T cell line specificity assay was performed as described elsewhere. (Fling et al, 1991). Briefly, 10^4 T cells/50 μl (after 12 days of stimulation) were incubated with irradiated (2000 rads) syngeneic (F344) or allogeneic (WKY or WF) rat thymocytes as APC at 10^6 cells/50 μl in the presence or absence of rTgP2 or hTgP2 peptides or 12mer overlapping peptides of rTgP2 **(Section 2.3)** or intact rTg in a final volume of 200 μl/well in 96-well, flat-bottomed microculture plates. The culture medium used was DMEM-10 **(Section 2.12)**. Later, the cultures were incubated at 37^0C/10% CO_2/95% humidified air for 72 hr. The cells were pulsed during the last 18 hr of culture with 1 μCi of ^{3}H-TdR/well in a total volume of 25 μl DMEM medium. The cultures were harvested onto glass fibre filters and ^{3}H-TdR uptake was assessed in a ß scintillation counter. Proliferative responses were expressed as SI +/ SD or mean cpm +/- SD of triplicate cultures.

2.15 Adoptive transfer of thyroiditis

For adoptive induction of thyroiditis in rats, a protocol was established. Adoptive EAT was induced by i.p. injection

of viable rTgP2-primed and rTgP2-boosted LNC at 10^8 LNC/ml HBSS/rat. These cells were obtained from F344 rats after 14 days of in vitro priming with 100 µl of CFA:PBS emulsion containing 100 nmol (210 µg) TgP2 and subsequent in vitro boosting with 10 µg/ml TgP2 for 3.5 to 4 days. As a control, F344 rats were primed with 100 µl CFA:PBS emulsion alone or containing 200 µg ovalbumin (OVA). Fourteen days later, primed LNC were in vitro boosted with 10 µg/ml Con A or 25 µg/ml OVA, respectively.

In addition, rTgP2-specific T line cells (**Section 2.12**) were in vitro boosted for 3.5 days at 37^0C in a 10% CO_2, 95% humidified air incubator with 10 µg/ml TgP2 in the presence of irradiated (2000 rads) syngeneic thymocytes as APC. The viable T cells were separated from dead cells by Ficoll-Paque density gradient centrifugation and washed three times with HBSS before injected at 10^8 cells/rat. Rats were sacrificed 10 days post-transfer and thyroids were collected in 10% buffered formalin for histological examination.

2.16 Generation of T cell hybridomas from peptide-primed LNC

The T cell hybridomas were generated following a modified method of Perkins et al (1991). Rats were immunized by injecting into one hind footpad 100 nmol (210 µg) rTgP2 in 100 µl of CFA:PBS (1:1) emulsion. Fourteen to fifteen days later, the popliteal LNC were collected aseptically and single cell

suspensions were prepared in DMEM-10 culture medium. After washing thrice with DMEM-10, 4 x 10^6 cells/ml/well were cultured in a 24-well, flat-bottomed plates(s) in the presence of 10 µg/ml TgP2 at 370C/10% CO_2/95% humidified air. Four days after the first <u>in vitro</u> boosting with the peptide, viable T cells were fused with BW5147 α^-/β^- at 1:2 ratio (lymphocytes: tumor cells) by adding 1 ml of PEG 1500. This was followed by a slow addition of DMEM without FBS and 40 mls DMEM-20. The preparation was incubated for 2 hr at 37^0C/10% CO_2/95% humidified air. Subsequently, 40 mls of DMEM-20 containing 1X fungizone (as per specifications) was added. The cell suspension was plated gently at 100 µl/well in 96-well, flat-bottomed microculture plates. The cultures were incubated at 37^0C/10% CO_2/95% humidified air. After 24 hr of incubation, 100 µl of 2X HAT (hypoxanthine, aminopterin, thymidine) was added/well. Gradually, the cells were changed from HAT to medium containing hypoxanthine and thymidine and finally to DMEM-20. Hybridomas usually appeared in microwells after 7-10 days of culturing. The contents of the wells with T cell hybridomas showing > 50% confluency were transferred to 24-well plates and subsequently screened for TgP2-specificity as described under **Section 2.18.**

2.17 Generation and cloning of T cell hybridomas from T line cells

Ficoll-cut $CD4^+$ rTgP2-specific T line cells were *in vitro* boosted with 10 μg/ml TgP2 in the presence of irradiated (2000) rad syngeneic thymic APC for 4 days. The fusion, post-fusion, and screening methods were as described under **Section 2.16.** Antigen-specific T cell hybridomas were cloned by limiting dilution at 0.33 cells/100 μl/well in 96-well, flat-bottomed microculture plates using DMEM-20 containing 1% syngeneic rat RBC suspension. The cultures were incubated at 37^0C/10% CO_2/95% humidified air. The hybridoma clones started appearing after 9-10 days of culturing. At this stage, 100 μl of spent medium was replaced with fresh DMEM-20 and this step was repeated 5 days later. When the hybridoma growth was >50% confluency, the contents of the wells from 96-well, flat-bottomed plates were transferred to 24-well, flat-bottomed plates and cultured in 1 ml of DMEM-20. The cells were subcultured once again and subsequently screened for TgP2-specificity as described under **Section 2.18.**

2.18 T cell hybridoma activation and CTLL assays

T cell hybridomas were plated at 10^5 cells/well (in 24 hr activation assay) or 1.5 x 10^4/well (in 48 hr activation assay) in 96-well, flat-bottomed microculture plates containing 5 x 10^5/well of irradiated (1500 rad) syngeneic spleen cells. Live splenocytes from syngeneic (F344) or allogeneic (WKY and WF) rats were used in the MHC-restriction

assay. Glutaraldehyde-fixed cells were prepared according to the protocol as described (Shimonkevitz et al, 1983). Preparation of single cell suspensions of thyrocytes (MHC class II^+ or MHC class II^-) was done as described under **Section 2.21.** The cultures were incubated with or without antigen either for 24 hr or 48 hr at 37^0C/10% CO_2/95% humidified air. Subsequently, 50 µl supernatant was transferred from the test plate to a new plate for IL-2 release assay on the IL-2 dependent CTLL-2 line and frozen for a minimum of 2 hr at -70^0C.

CTLL assay was performed in the following manner. After thawing of the above frozen plates(s), 10^4 CTLL cells/100 µl/well added. The medium used was DMEM-10. The cultures were incubated for 24 hr at 37^0C/10% CO_2/95% humidified air, pulsed during the last 6 hr of incubation with 1 µCi/well of ^3H-TdR. Later, the cells were harvested onto glass fibre filters and incorporated ^3H-TdR was counted in a liquid scintillation counter. The values were expressed as mean cpm +/- SD of duplicate or triplicate cultures.

2.19 Direct enzyme-linked immunosorbent assay (ELISA)

Serological analysis of peptide- or rTg-primed rat sera for peptide- and Tg-specific IgG was performed by a direct alkaline phosphatase-based ELISA. Heat denatured Tg was prepared according to a method of Shimojo et al (1988) by

boiling an aqueous Tg solution for 30 min. The wells of 96-well, flat-bottomed polyvinyl chloride (PVC) microtiter plates were coated with various antigens (0.2 μg of 17mer TgP1 or 0.21 μg of 18mer rTgP2 peptide or 8 μg of the truncated 12mer peptides of rTgP2 or 0.21 μg of hTgP2 or 1 μg of intact or heat denatured Tg) in 100 μl of coating buffer (carbonate buffer, pH 9.6) and incubated at 4^0C overnight. Ovalbumin was used as a control antigen. The plates were washed once with PBS and incubated with 100 μl/well of serum samples for 1 hr at room temperature (RT). Following three washes with PBS-Tween, 100 μl of 1/1000 diluted alkaline phosphatase-conjugated goat anti-rat IgG was washed three times with PBS-tween and p-phosphate substrate (1 mg/ml in 10% diethanolamine, 100 μl/well) was added and incubated at RT for 1 hr. The reaction was stopped by adding 25 μl/well of 5N NaOH. Absorbance of p-nitrophenolate product at 405 nm was measured using a Microplate reader. The values (experimental values minus the background values) were expressed as mean +/- SD of duplicate wells. Background values are the values obtained in the absence of immune sera.

2.20 Competitive inhibition ELISA

The protocol for competitive inhibition ELISA was as follows. The PVC plates were coated overnight at 4^0C with 1 μg of intact rTg/well in 100 μl of coating buffer (carbonate

buffer, PH 9.6) and then washed once with PBS. The unreactive sites were blocked with 1% BSA in PBS for 2 hr at RT. The wells were incubated with 50 µl of day 28 TgP-primed F344 rat (3/group) sera of Table 5.1 at 1/512 dilution per well. Simultaneously, the plates were incubated with or without competitor peptides (TgP2 as specific competitor; TgP1 as non-specific competitor). The competitor peptides were used at 200 molar excess of rTg coated on the plate. TgP2 (12.6 µg/ml) or TgP1 (11.4 µg/ml) or dilution buffer as such 50 µl/well were added and incubated at RT for 1 hr. Subsequently, the plates were washed three times with PBS-Tween and incubated with 100 µl/well of alkaline phosphatase-conjugated goat anti-rat IgG at RT for 1 hr. Later, the plates were washed three times with PBS-Tween and incubated with 100 µl/well of substrate solution (**Section 2.19**) at RT for 1 hr. The reaction was stopped by adding 25 µl/well of 5N NaOH and O. D. readings at 405 nm were taken. The values (experimental values minus the mean background values) were expressed as mean +/- SD of triplicate wells.

2.21 Induction and assessment of MHC class II antigen expression on Fisher rat thyroid epithelial cell line-5 (FRTL-5)

FRTL-5 cells were cultured in Ham's modified F-12 medium supplemented with a six-hormone mix and 5% FBS (culture

medium) as described (Ambesi-Impiobato et al, 1980). Induction of MHC class II antigens on FRTL-5 cells was performed following a modified protocol of Platzer et al (1987). Briefly, FRTL-5 cells were cultured either in 25 cm^2 or in 80 cm^2 tissue culture flasks and were treated with recombinant rat interferon-γ (IFN-γ) at 100 units/ml for 5 days. Later, the cell layer was rinsed once with HBSS and a cell suspension was prepared by adding 5 to 10 mls of filter sterilized PEB (PBS containing 0.02% EDTA and 1% BSA) and incubating at 37^0C for 15 min. The cells were washed thoroughly with HBSS before use.

MHC class I and II and antigen expression on thyrocytes was monitored by immunofluorescence. All washings were done with washing buffer (PAB) (see **Section 2.13**) at 4^0C/2500 rpm for 3 min. Briefly, 2.5×10^5 cells were incubated with 50 µl of 1/100 diluted OX-6 , OX-17 and OX-18 MAbs (**Section 2.2**) on ice for 30 min. As a control, isotype-matched MAb (HB 64) (10 µg/ml stock) was used. The cells were washed three times with washing buffer and incubated with 50 µl of 1/80 diluted goat anti-mouse IgG FITC conjugate on ice for 30 min. Later, the cells were washed three times as above and resuspended in 0.4 ml of dilution buffer and 100 µl (5 µg) of propidum iodide solution. The cells were subjected to FACS analysis. Dead cells were gated out. A minimum of 10,000 cells were counted by FACStar plus analyzer as mentioned earlier (**Section 2.13**).

CHAPTER 3

Immunotargeting of Thyroglobulin on Antigen-Presenting Cells Abrogates Natural Tolerance in the Absence of Adjuvant

3.1 Introduction

For decades, thyroglobulin (Tg), the major autoantigen of the thyroid gland, has been used mostly with complete Freund's adjuvant (CFA) for induction of experimental autoimmune thyroiditis (EAT) in mice (Charreire et al, 1989). Apart from CFA, several adjuvants such as lipopolysaccharide (LPS) (Esquivel et al, 1977), muramyl dipeptide (MDP) (Kong et al, 1985), poly A:U (Esquivel et al, 1978), and SGP (a synthetic copolymer of starch, acrylamide, and sodium acrylate) (Williams et al, 1987) were used to enhance the immunogenicity of Tg leading to induction of EAT in mice. Haptenization of Tg was also shown to enhance its immunogenicity (Weigle et al, 1965). Previously, adjuvant free induction of thyroiditis and autoantibodies to Tg in mice had been achieved only through the use of large amounts (several hundred micrograms) of mouse Tg (mTg) administered in repeated injections over a period of 4 weeks (EIRehewy et al, 1981). Subsequently, it was reported that, in the absence of adjuvant, extracorporeally antigen (Ag)-pulsed dendritic cells when injected into mice could also enhance the immunogenicity of mTg (Knight et al, 1988).

Several investigators have described that the immunogenicity of foreign proteins (Kawamura et al, 1986; Carayanniotis et al, 1987; 1990; Casten and Pierce 1988; Snider and Segal, 1987 and 1989) or peptides (Carayanniotis et al, 1988; Casten et al, 1988, Wyss et al 1991), or haptens (Mjaaland and Fossum, 1990 and 1991) is dramatically enhanced either in vivo or in vitro when such Ags are conjugated to MAbs specific for determinants expressed on the surface of antigen-presenting cells (APC). The outcome of these findings was that MAb-mediated delivery of antigen onto APC facilitated the uptake and further processing of Ag by APC leading to enhanced immunogenicity of Ags either in vivo or in vitro.

Taking into account the above reports on immunotargeting, the adjuvant free induction of EAT in mice was reexamined. In this chapter, I have asked whether or not the immunogenicity of mTg would similarly increase in vivo in the absence of adjuvant, by immunotargeting small doses of this autoantigen onto APC using MAbs specific for MHC class II Ags. To address this question, I have selected CBA/J ($H\text{-}2^k$) and C57B6/6J ($H\text{-}2^b$) mice that are known to be high and low responders to mTg when mTg is injected in CFA (Charreire, 1989). Induction of Tg-specific IgG responses was monitored by ELISA and thyroiditis was assessed by concomitant histological examination of the thyroid glands.

3.2 Results

3.2.1 Separation of immunoconjugates by HPLC gel filtration

Analysis of a simple mixture of mTg and MAb specific for MHC class II Ag (I-A^k) by gel filtration through a TSK-4000SW size-exclusion column (**Section 2.5**) indicated that free MAb eluted between 20.5 and 21.4 min, and free mTg at 15.6 min. (Figure 3.1A). Similar analysis of a conjugate mixture by gel filtration, obtained after cross-linking of mTg with MAb (anti-I-A^k MAb) by glutaraldehyde (**Section 2.5**), revealed a single high molecular weight peak containing soluble conjugates at 10.6 min, whereas small amounts of free mTg or MAb remained in the reaction mixture (Figure 3.1B). A similar HPLC profile was obtained with a simple mixture of mTg and control MAb specific for influenza NP (Figure 3.2A) and after cross-linking mTg to a MAb specific for influenza NP (Figure 3.2B). HPLC purified protein from each conjugate peak was subsequently used for immunization.

3.2.2 Immunotargeting of mTg-(I-A^k MAb) conjugate <u>in vivo</u> elicits mTg-specific IgG response in H-2^k but not in H-2^b mice

CBA (H-2^k) and B6 (H-2^b) mice were selected for the present study since they are known to elicit significant mTg-specific IgG responses when challenged with mTg in CFA, although the Ab titer and thyroiditis induction is higher in CBA than in B6 mice (Vladutiu and Rose 1971). Mice from each

Figure 3.1A-B Comparison of HPLC profile of (A) a simple mixture of mTg and anti-I-A^k MAb, (B) a sample following conjugation with glutaraldehyde of mTg with anti-I-A^k MAb. (A) 50 μl of simple mixture of mTg and anti-I-A^k MAb or (B) 50 μl of conjugate of mTg with anti-I-A^k MAb were passed through TSK-4000SW size-exclusion column using phosphate buffer, pH 7.0, at a flow rate of 1.0 ml/min. Horizontal axis represents time in minutes, vertical axis represents O.D. at 280nm.

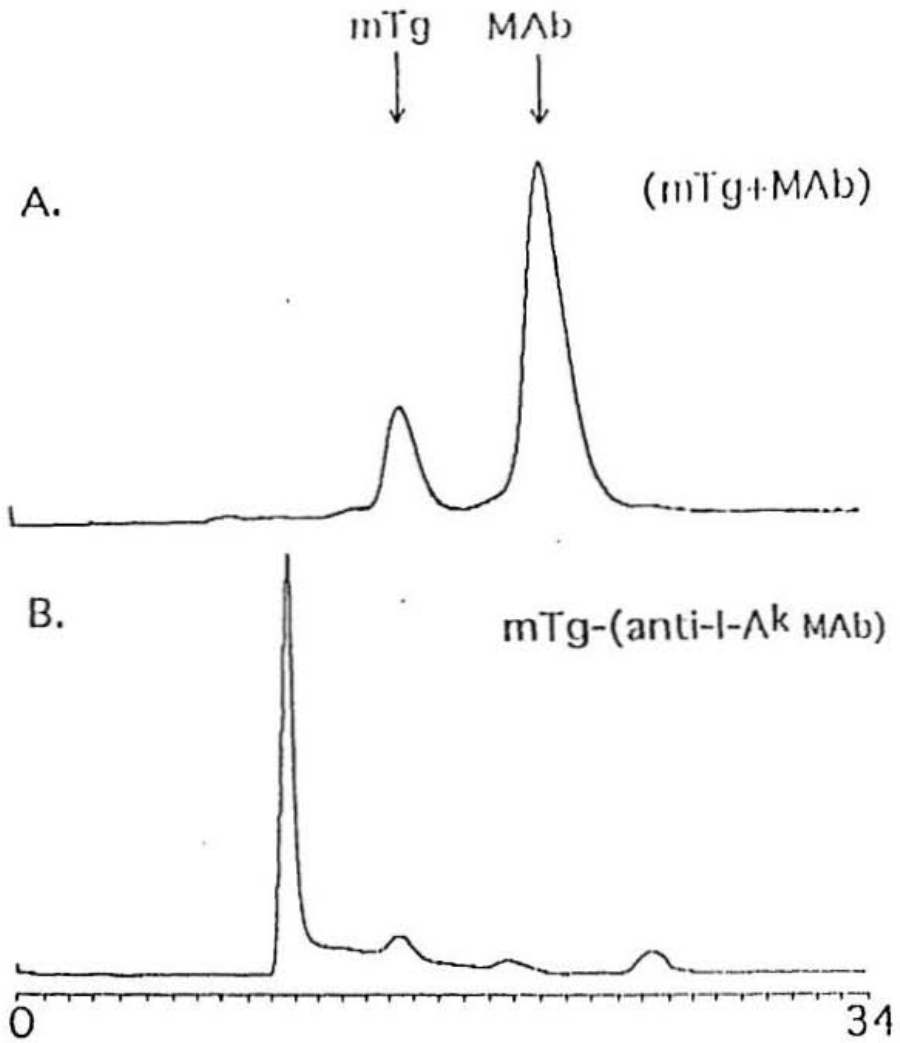
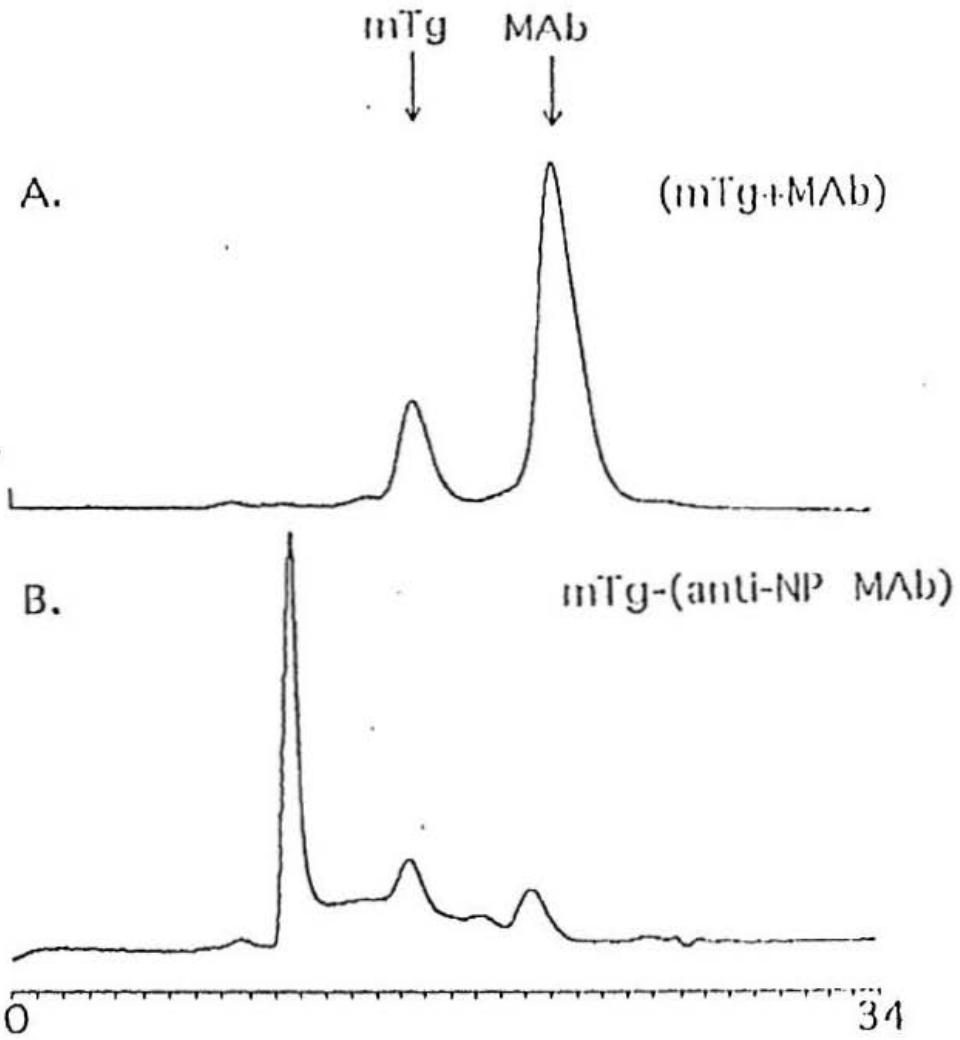

mTg
MAb
A.
(mTg+MAb)
B.
mTg-(anti-I-Ak MAb)
0
34

Figure 3.2A-B Comparison of HPLC profile of (A) a simple mixture of mTg and anti-Influenza nucleoprotein (NP) MAb, (B) a sample following conjugation with glutaraldehyde of mTg with anti-Influenza NP MAb. (A) 50 μl of simple mixture of mTg and anti-influenza NP MAb or (B) 50 μl of conjugate of mTg with influenza NP-specific MAb were passed through a TSK-4000SW size-exclusion column using phosphate buffer, pH 7.0, at a flow rate of 1.0 ml/min. Horizontal axis represents time in minutes, vertical axis represents O.D. at 280nm (as in Figure 3.1).

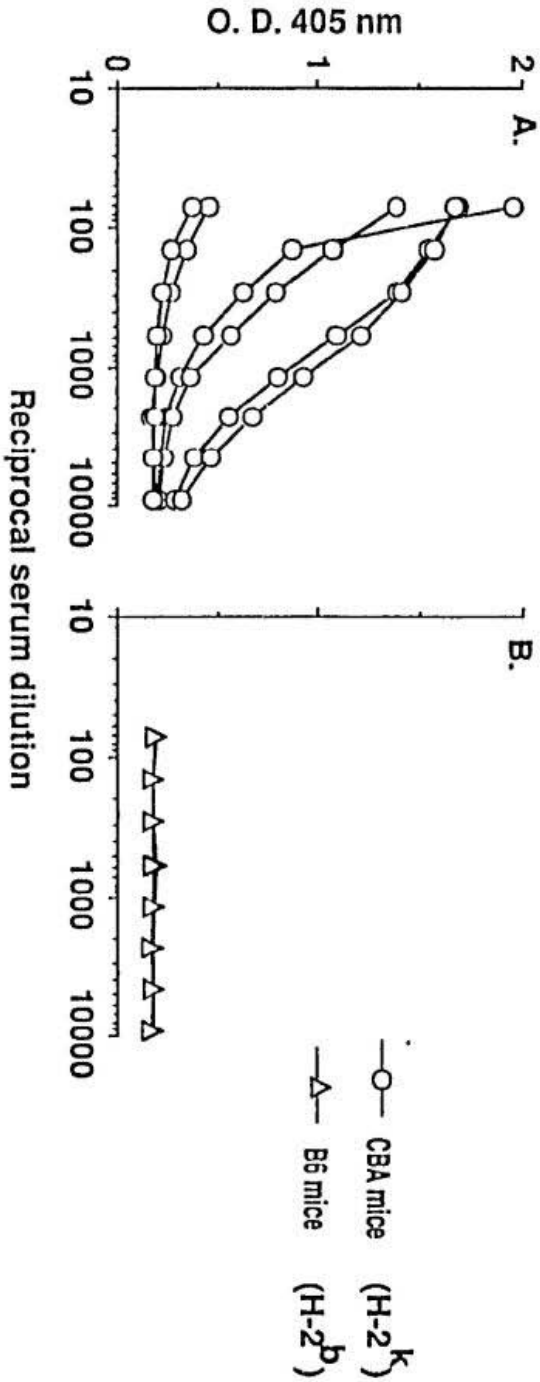

mTg
MAb
A.
(mTg+MAb)
B.
mTg-(anti-NP MAb)
0
34

strain (6/group) were primed s.c. at the base of the tail with 5 μg of mTg-(anti-I-A^k MAb) conjugate in 100 μl PBS on day zero and boosted i.p. with 50 μg of mTg in 100 μl PBS on day 21. Serum samples were collected on day 35 after the priming injection (**Section 2.6**). Significant IgG responses were observed only in CBA mice expressing I-A^k (Figure 3.3A) but not in B6 mice expressing I-A^b (Figure 3.3B). These findings are interpreted to be a result of focusing of mTg onto APC by the immunotargeting approach during the immunogenic challenge, because IgG responses were elicited in mice bearing appropriate MHC class II alleles only.

3.2.3 Priming with control immunoconjugate mTg-(anti-NP MAb) <u>in vivo</u> does not elicit an mTg-specific IgG response in H-2^k mice

To further test the effect/specificity of targeting of mTg onto APC by the immunotargeting approach, control CBA mice (2 mice/group) were injected with either 25 μg of purified mTg-(anti-NP MAb) conjugate in 100 μl PBS or 50 μg of mTg in CFA (as a positive control) s.c. at the base of the tail and were boosted with 50 μg of free mTg in 100 μl PBS as described (**Section 2.6**). Testing of the immune sera 35 days after the initial (priming) injection failed to reveal the presence of mTg-specific IgG in CBA mice that received the mTg-(anti-NP MAb) conjugate (Figure 3.4). In contrast, CBA mice that were

Figure 3.3A-B Determination of mTg-specific secondary IgG responses to immunotargeted mTg in CBA (A), and B6 (B) mice. IgG reactivity to mTg in individual mouse sera (Day 35) from CBA (6/group) but not B6 (6/group) mice primed on Day 0 with the indicated antigens and boosted on Day 21 with free mTg as described under **Section 2.6.** O.D. 405 nm values from an alkaline phosphatase-based ELISA represent mean +/- SD of triplicate wells. The assay was performed as described earlier (Chronopoulou and Carayanniotis, 1992).

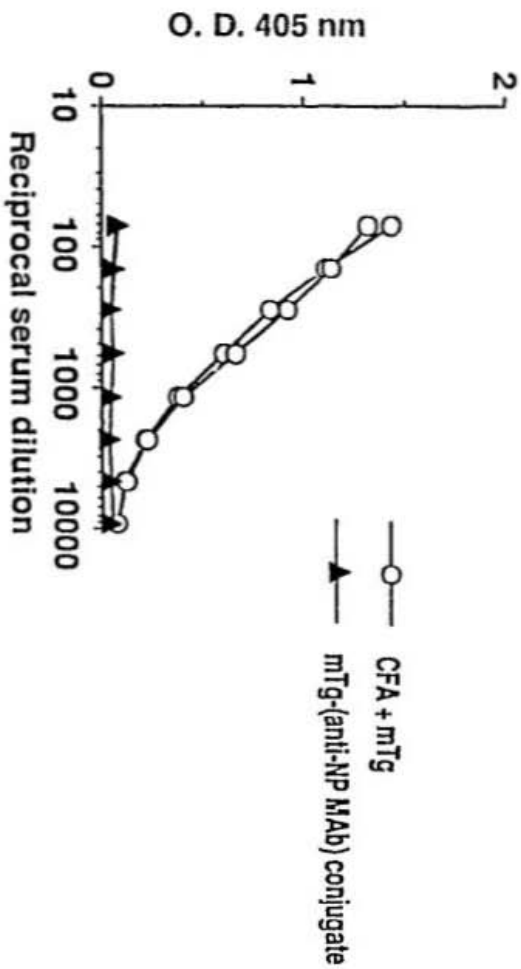

O. D. 405 nm
2
1
0
A.
B.
10
100
1000
10000
Reciprocal serum dilution
CBA mice (H-2k)
B6 mice (H-2b)

primed with mTg in CFA mounted a strong mTg-specific IgG response (Figure 3.4). The Ab titers induced after priming of mice with CFA + mTg were equivalent to those obtained after immunotargeting of mTg in the absence of adjuvant in CBA mice (Figure 3.3A). These findings further suggest that the enhancement of mTg immunogenicity in CBA mice after its conjugation to the anti-class II MHC MAb is not due to a possible aggregation effect, chemical modification of mTg, or non-specific uptake of immunoconjugate. These data are compatible with the hypothesis that specific delivery of mTg by MAb onto APC bearing appropriate MHC class II Ags leads to the abrogation of natural tolerance to mTg.

3.2.4 Immunotargeting of mTg favors induction of an IgG response but not thyroiditis in CBA mice

Histological examination of the thyroid glands obtained from CBA mice that received mTg-(anti-I-A^k MAb) conjugate for priming (from the experiment shown in Figure 3.3A) did not reveal infiltration with mononuclear cells (0/6). The data clearly indicated that, despite the presence of an mTg-specific IgG response, thyroid infiltration with mononuclear cells did not occur. The data also indicate that mTg-specific antibodies raised by this particular procedure were not pathogenic in mice.

Figure 3.4 Determination of mTg-specific secondary IgG responses in CBA mice primed with CFA + mTG (A) and mTg-(anti-NP MAb) conjugate (B). IgG reactivity to mTg in individual mouse sera collected on day 35 from CBA mice (2 mice/group) that were primed on Day 0 with the indicated antigens and boosted on Day 21 with free mTg as described under **Section 2.6.** O.D. 405 nm values from an alkaline phosphatase-based ELISA represent mean +/- SD of triplicate wells. The assay was performed as described earlier (Chronopoulou and Carayanniotis, 1992).


O. D. 405 nm
0
1
2
10
100
1000
10000
Reciprocal serum dilution
CFA + mTg
mTg-(anti-NP MAb) conjugate

3.3 Discussion

The results of this study demonstrate for the first time in EAT that adjuvant-free challenge of mice with mouse Tg (mTg) abrogates natural tolerance to mTg. This was achieved by targeting small doses of mTg onto APC expressing appropriate MHC class II Ags using MAbs (anti-MHC class II) as delivery vehicles.

The precise mechanism(s) underlying the observed MAb-mediated enhancement in the immunogenicity of conjugated Tg is (are) not clear. The most plausible reason for the observed effect may be due to the direct focusing of or targeting of Tg onto various APC (dendritic cells, B cells and macrophages) expressing appropriate MHC class II determinants. This would lead to internalization, processing and surface expression of Tg epitopes in the context of MHC class II molecules on APC. The peptide-MHC class II complexes would then be recognized by T helper (TH) cells, which in turn provide the second signal to mTg-specific B cells. Subsequently, this would result in the induction of a humoral immune response since IgG responses to Tg are T cell-dependent (Vladutiu and Rose, 1975). In this model, B cells (compared to macrophages and dendritic cells) are expected to act as APC by internalizing the conjugate either via their MHC class II Ags (specific pathway) or IgG receptors (non-specific pathway) expressed on their surface as suggested earlier (Carayanniotis and Barber,

1987; Skea and Barber, 1993).

Alternatively, the targeted MAb may also enhance the immunogenicity of the Ag non-specifically by inducing immunoglobulin (anti-I-A^k MAb)-specific TH cells that help mTg-specific B cells and promote an mTg-specific IgG response by a process called "linked recognition". This mechanism of immune recognition was described earlier (Janeway, 1983; Lesserman, 1985). The present findings, however, do not support the above possibility (linked-recognition mechanism), because mTg-specific IgG responses were not observed in H-2^b mice immunized with mTg-(anti-I-A^k MAb) conjugate or CBA mice immunized with mTg-(anti-NP MAb) conjugate (control).

The mechanism(s) by which the targeted MAbs specific for MHC class II Ags confer their adjuvanticity in vivo is/are not clear. It is possible that during cognate T-B cell interaction, recognition of MHC-peptide complexes on B cells by TcR of Tg-specific TH cells, leads to induction of costimulatory molecules, such as B7 on naive B cells, and these $B7^+$ B cells may effectively present the autoantigen to T cells by providing a second signal to T cells, leading to T cell activation. These activated T cells in turn may help Tg-specific B cells in vivo analogous to the results obtained in vitro as shown earlier (Nabavi et al, 1992). Alternatively, the adjuvanticity conferred by targeted MAb specific for MHC class II determinants may also be attributed to the ability of

targeted MAb to activate APC leading to secretion by APC of endogenous mediators such as interleukin-1 (IL-1) (Placois, 1985). In that regard, human recombinant IL-1α was shown earlier to enhance splenic plaque-forming cells to sheep RBC both in vitro and in vivo (Reed et al, 1989). In the murine system, evidence to support this possibility is not documented and remains to be established. It is worth noting that adjuvants such as LPS and MDP (a component of CFA), that have been used for induction of EAT, exert their adjuvanticity through the induction of IL-1 secretion by APC (Lasfargues et al, 1987; Lise and Audibert, 1989).

In the present study, despite the adjuvant free induction of an mTg-specific IgG response by immunotargeting, concomitant mononuclear cell infiltration of the thyroid was not observed in the responder CBA mice. In EAT studies, it is known that mTg-specific IgG responses are more easily induced than thyroid lesions (Charreire, 1989; Rose et al, 1971) and do not correlate with disease (thyroiditis) development. These data suggested that these two autoimmune manifestations are under different immunoregulatory control. Thus, the possibility that immunotargeting favours an autoantibody (AAb) response rather than thyroiditis induction cannot be excluded. Inability to induce thyroiditis in CBA mice by this approach may either be due to the low doses of immunoconjugates (5 μg of the conjugate/mouse) employed or failure to overcome the

immune mechanisms that regulate the induction of T cells associated with pathogenic thyroid autoimmunity.

Adjuvant free induction of EAT in mice by injecting large doses of mTg in saline at repeated intervals was reported earlier (EIRehewy et al, 1981). Similarly, Jermy et al (1993) also reported the adjuvant-free induction of experimental autoimmune myasthenia gravis (EAMG) in mice by injecting very small amounts of Ag, equivalent to 1 μg of affinity purified mouse AChR, in saline. The present observations that induction of Ab responses but not thyroid lesions by immunotargeting of mTg in mice suggests that the immunoregulatory mechanism(s) involved in the breakdown of tolerance leading to induction of autoreactive IgG response in mice is different from those involved in the induction of mononuclear cell infiltration of thyroid (pathogenic autoimmune response).

Carayanniotis and Barber (1987) have demonstrated adjuvant free-induction of IgG responses to avidin by targeting conjugate onto APC using MHC class II Ags as targets. In that study, the authors have used biotin as a cross-linker to produce effective Ag-Ab conjugates. In the present study, I have also demonstrated a targeting effect at low doses of injected Tg-MAb conjugate (Figure 3.3A-B). In contrast to the method used in the study by Carayanniotis and Barber (1987), I have used glutaraldehyde as a cross-linker

to produce effective immunoconjugates.

The present findings extend the previous reports describing the methods for enhancement of immunogenicity of foreign proteins such as avidin (Carayanniotis and Barber 1987; 1990; Carayanniotis et al, 1991), ferritin (Kawamura and Berzofsky, 1986), influenza haemagglutinin (Barber and Carayanniotis, 1988), hen egg lysozyme (Snider and Segal 1989; Snider et al, 1990), pigeon cytochrome C (Casten and Pierce,1988), foreign peptides (Carayanniotis et al 1988; Casten et al, 1988; Wyss et al, 1991) and haptens (Mjaaland and Fossum, 1990 and 1991).

The results described in the present one and other studies (Carayanniotis et al, 1987; 1988; 1990; 1991., Skea and Barber, 1993) suggest that professional APC such as dendritic cells, B cells, and macrophages play a role in the enhanced immunogenicity of mTg. However, the contribution towards Ag presentation by each of these APC populations is unknown. It is worth noting the report of Berg et al (1994) wherein the authors show that targeting of FITC onto APC expressing appropriate MHC class II Ags induced strong Ag-specific primary and secondary IgG responses in rats. These authors also showed that dendritic cells but not B cells or macrophages initiate the humoral immune response in MHC class II-mediated Ag delivery. These latter data furthermore support previous findings, implicating a primary role for

dendritic cells in the induction of immune response, obtained with a different methodology in which extracorporeally Ag-pulsed dendritic cells, when injected in saline into mice, initiated either humoral (Sornasse et al, 1990) or cell-mediated immune responses (Knight et al, 1988).

In the present study, MHC class II Ags have been exploited as targets to deliver the autoantigen (mTg) onto diverse APC populations, whereas in other studies IgD molecules have been exploited as targets to deliver diverse Ags such as bovine serum albumin (BSA) and fluorescein isothiocyanate (FITC) specifically onto B cells (Lees et al, 1990; Berg et al, 1994). Lees et al (1990) have reported the enhancement of the immunogenicity of BSA, in mice in the absence of adjuvant, by targeting BSA (100 μg conjugate /mouse) onto B cells using goat-anti-mouse IgD MAb. In contrast, Berg et al (1994) reported recently that targeting of FITC (25 μg conjugate/mouse) onto B cells using goat-anti rat IgD MAbs as delivery vehicles induced a weak specific-primary IgG response in rats. Thus, the efficacy of immunotargeting at enhancing the immunogenicity of Ags might be influenced by various factors such as the type of Ag (multivalent versus hapten) or the dose of the Ag or the species (rat versus mouse) or a combination of these parameters.

While the results obtained in the present study

demonstrate the induction of autoreactivity by targeting self-antigen onto APC, recent studies have also demonstrated the potential of the immunotargeting approach in suppressing autoimmunity. Day et al (1992) have described the suppression of experimental autoimmune encephalomyelitis (EAE) by targeting a pathogenic peptide of myelin basic protein (MBP) onto B cells with IgD-specific MAbs in Lewis rats. In this model, although EAE was suppressed, MBP-specific T cells were not anergized. In another report, Reim et al (1992) have shown that targeting of AChR on B cells in vitro normally enhances its presentation to specific T cells, whereas targeting followed by fixation of APC induced specific T cell anergy. Therefore, key factors such as the kind of Ag and of targeting MAb used, the type of targeted APC, the conjugate dose, and the route of administration can greatly influence the outcome of the immune response. The relative importance of each of these parameters remains to be established.

The difficulty in eliciting an autoimmune response with soluble autoantigen in saline necessitates the use of potent adjuvants. Most commonly used CFA is known to present problems such as inflammatory lesions (granulomas and open sores) at the immunization site (Broderson, 1989; Wiedman et al 1991) and exerts poorly understood effects on the immune system. The value of the immunotargeting approach in the studies of autoimmunity lies in its capacity to direct

autoantigen onto selected APC populations either in vitro or in vivo and to avoid initiation of autoreactivity in the context of simulated infection (Cohen, 1992) provided by CFA. In conjunction with the use of defined pathogenic epitopes, immunotargeting can provide new impetus in the study of regulatory mechanisms of adjuvant-free activation or suppression of an autoimmune response.

CHAPTER 4

Induction of Experimental Autoimmune Thyroiditis in Rats with the Synthetic Peptide (2495-2511) of Thyroglobulin

4.1 Introduction

Experimental autoimmune thyroiditis (EAT) in rats, induced after challenge with rat thyroid extract or rat thyroglobulin (rTg) in adjuvant (Jones and Roitt, 1961; Paterson and Drobish, 1968; Twarog and Rose, 1969, Rose, 1975; Lillehoj et al, 1981; De Assis-Paiva,1989), has served for decades as a model for Hashimoto's thyroiditis (HT) in humans. Various inbred rat strains differ in their susceptibility to EAT and it appears that MHC genes play a major role in determining the induction of the disease (De Assis Paiva, 1989) as observed in mouse EAT (Charreire, 1989). Significant non-MHC gene effects in rat EAT have also been reported (Lillehoj et al, 1981). Thyroid lesions induced in rats following Tg challenge have been found to correlate with lymph node cell (LNC) proliferative responses to rTg <u>in vitro</u> or delayed hypersensitivity (DTH) responses to rTg <u>in vivo</u> (Lillehoj and Rose, 1982). In contrast, no significant correlation has been observed between rTg-specific Ab levels and the degree of thyroid damage (Rose, 1975; Lillehoj and Rose, 1982).

Interpretation of genetic analyses and studies at the T-cell clonal level in EAT have been hampered by the fact that the autoantigenic determinants on Tg remain unknown, mainly because of the large size of Tg molecule (dimeric Tg MW = 660 kDa). Recently, there have been attempts from several laboratories to delineate T-cell epitopes on Tg. Champion et al (1991) identified an immunodominant and phylogenetically conserved 9mer Tg peptide 2551-2559 containing thyroxine at position 2553 and demonstrated that this peptide could trigger the activation of murine T-cell hybridomas. Subsequently, Hutchings et al (1992) also demonstrated that Tg peptide (2551-2559)-specific T cells could adoptively transfer thyroiditis to naive syngeneic mice. In addition, Texier et al (1992) identified an immunodominant and phylogenetically conserved 40mer human Tg (hTg) peptide that induced mild thyroiditis in mice. This Tg peptide was recognized by a cytotoxic T cell hybridoma ($CD4^-$, $CD8^+$, TcR α/β^+) in the context of MHC class I molecules.

Chronopoulou and Carayanniotis (1992) have also recently reported that the 17mer rat Tg (rTg) peptide 2495-2511 (rTgP1) induced thyroiditis in a heterologous species (mice) and elicited T cell and IgG responses. TgP1-induced IgG cross-reacted with Tgs from various species. These authors identified the TgP1 sequence by using algorithms (Margalit et al, 1981; Rothbard and Taylor, 1988) that scanned the known

portion of rTg (Di Lauro et al, 1985) for putative T cell epitopes since the primary amino acid sequence of mouse Tg (mTg) is unknown. Since TgP1 is phylogenetically conserved among rat, bovine and human Tgs, and causes thyroiditis in mice, it is possible that TgP1 constitutes a self peptide in mice. However, the lack of data concerning the primary amino acid sequence of mTg raises the possibility that recognition of xenogeneic determinants on TgP1 contributed to its immunopathogenicity.

The present study was undertaken to examine EAT induction with TgP1 in a homologous system using WKY, F344 and WF rat strains that are known to be EAT-susceptible after Tg challenge (Lillehoj and Rose, 1982). EAT was monitored by mononuclear cell infiltration of the thyroid and T-cell or B-cell autoreactivity.

4.2 Results

4.2.1 TgP1 is thyroiditogenic in rats

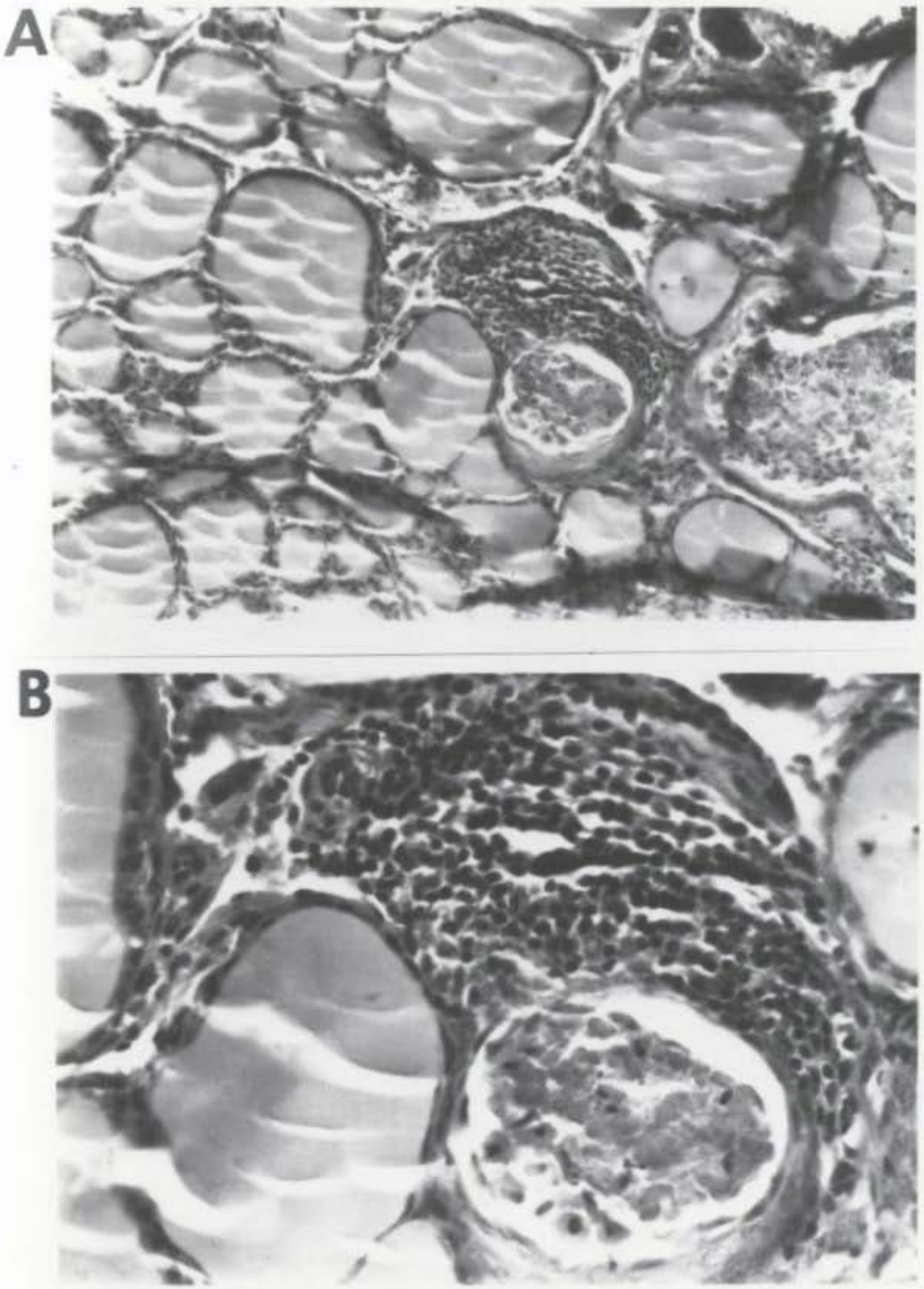
Since the Tg sequence 2495-2511 (TgP1) can induce EAT in mice, an attempt was made in the present study to determine whether it could similarly induce thyroiditis in the EAT-susceptible rat strains WF, F344, and WKY. Four rats per strain were s.c. primed with 200 nmol (380 μg) TgP1 in CFA, followed immediately by an i.v. challenge with Bordetella pertussis organisms. All animals were boosted with 100 nmol (190 μg) TgP1 in IFA on day 7 as described under **Section 2.9.** Twenty eight days after the first challenge, the thyroids were removed and subjected to histologic examination. Rats that received CFA plus Bordetella pertussis only served as controls. As shown in Table 4.1, three out of four (75%) WKY and WF rats and one out of four (25%) F344 rats developed mononuclear cell infiltration of the thyroid gland after peptide challenge. Infiltration had the appearance of either discrete perivascular foci (Figure 4.1) or diffuse interstitial accumulation of mononuclear cells surrounding the thyroid follicles. On the other hand, thyroids collected from adjuvant-primed rats did not reveal mononuclear infiltration. The results revealed the pathogenicity of TgP1 in rats of diverse MHC haplotypes and confirmed that TgP1 can induce thyroiditis in a homologous species.

Table 4.1 EAT induction by TgP1 in various rat strains[a]

Strain	Haplotype	Infiltration index 0	0.5	1	2	3	4	Rats with thyroid lesions	Incidence
WKY	*k*	1	–	2	1	–	–	3/4	75%
F344	*l*	3	–	1	–	–	–	1/4	25%
WF	*u*	1	1	1	1	–	–	3/4	75%

[a] Rats were primed with 200 nmol of TgP1 and *B.pertussis* and one week later, they were boosted with 100 nmol of peptide. Three weeks after the second challenge the thyroid glands were removed and mononuclear cell infiltration of the thyroid was assessed. Immunization and histological analysis was performed as described in Materials and Methods (Sections 2.8 and 2.9). No infiltration was observed in control animals (2 rats / group) that received CFA and *B. pertussis* only (not shown).

Figure 4.1 Photomicrographs showing TgP1-induced mononuclear cell infiltration in the thyroid. Focal mononuclear cell infiltration of the thyroid was induced after challenge of WKY rats with TgP1 (infiltration index of 1.0); (A) 100X, (B) 250X.


A
B

4.2.2 TgP1 induces strong secondary proliferative responses of lymph node cells (LNC) *in vitro*

The relative immunogenicity of TgP1 was examined in WF, F344 and WKY rat strains by proliferative assays. Rats were immunized in one hind footpad on day 0 with 200 nmol (380 μg) TgP1. Fourteen days later, the draining popliteal LNC were obtained and cultured in the presence or absence of TgP1. As shown in Figure 4.2A, LNC from WKY rats proliferated most strongly *in vitro* to TgP1 at the peptide concentration range tested (22.7 - 0.3 μM). Popliteal LNC from F344 and WF rats responded significantly as well. The responses were specific since no LNC proliferation was observed in cultures containing control Ag, ovalbumin (see legend to Figure 4.2A). Analysis of TgP1-primed sera from the above rat strains (of Figure 4.2A) by direct ELISA, did not reveal the presence of TgP1-specific primary IgG responses (Figure 4.2B).

Challenge of WKY rats with titrated amounts of peptide revealed that as little as 5 nmol (9.5 μg) of TgP1 can prime the rats for a significant popliteal LNC proliferative response to TgP1 *in vitro* (Table 4.2). LNC from any individual rat group did not respond to an unrelated Tg peptide (2550-2567), but proliferated strongly to PPD. The data indicated that TgP1 is strongly immunogenic at the T cell level, and TgP1-specific LNC proliferative responses correlate with susceptibility of rats to thyroiditis.

Figure 4.2A TgP1-specific LNC proliferative responses in rats of various RT1 haplotypes. Rats (2 rats/group) were injected with 100 μl of CFA emulsion containing 200 nmol (380 μg) TgP1 into one hind footpad and 14 days later the draining popliteal LNC were removed and incubated with the indicated concentrations of TgP1 for 4 days. ^{3}H-TdR was added during the last 18 hr of the culture. The assay was performed as described under **Section 2.7.** The values were expressed as SI +/- SD of quadruplicate wells. Background mean cpm of quadruplicate wells were WKY = 6,893, F344 = 11,632, and WF = 3,472. Proliferative responses (mean cpm) of TgP1-primed LNC to control antigen, ovalbumin (10 μg/ml) as follows: F344 = 15, 422; WKY = 9,762; WF = 5,341.

Figure 4.2B TgP1-priming does not elicit specific-primary IgG responses in rats. The pooled serum samples of TgP1-immunized rats that were used in the priming experiment (of Figure 4.2A) were analyzed for TgP1-specific IgG in an alkaline phosphatase-based ELISA as described under **Section 2.19.** O.D. 405 nm readings were expressed as mean +/- SD of triplicate wells at the indicated dilutions of the serum.

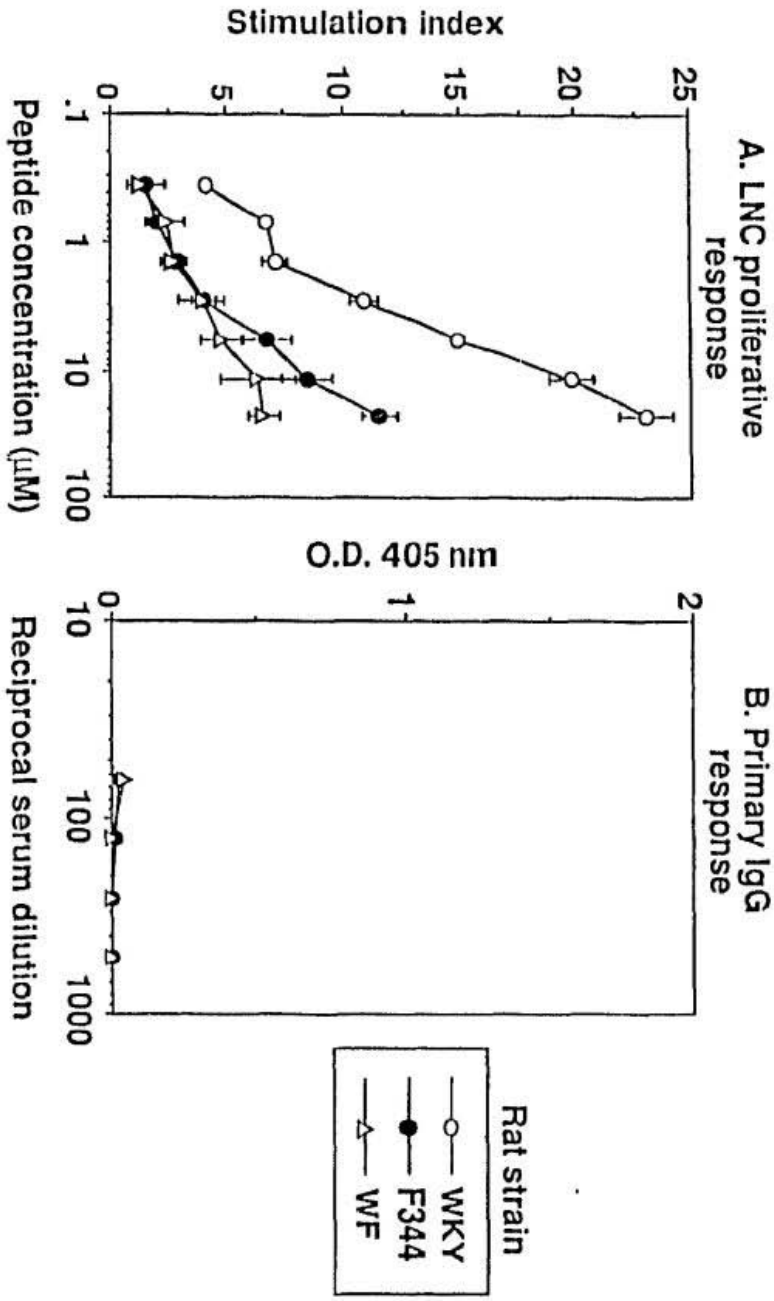

A. LNC proliferative response
Stimulation index
0
5
10
15
20
25
.1
1
10
100
Peptide concentration (μM)
B. Primary IgG response
O.D. 405 nm
0
1
2
10
100
1000
Reciprocal serum dilution
Rat strain
WKY
F344
WF

Table 4.2 Effect of antigen dose *in vivo* on TgP1 immunogenicity

TgP1 dose *in vivo* (nmol)	LNC proliferation in vitro (Δcpm +/- SD) in the presence of[a]		
	TgP1	Tg peptide (2550-2567)	PPD
25	21,305 +/- 2,889	1,675 +/- 612	73,235 +/- 6,386
5	18,480 +/- 4,286	1,056 +/- 2.195	81,332 +/- 3,483
1	2,287 +/- 1,848	3,655 +/- 3,804	85,437 +/- 4,231
0.2	4,915 +/- 436	635 +/- 1,796	79,867 +/- 1,989

[a] Popliteal LNC from WKY rats primed *in vivo* with the indicated dose of TgP1 were stimulated for 4 days *in vitro* with TgP1 (12 µg/ml), Tg peptide (2550-2567) (12 µg/ml) or PPD (10 µg/ml). ^{3}H-TdR was added during the last 18 hr of culture. The assay was done as described under Materials and Methods (Section 2.6). Background cpm in the absence of antigen were : 25 nmol =11.201, 5 nmol = 12.089, 1 nmol = 25,933 and 0.2 nmol = 8,283.

4.2.3 TgP1 is recognized by CD4$^+$ T cells in the context of both RT1-B and RT1-D MHC class II molecules

To address whether TgP1 is recognized in the context of RT1-B (I-A equivalent) or RT1-D (I-E equivalent) MHC class II molecules, various MAbs were used to block the proliferative response of TgP1-primed rat LNC to TgP1 in vitro. As shown in Table 4.3, peptide-specific proliferation of LNC from all rat strains was strongly inhibited (60-88%) in the presence of W3/25 (anti-CD4) MAb, suggesting that the response was mainly dependent on CD4$^+$ T cells. Addition of OX-6 MAb, specific for RT1-B antigens (I-A equivalent), significantly blocked TgP1-specific proliferation of LNC from F344 rats (86%), and to a lesser extent of LNC proliferation from WKY and WF rats (57% and 37% respectively). In the presence of OX-17 MAb, specific for RT1-D antigens (I-E equivalent), the response of F344 rat LNC was again strongly inhibited (67%) while LNC from WKY and WF rats were also significantly inhibited (31% and 42% respectively). No significant blocking was observed by the control MAb (H16-L10-4R5) specific for influenza type A virus nucleoprotein. The data suggested the presence of TgP1 epitope(s) that could be recognized in the context of either RT1-B or RT1-D gene products by CD4$^+$ T cells.

4.2.4 TgP1 contains cryptic T cell epitope(s)

The non-immunodominant nature of TgP1 was demonstrated

Table 4.3 MHC-restriction of TgP1-specific LNC proliferative response *in vitro*

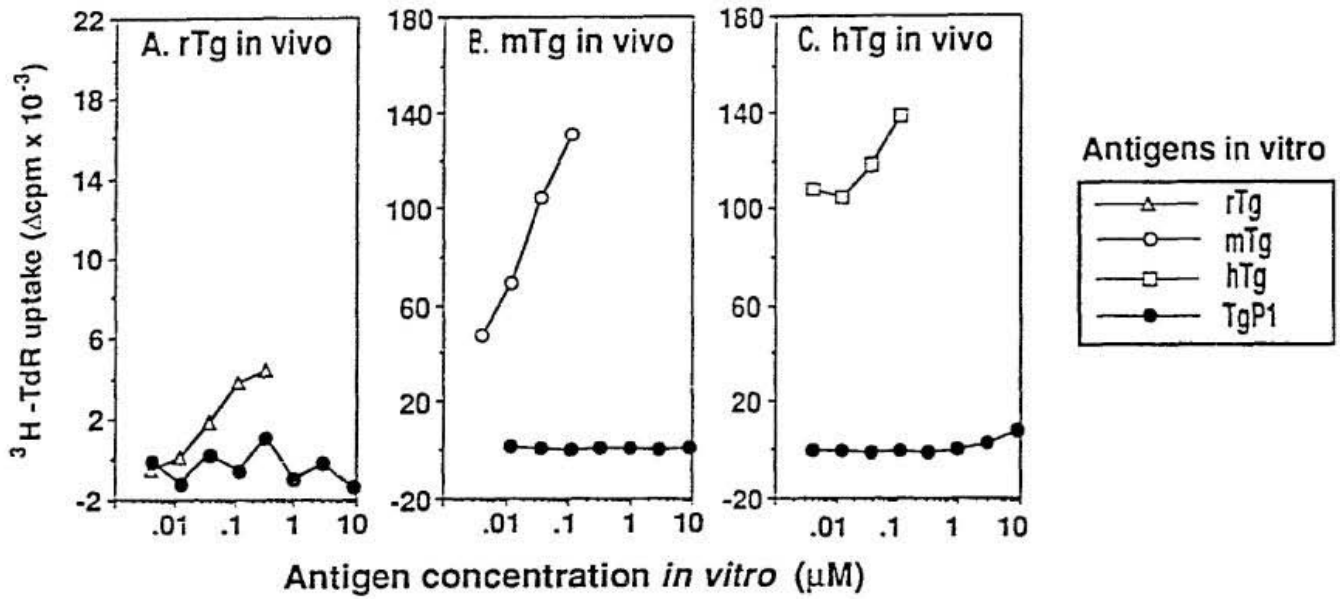
Blocking MAb	In vitro proliferation (cpm +/- S.D.) of TgP1-primed LNC from [a]		
	WKY	F344	WF
none	18,790 +/- 1,421 (0)[b]	22,675 +/- 2,027 (0)	12,013 +/- 2,056 (0)
W3/25	7,520 +/- 507 (60)	2,802 +/- 585 (88)	3,372 +/- 375 (72)
OX-6	7,996 +/- 1,117 (57)	3,091 +/- 935 (86)	7,531 +/- 1,030 (37)
OX-17	13,048 +/- 1,185 (31)	7,581 +/- 2,130 (67)	6,953 +/- 662 (42)
HB-65	N.D.	25,357 +/- 3,543 (-12)	15,149 +/- 3,270 (-26)

[a] Popliteal LNC from the indicated rat strains were primed *in vivo* with 12.5 nmol TgP1 in CFA and 14 days later were cultured *in vitro* with 6.2 µg/ml peptide and the indicated MAb. W3/25 (anti-CD4), OX-6, (anti-I-A) and HB-65 (H16-L10-4R5, anti- influenza A nucleoprotein) were used at 3.2 µg/ml and OX-17 (anti-I-E) at 1.6 µg/ml. At these concentrations, the indicated MAbs were non toxic since they did not inhibit Con A stimulation of rat splenic cells (data not shown). Tne assay was done as described under Materials and Methods (Section 2.7). The background cpm were : WKY = 6,704, F344 = 4,110, WF = 2,717.

[b] Percent inhibition of proliferation calculated as described in Materials and Methods (2.7)

earlier in a heterologous species (mice) (Chronopoulou and Carayanniotis, 1992) in the context of homologous (mTg) and heterologous Tg (rTg). The non-dominance of TgP1 on Tg reported earlier in mice may not apply in rats by virtue of possible differences in the Ag processing and presentation by lymph node APC of mice and rats. Therefore, it was important to reexamine the relative immunodominance of this peptide in the context of both homologous (rat) and heterologous (mouse and human) Tgs in rats. WKY rats (2/group) were primed by injection into one hind footpad of rat or mouse or human Tgs in CFA. Fourteen days later, the draining popliteal LNC were challenged in vitro with the corresponding Tg or equimolar concentrations of TgP1. The results are shown in Figure 4.3. The results demonstrated that priming with homologous or heterologous Tgs induced significant LNC proliferative responses to the corresponding Tg. However, no response was detected against TgP1 in vitro (Figure 4.3A-C). Lack of TgP1 reactivity was observed over a wide range of peptide concentrations in vitro that were clearly stimulatory to LNC primed with TgP1 in vivo (Figure 4.2A). Thus, even though TgP1 alone is immunogenic and pathogenic in rats, it cannot prime the host when it is given in the context of intact Tg, suggests that TgP1 is a cryptic determinant within Tg in rats.

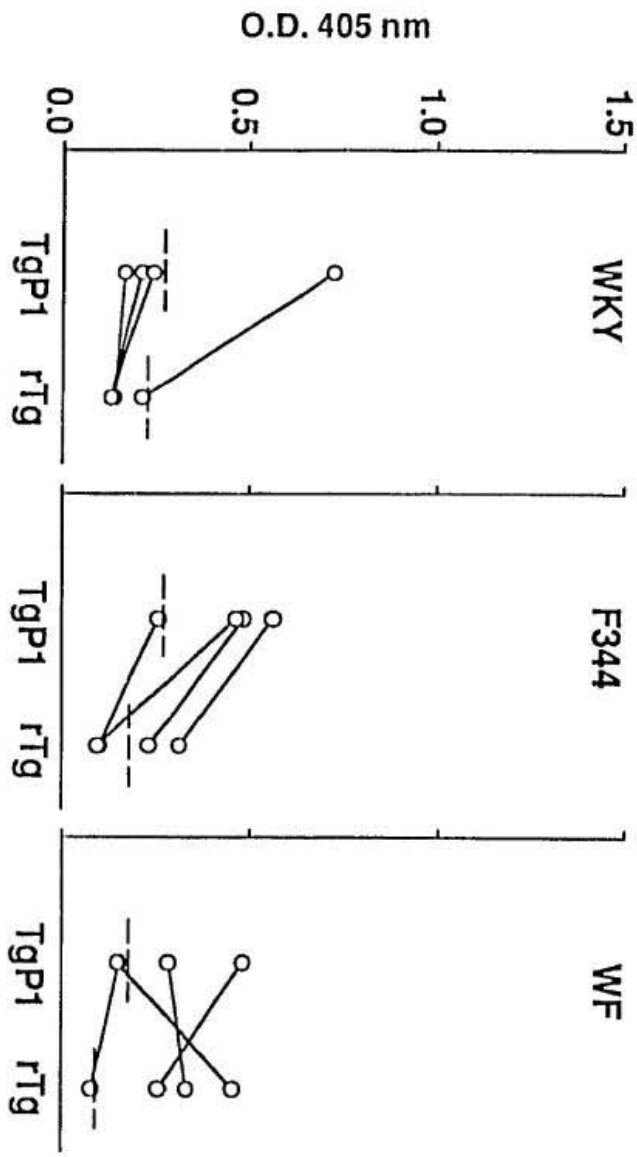
Figure 4.3 Rat LNC primed with homologous or heterologous Tg <u>in vivo</u> do not respond to TgP1 <u>in vitro</u>. WKY rats (2 rats/ group) were primed in one hind footpad with 200 μg of the indicated Tg in 100 μl CFA emulsion and 14 days later popliteal LNC were removed and cultured in the presence of the respective Tg or TgP1 for four days. ^{3}H-TdR was added during the last 18 hr of the culture. The assay was done as described under **Section 2.7.** The values were expressed as mean cpm +/- SD of quadruplicate wells. Background mean cpm of quadruplicate wells were rTg = 7,109, mTg = 3,903, and hTg = 23,439.


A. rTg in vivo
B. mTg in vivo
C. hTg in vivo
^{3}H -TdR uptake (Δcpm x 10^{-3})
Antigen concentration *in vitro* (μM)
Antigens in vitro
rTg
mTg
hTg
TgP1

4.2.5 TgP1-priming of rats does not elicit strong peptide-specific IgG responses

On day 28, sera were collected from rats that had been used in the experiment shown in Table 4.1. Sera were tested individually for the presence of Abs against the peptide or rTg by alkaline phosphatase-based ELISA. As shown in Figure 4.4, one out of four WKY, two out of four WF, and three out of four F344 rats showed a weak but detectable IgG response to the peptide. Sera from all rat strains showed weak or no cross-reactivity to rTg. Thus, strong peptide-specific IgG responses were not detected despite the presence of thyroiditis in rats.

Figure 4.4 Determination of TgP1-specific IgG responses by ELISA. Individual sera (at 1/32 dilution) from the indicated rat strains were tested for the presence of IgG specific for TgP1 and for IgG cross-reactive with rTg. Open circles represent individual rats. Data are obtained from complete serum titration curves. Dotted lines represent background O.D. values obtained with sera (at 1/32 dilution) of control rats (2 rats/strain) challenged with CFA and heat-killed Bordetella pertussis organisms. Standard deviation (SD) of triplicate values did not exceed 20% of the mean. The assay was done as described under **Section 2.19.**


O.D. 405 nm
0.0
0.5
1.0
1.5
WKY
F344
WF
TgP1
rTg
TgP1
rTg
TgP1
rTg

4.3 Discussion

The results of this chapter demonstrate that the homologous synthetic 17mer TgP1 sequence (2495-2511) induces thyroiditis, and elicits strong T cell but weak IgG responses in rats of diverse MHC haplotypes. These data support the previous findings reported in an heterologous species (mice) (Chronopoulou and Carayanniotis, 1992).

Since TgP1 causes thyroiditis in both rats and mice, it raised the possibility that this cryptic pathogenic sequence may also be important in the development of autoimmune thyroid disease (AITD) in humans. It should be noted that thyroiditis induction in rats was achieved with a 33-fold molar excess of peptide (380 μg TgP1) over known thyroiditogenic doses of Tg (2 mg Tg) (Jones and Roitt, 1961, De Assis-Paiva et al, 1989). However, an attempt was not made to determine whether or not lower immunogenic doses of TgP1 such as 5 nmol (9.5 μg) (Table 4.2) can also induce lymphocytic infiltration of the thyroid. It is noteworthy that TgP1 contains T cell epitopes that are restricted in the context of both RT1-B (I-A) and RT1-D (I-E) MHC class II molecules in various strains, but does not possess the putative MHC class II binding motif (S-----E) for $RT1\text{-}B^{l}$ class II MHC (Wegmann et al, 1994). Analogous findings which confirm the ability of a peptide to induce both I-A and I-E restricted autoreactive T cell responses in rats have been made with encephalitogenic T cell clones reacting to

the peptide 85-99 of myelin basic protein (MBP) (Offner et al, 1992).

The capacity of TgP1 to induce EAT in different rat strains and be recognized by T cells in the context of disparate MHC class II molecules is somewhat unexpected. These findings may be attributed to the presence of various MHC-binding motifs within the TgP1 sequence as predicted earlier (Chronopoulou and Carayanniotis, 1992). The capacity of TgP1 to induce EAT in both rats and mice may also be attributed to a probable identity between the rat and mouse Tgs at the TgP1 sequence and to the extensive homology between the rat and mouse MHC (Gill et al, 1987; Diamond et al, 1989). Nucleotide sequencing of the gene(s) coding for mTg and establishing its amino acid sequence are necessary to clarify the degree of homology between the rat and mouse Tgs at the TgP1 site.

Two striking correlates between Tg- and TgP1-mediated EAT emerge from the present findings in rats and from earlier findings in mice (Chronopoulou and Carayanniotis, 1992) : First, after TgP1 challenge, lymphocytic infiltration of the thyroid has been observed in all the rat strains that were tested and in mouse strains ($H\text{-}2^k$ and $H\text{-}2^s$) that are known to be susceptible to Tg-mediated EAT. Second, following priming with TgP1 <u>in vivo</u>, all of these strains have exhibited significant proliferative T-cell responses to TgP1 <u>in vitro,</u>

analogous to the findings with Tg in high responder rat strains (Lillehoj and Rose, 1982). Previously, it was demonstrated that in vitro proliferative responses correlated with the pathogenic potential of TgP1 (Chronopoulou and Carayanniotis, 1992). Similarly a correlation was also found between TgP1-specific LNC proliferative responses in vitro and susceptibility of rat (WKY, WF, and F344) strains to thyroiditis.

The recognition of TgP1 by proliferative $CD4^+$ T cells in the context of RT1-B and RT1-D MHC class II molecules in diverse rat strains (Table 4.3) may suggest that MHC class II-restricted T cells play a role in the thyroid disease development. In a collaborative study, we have recently shown the existence of MHC class II-restricted TgP1-specific $CD4^+$ T cells in mice and that MHC class II-restricted 9mer epitopes of TgP1 induce thyroiditis (Rao et al, 1994). However, I have not attempted to map the minimal T cell epitopes within TgP1 and also not addressed the functional role of MHC class II-restricted T cells specific for epitopes within TgP1 in rats. It is possible that these T cells may include Th cells that (a) help B cells in the generation of TgP1-specific IgG, (b) function as "final effector cells themselves" or "inducers of final effectors" in mediating thyroid lesions or, (c) play an effector role as mediators of delayed-type hypersensitivity (DTH) (Lillehoj and Rose, 1982).

Despite the similarities between Tg- and TgP1-mediated EAT in rats, the TgP1-mediated disease may represent a new EAT model unrelated to the one resulting from Tg use. The basis for this conclusion stems from the lack of immunodominance of TgP1 in the context of both homologous (rat) and heterologous (mouse and human) Tgs in rats (Figure 4.3), and in the context of homologous Tg (mTg) and heterologous (rTg) Tgs as shown earlier in mice (Chronopoulou and Carayanniotis, 1992). The lack of immunodominance of TgP1 may be explained in various ways: TgP1 may not be generated after Tg processing in vivo by APC in the peripheral lymph nodes or TgP1 may be generated but fail to compete with higher-affinity Tg peptides for binding to the MHC in a manner shown earlier with synthetic peptides of a variety of Ags (Sercarz et al, 1993). Alternatively, TgP1 may be generated in vivo after Tg challenge over the course of time, as a result of spreading of autoreactivity to cryptic T cell determinants, analogous to the one observed for cryptic determinants of MBP in MBP-primed or MBP-peptide primed mice (Lehmann et al, 1992).

At present, it is not clear, how the priming with the cryptic TgP1 sequence results in the induction of thyroiditis in rats. It is likely that specific T cells recognize TgP1 that may be expressed on thyrocytes in the context of MHC class II molecules as a product of thyroid proteases that

digest Tg differently from enzymes in lymph node APC. Thyroidal cathepsins are known to generate 27-33 kDa fragments of Tg that position TgP1 very close to their N-terminus (Dunn et al, 1991). It was previously hypothesized that leakage of such Tg fragments to the periphery may also lead to generation of TgP1 in APC of lymphoid tissues, assuming that these fragments were processed differently from that of intact Tg (Chronopoulou and Carayanniotis, 1992). Yet another attractive hypothesis is that TgP1-reactivity may be elicited via "molecular mimicry" of this peptide with peptidic sequences on proteins derived from various pathogens such as the reovirus that is known to cause thyroiditis in mice (Srinivasappa et al, 1988). The existence of pathogenic T cells specific to cryptic epitopes exist in the periphery suggest that thymic tolerance to TgP1 has not been established in rats. This is possibly due to inability of the APC to generate cryptic epitopes from intact Ags anologous the results reported for a cryptic peptide on self-cytochrome C in mice (Mamula, 1993). It is also possible that the molecular mimic of cryptic TgP1 on infectious agents may become immunodominant anlaogous to the results reported for a peptide on homologous and heterologous lysozymes (Moudgil and Sercarz, 1993). It is likely that when an individual comes in contact with the infectious agents that harbour these "homologous mimics", the autoreactive T cells specific for cryptic

epitopes of autoantigens may be recognized thus contributing to the triggering of pathogenic thyroid autoimmunity. The fact that TgP1 at the T cell level remained cryptic or non-dominant after Tg challenge of rats as observed in the present study and of mice reported previously (Chronopoulou and Carayanniotis, 1992) represents a difference between the TgP1 and the previously identified thyroiditis-causing Tg peptides (Champion et al,1991; Hutchings et al, 1992; Texier et al, 1992) which are immunodominant.

The observation that challenge of rats with TgP1 elicited weak TgP1-specific IgG responses (Figure 4.4) differs from the previous findings in mice where challenge of mice with TgP1 elicited strong peptide-specific IgG responses (Chronopoulou and Carayanniotis, 1992). It should be noted however that TgP1 used throughout the present study did not contain the extraneous amino acids such as N-terminal cysteine (Cys) and C-terminal tyrosine (Tyr) as opposed to the "Cys-TgP1-Tyr" used in the earlier studies in mice (Chronopoulou and Carayanniotis, 1992). Moreover, it would be interesting to know whether or not challenge of rats with Cys-TgP1-Tyr would enhance the TgP1-specific IgG response in different rat strains. However, the present findings are analogous to those reported earlier on Tg-mediated rat EAT (Rose, 1975 and Lillehoj and Rose, 1982), where Ab titers did not correlate with disease severity in various rat strains. In contrast to

the previous findings in mice, TgP1-specific IgG did not cross-react significantly with rTg (Figure 4.4). Two possibilities can be entertained for the observed differences in the pattern of reactivity of TgP1-primed sera to homologous Tg in rats and mice: It is possible that rat sera recognize TgP1 epitope(s) distinct from the ones recognized by mouse sera, and the tertiary folding or post-translational modifications of Tg (Charreire, 1989) may block the binding of IgG Abs to these epitopes.

EAT induction with TgP1 or other defined Tg peptides (Champion et al, 1991; Hutchings et al, 1992; Texier et al, 1992) opens a new field to study immunoregulatory mechanisms of this disease at the cellular or molecular level. In addition, it facilitates the easy establishment of phenotypic or functional profile of autoreactive T cell clones with defined specificity. This may eventually aid in the assessment and development of various immunotherapeutic strategies for human autoimmune thyroiditis.

CHAPTER 5

Homologous Thyroglobulin Peptide (2695-2713)-Mediated Experimental Autoimmune Thyroiditis (EAT) in Rats: Peptide-Specific MHC-Unrestricted $CD4^+$ T Cell Line Adoptively Transfers Thyroiditis to Naive F344 Rats

5.1 Introduction

Experimental autoimmune thyroiditis (EAT) is induced in susceptible strains of rats by injection of homologous thyroglobulin (rat Tg) (Lillehoj and Rose 1982) or of a synthetic 17mer Tg peptide, TgP1 (2495-2511) (**Chapter 4**) in adjuvant. EAT in rats can also be induced in naive syngeneic rats by adoptive transfer of thyroiditis with homologous or heterologous Tg-primed lymph node cells (LNC) (Twarog and Rose, 1970; Jankovic et al, 1969) but not by serum (Rose et al, 1973a). The studies performed in mice clearly indicate that EAT is a $CD4^+$ T cell-mediated disease since Tg-specific $CD4^+$ T cells transferred thyroiditis to high (Maron et al, 1983; Romball and Weigle, 1987) and low (Zerubavel-Weiss, 1992; Hiyama et al, 1993; Sugihara et al, 1993) responder syngeneic naive mice. This was further supported by experiments in which the induction of thyroiditis in mice was prevented with non-depleting anti-CD4 antibodies (Hutchings et al, 1993).

Previously described Tg-specific T cell lines or clones recognize Tg determinants in an MHC-restricted fashion but their fine epitope specificity is not defined (Champion et al, 1985; Zerubavel-Weiss et al, 1992; Hiyama et al, 1993; Sugihara et al, 1993). Furthermore, T cells associated with thyroid disease recognize evolutionarily conserved determinants on Tgs of various species (Maron et al, 1983; Romball and Weigle, 1987; Zerubavel-Weiss et al, 1992; Hiyama et al, 1993; Sugihara et al, 1993; Hutchings et al 1992; Texier et al, 1992; Rao et al, 1994). Recently, it was shown that an autologous (rat) homologue of an immunopathogenic epitope of bovine interphotoreceptor retinoid-binding protein (IRBP) failed to induce experimental autoimmune uveoretinitis (EAU) in Lewis rats (Kozhich et al, 1994). The pathogenic autoimmune response induced by the heterologous bovine IRBP peptide (1181-1191) was directed to a "surrogate epitope" (273-283) but not its homologue (1181-1191) on rat IRBP, highlighting the molecular mechanism involved in heterologous peptide-mediated autoimmune disease.

Previously it was shown that an 18mer rat Tg peptide, rTgP2 (2695-2713) causes EAT in an heterologous species (mice) (Carayanniotis et al, 1994). This peptide was identified by using algorithms (Margalit et al, 1989; Rothbard and Taylor, 1988) that scanned the known portion of the rTg. The rTgP2-mediated EAT in mice is characterized by specific T cell and

IgG responses and mononuclear infiltration of the thyroid induced by both direct challenge with the peptide and adoptive transfer with peptide-primed LNC. Since the primary amino acid sequence of mTg is not known, at present, it is uncertain whether or not rTgP2 constitutes a self epitope on mTg. Since its immunopathogenicity in an homologous species is unknown, I have addressed whether or not rTgP2 (1) is immunogenic at the T- and B- cell levels and causes thyroiditis in rats, (2) contains dominant T cell determinants in the context of homologous Tg, and (3) generates MHC-restricted peptide-specific $CD4^+$ T cells that can transfer thyroiditis to naive syngeneic rats.

5.2 Results

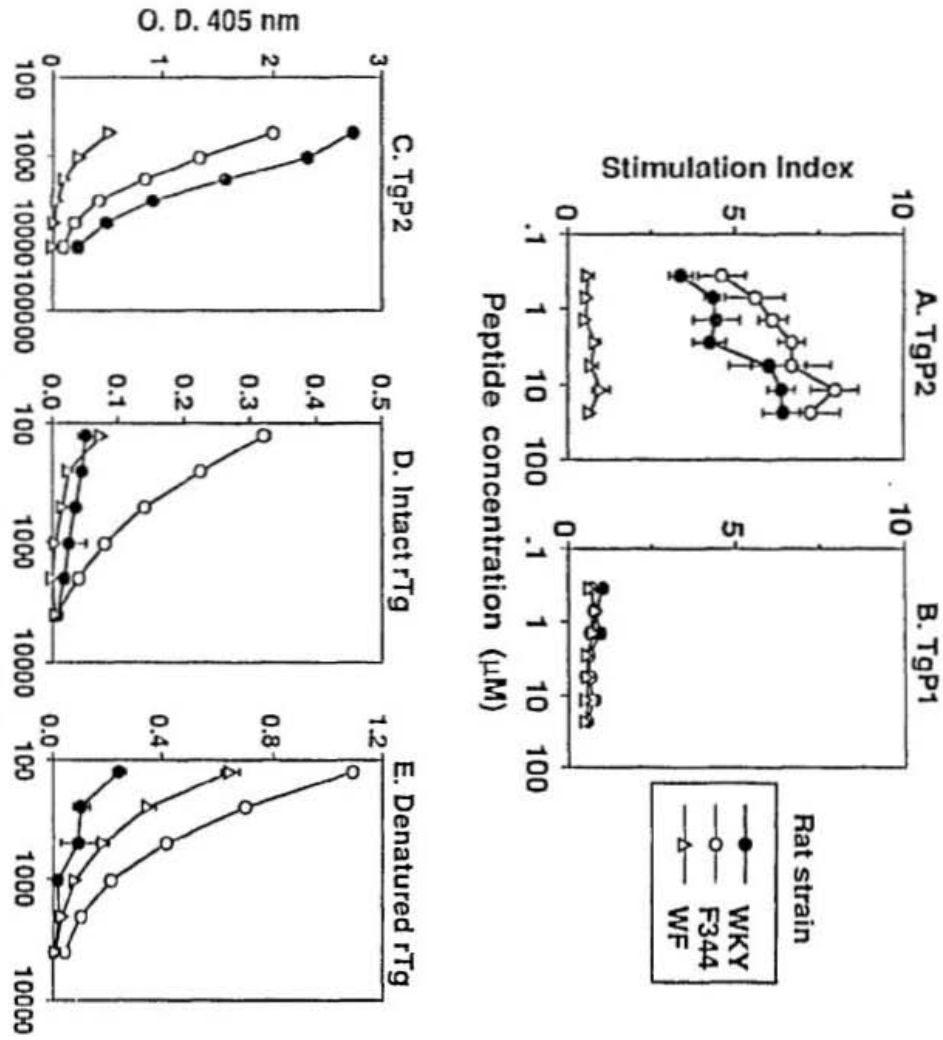
5.2.1 Priming with TgP2 induces specific LNC proliferative and IgG responses in different rat strains

To perform EAT studies with rTgP2 in rats, it was necessary to identify the responder rat strain in which the peptide induces LNC proliferative responses, because Tg-specific in vitro LNC proliferative responses correlate with susceptibility of rats to thyroiditis (Lillehoj and Rose, 1982). Three different rat strains namely F344 (RT-1^l), WKY (RT-1^k) and WF (RT-1^u) that were used in TgP1 (2495-2511)-mediated EAT studies earlier (**Chapter 4**), were injected with 200 nmol TgP2 in CFA in one hind footpad. Fourteen days later, the proliferative response of primed popliteal LNC was determined in the presence TgP2 (specific Ag) and TgP1 (control Ag). As shown in Figure 5.1A, TgP2 could induce proliferative responses in a dose dependent manner of LNC from F344 and WKY but not WF rats. The responses are specific because only background LNC proliferative responses were observed to control peptide, rTgP1 (Figure 5.1B). These data indicate the permissive recognition of rTgP2 in different RT-1 haplotypes, and rTgP2 induces LNC proliferative responses in rats of particular MHC haplotype only.

Serological analysis of the TgP2-primed sera from the same rats that were used in the priming experiment (of Figure

Figure 5.1A-B Proliferative LNC responses in rats of various RT1 haplotypes to TgP2 (A) and TgP1 (B) in vitro. Rats (2 rats/group) were injected with 100 µl of CFA emulsion containing 200 nmol TgP2 into one hind footpad. Fourteen days later, the draining popliteal LNC were removed and incubated with the indicated doses of TgP2 or TgP1 for 4 days. ^{3}[H]-TdR was added during the last 18 hr of the culture. The assay was done as described **(Sections 2.7)**. Background LNC proliferative responses (mean cpm of quadruplicate wells) in the absence of antigen were as follows: WKY = 19,340; F344 = 12,178; WF = 6,427.

Figure 5.1C-E Reactivity with TgP2 (C), intact rTg (D), and heat denatured rTg (E) of day 14 sera from TgP2-primed rats of different strains. Analysis of pooled serum samples from TgP2-primed rats (2 rats/group) from different rat strains obtained 14 days after footpad challenge with 100 µl of CFA emulsion containing 200 nmol TgP2 (of Figure 5.1A). Specific IgG reactivity against the indicated Ags was measured by an alkaline phosphatase-based ELISA **(Section 2.19)**. O.D. 405 nm readings are mean of triplicate wells and are expressed as mean +/- SD. Mean of the background values at O.D. 405 nm was 0.121. TgP1 or ovalbumin was used as a control antigen.


A. TgP2
B. TgP1
Stimulation Index
Peptide concentration (μM)
Rat strain
WKY
F344
WF
C. TgP2
D. Intact rTg
E. Denatured rTg
O. D. 405 nm
Reciprocal serum dilution

5.1A), revealed the presence of TgP2-specific IgG in all three rat strains. It was observed that, at the serological level, TgP2 is more immunogenic in F344 and WKY rats than in WF rats. Subsequent analysis of the reactivity of peptide-primed sera with rTg revealed that TgP2-specific IgG of F344 but not WKY and WF rats reacted with intact rTg (Figure 5.1D), although the same sera from all of the above three rat strains reacted with heat denatured rTg (Figure 5.1C). These data show that TgP2-specific IgG of different rat strains recognize distinct epitopes within the TgP2 site on rTg, and TgP2-specific IgG recognize linear B cell epitopes. The data clearly demonstrate that the homologous rTgP2 is immunogenic at both T- and B- cell levels in rats.

5.2.2 Direct challenge of F344 rats with TgP2 induces mild thyroiditis

To test whether or not TgP2-induced LNC proliferative responses correlate with susceptibility of rats to thyroiditis, F344 rats were selected for further EAT studies. Thyroids collected on day 28 from rats (3/group) challenged with TgP2 (immunization procedure is described under **Section 2.9**) showed a mild degree of mononuclear infiltration in 2 out of 3 rats (66% incidence) (Table 5.1). In contrast, thyroids from control group rats (adjuvant-primed) did not show thyroid lesions. These data indicate that rTgP2 causes thyroiditis in

Table 5.1 Direct challenge of F344 rats with TgP2 induces mild thyroiditis*

In vivo	Infiltration index						Rats with thyroid lesions	% incidence
	0	0.5	1.0	2.0	3.0	4.0		
Expt. No. 1								
CFA+TgP2	1	-	2	-	-	-	2/3	66%
CFA	3	-	-	-	-	-	0/3	0%
Expt. No. 2								
CFA+TgP2	1	1	2	-	-	-	3/4	75%
CFA	4	-	-	-	-	-	0/4	0%

* Rats were primed with 200 nmol TgP2 and *Bordetella pertussis* and 1 week later they were boosted with 100 nmol peptide. Control animals were injected with CFA and *Bordetella pertussis* only as described under Materials and Methods (Section 2.8). Three weeks after the second challenge, the thyroid glands were removed and mononuclear cell infiltration of the thyroids was assessed as described under Materials and Methods (Section 2.9).

rats. Specific LNC proliferative responses correlate with susceptibility of rats to thyroiditis and are in agreement with earlier data using Tg (Lillehoj and Rose, 1982).

5.2.3 *In vivo* (efficient) but not *in vitro* (inefficient) processing of homologous thyroglobulin results in activation of TgP2-specific proliferative T cells

To address whether or not TgP2 contains dominant T cell determinants in the context of homologous Tg (rTg) in rats, F344 rats (2/group) were challenged with 100 μl of emulsion containing 200 μg rTg or 100 nmol TgP2 or no antigen into one hind footpad and, 14 days later, LNC proliferative assays were performed. The results are shown in Table 5.2. The rTg-primed LNC proliferated strongly to rTgP2 but not rTg itself. Furthermore, TgP2-primed LNC proliferated strongly to TgP2 but not rTg at equimolar doses. CFA-primed LNC failed to proliferate in the presence of Tg-peptides. Proliferative responses were specific, because no responses were detected to the control peptide, rTgP1. These results indicate that (1) *in vivo* processing of rTg leads to generation of the TgP2 sequence and activation of peptide-specific T cells, (2) processing of rTg *in vitro* is inefficient, and (3) the TgP2 sequence contains non-dominant T cell determinants in rats since *in vitro* pulsing of TgP2-primed LNC with rTg did not

Table 5.2 TgP2 contains non-dominant T cell determinants

In vivo	*In vitro* popliteal LNC proliferative responses (SI +/- SD)[a]					
	TgP1		TgP2		rTg	
	9 μM	1 μM	1 μM	0.1 μM	1 μM	0. 1 μM
CFA + TgP2	1.0 +/- 0.3	0.8 +/- 0.2	3.7 +/- 0.6*	1.8 +/- 0.1	0.9 +/- 0.0	1.2 +/- 0.3
CFA + rTg	0.9 +/- 0.1	0.9 +/- 0.1	4.1 +/- 0.0	3.6 +/- 0.2	1.4 +/- 0.2	1.5 +/- 0.1
CFA	0.9 +/- 0.1	1.0 +/- 0.1	1.1 +/- 0.4	0.8 +/- 0.2	1.0 +/- 0.3	0.9 +/- 0.2

[a] F344 rats were injected into one hind footpad with 100 μl of CFA emulsion containing 200 μg rTg or 100 nmol TgP2 (210 μg) or no antigen. Fourteen days later, their popliteal LNC were collected and stimulated for 4 days *in vitro* with the indicated antigens as shown: 1 μM TgP1 = 2.1 μg/ml; 1 μM TgP2 = 2.1 μg/ml; 1 μg rTg = 660 μg/ml. The background proliferative responses (mean cpm) in the absence of antigen for different groups were as follows : CFA + TgP2 = 5,409; CFA + rTg = 14,057; CFA = 11,611. The proliferative responses (cpm x 10^{-3}) to PPD *in vitro* for different groups were as follows : CFA + TgP2 = 8.3 +/- 0.7; CFA + rTg = 5.9 +/- 0.5; CFA = 3.9 +/- 0.6.

* Underlined values denote significant proliferative responses (SI > 2.0) to TgP2.

result in TgP2-specific LNC proliferative responses.

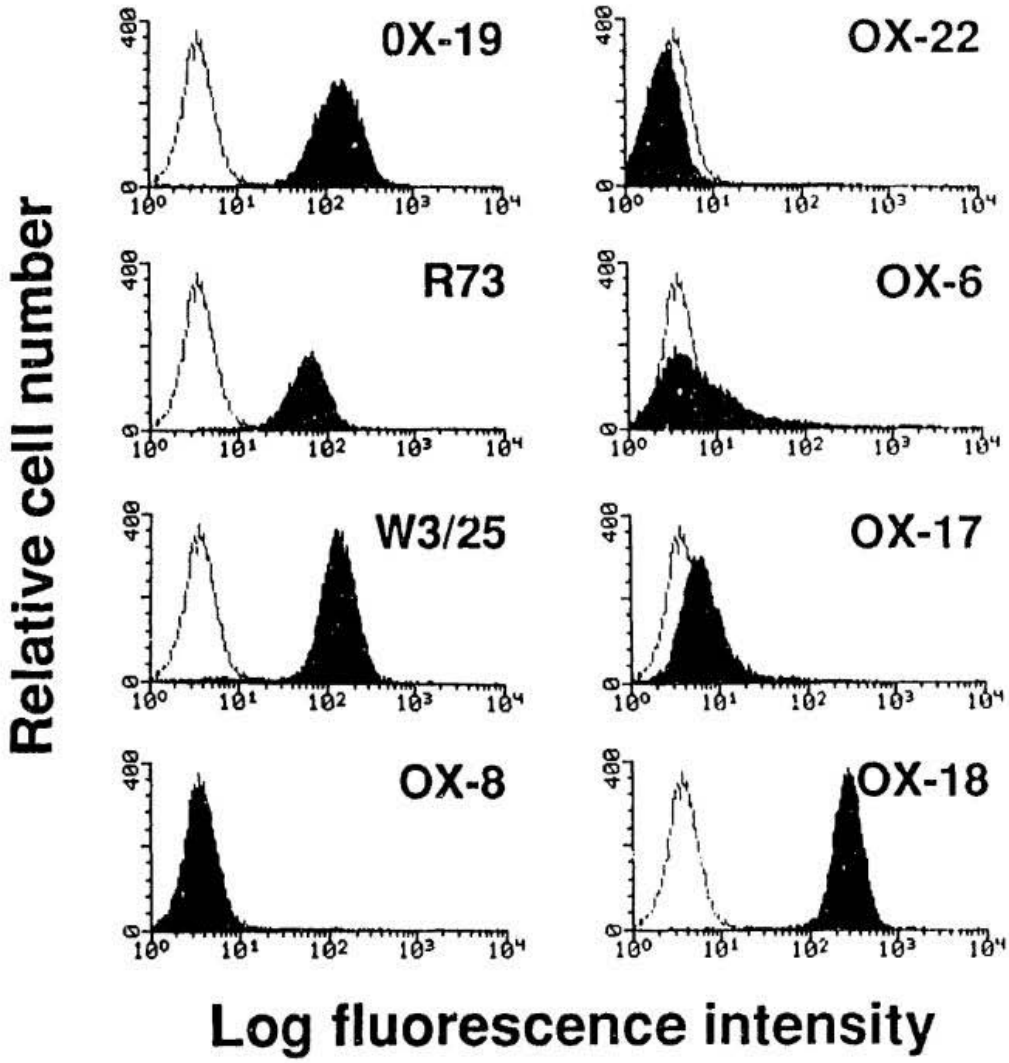
5.2.4 The TgP2-specific T line cells are of $CD4^+$, $CD8^-$, TCR-α/β^+, and $CD45RC^-$ phenotype

To characterize the TgP2-specific T cells in F344 rats, a short term TgP2-stimulated T cell line was generated and stained with various MAbs specific for rat lymphocytes. The results of the FACS analysis of the T cell line are shown in Figure 5.2. The T line cells are OX-19^+ (99.7%), W3/25^+ (98%), OX-8^- (2%) and $R73^+$ (99%), a phenotype characteristic of rat $CD4^+$, TCR α/β^+ T helper/inducer cells. All of the T cells were OX-22^- (100%), a finding consistent with the notion that the T cell line predominantly consists of memory T lymphocytes (Spickett et al, 1983; Powerie and Mason, 1989). A minor population of T cells expressed RT1-B (OX-6^+) (15 %) and RT1-D (OX-17^+) (6%) MHC class II molecules which suggests that activated rat T cells express MHC class II determinants (Reizis et al, 1994).

5.2.5 The TgP2-specific T line cells recognize the peptide in the context of MHC class II molecules

To further characterize the above TgP2-stimulated T cell line, a proliferative assay was performed. In the specificity assay (Table 5.3), the T cells responded to rTgP2 but not to rTgP1 (control peptide) or PPD. However, the T cell line

Figure 5.2 Flow cytometric analysis of TgP2-specific T cell line. Cultured F344 rat T line cells (obtained after 4 days of a third in vitro boosting with rTgP2) were incubated with appropriately diluted primary MAb against rat T cell surface markers (OX-19, W3/25, OX-8, R73, OX-6, OX-17 and OX-18) or with an isotype-matched control MAb (HB 64) and then with FITC-conjugated goat secondary Ab. Flow cytometric analysis was performed as described under **Section 2.13** using a FACSstar plus analyzer (Becton Dickinson, CA). Data are presented as histograms of log fluorescence intensity versus relative cell number. Shaded areas represent T cells incubated with both primary MAb specific for rat T cell markers and secondary Abs; white areas represent T cells incubated with primary control MAb and secondary Ab.


0X-19
OX-22
R73
OX-6
W3/25
OX-17
OX-8
OX-18
Relative cell number
Log fluorescence intensity

exhibited a weak but significant proliferative response to heterologous TgP2 (human TgP2 = hTgP2) that shares 79% a.a.sequence homology with rTgP2. These results indicate that the T cell line is TgP2-specific and recognizes conserved T cell determinants on heterologous hTgP2. Furthermore, the T cell line failed to recognize rTg itself in the culture, a finding confirming the earlier data obtained in LNC proliferative assays (Table 5.2) that in vitro processing of rTg does not generate the TgP2 sequence. Furthermore, when rTg-pulsed splenic APC were used, no proliferative response of the T cell line was observed (data not shown).

To test whether or not rTgP2 is recognized in the context of RT1-B or RT1-D MHC class II molecules, antibody blocking studies were performed. The results are shown in Table 5.3. Addition of either OX-6 or OX-17 MAb but not the isotype-matched control MAb (HB 64) (anti-matrix protein of Influenza type A MAb) to the culture, significantly inhibited (34%) TgP2-induced proliferation of specific T line cells. These results indicate that TgP2-specific T cell line recognizes rTgP2 in the context of both RT1-B^l and RTI-D^l MHC class II molecules. The results can also be interpreted as TgP2 is presented by both RT1-B^l and RTI-D^l MHC class II molecules and the T cell line has a mixture of specificities.

Table 5.3 Specific T cell line recognizes TgP2 in the context of MHC class II molecules

In vitro	*In vitro* lymphocyte proliferative responses (mean cpm +/- SD)
Specificity assay[a]	
rTgP2 (2 µg/ml)	46,011 +/- 2,215 (21.2 +/- 1.0)[b]
hTgP2 (20 µg/ml)	6,605 +/- 429 (3.1 +/- 0.2)
(2 µg/ml)	3,399 +/- 457 (1.6 +/- 0.2)
rTg (660 µg/ml)	1,789 +/- 601 (0.8 +/- 0.3)
TgP1 (2 µg/ml)	1,945 +/- 102 (0.9 +/- 0.1)
PPD (10 µg/ml)	1,897 +/- 284 (0.9 +/- 0.1)
No antigen	2,167 +/- 581
Blocking Assay[c]	
NIL	49,404 +/- 2,053 (0%)[d]
OX-6 (anti-I-A)	32,650 +/- 891 (34%)
OX-17 (anti-I-E)	32,376 +/- 1,988 (34%)
HB64 (control MAb)	47,973 +/- 4,025 (3%)
No antigen	2,367 +/- 162

[a] T line cells (10^4/well) were incubated in the presence or absence of indicated antigens and irradiated thymic APC (10^6/well) for 3 days. During the last 18 hr of culture, the cells were pulsed with 1 µCi/well of ^{3}H-TdR. The rest of the assay was done as described under Materials and Methods (Section 2.13).

[b] Values in the parenthesis represent SI +/- SD.

[c] T line cells were incubated with irradiated APC as above in the presence or absence of TgP2 at 5 µg/ml and the indicated blocking MAb: OX-6 (12.5 µg/ml), OX-17 (1.56 µg/ml) and HB64 (3.125 µg/ml). At these concentrations, the OX-6 and OX-17 MAbs were non toxic since they did not inhibit con A stimulation of rat splenic cells.

[d] Percent inhibition of proliferation was calculated as described under Materials and Methods (Section 2.7).

5.2.6 The TgP2-specific CD4$^+$ T cells are MHC-unrestricted

To address whether rTgP2-specific T cells of F344 rats are MHC-restricted, an MHC-restriction assay was performed using syngeneic (F344) as well as allogeneic (WKY and WF) rat thymic APC to present TgP2 or its overlapping N- and C-terminal 12mer peptides to TgP2-specific T cell line. The results are shown in Figure 5.3. The T cell line proliferated strongly to an 18mer rTgP2 or an N-terminal 12mer (2695-2706) but not to a C-terminal 12mer (2702-2713) peptide in the presence of syngeneic (F344 rat) APC. However, the intensity of proliferation was stronger in the presence of the 18mer TgP2 than the N-terminal 12mer peptide. These results indicate that the specificity of the T cell line is directed to determinants within the N-terminal 12mer (2695-2706) peptide. Unexpectedly, T line cells also proliferated when allogeneic APC from WKY rats were used to present TgP2 or the N-terminal but not C-terminal 12mer peptide of rTgP2. Presentation of the C-terminal 12mer peptide of rTgP2 by APC from WKY rats to the TgP2 specific T cells from F344 rats did not induce proliferation. In contrast, the T cell line failed to proliferate to the above Tg peptides when allogeneic APC from WF rats were used. The observed proliferative responses when WKY rat APC were used are not due to allorecognition because allogeneic APC either unpulsed or pulsed with the control peptide (TgP1) failed to induce

Figure 5.3 The TgP2-specific T cell line recognizes TgP2 or an N-terminal 12mer (2695-2706) peptide in an MHC-unrestricted fashion. F344 rat T cells (1 x 10^4/well) were cultured with irradiated (2000 rad) thymic APC (1 x 10^6/well) from different rat strains in triplicate wells of 96-well flat-bottomed micro culture plates for 72 hrs. The cultures were pulsed with 1 μCi of ^{3}H-TdR/well during the last 18 hr of culture. The MHC-restriction assay was done as described under **Section 2.14.** The values are expressed as SI +/- SD of triplicate cultures. The stimulation indices (SI) obtained when T cells alone were incubated with TgP2 at 20 μg/ml = 0.3 +/- 0.1 ; 2 μg/ml = 0.3 +/- 0.2; 0.2 μg/ml = 0.2 +/- 0.2; 0.02 μg/ml = 0.4 +/- 0.1. Background proliferative response of T line cells when incubated with APC from different rat strains are as follows: F344 = 2,167; WKY = 1,070; WF = 357.

177 b

A. F344 rat APC
(RT-1l)
B. WKY rat APC
(RT-1k)
C. WF rat APC
(RT-1u)
Stimulation Index
40
30
20
10
0
.01 .1 1 10 100 .01 .1 1 10 100 .01 .1 1 10 100
Peptide concentration (µg/ml)
Peptides in vitro
2695-2713 (18mer)
2695-2706 (N-terminal 12mer)
2702-2713 (C-terminal 12mer)

significant T cell proliferative responses. The proliferative responses were also not due to the mitogenic effects of TgP2 on T cells, because T cells incubated with graded doses of the peptide did not exhibit significant T cell proliferative responses (see legend to Figure 5.3). These data clearly indicate that the rTgP2-specific T cell line is MHC-unrestricted, is APC dependent for its activation, and recognizes determinants within the N-terminal 12mer peptide (2695-2706).

5.2.7 The TgP2-specific CD4$^+$ T cell line adoptively transfers thyroiditis to naive syngeneic rats

Next, we sought to address whether or not rTgP2-mediated EAT in F344 rats is a CD4$^+$ T cell-mediated disease. Following adoptive transfer of 1 x 10^8 TgP2-primed and TgP2-boosted (in vitro) LNC, a moderate degree of mononuclear infiltration of the thyroid (mild thyroiditis) was observed in 50% (3/6) of the recipient rats 10 days post-transfer (Table 5.4 and Figure 5.4 A-B). In contrast, a control group of rats (3/group) that received LNC either from CFA-primed rats that were con A-boosted in vitro or ovalbumin-primed rats that were ovalbumin-boosted in vitro, did not show thyroid lesions (Table 5.4). Furthermore, histological examination of the thyroids from rats that were injected with T line cells (CD4$^+$ CD8$^-$ TCR α/β^+) (Figure 5.3) at 10^8 cells /rat (4 rats/group)

revealed extensive infiltration with mononuclear cells in 100% (4/4) of the rats (Table 5.4 and Figure 5.4 C-D). The data clearly demonstrate for the first time that TgP2-induced rat EAT is mediated by $CD4^+$, $CD8^-$, TcR α/β^+ T cells.

5.2.8 The TgP2-induced Tg-reactive IgG is directed to determinants within but not outside the TgP2 site on Tg

Since rTgP2 is serologically immunogenic (5.1C), it was of interest to see whether or not secondary immune response sera would similarly react with intact rTg and whether the elicited IgG was directed to determinants within the TgP2 site on rTg. The results are shown in Figure 5.5. Analysis of pooled sera from the same rats as used in Table 5.1 (Expt. No. 1) by ELISA demonstrated the presence of rTgP2-specific IgG that react with intact rTg but not TgP1 (control peptide) (Figure 5.5A). Increased reactivity was also observed against heat denatured rTg, a finding compatible with the notion that peptide-induced Tg-reactive IgG recognizes linear B cell determinants. These data confirmed the results obtained earlier with TgP2-primed primary immune response sera (day 14) as shown in Figure 5.1D-E. Day 28 sera obtained from control group rats (primed with CFA and Bordetella pertussis on day 0 and boosted with IFA on day 7) did not show reactivity to rTgP2.

To determine whether or not TgP2-induced Tg-reactive IgG

is directed to determinants within the TgP2 site on Tg, we performed a competitive inhibition ELISA. The results are shown in Figure 5.5B. The results indicate that the rTgP2-specific IgG that binds to intact rTg was not composed of Abs specific for determinants outside the rTgP2 site, because free rTgP2 but not TgP1 inhibited near to completion the binding of rTgP2-specific IgG to rTg (Figure 5.5B). This observation reflects the absence of "intramolecular determinant spreading" at the serological level in rTgP2-induced rat EAT.

Table 5.4 TgP2-specific $CD4^+$ T cells adoptively transfer thyroiditis to naive F344 rats

In vivo	*In vitro*	Infiltration index						Rats with thyroid lesions	Incidence
		0	0.5	1.0	2.0	3.0	4.0		
Bulk Culture LNC[a]									
CFA+TgP2	TgP2	3	-	3	-	-	-	3/6	50 %
CFA+Ova	Ova	3	-	-	-	-	-	0/3	0 %
CFA	ConA	3	-	-	-	-	-	0/3	0 %
T cell line[b]									
TgP2-specific	TgP2	0	-	-	-	3	1	4/4	100 %

[a] Primed-popliteal LNC from F344 rats were boosted *in vitro* with appropriate antigens at the indicated doses (TgP2 = 10 µg/ml; ConA = 10 µg/ml; Ovalbumin = 25 µg/ml) for 3.5 to 4 days, washed three times with HBSS and injected i.p. into syngeneic rats at 1×10^8 viable cells/rat as described under Materials and Methods (Section 2.14). The thyroids were obtained after 10 days post-transfer and histological examination was done as described under Materials and Methods (Section 2.9).

[b] Ficoll-cut TgP2-specific $CD4^+$ T line cells after 3.5 days of *in vitro* stimulation with TgP2, were injected into naive syngeneic rats at 1×10^8 viable cells/rat. Thyroids were collected 10 days post-transfer and histological examination was performed as described in Materials and Methods (section 2.9).

Figure 5.4A-D Photomicrographs showing the TgP2-induced mononuclear infiltration in the thyroid. Mononuclear cell infiltration of the thyroid in F344 rats observed after i.p. adoptive transfer of 1 x 10^8 syngeneic TgP2-primed LNC [**A** (X 12.5) or **B** (X 200)] or i. p. adoptive transfer of 1 x 10^8 syngeneic TgP2-specific T line cells [**C** (X 12.5) or **D** (X 200)]. Arrows indicate the accumulation of mononuclear cells in the thyroid.

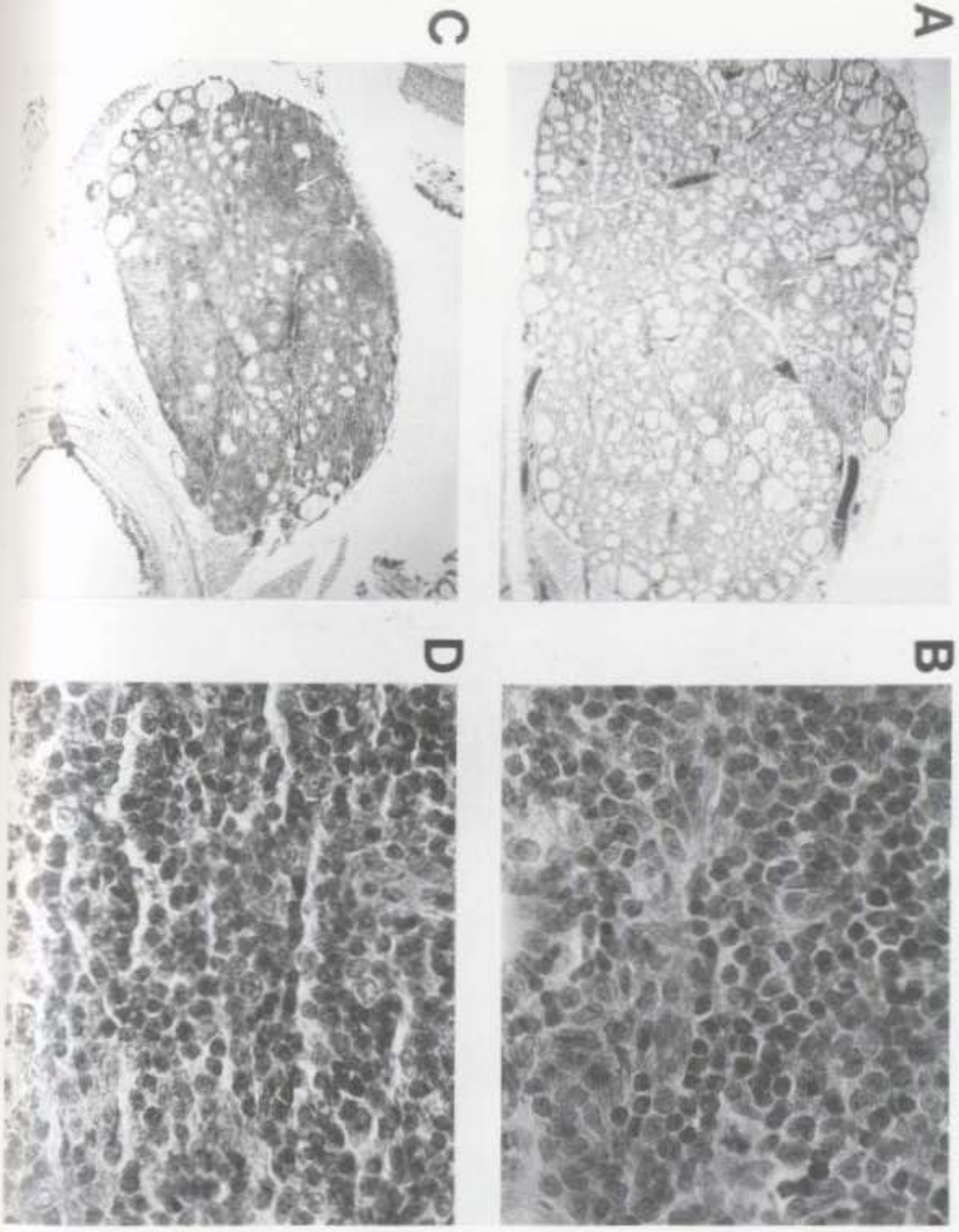
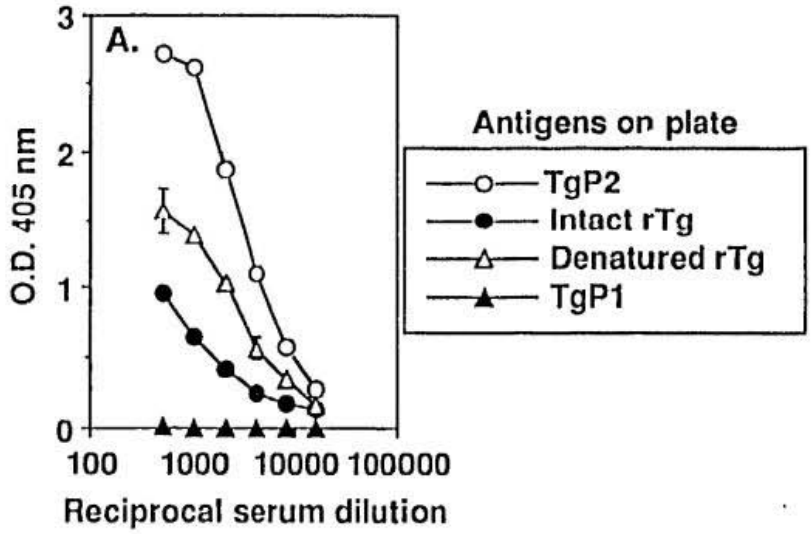
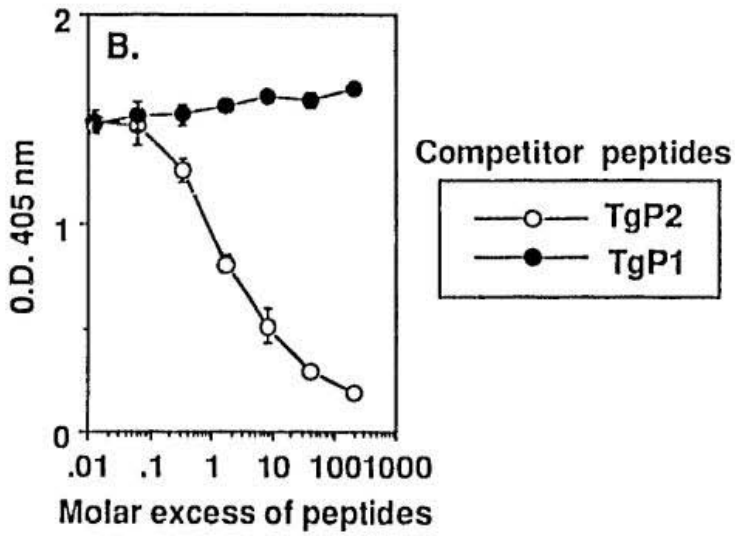

A
B
C
D

Figure 5.5A TgP2-specific IgG recognize linear B cell determinants on rTg. Demonstration of immunoreactivity of pooled TgP2-primed rat sera obtained 28 days after TgP2-priming (primed on day 0 and boosted on day 7) of F344 rats (3/group) (of Table 5.1; Expt. No.1) to TgP2 (0.21 µg/well), intact and heat denatured rTgs (1 µg/well) by a direct ELISA as described under **Section 2.19.** The values are expressed as mean +/- SD of duplicate wells. Mean of background values was 0.149.

Figure 5.5B Competitive ELISA of serum IgG-binding to rTg using the 18mer TgP2 or the 17mer TgP1 as inhibitor(s). Inhibition of binding of TgP2-induced Tg-reactive IgG (to rTg coated on the plate) present in TgP2-primed sera by free TgP2 but not TgP1. TgP2-primed serum (of Figure 5.5A) at 1/512 dilution was added to the wells with simultaneous addition of five-fold dilutions of TgP2 or TgP1 starting at 200 molar excess of rTg coated (1 µg/well) on the PVC plate. The values are expressed as mean +/- SD of triplicates. The assay was done as described under **Section 2.20.** Maximum O.D. 405 reading at 1/512 dilution of TgP2-primed serum in the absence of any competitor peptide was 1.62 +/- 0.03. Mean of the background values is 0.106.


A.
O.D. 405 nm
3
2
1
0
100
1000
10000
100000
Reciprocal serum dilution
Antigens on plate
TgP2
Intact rTg
Denatured rTg
TgP1


B.
O.D. 405 nm
2
1
0
.01
.1
1
10
1001000
Molar excess of peptides
Competitor peptides
TgP2
TgP1

5.3 Discussion

The results presented in this chapter demonstrate that the 18mer homologous Tg sequence, TgP2 (2695-2713) is thyroiditogenic in rats and show for the first time that Tg peptide-specific MHC-unrestricted $CD4^+$ T cells adoptively transfer organ-specific autoimmune thyroiditis to naive syngeneic rats.

The recognition of the 18mer TgP2 by F344 rat T cells in the context of both RT1-B and RT1-D MHC class II molecules (Table 5.3) is analogous to the recognition of 17mer TgP1 described earlier (Table 4.3). However, neither of these Tg sequences (TgP1 and TgP2) encompass the putative MHC class II ($RT1\text{-}B^l$) binding motif (S-----E) (Wegmann et al, 1994; Zhao et al, 1994), but both of these Tg peptides are pathogenic in F344 rats (Table 4.1 and Table 5.1). Thus, the data indicate the lack of a correlation between haplotype-specific MHC class II binding motif and $RT1\text{-}B^l$-restricted pathogenic TgP1 (Table 4.3) and TgP2 (Table 5.3) sequences. The present (Table 5.3) and previous (Table 4.3) data bring into question the generality of the haplotype-specific MHC class II ($RT1\text{-}B^l$) binding motif (S-----E) in identifying pathogenic epitopes. Moreover, the present data highlight the utility of haplotype-independent algorithms that search for putative T cell epitopes (Margalit et al, 1987; Rothbard and Taylor, 1988). In contrast, other investigators have identified T cell epitopes

on autoantigens using Rothbard and Taylor (1988) tetramer motif with variable rates of success (Fuji and Lindstrom, 1988).

Distinct effector functions (Figure 5.1A and 5.1C) i.e, T cell versus Ab responses, elicited by TgP2-priming in rats of diverse MHC haplotypes may be a consequence of MHC control of $CD4^+$ T cell subset (Th1 or Th2) activation. These data are analogous to those reported for various Ags such as the human collagen type IV (hCol IV) (Murray et al, 1989) and a 30 mer peptide of α chain of hCol IV (Murray et al, 1992) in mice and a 26mer peptide of MBP (Mustafa et al, 1993) in rats. Alternatively, the present findings (Figure 5.1) may also be an effect of MHC-peptide ligand density (Murray et al, 1994) i.e., high MHC class II/peptide density on the APC surface favours Th1-like (proliferative) responses, while low MHC class II/peptide density favours Th2-like (non-proliferative) responses. Additional experiments such as epitope specificity, MHC-restriction, lymphokine analysis and functional profile of TgP2-specific T cell clones in various rat strains are needed to extrapolate the phenomenon of MHC-peptide ligand density to the present findings.

The induction of TgP2-specific IgG (Figure 5.1C) but not T cell proliferative responses in WF rats is in agreement with the concept that peptide-specific non-proliferative Th cells can help B cells to produce IgG (Evavold and Allen, 1991).

The current results (Figure 5.1) also suggest that TgP2 may be recognized by both proliferative and non-proliferative (Th2) T cells in F344 and WKY rats, because TgP2-priming of these two rat strains readily elicited both T- and B-cell responses. It is also possible that specific proliferative and non-proliferative T cells may recognize distinct epitopes within TgP2 analogous to the distinct epitope recognition within a fibrinopeptide by specific proliferative and non-proliferative T cells (Peterson et al, 1983). The present findings (Figure 5.1A and 5.1C) are in apparent contrast to the immunogenicity of TgP1 (2495-2511) described earlier in the above rat strains in which TgP1-priming of rats induced specific proliferative T cell but not primary IgG responses (Figure 4.2A-B). Thus, it is reasonable to speculate that TgP1- and TgP2-priming of rats elicit T cells with distinct effector functions. The present (Figure 5.1) and previous (Figure 4.2) data also indicate that similar or distinct Tg peptides bind various MHC class II molecules. Analogous findings were reported for peptides of AChR (Fuji and Lindstrom, 1988), and of MBP (Vandenbark et al, 1985) in rat strains of various MHC haplotypes. Thus, recognition of Tg epitopes in various inbred rat strains suggests that similar (due to their promiscuity in MHC binding) or distinct Tg epitopes (due to lack of promiscuity in MHC binding) may be recognized by autoreactive T cells in the context of distinct or similar MHC

class II molecules in patients with AITD.

Demonstration of *in vivo* but not *in vitro* processing of homologous Tg (rTg) as assessed by activation of rTg-primed LNC by TgP2 *in vitro* (Table 5.2) is particularly surprising. Analogous findings were reported earlier in mice with diverse Ags such as acetylcholine receptor (AChR) and renal tubular Ag (RTA) (Yokoi et al, 1987; Heeger et al, 1994). It was shown earlier that AChR-primed LNC from C3H/He mice failed to respond to the intact Ag *in vitro*, although the same LNC proliferated significantly to three distinct AChR peptides (67-82; 146-162; 170-186) (Yokoi et al, 1987). In another study, it was shown that some of the T cell clones isolated from RTA-primed mice responded vigorously to a 22mer P2 peptide of RTA but not to an intact RTA *in vitro* (Heeger et al, 1994).

The underlying mechanism for the results obtained in the present one and previous studies (Yokoi et al, 1987; Heeger et al, 1994) is not clear. Several mechanisms have been proposed or shown to explain how T-cell determinants are selected for presentation in the context of class II MHC molecules (Sercarz et al, 1993). However, these mechanisms remain poorly understood. It can be proposed that the present findings may be a result of altered Ag processing and presentation of Tg epitopes by activated APC. These APC will be influenced by the cytokines released as a result of the inflammation

generated at the site of immunization with CFA emulsion containing rTg. This will subsequently lead to *in vivo* activation of peptide-specific T cells. Consistent with this possibility are the recent findings in the EAE model in which T cell responses to cryptic T cell determinants of MBP are induced following challenge of mice with a dominant MBP peptide (Ac1-11) (Lehmann et al, 1992). The failure of TgP2-primed LNC to proliferate to rTg *in vitro* suggests that *in vitro* (inefficient) processing of Tg may not result in the generation of TgP2 sequence but rather leads to its masking or destruction.

The *in vivo* activation of TgP2-specific T cells following rTg-priming of rats (Table 5.1) suggests that TgP2 but not TgP1 may be one of the few immunopathogenic determinants within Tg participating in Tg-mediated rat EAT. This finding is analogous to previously known pathogenic Tg peptides in mice (Hutchings et al, 1992; Texier et al, 1992). Thus, it would be interesting to test whether rTg-primed LNC that were TgP2-boosted *in vitro* could transfer thyroiditis to naive rats in view of the findings that TgP2-primed and TgP2-boosted LNC adoptively transfer thyroiditis to naive syngeneic rats (Table 5.4).

The present demonstration of the failure of rTg-primed LNC to proliferate to rTg *in vitro* (Table 5.2) is not new in thyroid autoimmunity. This finding confirms (Hirose et al,

1988b) and extends (Hunter, 1986; Williams et al, 1986) previous data reported for various autoantigens such as rTg, MBP and mTg in diverse species. In both of the latter studies, it was shown that adoptive transfer of antigen-primed LNC to naive syngeneic animals mediated pathogenic autoimmunity, although the Ag-primed LNC failed to proliferate to corresponding Ag in vitro. In the present study, as to why the rTg-primed LNC from F344 rats fail to proliferate against rTg in vitro is unknown. It is possible that self-antigen priming may elicit specific T suppressor (Ts) or immunoregulatory cells that prevent or inhibit the proliferative responses of self-Tg reactive T cells in vitro as suggested earlier (Rose et al, 1981). Alternatively, F344 rat T cells may be hyporesponsive to homologous Tg (rTg) as a result of these cells being anergized in the periphery by Tg epitope presenting B cells as hypothesized earlier (Weigle et al, 1980). However, it is clear that tolerance to rTg is incomplete, because challenge of F344 rats with rTg in adjuvants readily induces thyroiditis (Lillehoj et al, 1981 and Lillehoj and Rose, 1982). It would be interesting to examine whether or not rTg-primed LNC that were rTg-boosted in vitro will transfer thyroiditis to naive syngeneic rats similar to other studies (Hunter, 1986; Williams et al, 1986).

Adoptive induction of severe to mild thyroiditis with TgP2-specific T line cells and TgP2-primed LNC (bulk LNC

culture) respectively, may be due to the difference in the number of pathogenic T cells present in the T cell line as opposed to bulk LNC culture. This interpretation is consistent with previous findings that cell dose used in adoptive experiments critically influences the adoptive-transfer of thyroiditis in mice (Maron et al, 1983). The demonstration of TgP2-induced rat EAT as a $CD4^+$ T cell-mediated disease (Table 5.4 and Figure 5.4) is in agreement with previous reports on adoptively-mediated thyroiditis with rTg-primed LNC in rats (10^9 LNC/rat) (Twarog and Rose, 1970) and with Tg-specific $CD4^+$ T cell lines and clones in mice (Maron et al, 1983; Romball and Weigle, 1987; Sugihara et al, 1993). Induction of autoimmune (thyroid) lesions by the TgP2-specific T cell line is organ-specific, because no lymphocytic infiltration was observed in other organs such as livers and kidneys of the recipient rats (data not shown).

The N-terminal 12mer Tg peptide (2695-2706) (Table 5.3) recognized by the pathogenic $CD4^+$ T line cells encompasses the tetramer motif "KYIQ" (Rothbard and Taylor, 1988). This observation further confirms the utility of haplotype-independent algorithm (Rothbard and Taylor, 1988) for identification of T cell epitopes on known autoantigens. The stimulation of the TgP2-specific T line cells (Table 5.3) with heterologous TgP2 (hTgP2) (Malthiery et al, 1987) [which also contains the tetramer motif "KYIS" (Rothbard and Taylor,

1988)] is in general agreement with earlier reports that thyroiditis-inducing T cell lines and clones recognize conserved T cell epitopes on heterologous Tgs (Romball and Weigle, 1987; Sugihara et al, 1993; Champion et al, 1987). This observation, analogous to TgP1 (**Chapter4**), has raised the possibility that interspecies-conservative Tg epitopes may be involved or important in the induction of AITD in humans.

The significant abrogation of the TgP2-specific T cell proliferative responses by MHC class II-specific MAb (Table 5.3) may imply a role for MHC class II antigens in TgP2-mediated rat EAT. However, it remains to be established whether disease transfer requires TgP2-specific T cells restricted by RT1-B or RT1-D class II molecules alone or both. The ability of TgP2-specific T cell line to proliferate at a higher intensity against the 18mer TgP2 than the N-terminal 12mer Tg peptide (2695-2706) suggests that amino acid residues flanking the core determinant positively influence the peptide recognition by T cells (Bhayani et al, 1988; Vacchio et al, 1989; Nelson et al, 1994).

Although the TgP2-specific $CD4^+$ T cell line adoptively transfers severe thyroiditis to naive syngeneic rats (Table 5.4), the putative APC that present Tg epitopes to these T cells <u>in vivo</u> remain unknown. This question was raised because these T cells failed to proliferate in the presence of rTg-pulsed thymic APC (Table 5.3) or splenic APC <u>in vitro</u>

(data not shown). Though TgP2 is generated by processing of rTg by draining lymph node APC *in vivo* (Table 5.2), it is not known whether TgP2 is similarly generated by class II^+ thyrocytes by themselves in the target organ (Hirose et al, 1988a) leading to subsequent activation of thyroid-homing T cells *in vivo*. This can be tested by using untreated thyrocytes (MHC class$^-$) obtained from unimmunized rats or IFN-γ treated thyrocytes (MHC class II^+) to activate the TgP2-specific T cell hybridomas and assaying for IL-2 release analogous to the experiments performed previously with an immunodominant thyroxine containing 9mer pathogenic Tg peptide (Champion et al, 1991).

The mechanism(s) by which the TgP2-specific $CD4^+$ T cells mediate thyroid pathology remain(s) unknown. Several possibilities can be entertained to explain it. TgP2-specific $CD4^+$ T line cells may have direct cytolytic activity against thyrocytes without any assistance from the endogenous T cell pool (Maron et al, 1983) analogous to the cytotoxic potential of encephalitogenic $CD4^+$ T cells against MBP-pulsed astrocytes *in vitro* (Sun and Wekerle, 1986). Alternatively, these $CD4^+$ T cells may activate distinct final effector cells, such as Tg-specific MHC class I-restricted $CD8^+$ T cells from the recipient lymphocyte pool, through secretion of lymphokines as suggested (Cohen and Livant, 1976; Sugihara et al, 1993). In that regard, murine Tg-specific MHC class I-

restricted $CD8^+$ T cells have been shown to have cytolytic activity against TEC *in vitro* in mice (Creemers et al, 1983; Remy et al, 1989). This interpretation is also compatible with previous reports on the incidence of EAT in H-2 mutant mice in which a critical role for the H-2K (Tomazic and Rose, 1974; Maron and Cohen, 1979; 1980; Maron et al, 1982) or H-2D (Maron et al, 1982) gene products was suggested. However, such data are not available in rats to draw any correlation. Moreover, it is also possible that these thyroiditogenic $CD4^+$ T cells may mediate their effector functions through a DTH mechanism by secretion of lymphokines and activation of macrophages *in vivo*. A role in thyroid pathology for TNF-α (Taverne et al, 1987) and IL-1β (Wilson et al, 1990), cytokines secreted by macrophages, has been suggested. Analysis of lymphokine secretion profile or lymphokine mRNA expression of the pathogenic TgP2-specific T cell line and depletion of $CD4^+$ or $CD8^+$ T cells or both in the recipient rats may further the understanding of the mechanisms by which TgP2-specific T cells mediate thyroid lesions in rats.

The results demonstrating the MHC-unrestricted nature of the pathogenic TgP2-specific $CD4^+$ T cell line (Figure 5.3) with an epitope specificity for Tg sequence 2695-2706 are unexpected. These T cells are called promiscuous T cells. These data are in apparent contrast to the MHC-restricted nature of previously described thyroiditis-inducing T cell

lines or clones (Sugihara et al, 1993; Hiyama et al, 1993; Zerubavel- Weiss et al, 1992) and T cells specific for other conventional Ags (Zinkernagel and Doherty, 1974) in mice. The present findings (Figure 5.3) are in line with previous findings on MHC-unrestricted recognition of AChR by specific rat x mouse T-T hybridomas in Lewis rats in the context of syngeneic (Lewis) and allogeneic (Sprague-Dawley) rat APC (Tami et al, 1987). However, whether or not, these AchR-specific T cell hybridomas recognize similar epitopes in an MHC-unrestricted fashion was not investigated.

It is of interest to note that the activation of the MHC-unrestricted TgP2-specific T cell line is MHC class II-dependent (Table 5.3) and APC-dependent (Figure 5.3). These data are in contrast to an earlier report in which the authors have shown that activation of the MHC-unrestricted hapten (FITC)-specific $CD4^+$ T cells is MHC class II- and APC-independent (Silciano et al, 1986). T cell promiscuity has also been reported in diverse species with $CD4^+$ T cells specific for 9mer or 15mer peptides of tetanus toxin in the context of distinct HLA-DR alleles (Panina-Bordignon et al, 1989; Ho et al, 1990), for a 16mer peptide of herpes simplex virus glycoprotein D in the context of H-2 I-E alleles (Heber-katz et al, 1988), for a 17mer AChR peptide in the context of H-2 I-A alleles (Infante et al, 1991), and for a 20mer mycobacterial epitope in the context of H-2 I-A alleles

(Vordemeier, 1994).

Furthermore, the MHC-unrestricted recognition of the peptide reported in the present one and in other studies is not unique to $CD4^+$ T cells. This phenomenon was also reported by several investigators for cytotoxic T lymphocytes (CTL) against diverse Ags such as the tetanus toxoid (Schmitt et al, 1984), cyanogen bromide peptide fragments of ovalbumin (Carbone et al, 1988), the nonprotein Ag hemin (Sherman and Lara, 1989), the polyvalent Ag mucin (Barnd et al, 1989; Jerome et al, 1991), 15mer peptides of HIV-1 gp160 (Shirai et al, 1993), and a 23mer ras peptide (Yin et al, 1994).

The mechanism(s) of T cell promiscuity i.e., how T cells recognize peptide in an MHC-unrestricted fashion, is not known. Several possibilities can be entertained to explain it. One possibility is that the 18mer TgP2 or the 12mer Tg peptide (2695-2706) might bind to rat MHC class II molecules outside the peptide binding groove in an unconventional way and be recognized by T cells analogous to bacterial superantigens (SAg) (Dellabona et al, 1989). Additional experiments such as analysis of TcR Vß usage by the TgP2-specific T cells and peptide competition studies with the known SAg and the MHC class II bound peptides are needed to address this possibility. In contrast to SAg (Peavy et al, 1970), TgP2 is not mitogenic on unprimed rat splenocytes or LNC (data not shown). Analogous to SAg (Janeway and Katz,

1985; Lynch et al, 1985), recognition of TgP2 by promiscuous T cells is MHC class II- (Table 5.3) and APC-dependent (Figure 5.3).

Alternatively, T cell promiscuity may involve the recognition, by TcR of a single T cell clone, of a similar "peptide conformation" assumed upon interaction of the peptide with non-polymorphic residues of MHC class II molecules as hypothesized earlier (Panina-Bordignon et al, 1989). Thus, it is possible that F344 rat T cells may recognize a particular conformation of the 12mer Tg peptide (2695-2706) adopted upon its binding to MHC class II molecules on both F344 (syngeneic) and WKY (allogeneic) rat APC (Figure 5.3). On the other hand, T cell promiscuity may also involve the recognition of the peptide in a conventional way in which the peptide interacts with the polymorphic residues of MHC class II molecules (Vordemeier et al, 1994). The observations that the 18mer TgP2 or the 12mer Tg peptide-pulsed WF rat APC fail to stimulate the T cell line indicate that T cell promiscuity described in this study is incomplete. i.e., some (F344; WKY) but not all (WF) rat APC could trigger proliferation of the TgP2-specific T cell line (Figure 5.3). These results are analogous to those results obtained in previous studies for human (Panina-Bordignon et al, 1989) and murine (Vordemeier, 1994) T cells specific for peptides of tetanus toxin and mycobacterial protein, respectively. Two possibilities can be

entertained for the observed failure of TgP2-pulsed WF rat APC to trigger the activation of the specific T cell line: One possibility may be that the 12mer peptide may not bind to WF rat MHC class II and thereby fail to activate the T cell line. Alternatively, the 12mer peptide may bind to WF rat MHC class II but adopts a different conformation from that formed on F344 and WKY rat APC. The biological significance for the generation of promiscuous T cells over MHC-restricted T cells is unknown and remains conjectural.

As opposed to experimentally induced autoimmune thyroiditis in rats with Tg emulsion in adjuvant, the initial triggering event in the spontaneous form of Hashimoto's thyroiditis in humans is unknown. Among the several mechanisms that have been proposed, molecular mimicry (Oldstone, 1987) has been attributed to be one of the mechanisms that could trigger pathogenic autoreactivity. It is noteworthy that the N-terminal 12mer Tg epitope (2695-2706) which is recognized by the thyroiditis-inducing TgP2-specific T cell line possess 67% a.a. sequence homology with E1B peptide (V**S**F**Y**S**S**Y**I**Q**T**L**T**) (underlined residues are conserved amino acids) of adeno virus. This sequence homology has raised the possibility that thyroiditogenic T cells may be triggered by "molecular mimicry", however, its relevance to pathogenic thyroid autoimmunity remains to be determined. Thus, identification of pathogenic T epitopes within Tg in

experimental animal models will eventually aid in the definition of cross-reactive T cell epitopes or molecular mimics harboured in external microbial pathogens such as viruses and bacteria. This information will provide an important link between an environmental agent and an immunological effector system associated with formation of AITD in humans (Weetman and McGregor, 1994).

At present it is not known whether both TgP1 and TgP2 are important in the spontaneously induced thyroiditis either in animals or in humans. It would be interesting to address the relative importance of these pathogenic sequences, at both T and B cell levels, in spontaneously induced thyroiditis in rats and in HT patients. The results that will be obtained by these experiments will reveal the fundamental similarities or differences between the induced and spontaneous models of thyroiditis in terms of immunodominance and crypticity of Tg epitopes.

The findings of the absence of serological "intramolecular determinant spreading" in the TgP2-mediated rat EAT are in apparent contrast to a recent report on "intramolecular determinant spreading" of the autoimmune response to an ovarian self-peptide in a murine model of experimental autoimmune oophoritis (EAO) (Lou and Tung, 1993). These authors suggested that endogenous release of autoantigens subsequent to a pathological injury in the ovary

might be the cause for "determinant spreading" in this animal model (Lou and Tung, 1993). The findings of Lou and Tung (1993) have been extended to animal models of other human autoimmune diseases such as myasthenia gravis (Vincent et al, 1994), systemic lupus erythematosis (James et al, 1995; Topfer et al, 1995). Thus, the absence of serological "determinant spreading" in rat EAT suggest that "determinant spreading" may be governed by several parameters. These parameters may include the type of autoantigen and its concentration and availability in the circulation, the kinetics of the immune response, and the level of immunoregulatory mechanisms to that autoantigen.

In conclusion, the findings presented in this Chapter and in **Chapter 4** will provide a simplified model for studying/understanding immunoregulatory mechanisms of EAT.

CHAPTER 6

Mapping of Immunodominant B cell Epitopes within Thyroglobulin: Immunodominant shifts Among Heterologous Thyroglobulins Influence the Induction of IgG Responses to Distinct B epitopes within a Pathogenic Thyroglobulin Peptide (2695-2713) in F344 Rats

6.1 Introduction

Autoantibodies (AAbs) to thyroglobulin (Tg) are a common feature of experimental or clinical thyroid disease, but the nature and location of the epitopes recognized by these AAbs remain mostly undefined (Kuppers et al, 1992). Tg-specific IgG are present in unimmunized animals (Dighiero et al, 1983), healthy subjects (Ericsson et al, 1985; Guilbert et al, 1982; Kohno et al, 1988), and patients with thyroid disease (Roitt et al, 1956). These findings have spurred efforts to characterize Tg epitopes recognized only during the disease process and to distinguish them from those recognized by natural AAbs (Ruf et al, 1985; Piechaczyk et al, 1987; Bounani et al, 1989; Dietrich et al, 1991; Bresler et al, 1990b). Such information would aid in the development of diagnostic tools and in our understanding of the pathogenesis, progression, and immunoregulation of thyroid disease.

A commonly used approach for the delineation of Tg

determinants recognized by patients' sera or animal antisera involves three steps: First, a panel of mouse MAbs with distinct specificities are raised against human (hTg) or mouse Tg (mTg) and used to map the relative topolography of "clusters" of determinants on the Tg molecule. Second, in cross-inhibition studies, murine Tg-specific MAbs are tested for their capacity to inhibit the binding of patients' sera to intact hTg, and/or to chemically or enzymatically derived or recombinant hTg peptides (Dong et al, 1989; Kohno et al, 1985; Henry et al, 1990; 1992; Schultz et al, 1992; Bresler et al, 1990b; Piechaczyk et al, 1987; Ruf et al, 1983). Third, hTg-specific MAbs secreted by human-mouse B cell hybridomas are used as competitors with hTg-specific murine MAbs (Fukuma et al, 1991).

These efforts have led to the definition of a limited number (4-6) of Tg epitopes that are recognized by patients' sera (Nye et al, 1990; Chan et al, 1987; Fukuma et al, 1989) compared to the definition, by MAb panels, of 5-7 antigenic clusters on homologous Tg (mTg) (Gleason et al, 1990; Kotani et al, 1985), and of 6-12 antigenic clusters on heterologous Tg (hTg) (Bounani et al, 1992; Bresler et al, 1990a and 1990b; Piechaczyk et al, 1987; Ruf et al, 1983).

In spite of these advances made in defining the number of antigenic clusters on homologous <u>versus</u> heterologous Tg with mouse MAbs, the factors that influence the immunogenicity

of Tgs and the Tg peptides encompassing immunodominant B cell epitopes associated with AITD remain unknown. In this study, an attempt was made to address the above issue(s) by monitoring IgG responses to the pathogenic homologous Tg peptides, TgP1 (2695-2713) (**Chapter 4**) and TgP2 (2495-2511) (**Chapter 5**), by priming of F344 rats with homologous (rat) and heterologous (mouse, bovine, porcine and human) Tgs.

6.2 Results

6.2.1 The TgP2 but not the TgP1 contains immunodominant B cell epitope(s) in the context of homologous thyroglobulin

To address the serological immunodominance of the phylogenetically conserved pathogenic Tg peptides, TgP1 and TgP2, in the context of homologous Tg (rTg) in rats, day 28 sera from rTg-primed F344 rats (2/group) **(Section 2.11)** were obtained. Analysis of the pooled sera by direct ELISA for IgG responses to rTg, TgP1 (2495-2511), TgP2 (2695-2713), and ovalbumin (control Ag), showed immunoreactivity with intact rTg but not ovalbumin (Figure 6.2A). Surprisingly, the same sera reacted with pathogenic rat Tg peptide, TgP2 but not TgP1 (Figure 6.2B). The data indicate that the pathogenic TgP2 but not TgP1 sequence contains immunodominant B cell determinants in the context of homologous Tg (rTg) in rats. Furthermore, the rTg-induced IgG titer to TgP2 is only 128-fold lower than that observed against rTg itself (the end titer points of sera from rTg-primed rats against rTg and TgP2 are 1/64,000 and 1/512 dilution, respectively). These data further suggest that TgP2 is one of the few immunodominant B cell epitopes within the huge Tg molecule (660 kDa).

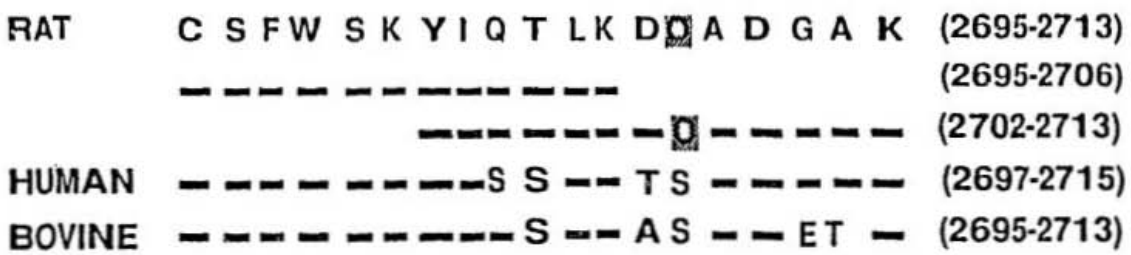
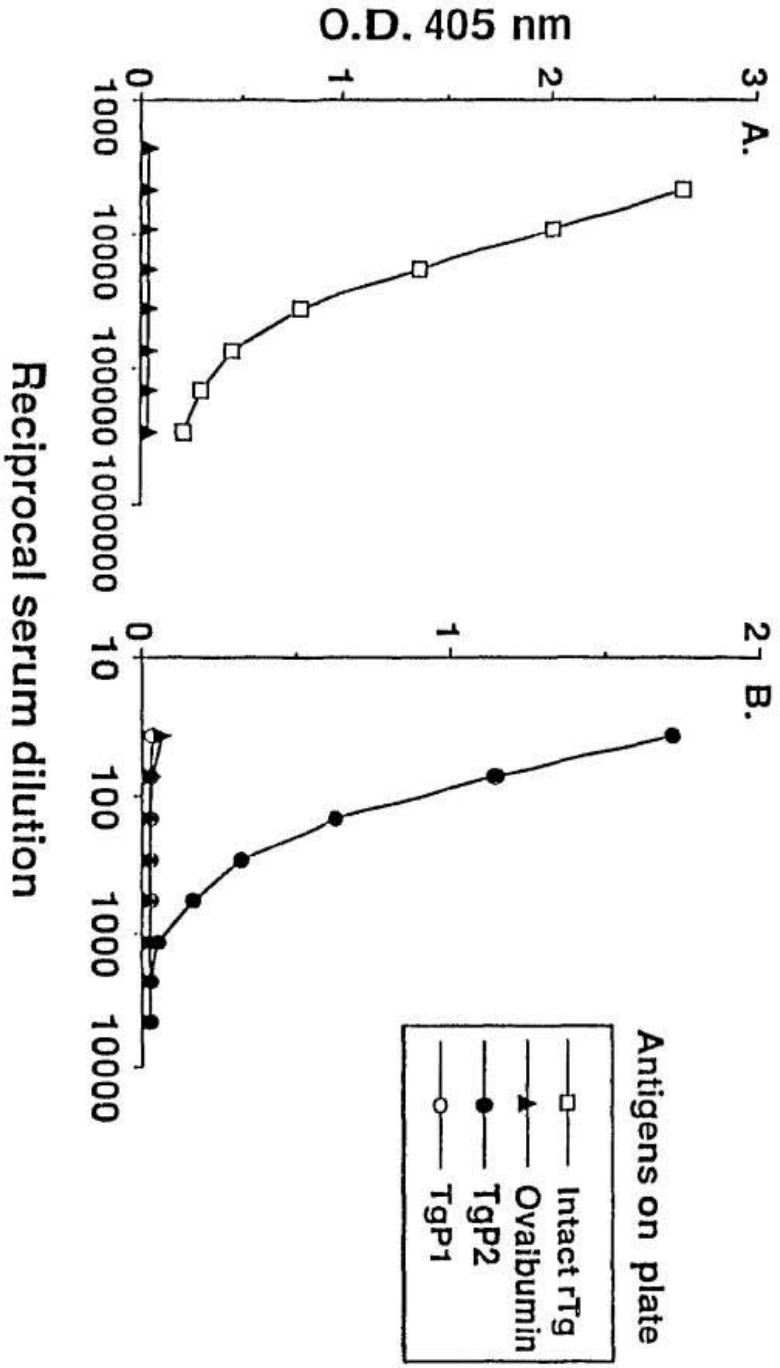

RAT C S F W S K Y I Q T L K D Q A D G A K (2695-2713)
(2695-2706)
(2702-2713)
HUMAN S S T S (2697-2715)
BOVINE S A S E T (2695-2713)

Figure 6.1 Comparison of primary amino acid sequence of TgP2 of rat, bovine, and human Tgs. For comparative purposes, the two 12mer overlapping truncated peptides of rTgP2 are also included in this figure (Carayanniotis et al, 1994).

* The shaded position indicates an amino acid deletion on rTgP2 corresponding to hTgP2.

Figure 6.2A Reactivity of sera from rTg-primed F344 rats with intact rTg but not OVA. Pooled sera (2/group) obtained 28 days after rTg-priming, as described under Materials and Methods (**Section 2.11**) were tested in serial two-fold dilutions against rTg (1 μg/well) or ovalbumin (1 μg/well) by a direct ELISA (**Section 2.19**). The readings were taken at O.D. 405 nm. The values are means of duplicate wells minus means of background values and expressed as mean +/- SD. Mean of back ground values was 0.190.

Figure 6.2B The TgP2 but not TgP1 contains immunodominant B cell epitopes. Pooled sera of rTg-primed F344 rats that were used in Figure 6.2B, were tested in serial two-fold dilutions against TgP1 (0.2 μg/well) or TgP2 (0.2 μg/well) by a direct ELISA (**Section 2.19**). The readings were taken at 405 nm. The values are means of duplicate wells minus means of background values. The values are expressed as mean +/- SD. Mean of back ground values was 0.204.


O.D. 405 nm
A.
B.
Reciprocal serum dilution
Antigens on plate
Intact rTg
Ovalbumin
TgP2
TgP1

6.2.2 The non-dominance of TgP1 remains constant, whereas the relative immunodominance of TgP2 varies significantly among heterologous Tgs

To address the serological immunodominance of phylogenetically conserved rat TgP1 and TgP2 sequences in the context of heterologous Tgs, day 28 sera from F344 rats (2/group) primed with Tgs of mouse, bovine, porcine, and human origin were analyzed by direct ELISA for IgG responses to TgP1 and TgP2. The results are shown in Figure 6.3. No IgG responses were elicited against TgP1, regardless of the heterologous Tg used for priming of F344 rats (Figure 6.3C). The data indicate that TgP1 is serologically non-dominant or cryptic on Tgs of various species. Surprisingly, heterologous Tgs varied dramatically in their capacity to elicit TgP2-specific IgG responses (Figure 6.3A). Bovine Tg (bTg) was as efficient as rTg, whereas mTg was moderately efficient and porcine or human Tg are not immunogenic at the TgP2 site (Figure 6.3A), although these Tg preparations elicited strong IgG responses to corresponding Tg (Figure 6.3D).

To address whether the failure of sera from hTg-primed rats to react with rTgP2 is due to amino acid sequence differences within the determinant site, in an analogy to T cell epitopes (Liu et al, 1993), the hTg-primed sera were tested against human TgP2 (hTgP2). The hTg-primed F344 rat sera failed to recognize hTgP2 itself (Figure 6.3C), thus

excluding the possibility that absence of immunoreactivity of these sera to rTgP2 is due to amino acid sequence differences between rTgP2 and hTgP2. Testing of the immuno-reactivity of sera from pTg-primed rats against the pTgP2 was not possible, because the primary amino acid sequence of pTg is not known. Thus, monitoring of the serological response to the 18mer TgP2 sequence presented in the context of diverse Tgs, revealed large variations in the relative immunogenicity of this peptide.

6.2.3 The fine epitope specificity of TgP2-specific IgG induced by thyroglobulins of various species varies dramatically

Since linear B cell epitopes are known to be in the range of 5 to 10 amino acids in length, it is not known whether TgP2-specific IgG induced by homologous and heterologous Tg-priming recognize similar or distinct B epitopes within the TgP2 site. Analysis of the reactivity of pooled serum samples from rTg, mTg, or bTg-primed rats (of Figure 6.3) against two overlapping 12mer truncated peptides of rTgP2 revealed an even more complex pattern of Ab responsiveness (Figure 6.5). The rTg-induced Abs recognized epitopes within the carboxy-terminal (2702-2713) but not the amino-terminal (2695-2706) 12 mer peptide indicating the localization of dominant B cell epitope(s) within the C-terminal peptide 2702-2713.

Figure 6.3A-D Immunodominance of TgP2 on heterologous Tgs varies dramatically. Reactivity of the sera from homologous or heterologous Tg-primed F344 rats (2/group) to (A) rTgP2, (B) hTgP2, (C) rTgP1, and (D) respective Tg in a direct ELISA **(Section 2.19)**. The sera from homologous Tg-primed animals employed in this experiment were the same that were used to establish the data in Figure 6.2. The values represent mean of triplicate wells minus the mean of the background values and SD values did not exceed 10% of the mean values. The mean of background values ranged from 0.144 - 0.152. The details of immunizations of rats with Tgs are described in **Section 2.11**.

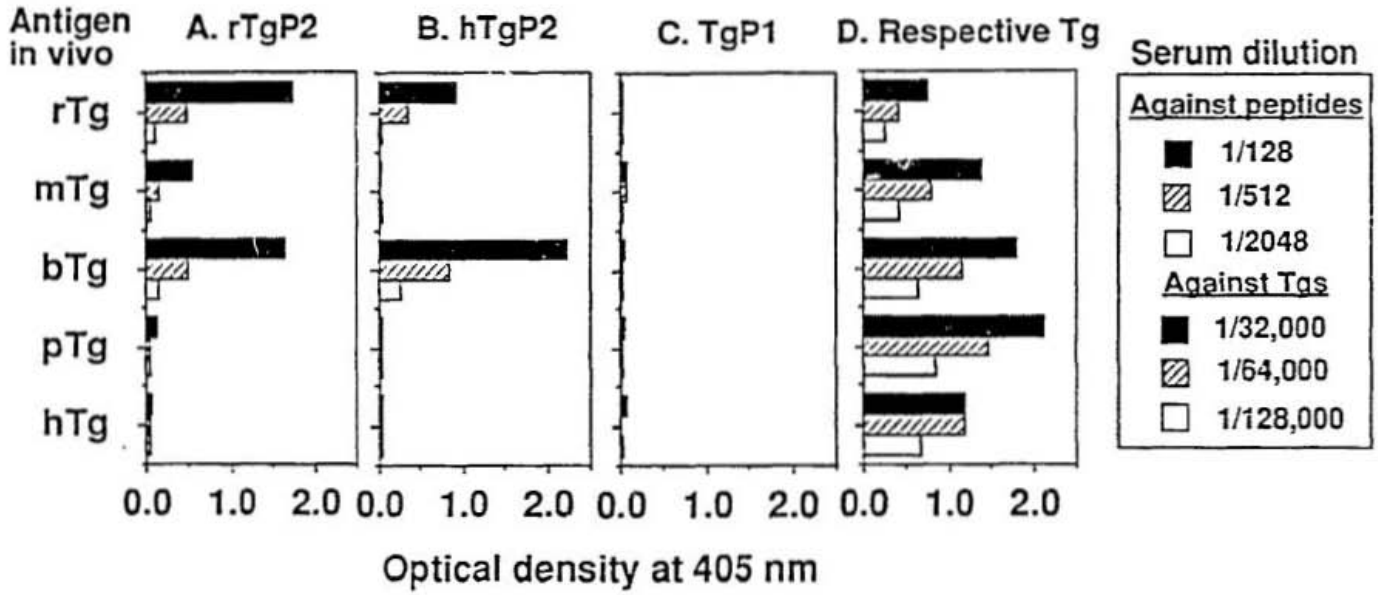
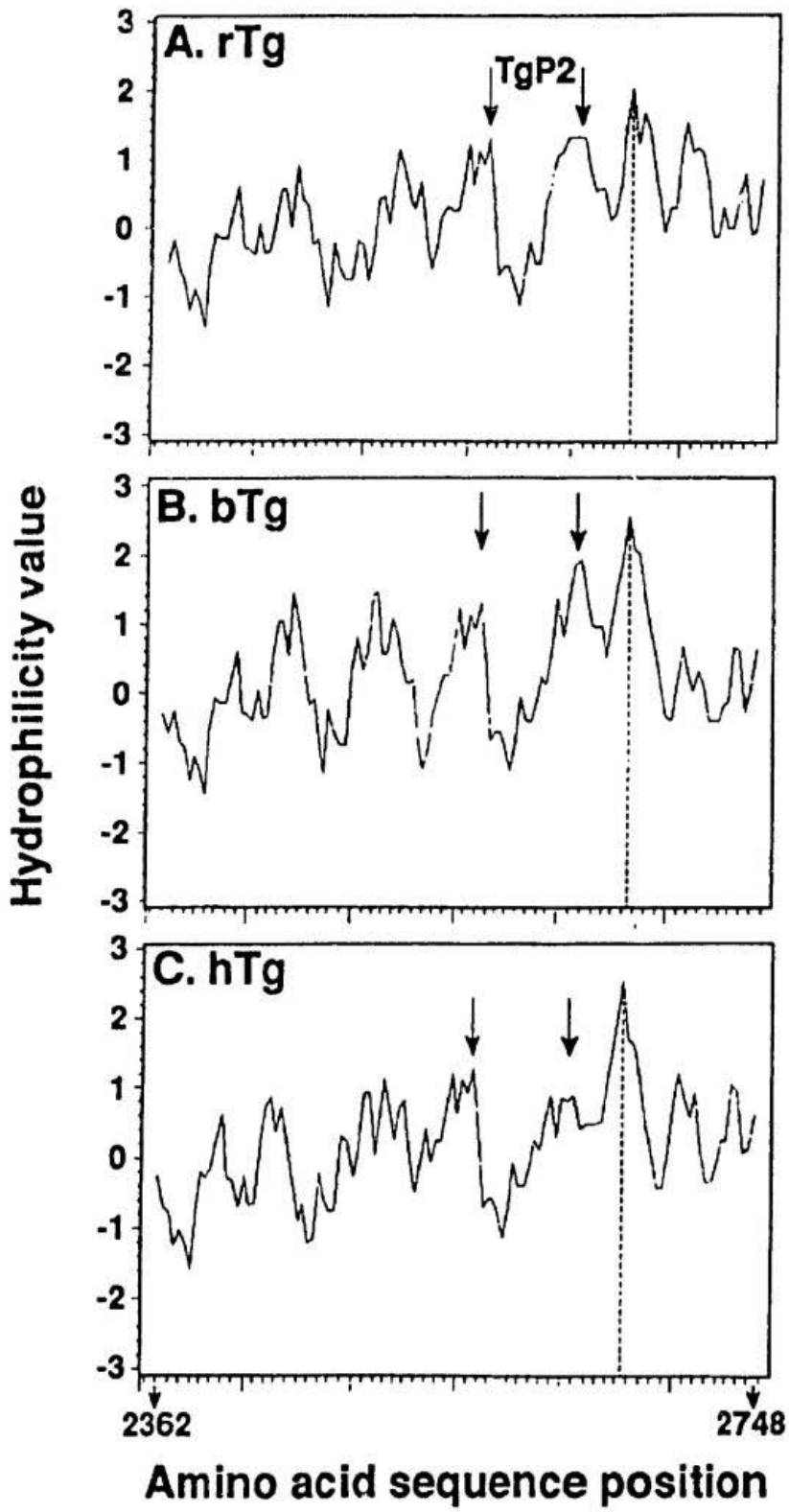

Antigen in vivo
A. rTgP2
B. hTgP2
C. TgP1
D. Respective Tg
rTg
mTg
bTg
pTg
hTg
0.0 1.0 2.0
Optical density at 405 nm
Serum dilution
Against peptides
1/128
1/512
1/2048
Against Tgs
1/32,000
1/64,000
1/128,000

Figure 6.4 Hydrophilicity profiles of corresponding TgP2 sequence on rat, bovine, and human Tgs. The hydrophilicity profiles were generated based on the method described by Hopp and Woods (1981). The arrows indicate the N- and C-terminii of the TgP2 sequences on rTg, bTg and hTg.


A. rTg
TgP2
B. bTg
C. hTg
Hydrophilicity value
3
2
1
0
-1
-2
-3
2362
2748
Amino acid sequence position

Surprisingly, bTg-induced IgG recognized determinants within the N-terminal (2695-2706) but not the C-terminal (2702-2713) 12mer peptide, indicating the localization of immunodominant B cell epitope(s) within the peptide 2695-2706. Interestingly, mTg-elicited IgG bound neither of the 12mer peptides of rTgP2 suggesting that it recognized a core determinant of rTgP2 which was lost by the splitting of rTgP2 into two 12mer peptides. This was further suggested by the lack of reactivity of mTg-induced IgG with hTgP2 (Figure 6.3B). Thus, within the TgP2 site, heterologous Tg-induced TgP2-specific IgG recognize B cell epitopes distinct from those of homologous Tg-induced TgP2-specific IgG and provides evidence for the presence of a cluster of species-specific immunodominant B cell epitopes at the TgP2 site.

It is of interest that at the T cell level there appear to be at least two T cell epitopes with in TgP2, one in its N-terminal half and the other epitope, likely to be, in its central region (Table 6.1).

6.2.4 The rTgP2 and hTgP2 sequences are mutually cross-reactive at the serological level

To examine the serological cross-reactivity among rat and human TgP2 peptides, F344 rats (2/group) were immunized with 12mer peptides of rTgP2 or 19mer hTgP2. The immune sera were examined by direct ELISA for peptide-specific IgG. In

Figure 6.5 Recognition of distinct B cell epitopes within TgP2 by sera from rat, mouse and bovine Tg-primed F344 rats. Pooled Tg-primed sera of F344 rats that were used in the experiment for Figure 6.2 and 6.3 were tested against two 12mer truncated peptides of rTgP2 in a direct ELISA as described under **Section 2.19**. The overlapping 12mer truncated peptides were used at 8 μg/well. The readings were taken at 405 nm. The values are expressed as mean of duplicate wells minus mean background values. The mean of the background values ranged from 0.147 - 0.152.

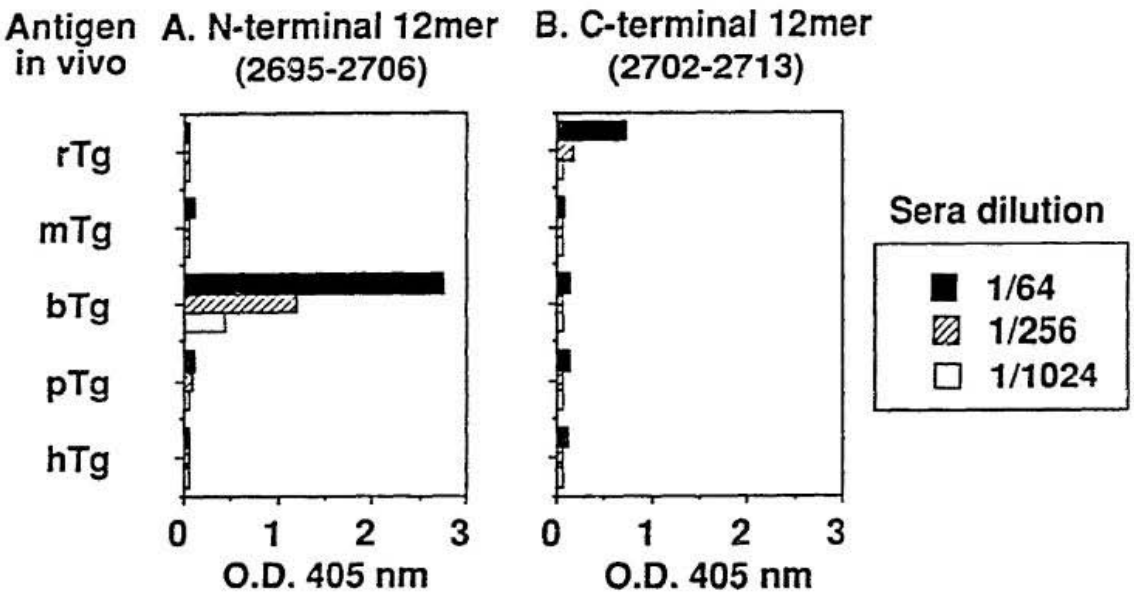

Antigen in vivo
A. N-terminal 12mer (2695-2706)
B. C-terminal 12mer (2702-2713)
rTg
mTg
bTg
pTg
hTg
0
1
2
3
O.D. 405 nm
0
1
2
3
O.D. 405 nm
Sera dilution
1/64
1/256
1/1024

Table 6.1 TgP2 sequence contains at least two distinct T cell epitopes

T cell hybrid (polyclone)	CTLL proliferation(cpm/1000)			
	No antigen	2695-2713	2695-2706	2702-2713
4G1	0.61 +/- 0.24	16.00 +/- 2.71	0.36 +/- 0.07	0.30 +/- 0.17
4H11	0.42 +/- 0.18	9.19 +/- 1.74	0.40 +/- 0.19	0.11 +/- 0.01
5D5	0.37 +/- 0.34	12.29 +/- 1.15	0.71 +/- 0.45	0.43 +/- 0.19
1F7	0.29 +/- 0.09	8.27 +/- 1.16	6.14 +/- 2.20	0.19 +/- 0.01
2A3	0.28 +/- 0.02	5.48 +/- 3.96	4.89 +/- 0.03	0.37 +/- 0.30
4G3	0.37 +/- 0.09	10.56 +/- 0.20	12.01 +/- 2.65	0.20 +/- 0.04

Polyclonal T cell hybridomas (1.5×10^4/well) were cultured with irradiated (1500 rad) syngeneic splenocytes (5×10^5/well) in the presence of indicated antigens for 48 hr in 200 µl volume of DMEM-10 in 96-well flat-bottomed micro culture plates. T cell hybridoma activation and CTLL assays were performed as described under Materials and Methods (section2.17). The peptide concentrations used were as follows: a.a. 2695-2713 = 10 µg/ml; a.a. 2695-2706 = 25 µg/ml; a.a. 2702-2713 = 25 µg/ml. The values are mean of duplicate wells +/- SD.

addition, sera from rTgP2-primed rats (Figure 5.5A) were also used. The results are shown in Table 6.2. IgG from rTgP2- and hTgP2-primed rats exhibited mutual cross-reactivity, though the peptides share only 79% primary amino acid sequence homology. The heterogeneity of the elicited rTgP2 and hTgP2-specific IgG was analyzed by testing their reactivity with the two 12mer truncated peptides representing the amino-end (2695-2706) and the carboxyl-end (2702-2713) of the rTgP2 sequence (see Figure 6.1). This analysis revealed that TgP2-specific IgG reacted more strongly with the C-terminal than with the N-terminal 12mer Tg peptide. These data indicate the relative immunodominance of the C-terminal 12mer over the N-terminal 12mer peptide of rTgP2.

Interestingly, the 12mer peptides are immunogenic themselves since the elicited IgG reacted with the corresponding peptide used for immune challenge (Table 6.2). However, the 12mer peptides were not mutually cross-reactive, although they share an hexamer overlapping amino acid sequence "YIQTLK". Furthermore, the inability of the C-terminal peptide (2695-2706)-specific IgG to react with the 18mer rTgP2 (Table 6.2) may be due, at least in part, to its ability to discriminate the different conformations adopted by the 18mer and 12mer peptides upon adsorption onto PVC plates as reported for peptides of Streptococcus mutans earlier (Lehner et al, 1989). The data indicate that rTgP2

Table 6.2 The rTgP2 and hTgP2 sequences are mutually cross-reactive at serological level

Antigen *in vivo*	Serum dilution	Secondary IgG responses (O.D.405 nm) against rTgP2 peptides			hTgP2
		2695-2713 (18mer)	2695-2706 (12mer)	2702-2713 (12mer)	(19mer)
rTgP2 (18mer) (2695-2713)	1/128	> 2.00	1.63	>2.00	>2.00
	1/512	>2.00	0.45	>2.00	>2.00
	1/2048	>2.00	0.13	>2.00	>2.00
N-terminal 12mer (2695-2706)	1/64	0.40	1.31	<0.05	1.24
	1/256	0.12	0.37	<0.05	0.38
	1/1024	<0.05	0.08	<0.05	0.10
C-terminal 12mer (2702-2713)	1/64	0.07	<0.05	0.62	0.09
	1/256	<0.05	<0.05	0.15	<0.05
	1/1024	<0.05	<0.05	<0.05	<0.05
hTgP2 (19mer)	1/128	> 2.00	1.09	2.18	>2.00
	1/512	> 2.00	0.37	0.80	>2.00
	1/2048	0.68	0.13	0.21	0.89

Pooled sera were obtained from F344 rats primed either with 200 nmol of 18 mer rTgP2 (3 rats/group) or N-terminal or C-terminal 12mer rTgP2 peptides (2 rats/group) or human TgP2 (2 rats/group) in CFA on day zero and boosted with 100 nmol of respective peptides on day 7. Sera were collected on day 28 and tested against indicated Ags by an alkaline phosphatase based ELISA as described under Materials and Methods (Section 2.18). The results are mean of duplicate or triplicate wells. The SD values did not exceed 10% of mean values. No significant IgG responses were detected against control peptide, rTgP1 (data not shown). Background values ranged from 0.12 to 0.14.

harbours two distinct immunogenic epitopes, of these, the C-terminal 12mer (2702-2713) is relatively dominant over the N-terminal 12mer (2695-2706) peptide.

6.2.5 Variation in TgP2 immunogenicity among heterologous thyroglobulins is not due to its inaccessibility to peptide-specific IgG

Since epitope accessibility to Ab is an intrinsic feature of antigenicity (Novotny et al, 1986), I asked whether the apparent lack of immunogenicity of TgP2 on porcine and human Tgs (Figure 6.3A and 6.3C) is due to its insufficient exposure on the surface of the Tg molecule. To address this, the reactivity of peptide-induced IgG (of Table 6.2) with Tgs from various species was assessed by direct ELISA. The results are shown in Table 6.3. Regardless of the immunogen used, significant IgG binding occurred on all Tgs tested (Table 6.3). However, Tg denaturation consistently allowed more efficient binding of specific IgG from hTgP2-primed rats, a finding consistent with the notion that the elicited Abs recognize linear Tg epitopes. In contrast, heat denaturation of Tgs did not generally increase its reactivity with IgG from sera of rats immunized with the shorter 12mer terminal peptides. An intriguing observation is that rTgP2-primed sera

reacted strongly with rat and mouse Tgs, weakly with bovine and porcine Tgs and not at all with hTg, although the same sera reacted with denatured Tgs from all the species.

These results indicate that: (a) the lack of immunogenicity of TgP2 on human and porcine Tgs cannot be attributed to lack of accessibility of the epitopes within the TgP2 site to Abs of predetermined specificity since specific IgG could readily bind to this Tg site, (b) the peptide (18mer versus 12mer)-induced IgG recognizes different epitopes on Tgs of various species and some of these epitopes are exposed while others are hidden within the TgP2 site, (c) the observed "skewness" of the serological response to the 12mer terminal peptides (Figure 6.5) also cannot be attributed to differential accessibility, since IgG raised against these peptides can bind to intact Tgs of various species tested, and (d) the fine epitope specificity of the 18mer rTgP2- versus the 19mer hTgP2-induced IgG is different.

Table 6.3 Variation in TgP2 immunogenicity among heterologous Tgs is not due to its differential accessibility to preformed antibodies*

Antigen in vivo	Serum dilution	Tg form	Serological cross-reactivity (O.D. 405 nm) against rTg	mTg	bTg	pTg	hTg
2695-2713 (18mer)	1/128	Native	1.80	1.96	0.20	0.16	0.08
	1/512	Native	1.00	1.14	0.04	0.07	0.02
	1/128	Denatured	>2.00	>2.00	1.58	1.86	1.59
	1/512	Denatured	>2.00	>2.00	0.85	0.97	0.87
2695-2706 (12mer)	1/128	Native	1.30	1.00	0.80	1.49	0.89
	1/512	Native	0.39	0.28	0.24	0.44	0.27
	1/128	Denatured	0.94	0.91	>2.00	0.84	0.76
	1/512	Denatured	0.29	0.26	0.99	0.21	0.19
2702-2713 (12mer)	1/128	Native	0.90	2.20	0.49	0.70	0.66
	1/512	Native	0.30	0.79	0.13	0.18	0.18
	1/128	Denatured	0.78	0.85	0.53	0.57	0.59
	1/512	Denatured	0.20	0.22	0.12	0.12	0.13
Human TgP2	1/128	Native	0.27	0.58	1.54	1.24	0.51
	1/512	Native	0.12	0.14	0.60	0.37	0.20
	1/128	Denatured	>2.00	>2.00	>2.00	>2.00	>2.00
	1/512	Denatured	1.38	1.82	>2.00	>2.00	1.34

*Cross-reactivity of pooled sera from peptide-primed F344 rats that were used in Table 6.1, against intact and heat denatured Tgs of various species as indicated by a direct ELISA (Section 2.18). O.D . 405 nm values represent means of duplicate wells. SD values did not exceed 5% of the mean values.

6.3 Discussion

The data described in this chapter (a) provide an explanation for the differences reported earlier in the mapping or location of immunodominant B cell epitopes on Tgs of various species, (b) provide the first evidence in EAT for an immunopathogenic Tg sequence (TgP2) that encompasses species-specific immuno-dominant B cell epitopes, (c) suggest that factors adjoining or distant from the epitopic site (TgP2) contribute to the variable immunodominance of Tgs at this site, and (d) reveal that the hierarchial immunodominance of B cell epitopes existing at the level of the complete Tg molecule (dimeric rTg has a m.wt. of 660 kDa) also exists at an 18mer (2.1 kDa) Tg peptide (rTgP2) level.

Features intrinsic to the protein structure such as surface accessibility (Novotny et al, 1986), hydrophilicity (Hopp and Woods, 1981), and segmental or local mobility (Westhof, 1984; Geysen et al, 1987) have been shown to determine the location of immunodominant B cell epitopes on large protein Ags. The present findings (Table 6.3), peptide-induced IgG reacting with intact heterologous Tgs, demonstrate that accessibility of epitopes (Novotny et al, 1986) is unlikely to be the intrinsic feature of Tgs which allows IgG responses to TgP2. These data are consistent with earlier results that accessibility of epitopes on intact protein Ag do not always correlate with their immunodominance (Wilson et al,

1984; Geysen et al, 1987). Furthermore, it is unlikely that the present data are influenced by the hydrophilic nature (Hopp and Woods, 1981) of Tgs at TgP2 site, because the hydrophilic index of the TgP2 sequence on rat, bovine and human Tgs are very similar (Figure 6.4). These findings are compatible with previous results which demonstrated that the hydrophilic nature of an epitope on a protein Ag does not always correlate with its immunodominance (Atassi, 1984). The lack of crystallographic resolution of Tg precludes us from determining the role of segmental mobility (Westhof, 1984) on the variable immunogenicity of heterologous Tgs at the TgP2 site. However, it has been suggested that if peptide-induced IgG reacts with both intact (Tainer, 1984; Westhof et al, 1984) and denatured Ag (Berzofsky, 1985), then the epitopic site on a given protein is likely to be mobile. The reactivity of peptide (12mers of rTgP2; hTgP2)-induced IgG with intact as well as heat denatured heterologous Tgs (Table 6.3), suggests that at least some epitopes within the TgP2 site on Tgs are mobile. These findings indicate that segmental mobility does not influence the immunodominant shifts among heterologous Tgs at the TgP2 site. Furthermore, the induction of Tg-reactive IgG by short synthetic peptides (Table 6.3) is consistent with the notion that the ability of short synthetic peptides to induce protein (intact Ag)-reactive Abs is not an uncommon event (Green et al, 1982) but occurs with high

frequency (Niman et al, 1983). On the other hand, the failure to induce hTgP2-specific IgG following hTg-priming is consistent with the notion that demonstration of peptide-reactive Abs following intact Ag-priming occurs with low frequency (Jemmerson, 1987).

The present analysis of the molecular context of the epitope within a given protein demonstrates that factors adjoining or distant from the epitopic site on heterologous Tgs influence its immunodominance. In this regard, Scheerlinck et al (1993) have demonstrated in mice that the presence of an immunodominant epitope within a recombinant protein used for immune challenge skewed the serological immune response away from the adjoining non-dominant epitope, whereas the absence of such an immunodominant epitope within the recombinant protein led to the induction of an IgG response to the non-dominant epitope. Rats similarly challenged with large recombinant fusion peptides of hTg containing hTgP2 will help to clarify this question. It has been shown earlier by several investigators that amino acid residues distant from or flanking the epitopic site (Shasthri et al, 1986; Dawe et al, 1993) or the presence of immunodominant epitopes away from the epitopic site (Nikcevich et al, 1994) in a given protein Ag profoundly influence the immunogenicity of MHC class II-restricted T cell epitopes.

The precise intrinsic features contributing to the

variable immunodominance of epitopes within the TgP2 site among heterologous Tgs are unknown. They may include differential tertiary folding and/or post-translational modifications. Tertiary folding may exert its effect either by masking the epitope(s) or by changing the hierarchy of immunodominant epitopes. The data as shown in Table 6.3, suggest that tertiary folding is unlikely to mediate its effect by masking epitopes within the TgP2 site to peptide-specific B cells in vivo. On the other hand, the differential tertiary folding among heterologous Tgs (as influenced by primary amino acid sequence differences) (Chan et al, 1986) may change the hierarchy of immunodominant B cell epitopes and thereby "skew" the immune response away from the epitopic site within the TgP2. However, evidence is lacking to support such a contention. Post-translational modifications of Tg such as iodination (Sundick et al, 1987) and glycosylation (Salable et al, 1976) have been shown to influence the immunogenicity of Tgs. At present, evidence for such differences among heterologous Tgs is lacking. However, overall, the present data suggest that factors intrinsic rather than extrinsic to Tgs contribute to the differential immunogenicity of heterologous Tgs at the TgP2 site.

Inconsistent detection of (Figure 6.3A) and distinct epitope recognition within (Figure 6.5) the TgP2 site by various Tg-induced IgG and consistent non-dominance of TgP1

(Figure 6.3C) suggest that by serological screening, TgP2 might be variably detected where as TgP1 might not be detected at all. Thus, the present approach indicate that by serological screening, some but not all Tg peptides associated with AITD will be identified.

Various investigators (Henry et al, 1990; 1992 ; Ruf et al, 1983; Dong et al, 1989) have demonstrated that the immunodominant Tg epitopes seen by autoantisera (HT patients' sera) are not consistently detected by heteroantisera (Tg-primed animal antisera). Henry et al (1992) demonstrated that epitopes within the 1149-1250 and 1258-1295 region on hTg are immuno-reactive with anti-hTg Abs from HT patients and rabbits respectively but not vice versa. In another study, Dong et al (1989) were able to localize immuno-reactive heteroepitopes within a larger hTg fragment (2644-2736) using anti-hTg Abs from rabbits (heteroantisera) but not with sera from HT patients (autoantisera).

The variable location or variation in the hierarchy of immunodominant epitopes on hTg as described above seems to be influenced by factors extrinsic to Tg such as species. Thus, it is possible that the non-dominance of hTgP2 or hTgP1 (Figure 6.3) in rats (heterologous species) in the context of hTg may become immunodominant in autoimmunized HT patients, a possibility that can be easily tested by screening the HT patients' sera directly against homologous TgP2 or TgP1

sequences. Thus, intrinsic (present study) and extrinsic (Henry et al, 1990;1992; Ruf et al, 1983; Dong et al, 1989) factors to Tg effect the location and mapping of the immunodominant B cell epitopes on Tgs. These findings need to be considered in the development of diagnostic MAb kits.

In the present study, an interesting correlation emerged between homologous Tg- and TgP2-induced IgG responses: the C-terminal 12mer (2702-2713) peptide is dominant in the context of both Tg and 18mer TP2. In contrast, the N-terminal 12 mer (2695-2706) peptide is relatively less dominant in the context of 18mer TgP2 and is non-dominant in the context of homologous Tg. It should be emphasized that the non-dominance of the N-terminal 12mer Tg peptide (2695-2706) in the context of Tg is not due to accessibility, hydrophilicity, and segmental mobility of this site within Tg (Table 6.3). However, it may be attributed to the presence of an immunodominant epitope adjacent to or away from it (Figure 5A-B) as suggested in other studies (Scheerlinck et al, 1993). The reason(s) for such an intramolecular immunodominance of B cell epitopes within the TgP2 site when rats were primed with TgP2 is/are unknown. The molecular mechanism by which T cells provide differential help for B cell clones with different specificities on the same 18mer peptide is difficult to explain in the context of existing models of T cell - B cell interaction (Berzofsky, 1983; Ozaki and Berzofsky et al,

1987). In contrast to Tg, it is likely that both extrinsic and intrinsic features to the peptide contribute to the intramolecular immunodominance of B cell epitopes within TgP2. These factors may be either the differences in the frequency of B cells specific for the two 12mer peptides in vivo and/or the differences in the quantity of T help provided to peptide-specific B cells in vivo. Additional experiments are warranted to address these possibilities.

The induction of lowered anti-peptide Ab titers following challenge with the 12-mer peptide(s) compared to an 18mer TgP2 challenge (Table 6.2) may be due to lowered receipt in quantity of T help by peptide-specific B cells. This may be an effect of lowered recruitment of Th cells because the 12mer peptides may contain fewer T cell epitopes than the 18mer TgP2. Recruitment of more T cell help may be attributable to the presence of at least two T cell epitopes within TgP2 (Table 6.2). Furthermore, the presence of a third T cell epitope within TgP2 is indirectly suggested by the serological immunogenicity of the C-terminal 12mer Tg peptide (2702-2713) (Table 6.1).

The induction of TgP2- but not TgP1-specific IgG (Figure 6.2), and T cell (Table 5.2) responses in Tg-primed rats suggest that TgP2 might be one of the few immunopathogenic determinants participating in rTg-mediated rat EAT. For these reasons, TgP2-mediated rat EAT may serve as an excellent

animal model for studying the immunopathogenesis of HT in humans. Although rTg contains linear B cell epitopes at the TgP2 site, the role of autoreactive B cells specific for such epitopes in the disease pathogenesis is unknown. It is possible that autoreactivity may be triggered by viral infection (Onedera, 1990; Srinivasappa et al, 1988) through induction of cross-reactive B cells to epitopes accessible on Tg. Autoreactive B cells thus generated might play a role either in steering Ag-processing (Davidson and Watts, 1989) or in enhancement of Ag-presentation (Hutchings et al, 1987; Lanzavecchia, 1990) or in shaping the T cell repertoire (Burkhardt et al, 1992; Watts and Lanzvecchia, 1993). Antigen processing and presentation events may contribute to the initiation or diversification of the autoimmune response (Mamula and Janeway, 1993; Lin et al, 1991; Mamula et al, 1994). Alternatively, it is also possible that some autoreactive B cells specific for Tg may tolerize rather than activate Tg-specific autoreactive T cells and thereby contribute to the peripheral tolerance mechanism (Fuchs and Matzinger, 1992; Gilbert and Weigle, 1994; Eynon and Parker, 1993).

It is worth noting that the N-terminal 12mer (2695-2706) peptide of TgP2 that is recognized by a pathogenic T cell line (Table 5.4) and is accessible to peptide-specific IgG (Table 6.3) has 67% amino acid sequence homology with a 12mer peptide

(V**S**F**Y**S**S**Y**I**Q**T**L**T**) of E1B protein of adeno virus. Whether this homologous sequence has any relevance to pathogenic thyroid autoimmunity remains to be clarified. The contribution of anti-Tg Abs to pathogenic thyroid autoimmunity is also not clearly established. Tg-specific Abs may potentiate T cell-mediated thyroiditis as shown earlier in rats (Rose et al, 1973). In this regard, the findings (Table 5.4), TgP2-induced rat EAT as a $CD4^+$ T cell-mediated disease, clearly demonstrate that peptide-specific T cells but not IgG play a pivotal role in the induction of thyroid lesions.

In conclusion, the present findings may have implications in the serological screening of Tg epitopes associated with autoimmune thyroid disease (AITD), and may enhance our understanding of the factors that influence the immunogenicity of Tg at the serological level.

CHAPTER 7

Thyrocytes Fail to Stimulate Thyroiditogenic Peptide (2695-2713)-Specific F344 Rat T cells: Implications for Understanding Antigen Presentation in Thyroid Autoimmunity

7.1 Introduction

A decade ago, it was hypothesized that ectopic or aberrant expression of MHC class II Ags on thyrocytes might lead to presentation of their own autoantigens to infiltrating T cells and in this way these class II$^+$ thyrocytes could play a crucial role in the initiation of autoimmune thyroid disease (AITD) (Botazzo et al, 1983). Class II Ag expression on thyrocytes and its role in thyroid autoimmunity have been investigated in different ways: by immunohisto-chemical studies of thyroid sections or FACS analysis of thyrocytes, by analysis of the signals required to induce class II Ags on thyrocytes in vitro, and by experiments aimed at the ability of these thyrocytes to activate autoreactive T cells in Ag presentation studies.

A direct role for class II$^+$ thyrocytes was shown in vitro by their ability either to induce proliferative responses of intrathyroidal T cell lines and clones from HT and GD patients (Londei et al, 1985 ; Dayan, 1991) or to present viral peptides to specific T cells (Londei et al, 1984).

Furthermore, several studies in rats (Hirose et al, 1988a; Kimura and Davies, 1991a and 1991b) and mice (Charreire and Michel-Bechet, 1982; Salamero and Charreire, 1983a; 1983b; 1985a; 1985b; Salamero et al, 1987a) have demonstrated the proliferation or activation of T cells upon incubation with primary thyroid monolayers or cultures. In contradiction, a few reports have also shown that class II$^+$ thyrocytes failed to activate the T cell clones or hybridomas (Ebner et al, 1987; Stein and Stadecker, 1987) and have suggested that thyrocytes by themselves do not function as APC. These conflicting results suggested that activation of T cells by primary thyroid monolayers as opposed to thyroid epithelial cell (TEC) lines or clones may be attributed to contamination of the former with APC such as dendritic cells. In addition, the Ag- and epitope-specificities of autoreactive T cells used in the previous studies are largely not delineated. Thus, it remains unknown whether class II$^+$ thyrocytes in the inflamed thyroid, without the assistance APC such as dendritic cells, present Tg epitopes to T cells and initiate the autoreactivity.

I have demonstrated earlier that an 18mer homologous (rat) Tg peptide, TgP2 (2695-2713) is thyroiditogenic in F344 rats, and consists of non-dominant T- but dominant B-cell determinants (Chapter 5). TgP2-specific TcR α/β^+, CD4$^+$ T line cells were also shown to adoptively transfer severe

thyroiditis to naive syngeneic rats (**Chapter 5**). Because of the failure of the rTgP2-specific T cell line to proliferate in vitro to rTg, I have hypothesized that Ia^+ thyrocytes in the target organ may function as APC by presenting the peptidic sequence (TgP2) to specific $CD4^+$ T cells. Therefore, in the present study, I have examined the possible APC function of thyrocytes by using a readily available Fisher rat thyroid epithelial cell line (FRTL-5) as APC. The thyrocytes were IFN-γ treated to induce MHC class II antigens on them. These APC were used to activate TgP2 (2695-2713)-specific, cloned rat x mouse T cell hybridoma derived from a $CD4^+$ TgP2-specific T cell line in F344 rats.

7.2 Results

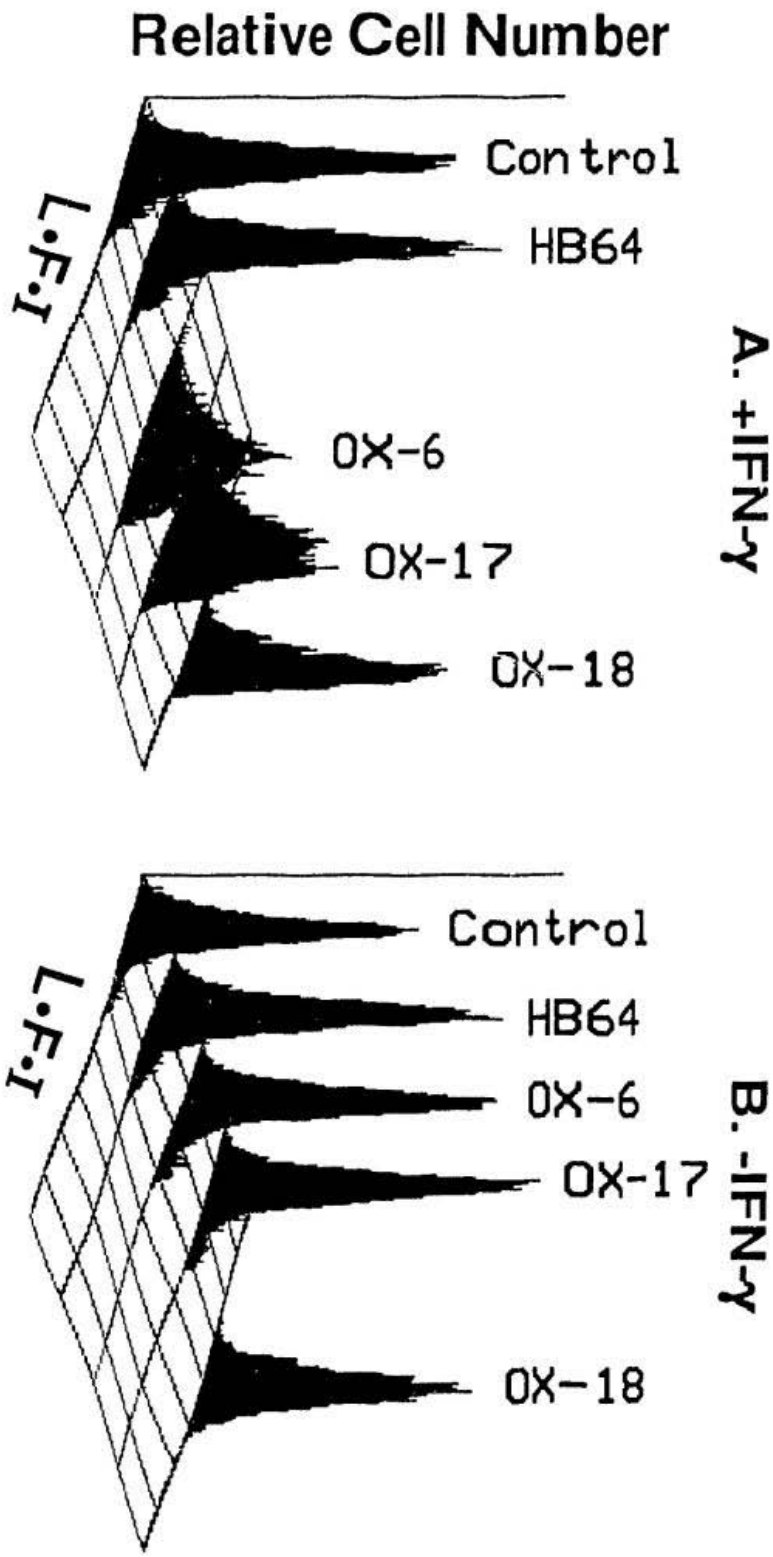
7.2.1 Treatment of FRTL-5 cells with recombinant IFN-γ induces MHC class II antigens

Previously it was reported that cloned FRTL-5 cells (thyrocytes) do not constitutively express MHC class II antigens (Ags), but do so upon treatment with recombinant IFN-γ for 5 days in vitro (Platzer et al, 1987). In the present study, treatment of FRTL-5 cells with 100 U/ml of IFN-γ induced the expression of both RT1-B (OX-6) (96%) and RT1-D (OX-17) (96.4%) class II Ags as assessed by FACS analysis (Figure 7.1A). In contrast, FRTL-5 cells that were not treated with IFN-γ failed to significantly express the class II Ags (5-6%) (Figure 7.1B). However, both IFN-γ untreated and treated FRTL-5 cells expressed MHC class I Ags (RT1-A) (OX-18) (99.8%)(Figure 7.1A-B). These data confirm that thyrocytes do not constitutively express class II (Ia) Ags but readily do so upon treatment with IFN-γ.

7.2.2 Phenotypic characterization of TgP2-specific 8F7-5 T cell hybridoma

Following the fusion of $CD4^+$, $CD8^-$ TgP2-specific T line cells with mouse BW5147 α^-/β^- thymoma cells, 265 of 768 seeded wells showed hybridoma growth and 43 hybrids were screened for TgP2-specificity. Out of 43 T cell hybridomas tested, 20

Figure 7.1 Flow cytometric analysis of FRTL-5 cells. FRTL-5 cells were incubated for 5 days in (A) the presence or (B) the absence of recombinant rat IFN-γ. Cells were detached from the plastic surface by EDTA treatment as described under Materials and Methods (**Section 2.21**). Cells were washed, and stained with OX-6 (RT1-B), OX-17 (RT1-D) and OX-18 (RT1-A) MAbs specific for MHC class II (RT1-B and RT1-D) and MHC class I (RT1-A) Ags respectively or with control isotype-matched MAb (HB-64) specific for matrix protein of influenza virus type A. Dead cells were gated out by incubating cells with 5 μg/tube of propidium iodide before subjecting the cells to FACS analysis. A minimum of 10,000 cells was counted. The results are expressed as histograms of relative cell number versus log fluorescence intensity (L.F.I).


Relative Cell Number
L.F.I.
Control
HB64
OX-6
OX-17
OX-18
A. +IFN-γ
L.F.I.
Control
HB64
OX-6
OX-17
OX-18
B. -IFN-γ

hybrids were TgP2-specific, and the rest of the 20 hybridomas were non-specific but responded to syngeneic splenic APC in the absence of the peptide itself (data not shown). The 8F7 T cell hybridoma was selected and cloned by limiting dilution (0.33 cells/well). The cloned 8F7-5 T cell hybridoma was used in <u>in vitro</u> Ag presentation studies.As shown in Figure 7.2A, the TgP2-specific T cell line after 4 days of <u>in vitro</u> stimulation with the peptide was W3/25$^+$ (98.9%), OX-8$^-$ (4%), OX-19$^+$ (98%), R73$^+$ (99.8%), OX-22$^-$ (1%) and expressed weakly OX-6 (RT1-B (13.3%) and OX-17 (RT1-D) (7.9%) class II Ags.

Surprisingly, the 8F7-5 cloned T cell hybridoma expressed both CD4 and CD8 markers near to 100%, suggesting that both CD4 and CD8 molecules are expressed on the same T cell in clonal T-T hybrid population. These data further suggest that after fusion with BW5147 α^-/β^- cells, there was activation of CD8 genes that had been silent in the parent T cell line, and release from stable repression of these loci in the long term rat CD4$^+$ T cell line. In contrast to the T cell line, the 8F7-5 T cell hybridoma did not express class II Ags. The rest of the phenotypic profile of the T cell hybridoma is similar to the parental T cell line.

7.2.3 The cloned 8F7-5 T cell hybridoma recognizes TgP2 in an MHC-unrestricted fashion

To address whether or not the cloned 8F7-5 T cell

Figure 7.2 Flow cytometric analysis of TgP2-specific (A) T cell line, and (B) cloned 8F7-5 T cell hybridoma. FACS analysis was performed as described under Materials and Methods (**Section 2.13**). Note that the hybridoma expresses both CD4 (W3/25) and CD8 (OX-8) molecules.

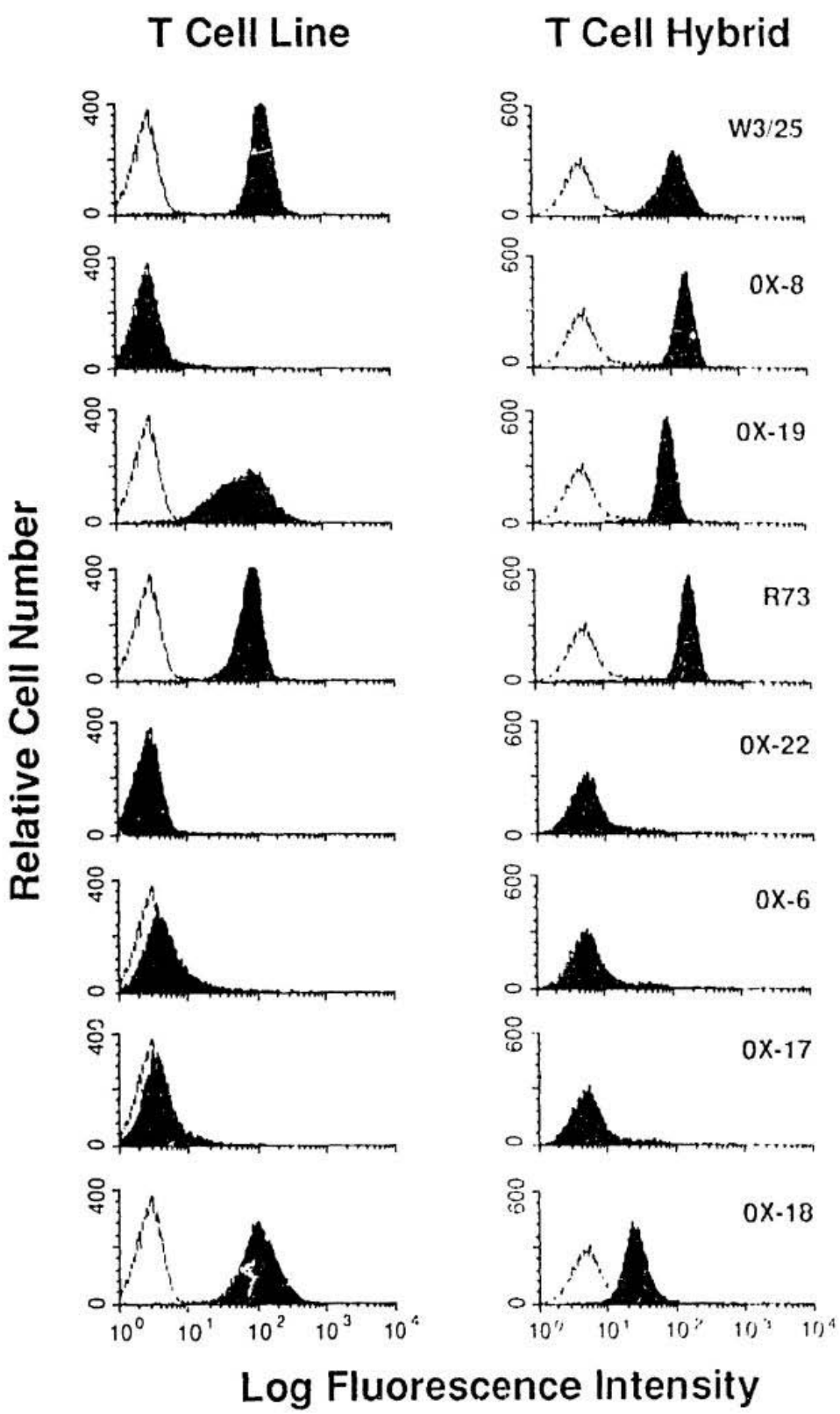

T Cell Line
T Cell Hybrid
W3/25
0X-8
0X-19
R73
0X-22
0X-6
0X-17
0X-18
Relative Cell Number
Log Fluorescence Intensity

Table 7.1 Specificity of the polyclonal (8F7) and clonal (8F7-5) T cell hybridomas derived from TgP2-specific $CD4^+$ T cell line

	CTLL proliferation (mean cpm/1000)		
T cell hybridoma	TgP2	TgP1	No antigen
8F7 (polyclone)	36.3 +/- 3.6	1.0 +/- 0.1	1.1 +/- 0.1
8F7-5 (clone)	31.2 +/- 3.3	1.2 +/- 0.3	0.7 +/- 0.5

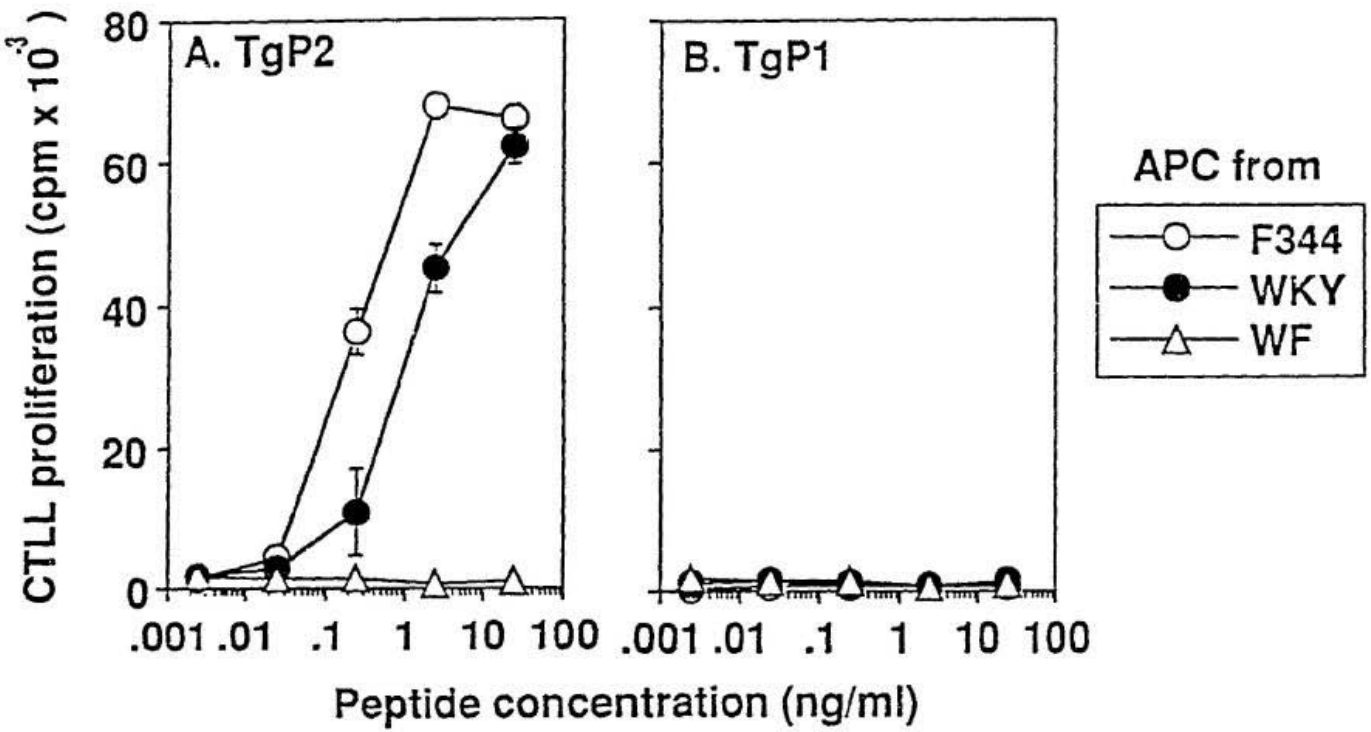
T cell hybridomas (1.5×10^4/well) were cultured with irradiated (1500 rad) syngeneic splenocytes (5×10^5/well) in the presence or absence of TgP2 (10 µg/ml) for 48 hr in 200 µl volume of DMEM-10 in 96-well flat-bottomed microculture plates. T cell hybridoma activation and CTLL assays were performed as described under Materials and Methods (Section 2.17). The values are mean of duplicate wells +/- SD.

hybridoma recognizes the peptide in an MHC-restricted fashion, specificity and MHC-restriction assays were performed. The results of the specificity assays are shown in Table 7.1 and of MHC-restriction assay in Figure 7.3. The 8F7-5 T cell hybridoma responded to TgP2 (Table 7.1) demonstrating the TgP2-specificity of the T cell hybridoma. In the MHC-restriction assay, splenic APC from syngeneic (F344) as well as allogeneic (WKY and WF) rats were employed. The results are shown in Figure 7.3. TgP2-pulsed F344, WKY but not WF rat APC could activate the T cell hybridoma (Figure 7.3A). The data indicate that the T cell hybridoma recognizes the peptide in an MHC-unrestricted fashion similar to the MHC-unrestricted nature of the TgP2-specific T cell line described earlier (Figure 5.3). The activation of the 8F7-5 T cell hybridoma with TgP2-pulsed WKY rat APC is equivalent to the degree of activation observed with TgP2-pulsed F344 rat APC. Activation of the T cell hybridoma by peptide-pulsed WKY rat APC is not attributed to allorecognition because no activation was observed in the presence of a control peptide, TgP1 (Figure 7.3B). These observations indicate that the TgP2-specific T cell hybridoma is MHC-unrestricted.

7.2.4 Both unpulsed or peptide-pulsed Ia$^+$ thyrocytes are ineffective at activating the cloned 8F7-5 T cell hybridoma

To test whether or not Ia$^+$ thyrocytes (IFN-γ treated

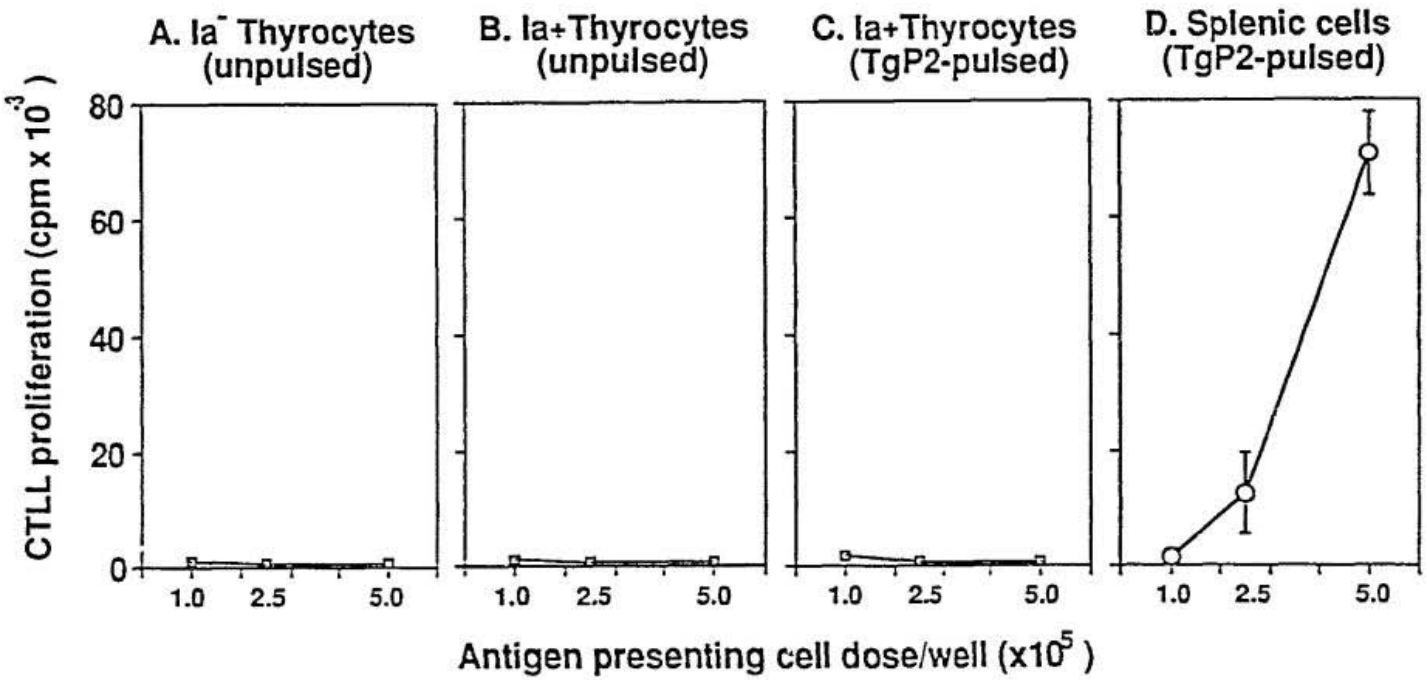
Figure 7.3 The 8F7-5 T cell hybridoma recognizes TgP2 in an MHC-unrestricted fashion. The 8F7-5 T cell hybridoma cells (10^5/well) were incubated with graded doses of (A) TgP2 or (B) TgP1 in the presence of syngeneic (F344) and allogeneic (WKY and WF) rat APC (5 x 10^5/well) for 24 hrs. Subsequently, the CTLL assay was performed by incubating 10^4 CTLL cells/well with 50 μl of supernatant from hybridoma activation cultures as described under **Section 2.18.** The results are expressed as mean cpm +/- SD of triplicate cultures. Background CTLL proliferative responses (mean cpm) when T cells were incubated with APC in the absence of peptide was as follows: F344 rat APC: 1739; WKY rat APC: 2010; WF rat APC: 1177.


A. TgP2
B. TgP1
CTLL proliferation (cpm x 10^-3)
80
60
40
20
0
.001 .01 .1 1 10 100
.001 .01 .1 1 10 100
Peptide concentration (ng/ml)
APC from
F344
WKY
WF

FRTL-5 cells) could act as APC by presenting the endogenously generated TgP2 sequence to the cloned 8F7-5 T cell hybridoma, hybridoma activation and CTLL assays were performed. As shown in Figure 7.4B, Ia^+ thyrocytes when used in graded doses (10^5/well to 5 x 10^5/well) failed to activate the TgP2-specific 8F7-5 T cell hybridoma, thus failing to act as APC as expected. This failure of activation of the hybridoma is not due to the low APC (thyrocytes) number used in the activation assay, because at similar cell numbers, TgP2-pulsed splenocytes could readily activate the T cell hybridoma (Figure 7.4D). Therefore, the observed results are likely to be due to the inability of TEC to present endogenously generated TgP2 sequence to the T cell hybridoma in the context of self-MHC.

Furthermore, an experiment was performed to test whether or not pulsing of Ia^+ thyrocytes with TgP2 could overcome this defect. The results are shown in Figure 7.4C. Graded doses of TgP2-pulsed Ia^+ thyrocytes did not activate the T cell hybridoma, whereas similarly treated splenocytes did. These results demonstrate that Ia^+ thyrocytes cannot present Tg epitopes, either endogenously generated or exogenously pulsed, by themselves, to autoreactive T cells in F344 rats. It is likely that the inability of Ia^+ thyrocytes to present exogenously pulsed TgP2 may be due to its inherent deficiency in the expression of certain costimulatory molecules.

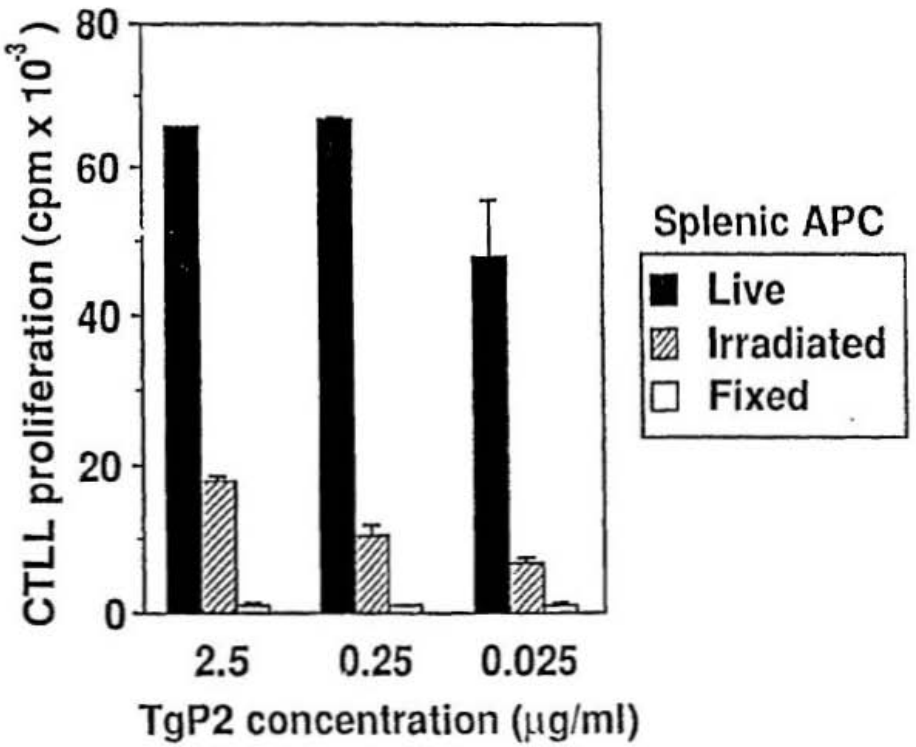
Figure 7.4 Neither unpulsed nor peptide-pulsed Ia$^+$ thyrocytes activate the cloned 8F7-5 TgP2-specific T cell hybridoma. Graded numbers of Ia$^-$ (IFN-γ untreated) or Ia$^+$ (IFN-γ treated) thyrocytes or splenocytes of F344 rat were cultured with T cell hybridoma cells (10^5/well) with (1 μg/ml) or without TgP2. After 24 hr of incubation, IL-2 content in the culture supernatant was assessed by the proliferation of the IL-2 dependent CTLL line as described under **Section 2.18**. Results are expressed as mean cpm +/- SD of triplicate cultures. Background CTLL proliferative responses (mean cpm) when T cells were incubated with APC in the absence of peptide was 1475.


A. Ia⁻ Thyrocytes (unpulsed)
B. Ia+Thyrocytes (unpulsed)
C. Ia+Thyrocytes (TgP2-pulsed)
D. Splenic cells (TgP2-pulsed)
CTLL proliferation (cpm x 10⁻³)
80
60
40
20
0
1.0
2.5
5.0
Antigen presenting cell dose/well (x10⁵)

7.2.5 Fixation or irradiation abrogates antigen-presentation capacity of splenocytes

It has been suggested that fixation or irradiation of splenic APC prior to pulsing with Ags abrogates activation of costimulatory dependent MBP-specific rat x mouse T-T cell hybridomas (Mannie et al, 1990; Watkins and Mannie, 1993). Chemical fixation of splenic APC is known to destroy mostly uncharacterized costimulatory activities (Jenkins et al, 1987 and 1988). Similarly, I tested whether or not 8F7-5 T cell hybridoma requires costimulatory molecules for its activation. For this purpose, splenic APC were either glutaraldehyde fixed or irradiated and pulsed with TgP2. Then these TgP2 pulsed APC were used to activate the 8F7-5 T cell hybridoma. The results are shown in Figure 7.5. Glutaraldehyde fixation completely abrogated the presentation of TgP2 by splenic APC since the T cell hybridoma response (assessed by the CTLL proliferative response) was similar to the background values. This observation raised the possibility that the 8F7-5 T cell hybridoma requires costimulatory molecules. Furthermore, irradiation of the splenic APC also dramatically reduced their capacity to present TgP2 to the 8F7-5 T cell hybridoma. These results further suggest that the activation of the T cell hybridoma requires yet undefined costimulatory molecules.

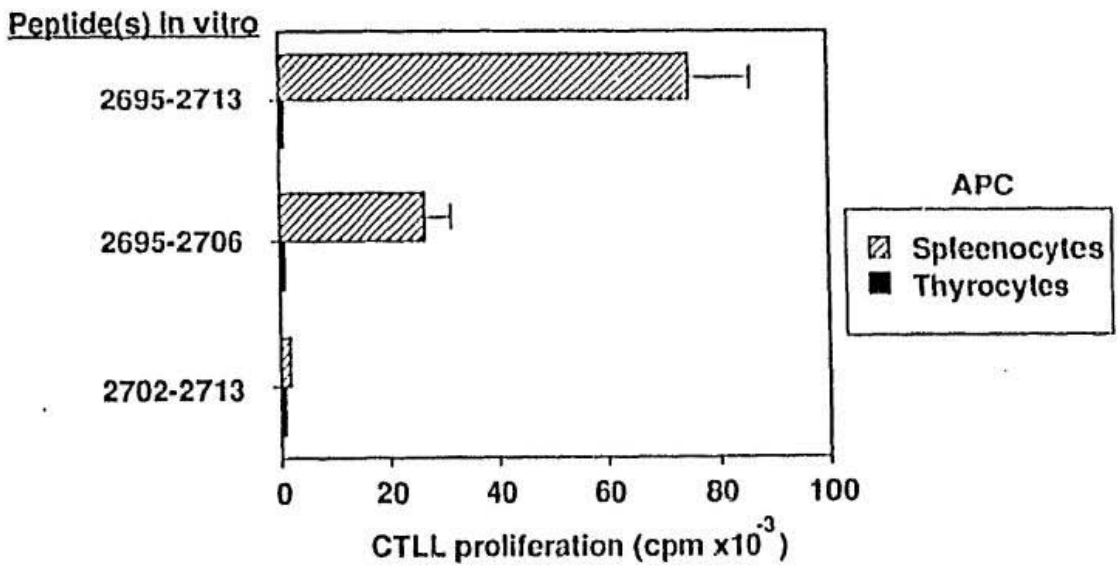
Figure 7.5 Fixation or irradiation abrogates antigen-presentation by splenocytes to the cloned 8F7-5 T cell hybridoma. Glutaraldehyde fixed or irradiated (1500 rad) or live splenocytes at 5 x 10^5 /well were added to the culture containing the 8F7-5 T cell hybridoma cells at 10^5/well in the presence or absence of peptide at the indicated doses for 24 hrs. Subsequently, a CTLL assay was performed as described under **Section 2.18.** Results are expressed as mean cpm +/- SD of triplicate cultures of proliferating CTLL cells. Background CTLL proliferative responses (mean cpm) when T cells were incubated with APC alone were as follows: 1720; 709; 809 when live; irradiated; and fixed splenocytes were used as APC respectively.


80
60
40
20
0
CTLL proliferation (cpm x 10^{-3})
2.5
0.25
0.025
TgP2 concentration (μg/ml)
Splenic APC
Live
Irradiated
Fixed

7.2.6 The N-terminal 12mer (2695-2706) peptide-pulsed splenocytes but not Ia$^+$ thyrocytes activate the cloned 8F7-5 T cell hybridoma

I have demonstrated earlier that F344 rat TgP2-specific CD4$^+$, CD8$^-$ T cell line recognizes T cell determinants within the N-terminal (2695-2706) but not C-terminal (2702-2713) 12mer peptide of TgP2 (2695-2713) (Figure 5.3). Therefore, I tested whether or not 8F7-5 T cell hybridoma generated from TgP2-specific T cells would similarly recognize the N-terminal but not the C-terminal 12mer peptide, and if so, whether the 12mer Tg peptide-pulsed Ia$^+$ thyrocytes could similarly activate the cloned 8F7-5 T cell hybridoma. The results are shown in Figure 7.6. The results demonstrate that the 8F7-5 T cell hybridoma recognizes the N-terminal 12mer (2695-2706) but not the C-terminal 12mer (2702-2713) Tg peptide. However, the N-terminal 12-mer Tg peptide-pulsed, IFN-γ treated (Ia$^+$) thyrocytes, like the 18mer TgP2-pulsed Ia$^+$ thyrocytes, also failed to activate the 8F7-5 T cell hybridoma. These data further emphasize that MHC-class II Ag expression alone on thyrocytes (APC) is not sufficient to present the Tg-peptide to the 8F7-5 T cell hybridoma but rather suggests that the T cell hybridoma requires the expression of costimulatory molecules on thyrocytes.

Figure 7.6 The 12mer Tg peptide-pulsed splenocytes but not Ia$^+$ thyrocytes activate the 8F7-5 T cell hybridoma. Either splenocytes (5 x 10^5/well) or Ia$^+$ thyrocytes (IFN-γ treated FRTL-5 cells)(5 x 10^5/well) were pulsed with the indicated peptides and incubated with 10^5 8F7-5 T cell hybridoma cells/well for 24 hr. A volume of 50 μl of the hybridoma culture supernatant was assayed for the support of CTLL proliferation. The results are expressed as mean +/- SD of triplicate cultures. The assay was done as described under **Section 2.18.** The peptides were used at 10 μg/ml when thyrocytes were used as APC and at 2.5 μg/ml when splenocytes were used as APC. Background CTLL proliferation (mean cpm) when T cells were incubated with APC alone was as follows: 1451 and 668 when splenocytes and thyrocytes respectively were used as APC.


Peptide(s) in vitro
2695-2713
2695-2706
2702-2713
APC
Splecnocytes
Thyrocytes
0
20
40
60
80
100
CTLL proliferation (cpm x10-3)

7.3 Discussion

The data described in this study (a) indicate that MHC class II$^+$ thyrocytes fail to function as APC in activating the TgP2 (2695-2713)-specific cloned 8F7-5 T cell hybridoma, (b) question the APC function of thyrocytes at initiating autoreactivity in the target organ, and (c) support the notion that particular APC selectively interact with Ag-specific T cells.

The failure of the 8F7-5 T cell hybridoma to be activated by MHC class II$^+$ thyrocytes is unlikely to be due to its co-expression of CD4 and CD8 molecules (Figure 7.2), because it could be readily activated by TgP2-pulsed splenocytes (Table 7.1). The expression of both CD4 and CD8 molecules by the 8F7-5 T cell hybridoma generated from CD4$^+$, CD8$^-$ TgP2-specific T cell line (Figure 7.2) is surprising, but is in good agreement with a previous report in which the authors reported the expression of both CD4 and CD8 molecules by the rat x mouse T cell hybridomas (Gocinski et al, 1993). The present findings (Figure 7.2) demonstrate that 100% of the 8F7-5 T cell hybridoma cells express CD8 molecules in contrast to the T cell hybridomas reported in the studies of Gocinski et al (1993) which express CD8 molecules in the range of 28% to 91%. The reason for this apparent discrepancy is unknown, but may be attributed to the activation status of the T cells at the time of their immortalization. Furthermore, the expression of

both CD4 and CD8 molecules is not unique to the 8F7-5 hybridoma alone, since a similar phenotypic profile was observed with another TgP2-specific T cell hybridoma (3H2) derived from the peptide-specific T cell line in the same fusion (data not shown).

The observation that unpulsed IFN-γ treated (Ia^+) thyrocytes fail to activate the 8F7-5 T cell hybridoma (Figure 7.4B) is unexpected. This finding disagrees with previous experimental findings (Hirose et al, 1988a). In the latter study, the authors have shown that the Ia^+ FRTL-5 clone (IB-6) could readily activate not only autoreactive (F344) but also alloreactive (Buffalo) rat $CD4^+$ T line cells. However, the T cells used in that study and the present one were derived from the same species (rat) and strain (F344). The 8F7-5 T cell hybridoma is specific for the 12mer Tg sequence (2695-2706) within the pathogenic 18mer TgP2 (2695-2713) sequence, whereas the T cells used in the studies of Hirose et al (1988a) are specific for non-thyroid Ags. Thus, the distinct results could reflect a difference in the activation requirements of the T cell hybridomas <u>versus</u> T cell lines employed. It is thus likely that the T cells used in the studies of Hirose et al (1988a) may have different costimulatory requirements for their activation from that of the 8F7-5 T cell hybridoma used in the present study. The present results (Figure 7.6), abrogation of Ag-presentation

capacity of splenocytes by both fixation and irradiation, support the notion that activation of the 8F7-5 T cell hybridoma requires costimulatory molecules. Similar to the present findings, Watkins and Mannie (1993) have recently demonstrated that activation of MBP-specific rat x mouse T-T hybridomas could be abrogated when irradiated splenic cells were used as APC to present MBP. In addition, these authors have shown that the activation of hybridomas was readily reconstituted when Ag-pulsed irradiated splenic APC were mixed with syngeneic live B cells or T cells or allogeneic APC but not with soluble exogenous cytokines. These results led them to suggest that the activation of T cell hybridomas require membrane-associated costimulatory molecules. Thus, it would be interesting to test whether activation of the TgP2-specific 8F7-5 T cell hybridoma, used in the present study, similarly requires membrane costimulatory molecules. This may be addressed by performing cell separation experiments with, for example, the use of polycarbonate microporous cell culture inserts as was employed earlier (Watkins and Mannie, 1993).

In contrast, the present findings (Figure 7.4B) that Ia^+ thyrocytes by themselves do not function as APC, are in good agreement with previous reports in mice (Ebner et al, 1987 and Stein and Stadecker, 1987). In that regard, Stein and Stadecker (1987) have suggested that the unresponsiveness of T cells with Ia^+ mouse TEC (M-5) was due to deficient

expression of certain membrane costimulatory molecules by Ia$^+$ mouse TEC. Thus, similar results in diverse species suggest that Ia$^+$ thyrocytes by themselves may not activate costimulation dependent T cells. Different investigators have shown that either PMA treatment (Stein and Stadecker, 1987) or irradiation (Czirjak et al, 1990) of murine thyrocytes (M-5 cell line) rendered these cells capable of activating both autoreactive T cell lines and alloreactive T cell hybridomas. These results suggested that either of these treatments might have induced undefined costimulatory molecules on thyrocytes. It would be interesting to examine whether similar treatment of FRTL-5 cells would render them capable of activating the 8F7-5 T cell hybridoma. It is likely that the 8F7-5 T cell hybridoma is not dependent on irradiation induced costimulatory molecules because irradiation of splenic APC abrogates their ability to present TgP2 leading to activation of the T cell hybridoma. Taken together, these various results strongly suggest that the pathways of activation of autoreactive T lymphocytes described here are different from those postulated by Botazzo et al (1983) in which epithelial cells express aberrant class II molecules and present Tg epitopes without the help of other APC such as dendritic cells. Furthermore, the current observations that both 18mer and 12mer Tg peptide-pulsed Ia$^+$ thyrocytes failed to stimulate the 8F7-5 T cell hybridoma (Figure 7.4C and Figure 7.6) are

surprising, given that 23mer hemagglutinin peptide-pulsed, HLA-matched DR^+ thyrocytes (primary thyroid monolayers) from a GD patient could stimulate the specific T cell line (Londei et al, 1984). Thus, the distinct results observed in diverse species with peptide-pulsed Ia^+ thyrocytes may either be due to differences in activation requirements of T cell clones versus T cell hybridomas or to the heterogeneity in the APC population used i.e., primary thyroid monolayers, but not FRTL-5 cells, may be contaminated with APC such as dendritic cells.

However, as to why the 18mer (2695-2713) or the 12mer Tg peptide (2695-2706)-pulsed, IFN-γ treated (Ia^+) thyrocytes have failed to stimulate specific T cells is unknown. It may be argued that the observed results may be due to defective Ag processing rather than mere failure to express costimulatory molecules by Ia^+ thyrocytes. I have not addressed the issue of whether these Tg peptides require processing before being presented by splenic APC or directly bind to class II molecules on splenic APC. However, the failure of the Ia^+ thyrocytes to present the 12mer Tg peptide (2695-2706) is probably not due to defective Ag processing, because this peptide is likley to bind directly to class II molecules. This possibility is suggested because this 12mer Tg peptide is smaller in size than most of the class II bound naturally processed peptides which are in the range of 13-25 amino acids

in length (Rudensky et al, 1991; 1992; Hunt et al, 1992; Nelson et al, 1992; Chicz et al, 1992; 1993).

Furthermore, the differences in the immunogenicity of the 18mer and 12mer Tg peptides (Figure 7.3A), as assessed by IL-2 release by the T cell hybridoma, are not unusual. These results may be a consequence of a positive effect of amino acid residues flanking the stimulatory 12mer peptide on the recognition and activation of the T cell hybridoma. This interpretation is compatible with the findings that flanking residues outside the minimal stimulatory peptide exert a positive effect on the recognition and activation of T cells (Bhayani et al, 1988; Vacchio et al, 1989; Nelson et al, 1994). In that regard, similar results were obtained earlier with the TgP2-specific T cell line (Figure 5.3).

Moreover, the inability of peptide-pulsed Ia^+ thyrocytes to activate specific T cells (Figure 7.4 and 7.6), also contrast with several previous reports that Ag-pulsed Ia^+ non-lymphoid cells can serve as APC for Ia-restricted Th cells <u>in vitro</u> (Fierz et al, 1985; Frei et al, 1987; McCarron, 1985; Umetsu et al, 1985). Thus, the discrepancies in the reported APC function of thyrocytes and other non-lymphoid cells may be attributable to a variety of factors such as the lineage and characteristics of APC, the heterogeneity of the final APC population, the species employed, and the nature and assay of the responding cells.

The differences in the handling of Tg peptide (2695-2706) by splenocytes and Ia^+ thyrocytes (Figure 7.6) as assessed by the ability of Tg peptide (2695-2706)-pulsed splenocytes but not Ia^+ thyrocytes to activate the specific T cells (Figure 7.6) suggest that activation of T cells does not depend entirely on the nature of the peptide Ag but also involves the inherent properties of APC. For example, APC differ in their expression of the putative accessory or costimulatory molecules with which they interact with T cell surface ligands (Liu and Linsley, 1993). The present findings (Figure 7.6) support the notion that a particular APC subset selectively activates Ag-specific T cells (Mannie et al, 1990; Sun et al, 1993; St. Louis et al, 1993). In this regard, Sun et al (1993) have demonstrated that non professional APC such as the Ia^+ T cell clone (LOA) or the Ia inducible rat glial cell clone (F10) can present MBP peptide analogs [69-84Gly and (P80) 68-86] to encephalitogenic T cells specific for MBP peptide (a.a 68-88), whereas the thymocytes could not. Mannie et al (1990) also have demonstrated that rat x mouse T cell hybridomas specific for MBP peptide (68-86) were activated by particular APC subsets within splenocytes. In another study, St. Louis et (1993) also demonstrated that costimulation deficient, Ag-pulsed, IFN-γ treated murine endothelial cells readily induced T cell proliferative response in Th2 but not in Th1 clones.

The observed failure of either unpulsed or Tg peptide-pulsed Ia$^+$ thyrocytes to activate the TgP2-specific 8F7-5 T cell hybridoma is difficult to reconcile with the finding that the CD4$^+$ TgP2-specific T cell line adoptively induces severe thyroiditis in naive syngeneic rats (Table 5.4). Thus, one might speculate that thyrocytes can express costimulatory molecules such as B7 physiologically *in vivo*, and are able to present Tg epitopes to T cells in natural or experimentally induced autoimmune disorders or models. However, the recent observation that Ia$^+$ thyrocytes do not express the B7 molecule (Tandon et al, 1994) raises the possibility that Ia$^+$ thyrocytes may not activate costimulatory dependent thyroid antigen-specific T cells, and that APC other than Ia$^+$ thyrocytes in the thyroid present Tg epitopes to T cells. On the other hand, thyrocytes may not express costimulatory molecule such as B7 under the influence of IFN-γ either *in vitro* or *in vivo*, because the expression of B7 is differently regulated in APC of different lineages or in different anatomical locations (Chelen et al, 1995). In contrast to B7 expression, thyrocytes of HT patients coexpress both ICAM-1 and LFA-1 ligands (Bagnsco et al, 1995). Therefore, it is necessary to examine the surface expression by Ia$^+$ FRTL-5 cells of different

costimulatory molecules such as B7 or its equivalent, ICAM-1, and HSA-1 (Liu and Linsley, 1992) and to correlate the

expression of costimulatory molecules with their ability to activate TgP2-specific T cell hybridoma. It should be noted that B7 expression has been reported on hematopoietic cells, but not on other cell types (St. Louis et al, 1993). It is thus unlikely that Ia^+ thyrocytes express this molecule, but this could not be verified for lack of antibodies against the rat homologue of murine B7 protein. It is likley that a role for ICAM-1 is not envisaged in the activation of the T cell hybridoma since FRTL-5 cells are known to express ICAM-1 under basal condition itself (Tandon et al, 1991c). It would also be interesting to examine whether in situ expression of IFN-γ in the thyroid, using a tissue-specific promoter, similar to IFN-γ transgenic mouse models described earlier (Sarvetnick et al, 1988; 1990), would lead to infiltration of thyroid by Tg-specific autoreactive T cells in rats.

Based on the present findings, I hypothesize that costimulation deficient class II^+ thyrocytes by presenting Tg epitopes may provide signal I to Tg peptide-specific T cells, whereas dendritic cells in the thyroid (Kabel et al, 1988; Hassman et al, 1988; Voorby et al, 1990) provide signal II in a trans manner. Thereby, these two APC populations synergistically activate the pathogenic Tg-specific T cells in the inflamed thyroid. Activation of T cells by such a mechanism has been shown earlier in in vitro model systems (Liu and Janeway, 1992; St. Louis et al, 1993).

Alternatively, dendritic cells in the thyroid by themselves could take up rTg secreted by thyrocytes or its cleavage fragments generated by thyroidal proteases (Dunn et al, 1991) and present Tg epitopes to thyroid-homing T cells. The ability of Ia^+ thyrocytes to activate costimulatory independent T cells should not be excluded, in view of the recent findings that $B7^-$ Ia^+ murine kidney tubule cells (KTC) readily activate costimulatory independent but not dependent T cell hybridomas (Hagerty et al, 1994).

It is well established by both in vitro (Gaspari et al, 1988; Markmann et al, 1988) and in vivo (Lo et al, 1988; Guerder et al, 1994) experimental models, that Ag-presentation by APC to T cells in the absence of co-stimulation (signal II) results in T cell anergy or unresponsiveness. Thus, it is tempting to speculate that Ia^+ thyrocytes with MHC class II-peptide complexes, may deliver tolerogenic rather than activation signals to thyroid-Ag-specific T cells and induce T cell paralysis. It remains to be established whether peptide-pulsed Ia^+ thyrocytes can similarly induce anergy in the peptide-specific T cell hybridoma or thyroiditis-inducing T line cells. This assumption can be tested by both in vitro (restimulation experiments) and in vivo (adoptive experiments) analogous to the recent experiments performed with IFN-γ-treated (Ia^+) microvessel endothelial cells and encephalitogenic T cells in Lewis rats (Boudoulous et al,

1995). If Ia^+ thyrocytes do induce anergy in thyroid-Ag specific T cells, then the ectopic or aberrant expression of class II on thyrocytes <u>in vivo</u> may serve as an extrathymic mechanism for induction of peripheral tolerance rather than activating Tg-reactive T cells leading to pathogenic thyroid autoimmunity.

In summary, the findings reported in this chapter suggest that the simple expression of class II molecules on thyrocytes does not confer APC function, thyrocytes may be deficit in the expression of certain membrane costimulatory molecules, and aberrant expression of class II on thyrocytes in the diseased thyroid may be a consequence but not the cause of an initial autoimmune insult. These findings will help us to understand the autoantigen-presentation in the thyroid gland.

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